

MULTIOMICS APPROACHES TO OVERCOME DRUG RESISTANCE IN CANCER

**A DISSERTATION SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MOLECULAR BIOLOGY AND GENETICS**

**By
Barış Küçükkaraduman
September 2021**

MULTIOMICS APPROACHES TO OVERCOME DRUG RESISTANCE IN CANCER

By Barış Küçükkaraduman

September 2021

We certify that we have read this dissertation and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

Ali Osmay Güre (Advisor)

Burçak Vural

Bala Gür Dedeoğlu

Bahar Değirmenci Uzun

Kerem Mert Şenses

Approved for the Graduate School of Engineering and Science:

Ezhan Karaşan

Director of the Graduate School

ABSTRACT

MULTIOMICS APPROACHES TO OVERCOME DRUG RESISTANCE IN CANCER

Bariş Küçükkaraduman
Ph.D. in Molecular Biology and Genetics
Supervisor: Ali Osmay Güre
September 2021

Chemotherapy resistance remains one of the major challenges in cancer treatment. Most of the studies on drug resistance have focused on genetic evolution of cancer cells; however, this focus has shifted to non-genetic and epigenetic mechanisms. There is accumulating evidence that mechanisms of drug resistance are not mutually exclusive but instead coexist within a given cancer to develop resistance and therapy failure. Hence, overcoming resistance requires the comprehension of these complex biological processes. Here, we aimed to characterize drug resistance mechanisms by performing both single omics interrogations and multi-omics integrative analysis. For this purpose, we conducted Gene Set Enrichment Analysis (GSEA), functional enrichment analysis on protein-protein interaction (PPI) networks and miRNA-target networks for interpreting gene and miRNA expression data. To gain further biological insights on resistance mechanisms, we focused on identifying a multi-omics molecular signature that discriminates cancer cells based on their drug response profiles. Collectively, these *in silico* analyses suggested the epithelial-to mesenchymal transition (EMT) as a mediator of 5-FU/irinotecan resistance in colon cancer and irinotecan/gemcitabine resistance in pancreatic cancer. Drug sensitive cancer cells exhibited a more epithelial phenotype with increased cell proliferation. Multi-omics integration analysis revealed some EMT-related genes such as TGM2 and FOSL1, to contribute differential drug response in cancer cells. On the other hand, response of breast cancer cells to doxorubicin exhibited an opposite profile in which mesenchymal phenotype is sensitive while resistant cells have epithelial phenotype.

Secondly, we aimed to induce mesenchymal-to-epithelial transition to overcome EMT-mediated drug resistance. We selected eight natural compounds and two re-purposed agents that have been shown to reverse EMT in various studies. We noted transcriptional changes suggesting a shift towards a more epithelial phenotype in 4 out of the 6 cell lines upon treatment with at least one compound tested. None of the natural compounds or re-purposed agents triggered MET in all cancer cells screened. In addition, compounds with clear or slight MET induction did cause these effects in a specific cell line or only in specific cancer type. We investigated next whether the treatment with natural compounds would result in chemosensitization. MET induction by natural compounds is not uniformly related to increased sensitivity to chemotherapeutics but can result in occasional synergistic or additive effects.

Lastly, based on cytotoxic activity of a novel c-Src inhibitor 10a in 15 melanoma cells, we report the identification of a new gene signature that can predict chemosensitivity to 10a. Two distinct phenotypes of cells, defined as sensitive and resistant, were further analyzed to reveal an underlying mechanism for this differential response to 10a. We found that proliferative or mesenchymal features of the cells are associated with distinct sensitivity of 10a. Through a protein–chemical interaction network analysis, we identified that three histone deacetylase inhibitors, valproic acid, entinostat, and trichostatin A, were predicted to synergize with 10a. The synergizing effect of valproic acid was validated *in vitro*. We also aimed to define a minimal number of genes that could be used as biomarkers of 10a sensitivity. We show that the expression level of four genes can be used to predict drug sensitivity against 10a.

Keywords: Chemotherapy resistance, Network analysis, 5-FU, Irinotecan, Gemcitabine, Doxorubicin, EMT, Plasticity

ÖZET

KANSERDE İLAÇ DİRENCİNİN ÜSTESİNDEN GELMEK İÇİN MULTİ-OMİK YAKLAŞIMLAR

Barış Küçükkaraduman
Moleküler Biyoloji ve Genetik, Doktora
Tez Danışmanı: Ali Osmay Güre
Eylül 2021

Kanser tedavisinde karşılaşılan temel zorluklardan biri kemoterapiye oluşan dirençtir. İlaç direnci üzerine yapılan çalışmalar genellikle kanser hücrelerinin genetik evrimi üzerine yoğunlaşmıştır, fakat epigenetik ve genetik olmayan mekanizmalar üzerine yapılan çalışmalar da ağırlık kazanmaya başlamıştır. Son bulgular ilaç direncinin tek bir mekanizmayla değil birden fazla direnç mekanizmasının bir araya gelmesiyle meydana geldiğini göstermektedir. Bu yüzden, oluşan ilaç direncini kırabilmek için kompleks biyolojik mekanizmaların daha iyi kavranması gerekmektedir. Bu çalışmada, omik verileri tek tek veya birleştirici algoritmalarla analiz ederek ilaç direnç mekanizmalarını karakterize etmeyi amaçladık. mRNA ve miRNA verisetleri yorumlamak için gen seti zenginleştirme ve bu verisetleriyle oluşturulmuş protein-protein etkileşim ağları ve miRNA-hedef etkileşim ağlarının fonksiyonel zenginleştirme analizlerini gerçekleştirdik. Kanser hücrelerini ilaç yanıt profillerine göre ayırt edebilen multi-omik moleküler profiller keşfetmeyi amaçladık. Yapılan analizler sonucunda epitelden mezenkimal'e geçiş (EMT) fenotipinin kolon kanserinde 5-FU ve irinotecan direnciyle ve pankreas kanserinde irinotecan ve gemcitabine direnciyle ilişkili olduğunu bulduk. Bu ilaçlara hassasiyet gösteren kanser hücrelerinin artan hücre çoğalması gösteren epitel fenotipe sahip olduğunu bulduk. Multi-omik entegrasyon analizimiz, EMT ilişkili TGM2 ve FOSL1 gibi genlerin ilaçlara oluşan yanıt farklılıklarıyla ilişkili olabileceğini gösterdi. Öte yandan, meme kanseri hücrelerinin doxorubicin ilacına gösterdiği yanıt tam aksi bir profil ortaya koydu. Bu hücrelerde mezenkimal fenotip ilaç hassasiyetiyle, epitel fenotip ilaç direnciyle ilişkili bulundu.

Bulgular ışığında EMT kaynaklı ilaç direncini kırmak için bu fenotipi tersine döndürmeyi hedefledik. Bunu yapabilmek için daha önceki çalışmalarda EMT'yi tersine döndürebildiği gösterilmiş sekiz doğal bileşik ve klinikte farklı amaçlarla kullanımda olan iki ilaç belirledik. Altı hücre hattı ile yapılan çalışmada dört hücre hattında en az bir ilaç ile epitel fenotipe geçiş gözlemlendi. Seçilen doğal bileşik ve ilaçlardan hiçbirinin taranan tüm hücre hatlarında EMT'yi tersine çeviremediği gözlemlendi. Mezenkimalden epitel fenotipe geçiş belirli hücre hatlarında yada belirli bir kanser türünde gözlenirken genel bir indüksiyon görülmedi. Sonrasında kanser hücrelerinin doğal bileşikler ve klinikte kullanımda olan iki ilaçla muamelesi sonrası kemoterapötik ilaçlara hassiyet kazanıp kazanmadığını inceledik. Kombinasyonel terapinin nadiren sinerjistik veya additif etki oluşturduğunu fakat bu etkinin tamamen mezenkimalden epitele geçişin indüklenmesiyle ilişkili olmadığını gördük.

Son olarak, yeni c-Src inhibitörü 10a'nın on beş melanoma hücre hattındaki sitotoksik etkilerini analiz ederek, 10a kemo-hassasiyetini tahmin edebilecek bir gen listesi belirledik. 10a ilacına hassasiyet ve direnç gösteren hücre hatlarını iki gruba ayırarak hassasiyet farklılıklarına sebep olan nedenleri araştırdık. Hassas olan grup proliferatif fenotip gösterirken dirençli hücre hatlarının mezenkimal fenotipe sahip olduklarını bulduk. Protein-kimyasal etkileşim ağ analizi sonucu üç histon deasetilaz inhibitörünün 10a ile sinerji gösterebileceği tahmininde bulunduk. Bir histon deasetilaz inhibitörü olan valproik asit'in sinerji profilini in vitro olarak doğruladık. Son olarak, 10a hassasiyetini tahmin edilecek minimum sayıda gen listesi oluşturduk ve bu genlerin ekspresyonunun 10a sensitivitesini başarıyla tahmin edebildiğini gösterdik.

Anahtar sözcükler: İlaç direnci, Ağ analizi, 5-FU, Gemcitabin, Irinotekan, Doksorubisin, Epitel, Mezenkimal, Plastisite



I dedicate my thesis to my family for their endless support and love...

Acknowledgements

First of all, I would like express my sincere gratitude to my advisor Prof. Dr. Ali Osmay Güre for his enduring support and remarkable scientific guidance during my scientific discoveries. His wide knowledge, support and inspiring suggestions helped me improve myself in the field of science.

I also would like to thank Dr. Bala Gür Dedeoğlu and Dr. Bahar Değirmenci Uzun for being part of my thesis committee for all these years and for sharing their constructive comments towards my project. I would also like to thank Dr. Burçak Vural and Dr. Kerem Mert Şenses for kindly accepting my invitation and sparing their time for evaluating my thesis and being part of my thesis defense committee.

I also want to thank to my friends and colleges for their help and support during my PhD studies. I received a lot of support during my PhD study in scientific manners as well as in friendship. I would like to acknowledge Muhammad Waqas Akbar, Seçil Demirkol, Ege Dedeoğlu, Murat İşbilen, Can Türk, Seyhan Türk for their remarkable efforts on our collaborative projects. I am also grateful to our relatively new group members Shila Azizolli, Marzana Ishraq, Isli Cela, Farid Ahadli and Farid Sadati for their help and friendship. I am sincerely thankful to Unal Metin Tokat, Pelin Ersan, Oğuzhan Tarman and İrem Evcili for sharing beautiful moments with me during this journey.

I would like to express my sincere appreciation to all Bilkent MBG family, especially Yavuz Abi, Okan Bey, Pelin and Seda Hanım made this department as a home for all of us.

Above all, I would like to thank my family, my father Şinasi Küçükkaraduman, my mother Elvan Küçükkaraduman, my sisters Burçin and Betül for their unconditional love and support by all means. I also want to express my deep gratitude and my love for my wife Büşr  Küçükkaraduman for his outstanding support, being great friend and life partner. But most of all I want to thank god and my wife for their precious gift to me, my son, Ediz.

I was supported by TÜB TAK 2211 scholarship program during my study, thereby I would like to thank TÜB TAK for giving me this opportunity.

Contents

1. Introduction.....	1
1.1. Analysis on drug resistance mechanisms by using multi-omics data	1
1.1.1. Gene set enrichment analysis (GSEA).....	2
1.1.2. NetworkAnalyst 3.0	2
1.1.3. miRNet 2.0.....	3
1.1.4. mixOmics.....	3
1.2. Cancer and treatment options.....	4
1.3. Cancer therapeutic resistance	4
1.3.1. Mechanisms for 5-fluorouracil resistance.....	5
1.3.2. Mechanisms for irinotecan resistance	6
1.3.3. Mechanisms for gemcitabine resistance	6
1.4. EMT as a drug resistance mechanism	7
1.5. Reversal of EMT to overcome drug resistance	8
1.6. Natural products and their roles in cancer.....	9
1.7. Natural products as an EMT modulator and chemotherapy enhancer	10
1.8. Melanoma.....	11
1.8.1. Treatment options and resistance mechanisms in melanoma	12
1.8.2. c-Src as a therapy target for melanoma.....	15
1.8.3. Dasatinib and saracatinib	15
1.8.4. Pyrazolo pyrimidine derivatives that targets c-Src	16
2. Aim of the study.....	17
3. Materials	18
3.1. General laboratory reagents.....	18
3.2. General laboratory solutions.....	18
3.3. General laboratory equipment	19
3.4. Cell culture equipment and reagents	19
3.5. Cell culture solutions	20
3.6. Primers.....	20

3.7. Drugs and natural compounds	21
4. Methods.....	22
4.1. Cancer cell lines and maintenance of cells in culture	22
4.2. Thawing, passaging and freezing cell lines.....	22
4.3. Cell viability analysis	23
4.4. RNA isolation.....	24
4.5. RNA quantification	24
4.6. DNase treatment.....	24
4.7. cDNA synthesis.....	25
4.8. End-point PCR analysis	25
4.9. Agarose gel electrophoresis	26
4.10. Quantitative real-time PCR analysis.....	26
4.11. Bioinformatic methods.....	27
4.11.1. Identification of sensitive and resistant cell lines	27
4.11.2. Functional enrichment analysis by GSEA, NetworkAnalyst and miRNet	27
4.11.3. Multi-omics data integration with mixOmics	28
4.11.4. Two-fold cross-validation with 1000 iteration	28
4.12. Statistical Analysis.....	29
5. Results.....	30
5.1. Characterization of resistance mechanisms for 5-FU in colon cancer	30
5.2. Characterization of resistance mechanisms for irinotecan in colon cancer.....	38
5.3. Characterization of resistance mechanisms for irinotecan in pancreatic cancer	46
5.4. Characterization of resistance mechanisms for gemcitabine in pancreatic cancer.....	53
5.5. Characterization of resistance mechanisms for doxorubicin in breast cancer.....	61
5.6. Reversal of EMT by natural compounds to overcome EMT-driven drug resistance	70
5.6.1. Use of natural products to enhance 5-FU and Oxaliplatin sensitivity in KM12 colon cancer cells.....	78
5.6.2. Effects of natural products on cancer cell viability	80
5.6.3. Combinatory effects of natural products and chemotherapy drugs on colon cancer cells	83
5.6.4. Combination of curcumin and resveratrol with irinotecan in pancreatic cancer cell lines	94

5.6.5.	Combinatory effects of natural compounds and gemcitabine in MIA PaCa-2 cells.	96
5.6.6.	Combination treatment of natural products and targeted agent Gefitinib in MIA PaCa-2 cells.....	98
5.6.7.	Combination treatment of natural products and targeted agent Selumetinib in MIA PaCa-2 cells.....	99
5.7.	Intrinsic resistance against c-Src inhibitor in melanoma and strategies for overcoming	101
5.7.1.	Inhibitory effects of c-Src inhibitor 10a on 15 primary melanoma cells.....	101
5.7.2.	mRNA based biomarkers to predict chemosensitivity against 10a	102
5.7.4.	Protein–chemical interaction network analysis to further investigate resistance mechanism	108
5.7.5.	Use of valproic acid to enhance chemotherapy against 10a	109
5.7.6.	Minimal number of predictive biomarkers of 10a sensitivity.....	111
6.	Discussion.....	113
6.1.	Characterization of chemotherapy resistance mechanisms	113
6.2.	Resistance mechanisms to 5-FU in colon cancer cells	114
6.3.	Resistance mechanisms to irinotecan in colon cancer.....	116
6.4.	Resistance mechanisms to irinotecan in pancreatic cancer	117
6.5.	Resistance mechanisms to gemcitabine in pancreatic cancer.....	118
6.6.	Resistance mechanisms to doxorubicin in breast cancer.....	119
6.7.	Targeting EMT to overcome drug resistance in cancer.....	120
6.8.	Mesenchymal/invasive phenotype in melanoma as a mechanism for c-Src inhibitor resistance	122
7.	Bibliography	124
8.	Publications.....	136

List of Figures

Figure 5. 1: Enriched gene sets in colon cancer cell lines sensitive to 5-FU.....	30
Figure 5. 2: Enriched gene sets in colon cancer cell lines resistant to 5-FU.	31
Figure 5. 3: PPI network analysis with 5-FU sensitivity associated gene list.	32
Figure 5. 4: PPI network analysis with 5-FU resistance associated gene list.	33
Figure 5. 5: 5-FU resistance associated miRNAs-target network analysis.....	34
Figure 5. 6: Sample scatter plot and pearson correlation coefficients of mixOmics analysis based on 5-FU sensitivity profiles of colon cancer cell lines.	35
Figure 5. 7: List of selected mRNA variables and their loading weights associated with 5- FU sensitivity.	36
Figure 5. 8: List of selected miRNA variables and their loading weights associated with 5-FU sensitivity.....	36
Figure 5. 9: List of selected protein variables and their loading weights associated with 5- FU sensitivity.	37
Figure 5. 10: List of selected methylation events and their loading weights associated with 5-FU sensitivity.....	37
Figure 5. 11: Differential transglutaminase mRNA and protein levels in colon cancer cells.	38
Figure 5. 12: Enriched gene sets in colon cancer cell lines sensitive to irinotecan.	39
Figure 5. 13: Enriched gene sets in colon cancer cell lines resistant to irinotecan.....	39
Figure 5. 14: PPI network analysis with irinotecan resistance associated gene list.	40
Figure 5. 15: miRNAs-target network analysis with irinotecan resistance associated miRNAs.	41
Figure 5. 16: Sample scatter plot and pearson correlation coefficients of mixOmics analysis based on irinotecan sensitivity profiles of colon cancer cell lines.....	42
Figure 5. 17: List of selected mRNA variables and their loading weights associated with irinotecan sensitivity.	43
Figure 5. 18: List of selected miRNA variables and their loading weights associated with irinotecan sensitivity.	43
Figure 5. 19: List of selected protein variables and their loading weights associated with irinotecan sensitivity.	44

Figure 5. 20: List of selected methylation events and their loading weights associated with irinotecan sensitivity.	44
Figure 5. 21: Protein expression levels and mRNA expression levels of FOSL1 in irinotecan-sensitive and –resistant cell lines.	45
Figure 5. 22: Enriched gene sets in pancreatic cancer cell lines sensitive to irinotecan...	46
Figure 5. 23: Enriched gene sets in pancreatic cancer cell lines resistant to irinotecan. ..	47
Figure 5. 24: PPI network analysis with irinotecan resistance associated gene list.	48
Figure 5. 25: PPI network analysis with irinotecan sensitivity associated miRNA list....	49
Figure 5. 26: Sample scatter plot and pearson correlation coefficients of mixOmics analysis based on irinotecan sensitivity profiles of pancreatic cancer cell lines.	50
Figure 5. 27: List of selected mRNA variables and their loading weights associated with irinotecan sensitivity.	51
Figure 5. 28: List of selected miRNA variables and their loading weights associated with irinotecan sensitivity.	51
Figure 5. 29: List of selected protein variables and their loading weights associated with irinotecan sensitivity.	52
Figure 5. 30: List of selected methylation events and their loading weights associated with irinotecan sensitivity.	53
Figure 5. 31: Enriched gene sets in pancreatic cancer cell lines sensitive to gemcitabine.	54
Figure 5. 32: Enriched gene sets in pancreatic cancer cell lines resistant to gemcitabine.	55
Figure 5. 33: PPI network analysis with gemcitabine resistance associated gene list.....	56
Figure 5. 34: Sample scatter plot and pearson correlation coefficients of mixOmics analysis based on irinotecan sensitivity profiles of pancreatic cancer cell lines.	57
Figure 5. 35: List of selected mRNA variables and their loading weights associated with gemcitabine sensitivity.....	58
Figure 5. 36: List of selected miRNA variables and their loading weights associated with gemcitabine sensitivity.....	59
Figure 5. 37: List of selected protein variables and their loading weights associated with gemcitabine sensitivity.....	60
Figure 5. 38: List of selected methylation events and their loading weights associated with irinotecan sensitivity.	60
Figure 5. 39: Enriched gene sets in pancreatic cancer cell lines sensitive to doxorubicin.	62
Figure 5. 40: Enriched gene sets in pancreatic cancer cell lines resistant to doxorubicin.	63

Figure 5. 41: PPI network analysis with doxorubicin sensitivity associated gene list.	64
Figure 5. 42: PPI network analysis with doxorubicin resistance associated gene list.	65
Figure 5. 43: PPI network analysis with doxorubicin resistance associated miRNA list.	65
Figure 5. 44: Sample scatter plot and pearson correlation coefficients of mixOmics analysis based on doxorubicin sensitivity profiles of breast cancer cell lines.	66
Figure 5. 45: List of selected mRNA variables and their loading weights associated with doxorubicin sensitivity.	67
Figure 5. 46: List of selected miRNA variables and their loading weights associated with doxorubicin sensitivity.	68
Figure 5. 47: List of selected protein variables and their loading weights associated with doxorubicin sensitivity.	69
Figure 5. 48: List of selected methylation events and their loading weights associated with irinotecan sensitivity.	69
Figure 5. 49: Expression levels of EMT marker genes in cancer cell lines.	71
Figure 5. 50: Effect of natural compounds and repurposed agents on the expression of EMT marker genes in Caco-2.	72
Figure 5. 51: Effect of natural compounds and repurposed agents on the expression of EMT marker genes in KM12.	73
Figure 5. 52: Effect of natural compounds and repurposed agents on the expression of EMT marker genes in LoVo.	74
Figure 5. 53: Effect of natural compounds and repurposed agents on the expression of EMT marker genes in WiDr.	75
Figure 5. 54: Effect of natural compounds and repurposed agents on the expression of EMT marker genes in MDA-MB-157.	76
Figure 5. 55: Effect of natural compounds and repurposed agents on the expression of EMT marker genes in MIA PaCa-2.	77
Figure 5. 56: Combination treatment of KM12 cells with 5-FU and natural compounds.	79
Figure 5. 57: Combination treatment of KM12 cells with oxaliplatin and natural compounds.	79
Figure 5. 58: Inhibitory effects of forskolin on different types of cancer cell lines.	81
Figure 5. 59: Combination treatment of curcumin and irinotecan in pancreatic cancer cell lines.	95
Figure 5. 60: Combination treatment of resveratrol and irinotecan in pancreatic cancer cell lines.	96

Figure 5. 61: Combination treatment of natural compounds and gemcitabine in MIA PaCa-2 pancreatic cancer cell line.	97
Figure 5. 62: Combination treatment of natural compounds and gefitinib in MIA PaCa-2 pancreatic cancer cell line.	99
Figure 5. 63: Combination treatment of natural compounds and Selumetinib in MIA PaCa-2 pancreatic cancer cell line.	100
Figure 5. 64: Hierarchical clustering of cell lines according to 10a chemosensitivity biomarker genes.	104
Figure 5. 65: Sensitivity profiles of fifteen melanoma cells.	105
Figure 5. 66: Comparative analysis of differentially enriched molecular gene sets among sensitive and resistant cells by GSEA.....	106
Figure 5. 67: Comparative analysis of differentially enriched molecular gene sets among sensitive and resistant cells by GSEA.....	106
Figure 5. 68: Protein-chemical interaction network with chemosensitivity associated genes.	108
Figure 5. 69: Combination treatment of 10a-resistant M221 melanoma cells with 10a and sodium valproate.....	109
Figure 5. 70: Combination treatment of 10a-resistant M193 melanoma cells with 10a and sodium valproate.....	111
Figure 5. 71: Gene expression analysis of candidate biomarkers for 10a chemosensitivity.	112

List of Tables

Table 3. 1: General use laboratory chemicals and kits	18
Table 3. 2: Cell culture equipment and reagents.....	19
Table 3. 3: List of primers and sequences.....	20
Table 3. 4: Drugs and natural compounds.	21
Table 5. 1: Summary table of MET induction experiments.....	78
Table 5. 2: In vitro cytotoxicity analyses of natural compounds and repurposed agents in breast cancer cell lines.	80
Table 5. 3: In vitro cytotoxicity analyses of natural compounds and repurposed agents in colon cancer cell lines.	81
Table 5. 4: In vitro cytotoxicity analyses of natural compounds and repurposed agents in pancreatic cancer cell lines.	82
Table 5. 5: Effects of apigenin treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.....	83
Table 5. 6: Effects of curcumin treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.....	83
Table 5. 7: Effects of EGCG treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.....	84
Table 5. 8: Effects of Fisetin treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.....	84
Table 5. 9: Effects of forskolin treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.....	85
Table 5. 10: Effects of procyanidin B2 treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.....	85
Table 5. 11: Effects of resveratrol treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.....	85
Table 5. 12: Effects of urolithin A treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.....	86
Table 5. 13: Effects of fulvestrant treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.....	86
Table 5. 14: Effects of metformin treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.....	87

Table 5. 15: Effects of apigenin treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.	87
Table 5. 16: Effects of curcumin treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.	87
Table 5. 17: Effects of EGCG treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.	88
Table 5. 18: Effects of fisetin treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.	88
Table 5. 19: Effects of apigenin treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.	88
Table 5. 20: Effects of procyanidin B2 treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.	89
Table 5. 21: Effects of resveratrol treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.	89
Table 5. 22: Effects of urolithin A treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.	89
Table 5. 23: Effects of fulvestrant treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.	90
Table 5. 24: Effects of metformin treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.	90
Table 5. 25: Effects of apigenin treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.	91
Table 5. 26: Effects of curcumin treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.	91
Table 5. 27: Effects of treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.	91
Table 5. 28: Effects of treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.	92
Table 5. 29: Effects of forskolin treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.	92
Table 5. 30: Effects of procyanidin B2 treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.	92
Table 5. 31: Effects of resveratrol treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.	93
Table 5. 32: Effects of urolithin A treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.	93

Table 5. 33: Effects of fulvestrant treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.	93
Table 5. 34: Effects of metformin treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.	94
Table 5. 35: Summary table of combinational treatments.	101
Table 5. 36: The half-maximal inhibitory concentration (IC ₅₀) values for c-Src inhibitor 10a in melanoma cells.....	101
Table 5. 37: Number of genes with a frequency of significance in more than 900, 800 and 700 iterations.....	102
Table 5. 38: List of 46 genes that were significantly associated with 10a cytotoxicity values.	102
Table 5. 39: Enriched gene sets in 10a-sensitive and –resistant melanoma cells.	107
Table 5. 40: Combination Index values of combinations in M221 melanoma cells.....	110
Table 5. 41: Combination Index values of combinations in M193 melanoma cells.....	111

Chapter 1

Introduction

1.1. Analysis on drug resistance mechanisms by using multi-omics data

With the help of recent developments, high throughput technologies has led to measure a large number of biological molecules, such as mRNAs, miRNAs, proteins, metabolites etc. Different methods are used to measure each type of such molecules which is called “omic”. Analysis of single type of omics seldom provide causal relations and correlations, however, combining such large-scale biological datasets can provide opportunities to understand various biological processes and complex human diseases, such as cancer. Additionally, multivariate analysis of biological datasets can lead to the discovery of important biological insights on complex processes like drug resistance in cancer therapy. Many multi-omics level analysis have been recently conducted by integrating genomics, proteomics, metabolomics and epigenomics data [1]. Several studies have shown that integrative analysis of multi-omics data contributes to elucidate complex biological mechanisms for drug resistance [2-4].

Many large consortia performed several experiments on the same biological sample, providing multi-omics datasets. TCGA (The Cancer Genome Atlas) and ICGC (International Cancer Genome Consortium) focuses on characterization of different types of human cancer, collecting multi-omics data from tens of thousands of tumors [5, 6]. CCLE (The Cancer Cell Line Encyclopedia) and Genomics of Drug Sensitivity in Cancer (GDSC) projects aimed to characterize genetic and pharmacological features of a large panel of human cancer cell lines [7, 8]. These datasets constitute an important tool in revealing resistance mechanisms against cancer therapeutics. Additionally, translation of cancer cell line integrative genomics into cancer patient’s stratification can further improve the understanding of cancer biology and provide novel cancer therapy.

1.1.1. Gene set enrichment analysis (GSEA)

GSEA is a powerful analytical method being used in interpretation of gene expression data. This computational method focuses on a collection of annotated gene sets that share common biological regulation, chromosomal location, or function. GSEA searches for the gene sets that show statistically significant, concordant differences between two phenotypes of interest [9]. In the method, the genes are ordered top to bottom according to their differential expressions between defined phenotypes. Then, the genes in the set that are over-represented at either the top or bottom of the list are counted to calculate enrichment score. By a phenotypic based permutation test, statistical significance of the enrichment score is calculated. Normalization is performed on enrichment scores and a false discovery rate is calculated [10]. Cell line drug response data sets offer a many of evaluable phenotypes and thus GSEA can be utilized to search for gene sets and pathways associated with a particular drug response.

1.1.2. NetworkAnalyst 3.0

NetworkAnalyst 3.0 is a web based platform offering statistical, visual and network based analytics for transcriptomic data. It accepts various types of data inputs including one or multiple gene lists. By using NetworkAnalyst 3.0, data inputs can be used to create generic protein-protein interaction networks, cell-type or tissue specific protein-protein interaction networks, gene co-expression networks, gene regulatory networks as well as networks for pharmacogenomics and toxicogenomic studies. Functional enrichment analysis can be also performed via integrated knowledgebase like KEGG, Reactome and PANTHER SLIM/GO [11, 12]. Consequently, NetworkAnalyst can be an user-friendly and powerful tool to gain system-level understanding of gene expression patterns in drug resistance.

1.1.3. miRNet 2.0

miRNet 2.0 is a web based tool designed to help understanding of microRNA functions with existing knowledge via visual and network based analytics. The tool depends on three main steps, data input, network creation and visual analysis of networks. The latest releases of miRNA annotation databases were embedded into miRNet's underlying interaction knowledgebase. It is also possible to include protein-protein interactions in the miRNA-target networks. miRNA-transcription factor and transcription factor-gene interaction networks can be also integrated into networks for visual exploration. Functional enrichment analysis can be also performed via integrated databases like KEGG, Reactome and PANTHER SLIM/GO [13, 14]. Therefore, miRNet 2.0 is highly important tool for understanding of various biological processes and disease conditions. The miRNet 2.0 can provide for adaptation of system biology approach to integrate all important players.

1.1.4. mixOmics

mixOmics is an R package consisting of multivariate methods that are applied to statistically integrate 'omics data coming from different sources to identify the most discriminant subset of biological features such as transcripts, proteins and metabolites. mixOmics provides functions to enable feature selection that are key predictors of the phenotypes of interest. In contrast to univariate methods, mixOmics provides the identification of a combination of discriminative features that meet biological assumptions such as drug-sensitive and -resistant subgroups [15].

In the context of supervised analysis, mixOmics methods include Partial Least Squares Discriminant Analysis, Generalized Canonical Correlation Discriminant Analysis and multi-group Partial Least Squares Discriminant Analysis. Feature selection is performed via L1 regularisation [16-18]. mixOmics can lead to the identification of crucial biological insights and can be extremely valuable in determining what molecular signatures are associated with drug resistance.

1.2. Cancer and treatment options

Cancer is one the leading causes of death. In 2020, new cancer cases were estimated as 19.3 million and almost 10 million people died due to the cancer worldwide. Incidence rate of cancer is expected to be almost 30 million cases in 2040, which infer ~50% increase from 2020 [19]. Cancer is a disease where cells divide in uncontrolled manner and spread into surrounding tissues and other parts of the body [20]. Inheritance, lifestyle habits, and environmental factors can play a role in the development of cancer. Mutations in tumor suppressor genes or oncogenes caused by these factors lead to abnormal protein homeostasis in the cell, in turn, results in cancer initiation, development, progression and metastasis [21, 22].

There are various types of cancer treatment. Types of treatment a patient receive depend on the type of cancer, how advanced it is and its molecular profile. Treatments for cancer include traditional techniques like surgery, radiotherapy and chemotherapy; and newer techniques like targeted therapy and immunotherapy. Although the trend for cancer treatment has evolved from non-selective cytotoxic agents to specific, mechanism-based therapeutics, cancer continuous to be a leading cause of mortality [23, 24]. Therefore, there is a need for more efficacious, tolerable and novel approaches to treat cancer.

1.3. Cancer therapeutic resistance

One of the reasons why cancer continues to be a leading cause of mortality is therapeutic resistance [25]. Various resistance mechanisms led cancers to escape therapy-induced cell death. However, many of resistance mechanisms remains incompletely understood [26]. Therapeutic resistance in cancer can be classified as intrinsic and acquired resistance. Intrinsic resistance refers to the ability of cancer cells to escape from therapy-induce cell killing due to pre-existing cellular phenotype or genetic alterations [27, 28]. On the other hand, acquired or adaptive resistance develops after an initial response to therapy by positive selection of cancer cells with pre-existing genetic alterations and/or by acquiring specific genetic abnormalities in the drug target itself, protein alterations in parallel or

downstream pathways, metabolic adaptations and epigenetic changes [29-32]. Increasing evidence also suggest that tumor microenvironment is involved in acquired resistance of cancer cells to various therapies [33].

Genetic evolution of cancer cells is often considered as the main driver of cancer drug resistance. However, recent studies suggest that resistance mechanisms are multifactorial and not solely consist of genetic alterations. Non-genetic and epigenetic mechanisms are gaining greater recognition. There is accumulating evidence that mechanisms of drug resistance are not mutually exclusive but instead coexist within a given cancer to drive resistance development and therapy failure. Lineage switching, phenotypic plasticity and epigenetic mechanisms that change gene expression patterns triggered by oncogenic stress are increasingly recognized in cancer drug resistance [34-36].

1.3.1. Mechanisms for 5-fluorouracil resistance

5-fluorouracil (5-FU) is one of the commonly used chemotherapeutic drugs in cancer treatments. It is a pyrimidine analog, which induces cytotoxicity through increasing DNA damage by inhibiting the action of thymidylate synthase [37]. Despite the several advantages, the 5-year survival in patients treated with 5-FU remains too low due to the development of drug resistance after chemotherapy [38].

Various mechanisms were suggested to explain 5-FU resistance. One of these mechanisms involves the alterations in thymidylate synthase levels or other enzymes that have roles in 5-FU metabolism and activation. High mRNA levels of thymidylate synthase levels were found to be associated with 5-FU resistance [39]. MDR transporters were also shown to be responsible with 5-FU resistance [40]. Another mechanism for 5-FU is quiescence and metabolic adaptation in cancer stem cells. Additionally, several transcription factors like FOXM1, TFAP2C and NRF2 were found to be associated with increased 5-FU resistance [41-43].

Another proposed resistance mechanism for 5-FU is resistance to DNA damage. Hence, the roles of mismatch repair and base excision repair in 5-FU resistance has been studied extensively [44, 45].

1.3.2. Mechanisms for irinotecan resistance

Irinotecan is an analog of camptothecin that is a DNA topoisomerase I inhibitor. Irinotecan stabilizes the topoisomerase/DNA complex, in turn, resulting in DNA replication and transcription. It is a promising cancer therapeutic but its clinical activity is limited due to the intrinsic and acquired drug resistance [46].

There are some reported mechanisms of irinotecan resistance. The main factor for the resistance is alterations in topoisomerase I expression levels [47]. Mutations in target binding site is another reason for irinotecan resistance [48]. Carboxylase is the enzyme required to create active metabolite from irinotecan. Decreased levels of carboxylase has been shown to be responsible factor for irinotecan resistance [49]. Another mechanism for irinotecan resistance is the increased expression of uridine diphosphate glucuronosyltransferase that inactivates active metabolite of irinotecan. Increased levels of multidrug resistance proteins were also shown to result in increased efflux of irinotecan leading to resistance [50].

1.3.3. Mechanisms for gemcitabine resistance

Gemcitabine is a deoxycytidine analogue and the first-line treatment for pancreatic ductal adenocarcinoma [51]. Despite initial responsiveness, pancreatic cancer eventually develops gemcitabine resistance resulting in limited clinical efficacy [52].

Major factors responsible for gemcitabine resistance includes drug transporters, drug metabolism by enzymes and competitive substrates to active metabolites of gemcitabine. Expression levels of nucleoside transporters hEN1, hCNT1 and hCNT3 are highly associated with sensitivity against gemcitabine. Downregulation of these proteins were shown to result in resistance [53, 54]. Another mechanism for gemcitabine resistance is related to deoxycytidine kinase that is required for intracellular drug activations. Studies have showed that reduced levels of deoxycytidine kinase are associated with acquired resistance to gemcitabine [55]. On the other hand, upregulation of cytidine deaminase,

responsible for gemcitabine degradation and excretion, was found to result in gemcitabine resistance [56]. Additionally, upregulations of ribonucleotide reductase and thymidylate synthase were shown to cause gemcitabine resistance by competing in DNA synthesis [57-59].

1.3.4. Mechanisms for doxorubicin resistance

Doxorubicin is an anthracycline antibiotic that is widely used as a cancer therapeutic. Proposed mechanisms for doxorubicin action in cancer cell include inhibition of topoisomerase II mediated DNA repair and damaging DNA, cellular proteins and membranes by generating free radicals [60]. Although it is one of the most effective anti-neoplastic agents, resistance against doxorubicin is a problem limiting its use [61].

Major factors responsible for doxorubicin resistance includes upregulation of efflux pump proteins, continued topoisomerase II function by mutation in binding site or upregulation of topoisomerase II, and suppression of downstream apoptosis signaling [60]. The amplification of ERBB2 was also shown to affect sensitivity against doxorubicin [62].

1.4. EMT as a drug resistance mechanism

Phenotype switching induced by cancer therapy is often thought as bidirectional in which cancer cells switch between a sensitive and resistant state. This phenomenon is largely regulated by reversible epithelial to mesenchymal transition (EMT/MET). Accumulating evidence has suggested that EMT is not a binary epithelial-mesenchymal state; indeed, there are several intermediate states showing distinct phenotypic characteristics [36]. The EMT/MET driven cellular plasticity is exploited by cancer cells resulting in therapy resistance [63].

Several factors affect EMT/MET plasticity in cancer. Genetic evolution of cancer cells may result in mutational activation of the signaling pathways that induce EMT-driven cellular plasticity. Similarly, growth factors and cytokines from tumor microenvironment, acidity

and hypoxic conditions in tumor core, inflammatory microenvironment or exposure to therapeutics regulate EMT-related gene expression changes in cancer cells [64].

EMT-mediated cellular plasticity has suggested as a mechanism that fuels resistance to various conventional chemotherapeutics and targeted drugs. In a study, differences between parental and 5-FU-resistant HT29 colon cancer cells has been characterized and molecular alterations showed a marked increase in the expression of EMT-related genes suggesting the role of EMT in 5-FU resistance [65]. Likewise, irinotecan-resistant LoVo colon cancer cells displayed invasive phenotype with cancer stem cell-like morphology and increased expression of Twist1, an EMT transcription factor [66]. Furthermore, resistance to another traditional chemotherapeutic gemcitabine has been associated with cadherin switching mediated by EMT signaling [67]. Gefitinib resistance was also associated with EMT and reversal of EMT has shown to result in recovery of tyrosine kinase inhibitor. [68] In lapatinib resistant breast cancer cell models, EMT-related markers, Snail and Vimentin were shown to be upregulated [69]. It was also shown that lapatinib-resistant gastric cancer cell line SNU216 exhibit an EMT phenotype [70]. Likewise, studies suggest that MEK inhibitor treatment results in phenotype switching in cancer [71]. Lung cancer studies showed that EMT activates different feedback mechanisms and MAPK activation in both epithelial like and mesenchymal-like cells following MEK inhibition [72]. Taken together, studies suggesting the role of EMT in drug resistance provide insights into approaches for preventing or overcoming this resistance in cancer.

1.5. Reversal of EMT to overcome drug resistance

The role of EMT in cancer therapeutic resistance has been widely investigated. Several EMT-associated signaling pathways are found to be involved in therapy resistance in cancer cells. Therefore, targeting EMT has been exhaustively discussed as a promising strategy for overcoming drug resistance in cancer [73]. Accordingly, studies focused on drugs targeting EMT to prevent and/or overcome drug resistance. Antibiotic salinomycin is one of the drugs that are shown to target EMT. A study showed that salinomycin inhibited doxorubicin-induced EMT in HCC cells and enhanced sensitivity to doxorubicin

[74]. In addition to salinomycin, mocetinostat, a histone deacetylase inhibitor, was shown to downregulate EMT-TF ZEB1 resulting in reversal of EMT phenotype in docetaxel-resistant pancreatic cancer cell lines and this resulted sensitization of cells [75]. Zidovudine, an antiviral drug, is another example in which zidovudine was shown to restrain Akt/GSK3B/Snail-driven EMT, thereby, sensitize pancreatic cells to gemcitabine [76]. In a similar approach, metformin exhibited great promise and several studies demonstrated that metformin inhibits EMT via different mechanisms in various cancer types [77-80]. There are also cancer therapeutics which are repurposed and used to enhance chemotherapy. Fulvestrant, a therapeutic targeting estrogen receptor in breast cancer, was shown to inhibit EMT and increases sensitivity of lung xenografts to docetaxel [81]. These and several other EMT-modulating molecules have been entered clinic in the hope of enhancing cancer therapy efficacy. Natural compounds are also good candidates since they are inexpensive, accessible, and readily applicable with minimum toxicity.

1.6. Natural products and their roles in cancer

Nutrition and diet have been considered as an effective strategy for reducing cancer risk. Numerous dietary natural products have shown a potential role in prevention and treatment of cancers [82-84]. Accumulating evidences showed that naturally occurring bioactive compounds interfere with carcinogenesis by inhibiting the tumor cell proliferation and targeting multiple abnormally activated signaling pathways [85, 86].

In clinics, conventional chemotherapy commonly leads to many-side effects caused by systemic toxicity. Currently, combination of chemotherapeutics has been applied to various cancer types, providing amelioration of side effects and enhancement of therapy with the lowering doses [87]. Furthermore, use of plant-derived natural compounds with minimal cytotoxicity has become more important in clinic. Recent studies show that naturally occurring bioactive molecules combined with anticancer drugs have the potential to enhance efficacy of chemotherapy and attenuate drug resistance [88]. An extract of *Solanum nigrum* leaves was shown to enhance efficacy of doxorubicin, docetaxel, cisplatin and 5-FU in colorectal cancer cells through autophagy induction [89]. Similarly, three

natural phenolic acids (caffeic, rosmarinic and ursolic) were shown to potentiate paclitaxel in ex vivo breast cancer cells by modulating tumor microenvironment [90]. Likewise, Kaempferol, a dietary flavonoid, was shown to sensitize hepatocellular carcinoma cells against sorafenib by decreasing the overexpression of p-glycoprotein that mediates multidrug resistance in HCC [91]. Capsaicin, the ingredient of hot chili peppers, was also tested in combination with docetaxel and combinational treatment were found to be synergistic by activating AMPK [92]. Furthermore, many natural products have been shown to modulate epithelial to mesenchymal cancerous transition. Therefore, impeding or reversal of EMT by natural products can be also an effective strategy to overcome EMT-induced chemoresistance.

1.7. Natural products as an EMT modulator and chemotherapy enhancer

Curcumin is the one of the most studied natural products, present in turmeric. Many studies showed that curcumin sensitizes cancer cells to chemotherapeutics by modulating EMT [93]. In a study, curcumin treatment was found to upregulate EMT-inducing miRNAs in 5-FU-resistant colon cancer cells. Major polycomb repressive complex subunits that mediate cancer stemness were also found to be downregulated by curcumin exposure resulting in EMT inhibition and sensitization of 5-FU-resistant colon cancer cells [94]. Interestingly, curcumin could not only enhance chemotherapy but also increases the efficiency of radiotherapy by modulating EMT. Curcumin was shown to inhibit γ -irradiation-induced EMT via the suppression of Gli1 and upregulation of Sufu, in turn, resulting in the enhancement of radiotherapy [95]. Likewise, curcumin was found to sensitize 5-FU-resistant HCT-116 colon cancer cells through reversal of EMT via inhibiting TET1 and NKD2 expression and suppression of WNT signaling pathway [96]. Resveratrol (phytoalexin) is a polyphenol, found in grapes, chocolate and peanuts [97]. Regulatory roles of resveratrol in EMT leading to chemosensitization have also been studied extensively. A recent study showed that resveratrol enhanced FL118-induced cell death via regulating EMT through upregulation of E-cadherin and downregulation of mesenchymal marker genes N-cadherin, Vimentin, Snail, Slug, Fibronectin, Twist and Zeb family [98]. Similarly, doxorubicin resistance in gastric cancer cells were reversed by

resveratrol through inhibition of Akt pathway achieving the reversal of EMT [99]. It was also shown that resveratrol inhibits the cisplatin-induced Snail expression in ovarian cancer cells; thus, reversal of EMT induced by resveratrol potentiates the efficacy of cisplatin in ovarian cancer cells [100]. In a similar way, resveratrol sensitized doxorubicin-resistant breast cancer cells by modulating EMT through SIRT1/ β -catenin signaling pathway [101]. Recently apigenin, a natural flavonoid, was shown to act as an inhibitor of Snail, which is a key regulator of EMT [102, 103]. Apigenin were shown to inhibit IL-6 linked downstream pathways resulting in epithelial marker E-cadherin upregulation and downregulation of the mesenchymal marker N-cadherin [104]. Another natural product EGCG, the most prevalent tea polyphenol, was also shown to reverse epithelial to mesenchymal transition and synergistic effects of EGCG were shown in pancreatic cancer cells when combined with gemcitabine through modulating EMT markers [105]. Fisetin, a natural flavanol found in various fruits and vegetables, was shown to reverse EMT *in vivo* in a xenograft model of the MDA-MB-231 breast cancer cell line [106]. Forskolin, a natural product derived from the root of the plant *Cleus Forskohlii*, was shown to induce tumor-initiating cells to undergo mesenchymal to epithelial transition, and causing them to lose their tumor initiating ability [107]. Procyanidin B2, an abundant polyphenol in red wine, was shown to reverse high glucose induced-EMT in a human renal epithelial cell line [108]. Additionally, urolithin A, a metabolite of ellagitannins, was shown to inhibit metastasis in SW620 colon cancer cell line, and increase sensitivity against 5-FU in Caco-2 colon cancer cell line [109, 110].

1.8. Melanoma

Melanoma is one of the most aggressive types of cancer with heterogeneous features [111]. There were an estimated 324,635 new cases of skin melanoma and 57,043 deaths in 2020. Moreover, the incidence of melanoma continues to increase. On the other hand, with the advancement in targeted therapies and immunotherapies, death rates were rapidly declined worldwide. In high-incidence rate countries like Australia and New Zealand, supportive environment and providing sun-protection products also helped decrease incidence rates [19].

The risk factors for melanoma include genetic predisposition and environmental exposure. The most important environmental risk for malignant melanoma is exposure to ultraviolet radiation because of its genotoxicity [112]. The number of melanocytic nevi and genetic predisposition are the most important host risk factors. The number of total nevus were found to be associated with increased risk of melanoma [113]. In familial melanoma cases, mutations in CDKN2A and CDK4 have been primarily linked to increased risk of melanoma [114]. Recently, another gene MC1R have been found to alter risk of familial melanoma [115]. Additionally, other cancer-related inherited genes were found to raise risk of developing melanoma [116].

According to WHO, melanoma can be divided into 9 distinct subtypes in relation to clinical, histological features and genomic characteristics. These includes superficial spreading melanoma, lentigo maligna melanoma, desmoplastic melanoma, spitz melanoma, acral melanoma, mucosal melanoma, melanomas arising in congenital nevi, melanomas arising in blue nevi and uveal melanoma. Superficial spreading melanoma is the most common subtype, it is almost 70% of the cases with the best prognosis. On the other hand, nodular melanomas which do not belong to any of the subtypes discussed above, are thought to be heterogeneous subtype with worse prognosis [117].

1.8.1. Treatment options and resistance mechanisms in melanoma

Melanoma is one the most aggressive types of cancer with poor prognosis. Treatment landscapes and success rates depend on factors, including applicability of surgery, stage of the tumor, rate of tumor growth and presence of melanoma-associated genetic mutations. While surgery remains the standard treatment for localized and early-stage melanoma, advanced stages of melanoma may require lymph node dissection, radiotherapy and systemic therapies including conventional chemotherapy, targeted therapy and immunotherapy [118, 119].

For early stages of melanoma, stages I-to-IIIB, surgical resection is the primary treatment [120]. Clinico-pathological features of the tumor help identify surgery procedures and safety margins differ according to it [121]. On the other hand, adjuvant therapies are advised for improved survival. For advanced stages of melanoma, conventional chemotherapy is recommended in cases with metastatic melanoma while standard

procedure includes surgical removal of metastases [122]. While use of radiotherapy is highly rare in primary tumor treatment, treatment of skin, bone and brain metastases may require radiotherapy [123].

It has been shown that adjuvant lymph node field radiotherapy can reduce the local recurrence rate with no improvement in long-term survival [124]. Likewise, locally treated brain metastases of melanoma were found to have no survival benefit from adjuvant brain radiation [125]. On the other hand, advancement in radiotherapy techniques such as stereotactic radiosurgery and stereotactic ablative radiotherapy show promising results in treatment of metastatic melanoma with the combination with immunotherapy or targeted therapy [126, 127].

First chemotherapeutic for metastatic melanoma was approved in 1974 by FDA, it is called Dacarbazine. Despite the fact that dacarbazine continues to be standard first-line treatment for metastatic malignant melanoma, studies reported very low efficacy in clinic with less than 5% complete response and 2%-6% of patients 5-year survival [128]. It is also shown that it has no survival benefit in brain metastases since dacarbazine is unable to cross blood-brain barrier [129]. Additionally, several resistance mechanisms were suggested for alkylating agent dacarbazine, its oral analog temozolomide. Most studied resistance mechanisms for dacarbazine is the increased levels of expression/activity of O⁶-methylguanine-DNA methyltransferase (MGMT) which removes the methyl and chloroethyl adducts generated by dacarbazine [130]. Another mechanism for chemotherapy resistance in melanoma were suggested as activation of Akt [131]. However, combination of temozolomide with PI3K/AKT/mTOR pathway inhibitor did not improve clinical efficacy of temozolomide in melanoma patients [132]. Still, several clinical trials are ongoing to improve overall survival of patients by using dacarbazine in combination with radiotherapy, targeted and immune-therapies since long-term efficacy of such treatments is limited [133].

Key signaling pathways that regulate cell growth and survival were found to be overactive in majority of melanomas. About half of all melanomas harbors mutations in BRAF [134]. Several BRAF inhibitors such as vemurafenib and dabrafenib were developed to inhibit this signaling pathway. Response rates to Vemurafenib were found to be 48% in compared

to dacarbazine alone with 5% [135]. Likewise, another BRAF inhibitor dabrafenib got approval by providing 5.1 months progression free survival compared to 2.1 months with dacarbazine alone [136]. However, clinical benefit of these therapies is limited due to rapid development of various resistance mechanisms. Downstream reactivation via MEK is one of the common resistance mechanisms against BRAF inhibition in melanoma. To overcome this drug resistance, several MEK inhibitors were developed and used in combination with BRAF inhibitors, resulting higher response rates compared to single agent targeted therapy [137, 138]. Unfortunately, efficacy of BRAF-MEK inhibition is not also long-lasting, mutational reactivation of MAPK pathway, non-genomic or immune evasion mechanisms contribute to BRAF-MEK inhibitor resistance [139-141].

Melanoma is particularly immunogenic cancer type with a high mutational burden compared to other cancers. Therefore, it is expected to be highly susceptible to immunotherapy. Immune regulatory checkpoints are the main targets in immunotherapy since cancer cells may evade the immune system by expressing ligands of these checkpoint molecules, which results in inactivation of T cells [118]. For melanoma, ipilimumab is the first immunotherapeutic that blocks CTLA-4 with great survival benefit. Then, another immune checkpoint inhibitor, nivolumab, were developed to block PD-1 with increased efficacy and lower cytotoxicity profile compared to ipilimumab monotherapy. Furthermore, dramatic and durable responses for patients with melanoma were achieved in combination immunotherapy with ipilimumab and nivolumab. 5-year survival was found to be 52% in combination immunotherapy, 44% in nivolumab monotherapy and 26% in ipilimumab monotherapy [142]. However, almost half of the patients with melanoma do not achieve any significant immunotherapy response and disease relapse are observed within 2 years in substantial proportion of patients with initial response [143]. Additionally, 50% of patients experienced grade 3-4 adverse effects of immunotherapy [144]. So, long-term efficacy of melanoma treatments is still limited by their adverse effects and innate or acquired resistance. Therefore, defining new targets for therapy may help improve patient's quality of life and increase survival time.

1.8.2. c-Src as a therapy target for melanoma

The cytoplasmic protein c-Src is a proto-oncogene and member of Src kinase family composed of nine non-receptor tyrosine kinases. Src kinase family includes Src, Yes, Fyn, Fgr, Lck, Hck, Blk, Lyn and Frk [145]. c-Src is activated by different classes of cellular receptors including adhesion receptors, cytokine receptors, G-protein coupled receptors and receptor tyrosine kinases. c-Src activation, in turn, results in a diverse spectrum of biological activities including cell adhesion, transformation, migration, apoptosis, cell cycle progression, and various immune responses [146].

In normal cells, c-Src is strictly regulated by CSK and PTP1B. CSK inactivates c-Src by phosphorylating a conserved tyrosine residue in c-terminal domain. Contrary, PTP1B dephosphorylates this residue resulting in c-Src activation. Additionally, active growth-factor receptor can bind SH2 domain of c-Src resulting in activation of c-Src. Similarly, binding of FAK and CAS to SH2 AND SH3 domains of c-Src can induce activation of c-Src. It is also strictly regulated at both transcriptional and post-transcriptional levels via various mechanisms. Disruption of any of these regulatory mechanisms may be associated with increased expression and activity of c-Src, in turn, advanced tumor progression, growth, advanced stages of cancer and metastasis [147].

In small subsets of colon and endometrial cancers, constitutive catalytic activity was associated with truncated c-terminal domain. Furthermore, mutational activation of c-Src is highly rare event in cancer types. More commonly, increased expression and/or activity of c-Src was observed in melanoma and other solid tumors [148]. Indeed, inhibition of c-Src with Dasatinib was shown to result in variable inhibition melanoma cell growth in vitro [149]. Hence, c-Src represent a promising therapeutic target for the treatment of melanoma.

1.8.3. Dasatinib and saracatinib

Several c-Src inhibitors have been available for clinical assessment, including dasatinib and saracatinib that are utilized in phase II clinical trials in melanoma. Dasatinib is a small molecule inhibitor of tyrosine kinases including EPHA2, BCR-ABL fusion, c-KIT,

PDGF β receptor and SRC family including c-Src, Yes, Lck and Fyn. Further, dasatinib is shown to inhibit c-Src in nanomolar levels and it was well tolerated in clinical trials for CML and gastrointestinal stromal tumors. Phase II trial of dasatinib in 39 melanoma patients showed that only two patients had partial responses lasting more than 24 weeks. Three patients had minor responses lasting more than 28 weeks. Overall, 6-months PFS rate was 13% suggesting minimal activity and dasatinib was poorly tolerated [150]. In phase II trial of saracatinib in patients with metastatic melanoma, 23 patients received oral saracatinib at a dose of 175 mg daily. Although it was well tolerated compared to dasatinib, no objective clinical responses were observed [151]. Hence, there is a need for more potent c-Src inhibitors, and/or a better definition of melanoma subtypes that would be suitable for Src inhibitor-based treatment protocols.

1.8.4. Pyrazolo pyrimidine derivatives that targets c-Src

Pyrazolo pyrimidine core provides drug-like scaffold that is nitrogen-containing heterocycle widely distributed in nature and playing important roles in the metabolism of all living cells. This scaffold present in various pharmacologically active compounds. Pyrazolo pyrimidine derivatives have been shown to have great potential as the basis of pharmacological compounds. Over the years, studies focused on chemical modifications of the pyrazolo pyrimidine scaffold to advance selective activity on kinases and biological availability [152]. In 1996, first pyrazolo pyrimidine kinase inhibitors were shown to inhibit SRC family of non-receptor kinases [153]. Additionally, many pyrazolo[3,4-d]pyrimidine derivatives were synthesized as serine/threonine and tyrosine kinase inhibitors showing antitumor activity. 10a is the 6-methylthio derivative of pyrazolo[3,4-d]pyrimidine, which was shown to inhibit c-Src selectively [154].

Chapter 2

Aim of the study

Innate and acquired resistance to chemotherapy are major obstacles to the effective treatment of cancer. There are various resistance mechanisms such as decreased drug accumulation and increased drug export, pre-existing genetic alterations and/or acquired genetic abnormalities in the drug target itself and signalling transduction molecules, metabolic adaptations, increased repair or tolerance to drug-induced DNA lesions, evasion of apoptosis and epigenetic changes. Phenotypic plasticity driven by reversible epithelial to mesenchymal transition (EMT/MET) are also increasingly recognized as one of the key factors in chemotherapeutic resistance mechanisms. My first aim was to characterize drug resistance mechanisms in colon, pancreatic and breast cancer cells by analyzing multi-omics data sets with advanced bioinformatics tools such as GSEA, NetworkAnalyst, miRNet, mixOmics.

We hypothesized that reversal of EMT with natural compounds can be effective strategy to overcome EMT-mediated drug resistance. Therefore, I next aimed to induce mesenchymal to epithelial transition in cancer cells by treatment with natural compounds that were shown to reverse EMT in several studies. I further aimed to combine chemotherapy with natural compounds to enhance sensitivity to chemotherapeutics.

In melanoma cells, an improved profiling of drug resistance is needed. For this purpose, I aimed to investigate underlying resistance mechanisms for novel c-Src inhibitor 10a. Our in silico analysis revealed that mesenchymal/invasive features of the cells are associated with increased resistance. We then aimed to identify a strategy to overcome 10a resistance and minimal number of biomarkers that could help classify melanoma subtypes into 10a sensitive and resistant subgroups.

Chapter 3

Materials

3.1. General laboratory reagents

Chemical and kits for general use in the laboratory were listed in Table 3.1 with the manufacturers.

Table 3. 1: General use laboratory chemicals and kits

Chemicals/Kits	Manufacturer
Ethanol	Sigma-Aldrich (St. Louis, MO, USA)
Isopropanol	Sigma-Aldrich (St. Louis, MO, USA)
Chloroform	Sigma-Aldrich (St. Louis, MO, USA)
PureZOL™ RNA Isolation Reagent	Bio-Rad Laboratories (Hercules, CA, USA)
RNase Zap	Ambion (Carlsbad, CA, USA)
Nuclease Free Water	Ambion (Carlsbad, CA, USA)
DNA-free™ Kit	Invitrogen (Carlsbad, CA, USA)
RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific (Waltham, MA, USA)
OneTaq Quick-Load 2X Master Mix with Standard Buffer	New England Biolabs (Ipswich, MA, USA)
iTaq Universal SYBR Green Supermix	Bio-Rad Laboratories (Hercules, CA, USA)

3.2. General laboratory solutions

Running buffer TAE was prepared as a 50X concentrated stock by dissolving 242 g of Tris Base (Sigma-Aldrich, St. Louis, MO, USA), 37.2 g of EDTA (Sigma-Aldrich, St. Louis, MO, USA) in 57.1 ml of glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA) and adding up to 1 liter of ddH₂O.

Gels for electrophoresis was prepared by dissolving 1.5 g of agarose (Sigma-Aldrich, St. Louis, MO, USA) in 100 ml of 1X TAE. Then, 5 µl of 10 mg/ml concentrated ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) was added and allowed to solidify.

3.3. General laboratory equipment

To check RNA quantity and quality, NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used before and after DNase treatment. Centrifugation steps were performed by using Centrifuge 5810R (Eppendorf, Hamburg, Germany). GeneAmp 9700 PCR system Thermo Scientific (Waltham, MA, USA) was used to qualify cDNA synthesis. qPCR experiments were performed by using LightCycler 480 II (Roche, Basel, Switzerland). Gel electrophoresis was performed in Mini-Sub Cell GT Cell (Bio-Rad Laboratories, Hercules, CA, USA). Gel imaging was performed by using ChemiDoc Imaging Systems (Bio-Rad Laboratories, Hercules, CA, USA). Synergy HTX multi-mode reader was used to monitor luminescence-based assay.

3.4. Cell culture equipment and reagents

Cell culture equipment and reagents were listed in Table 3.2 with the manufacturers.

Table 3. 2: Cell culture equipment and reagents.

Equipment/Reagents	Manufacturer
CO ₂ Incubator	Nuaire (Minnesota,USA)
Class II Biosafety Cabinet	Nuaire (Minnesota,USA)
Axiovert 25 Microscope	Zeiss (Oberkochen, Germany)
Dulbecco's Modified Eagle Medium (DMEM)	Biowest LLC (Nuaille, France)
Rosswell Park Memorial Institute Medium (RPMI-1640)	Biowest LLC (Nuaille, France)
Trypsin-EDTA	Sigma-Aldrich (St. Louis, MO, USA)
Fetal Bovine Serum (FBS)	Biowest LLC (Nuaille, France)
L-Glutamine-Penicillin-Streptomycin	PAN-Biotech (Aidenbach, Germany)
CellTiter-Glo® Luminescent Cell Viability Assay	Promega (Wisconsin, USA)

3.5. Cell culture solutions

10X phosphate- buffered saline (PBS) was prepared by dissolving 80g of NaCl (Sigma-Aldrich, St. Louis, MO, USA), 2g of KCl (Sigma-Aldrich, St. Louis, MO, USA), 14.4g of Na₂HPO₄(Sigma-Aldrich, St. Louis, MO, USA) and 2.4g of KH₂PO₄ (Sigma-Aldrich, St. Louis, MO, USA) in 800 ml of ddH₂O. Then, pH of the solution was adjusted to 7.4 by adding HCl (Sigma-Aldrich, St. Louis, MO, USA) and the final volume was completed to 1 liter by adding ddH₂O. 1X PBS was prepared by adding 900 ml of ddH₂O to 100 ml 10X PBS. It is autoclaved and filtered before use in cell culture.

Complete cell culture media was prepared by adding 5 ml of 100X L-Glutamine-Penicillin-Streptomycin solution and 50 ml FBS to 445 ml of DMEM or RPMI-1640 medium.

Freezing medium was prepared by adding 10 ml of DMSO to 90 ml of FBS.

3.6. Primers

Primers were designed by using NCBI's Primer-BLAST tool and purchased from Oligomer Biotechnology (Ankara, Turkey) [155]. 100 µM stock solution was prepared according to manufacturer's instructions and it is diluted to a 10 µM working stock. The sequences of primers were listed in Table 3.3.

Table 3. 3: List of primers and sequences.

CDH1_F	TCCAGGAACCTCTGTGATGGA
CDH1_R	CGTAGGGAAACTCTCTCGGTC
VIM_F	CGGGAGAAATTGCAGGAGGA
VIM_R	AAGGTCAAGACGTGCCAGAG
TMED7_F	CAGTGCTCTTACCCAGATGGA
TMED7_R	TTCGGCCTTGAGCTTCTCTT
XRCC5_F	GCAGTGTACCTCTGTTGGA
XRCC5_R	GCTGGTTACTCGCTTCCTCA
PLOD2_F	TCGAGCATCCCCACAGATAA
PLOD2_R	CCTTGACCAAGGACCTTCAC
NSUN5_F	GTGCGTGTGAACACTCTCAAG
NSUN5_R	TTGAGGGCTCGTAAGTCATCG
GAPDH_F	GCTCATTTCTGCTGATGACAACG
GAPDH_R	CTCTTGTGCTCTTGCTGGGG

3.7. Drugs and natural compounds

Drugs and natural compounds used in studies were listed in Table 3.4.

Table 3. 4: Drugs and natural compounds.

Drugs/Natural compounds	Manufacturer	Catalog Number
(-)-Epigallocatechin Gallate	Cayman Chemical (MI, USA)	70935
5-FU	Selleck Chemicals (TX, USA)	S1209
Apigenin	Selleck Chemicals (TX, USA)	S2262
Curcumin	Selleck Chemicals (TX, USA)	S1848
Fisetin	Selleck Chemicals (TX, USA)	S2298
Forskolin	Selleck Chemicals (TX, USA)	S2449
Fulvestrant	Selleck Chemicals (TX, USA)	S1191
Gefitinib	Selleck Chemicals (TX, USA)	S1025
Gemcitabine	Selleck Chemicals (TX, USA)	S1149
Irinotecan hydrochloride	Sigma-Aldrich	I1406
Lapatinib	Cayman Chemical (MI, USA)	11493
Metformin	Cayman Chemical (MI, USA)	13118
Oxaliplatin	Selleck Chemicals (TX, USA)	S1224
Procyanidin B2	Cayman Chemical (MI, USA)	19865
Resveratrol	Santa Cruz Biotechnology (TX, USA)	sc-200808
Selumetinib	Selleck Chemicals (TX, USA)	S1008
Sodium Valproate	Sanofi (Paris, France)	Depakin
Urolithin A	Cayman Chemical (MI, USA)	22607

Chapter 4

Methods

4.1. Cancer cell lines and maintenance of cells in culture

Breast cancer cell lines, CAL51, MDA-MB-157, MDA-MB-231, MDA-MB-436, MDA-MB-453 and MDA-MB-468 were cultured in DMEM medium. Colon cancer cell lines, Caco-2, HCT-8, KM-12, LS513, LoVo, SW837, and WiDr were cultured in RPMI-1640 medium. Pancreatic cancer cell lines, AsPC-1, BxPC-3, SU8686 were cultured in RPMI-1640 medium while MIA PaCa-2 cells were cultured in DMEM medium. M307, M133, M189, SHM20A2, M187, M144, M24, M34, M77, M7, M1, M433, M2025, M221, M193 melanoma cells were cultured in RPMI-1640 medium. Respective media was supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin- L-Glutamine. Cells were cultured in a humidified atmosphere with 5% CO₂ at 37 °C. The culture medium was replenished by passaging when cells reach to 70-80 % confluency.

4.2. Thawing, passaging and freezing cell lines

Each of cryovial of cell was thawed immediately at 37 °C in water bath. Half-melted cell content was dissolved in 1 ml of respective medium and transferred to a 15 ml falcon that have already 5 ml respective medium. The cells then were centrifuged at 900 rpm for 5 minutes. After removal of supernatant, the cell pellet was dissolved in 4-5 ml of DMEM/RPMI medium and transferred to 25 cm² cell culture flasks. Seeded cells were kept in a humidified atmosphere with 5% CO₂ at 37 °C. After an overnight incubation, cell culture medium was changed.

Confluency were followed to decide on passaging of cultured cells. If the confluency of cells reached to 80-90 %, cells were passaged to new flask. In passaging, stale media were aspirated and cells were washed with 1X PBS. After removal of PBS, 1 ml of Trypsin-EDTA

was added on cells to detach the cells from the flask. Then, the flask was incubated in cell culture incubator for 3-5 min. After detachment took place, 5 ml of fresh cell culture medium was added on cells to resuspend cells. Pipetting was performed to avoid clumping. 1-2 ml of the resuspension was transferred to new flask that contain 10 ml of fresh medium.

To cryopreserve cells in liquid nitrogen, cells were frozen in freezing medium. Firstly, cell media were aspirated and cells grown in the flask were washed with 1X PBS. After aspiration of PBS, cells were trypsinized by adding 1 ml of trypsin-EDTA. 5 ml medium was then added to trypsinized cells and it is transferred to 15 ml falcon. The mixture was centrifuged at 1000 rpm for 5 min. the supernatant was removed and cell pellet were resuspended in freezing media. The volume of freezing medium is determined according to the amount of cell content to be frozen. 1 ml of the resuspension was then transferred to cryovials. After labeling, the vials were stored at 20 °C for 2 hours then moved to liquid nitrogen.

4.3. Cell viability analysis

Cancer cell lines were seeded at 2000 cells per well in 96-well plates (Corning, NY, USA). After incubation for 24 hours, cells were treated with chemotherapeutics, targeted drugs and natural compounds using 6 different concentrations. Cell viability was quantified with CellTiter-Glo Luminescent Cell Viability Assay according to manufacturer's instructions after 72 hours of treatment. IC50 values were obtained by a method previously described. Different methods were used to assess combination treatments. First approach was the combination of single dose of a natural compound with six different doses of a chemotherapeutic or targeted agent. In the second approach, three different doses of natural compounds, two different doses of chemotherapeutics and their all combinations were administered to cancer cell lines. In the third approach, six different doses of natural compounds were combined with six different doses of chemotherapeutics. All combination treatments were administered to cancer cell lines in 96-well plates after the cell line were seeded and incubated for 24 hours. After 72 hours treatment, cell viability was quantified with CellTiter-Glo Luminescent Cell Viability Assay.

4.4. RNA isolation

Cell medium was aspirated and cells were washed with 5 ml of 1X PBS. After aspiration of PBS, 1 ml of PureZOL RNA isolation reagent was added immediately to flask. The lysate was pipetted up and down several times and incubated at room temperature for 5 minutes once cells have been disrupted in PureZOL. Then, lysate was transferred to RNase-free tube. 0.2 ml of chloroform was added to lysate and the sample was shaken vigorously for 15 sec. After 5 minutes incubation for 5 minutes at room temperature, the sample was centrifuges at 12000 g for 15 minutes at 4 °C. The aqueous phase containing RNA was immediately transferred to new RNase-free tube. Then, 0.5 ml of isopropanol was added to aqueous phase. After mixing thoroughly, the sample was incubated at room temperature for 5 minutes. The mixture was centrifuged at 12000 g for 10 minutes at 4 °C. The supernatant was discarded and the pellet (RNA) was washed with 1 ml of 75% ethanol. After vortexing, the sample was centrifuged at 7500 g for 5 minutes at 4 °C. The supernatant was discarded carefully and the RNA pellet was air-dried for about 5 minutes. Then, the pellet was resuspended in the certain amount of nuclease free water determined by the quantity of the pellet. In final, the resuspension was incubated at 55 °C for 10 minutes to completely dissolve the RNA pellet. RNA samples were stored at -80 °C for further experiments.

4.5. RNA quantification

Total RNA concentration and purity were measured in NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer. RNA samples with A260/A230 ratio between 2.0-2.2 and A260/A280 ratio between 1.8-2.1 were assigned as qualified.

4.6. DNase treatment

To remove residual DNA in RNA samples, DNA-free™ Kit were used. After RNA concentration was measured using NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer, RNA samples with concentration more than 200 ng/μl were diluted to 200 ng/μ or less. 44 μl of RNA sample was added to mixture of 1 μl DNase I and 5 μl DNase buffer. The mixture was then incubated at 37 °C for 30 minutes. Then, 5 μl DNase

inactivating reagent was added to mixture to terminate reaction. Mixing occasionally, reactions were incubated for 2 minutes in room temperatures. After centrifugation of the sample for 2 minutes at 1000 rpm, the supernatant containing DNA-free RNA was transferred to a new RNase-free tube.

4.7. cDNA synthesis

2000-4000 ng of total RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit. 1 µl of random hexamer primers were added to reaction tube containing 2000-4000 ng/11 µl of total RNA. This mixture was incubated at 65 °C for 5 minutes and placed on ice. Then, reaction master mix was prepared with 1 µl of 5X reaction buffer, 1 µl of Ribolock RNase inhibitor, 2 µl of 10 mM dNTP mix and 1 µl of M-MuLV reverse transcriptase for each sample. 8 µl of this reaction master mix was then added into the sample containing RNA and random hexamer primers. Each sample was mixed gently by pipetting and centrifuged briefly. The reaction tube was incubated at 25 °C for 5 minutes followed by 60 minutes at 42 °C. cDNA synthesis reaction was terminated by heating at 70 °C for 5 minutes. No template control reaction was performed using nuclease-free water instead of RNA samples to check whether reagents were contaminated or not. -RT control reaction was also performed using nuclease-free water instead of reverse transcriptase to check whether RNA samples were contaminated with genomic DNA. cDNAs were stored at -80 °C for further experiments.

4.8. End-point PCR analysis

All samples from cDNA synthesis, including control reactions, were amplified with primers of GAPDH supplied with RevertAid First Strand cDNA Synthesis Kit in order to check for cDNA synthesis and contamination. For PCR reaction, 12.5 µl of OneTaq Quick-Load 2X Master Mix with Standard Buffer, 0.5 µl of 10 µM forward and reverse primers, 9.5 µl of nuclease free water and 2 µl of samples were mixed. In no template control reaction, nuclease free water was used instead of samples in order to check any contamination risk in reaction setup. Initial denaturation with this PCR kit was at 94 °C for 30 seconds followed by 30

cycles of 94 °C for 20 seconds, 55 °C for 30 seconds, 68 °C for 30 seconds. Final extension was performed for 5 minutes at 68 °C.

4.9. Agarose gel electrophoresis

To check whether cDNA synthesis result in success and any reagents or samples are contaminated with genomic DNA, end-point PCR products were run on agarose gel and visualized in ChemiDoc Imaging Systems. 1.5 g of agarose was dissolved in 100 ml of 1X TAE buffer by using microwave. After solution was cooled down to 50-55 °C, 5 µl ethidium bromide was added and mixed well. The solution was then poured into gel tank and the comb was placed. After solidification, end-point PCR reactions with loading dye and 3 µl of Gene Ruler 50 bp DNA ladder (#SM0373 Thermo Scientific, MA, USA) were loaded into wells generated from removing the comb. The gel was run at 125 V for 25 minutes and visualized under UV via ChemiDoc Imaging Systems.

4.10. Quantitative real-time PCR analysis

Quantitative real-time PCR was performed Light Cycler 480 II instrument and iTaq Universal SYBR green supermix. Primers were designed using Primer-BLAST online tool and sequences were listed in Table 3.3. Each qRT-PCR reaction was performed in triplicates with no template control for each primer set in each plate. Each 10 µl reaction mix was prepared with 5 µl iTaq Universal SYBR green supermix, 0.5 µl of 10 µM forward and reverse primers, 2 µl of sample cDNA and 2 µl nuclease-free water. Thermal cycling protocol includes initial DNA denaturation and polymerase activation at 95 °C for 30 seconds and amplifications step with denaturation at 95 °C for 15 seconds, 60 °C for 60 seconds in 35-40 cycles. Melt-curve analysis were performed with 0.5 °C increment between 65 °C-95 °C in every 2-5 seconds/step. Cq values were then obtained by instrument, indicating quantification cycle. Based on Cq values, expression values of genes were then normalized to housekeeping gene, GAPDH. All relative expression values was calculated using the 2^{-DDCT} method [156].

4.11. Bioinformatic methods

4.11.1. Identification of sensitive and resistant cell lines

Drug-tissue response curves were retrieved from PharmacDB database [157]. Calculated Area Above Curve (AAC%) and IC50 values were downloaded from the database. Cell lines were ranked by AAC (%) or IC50 values from lowest to highest. Top-15 cell lines ranked by IC50 values and bottom-15 cell lines ranked by AAC% were assigned as sensitive for each drug response data sets (CCLE, CTRPv2, GDSC1000, gCSI). Bottom-15 cell lines ranked by IC50 values and top-15 cell lines ranked by AAC% were assigned as resistant for each drug response data sets. Then, common cell lines in at least two of drug response data sets were chosen as sensitive and resistant cell lines.

For the melanoma cells, sensitivity associated-46 genes were used to cluster cell lines and to generate a heat map by heatmapper online tool [158]. Based on hierarchical clustering and chemosensitivity profiles, two subgroups of melanoma cells were identified as relatively sensitive and resistant to 10a.

4.11.2. Functional enrichment analysis by GSEA, NetworkAnalyst and miRNet

GSEA was performed using default settings on GSEA 4.0.2 software, and gene sets were extracted from MysigDB v7.0 [9]. Phenotypes defined as sensitive and resistant, were further analyzed to explore the potential resistance mechanisms against 5-FU, irinotecan, gemcitabine, doxorubicin and c-Src inhibitor 10a.

For colon, pancreatic and breast cancer cell lines, sensitivity or resistance associated top-50 genes were selected from the differentially expressed gene list that was ranked by p-value obtained with t test. These gene lists then were used to build protein-protein interaction networks by using NetworkAnalyst 3.0 [12]. Functional enrichment analysis was then performed with built-in tools in NetworkAnalyst. To build miRNA-target interaction networks, significantly differentially expressed miRNA lists (p-value <0.05) were uploaded to miRNet 2.0. Functional enrichments analysis then performed by integrating protein-protein interaction networks to miRNA-target interactions. For melanoma cells, sensitivity and resistance associated gene list from 46-genes was used to build and protein-chemical

interaction networks by using NetworkAnalyst 3.0. Protein-chemical network data in NetworkAnalyst is based on the data from Comparative Toxicogenomics Database (CTD) [160].

4.11.3. Multi-omics data integration with mixOmics

Omics data sets including transcriptomics, mirnomics, proteomics and methylomics were downloaded from CCLE database (<https://sites.broadinstitute.org/ccle/>). Based on sensitivity subgrouping, data sets were pre-filtered according to differential expression determined with t test ($p\text{-value} < 0.05$). `perf.diablo` function was used to select the component number of data sets. In all analyses, first component resulted in the lowest error rate. Feature selection was then performed with `tune.block.splsda` function by restricting number of eligible variables to 5-12 for decreasing computational time. `plot.diablo` function was used to show correlations among first component of data sets. `plotLoadings` function was used to show loading weights of variables in the first components of data sets. Loading weights indicated as a contribution score below the figures suggesting the importance of each selected variable.

4.11.4. Two-fold cross-validation with 1000 iteration

Gene expression data of primary melanoma cell lines used were obtained previously by using landmark transcripts whose expression levels infer the remainder of the transcriptome computationally [159]. To identify individual genes whose expression profile shows significant correlation with 10a chemosensitivity, two-fold cross-validation with 1000 iteration method was used. In this method, cell lines were divided into two groups randomly with the same sample sizes. In each group, Pearson r and corresponding p -values were calculated between cytotoxicity values (IC_{50}) and expression of each gene. Significantly correlated genes in both groups in the same direction with either a positive or negative correlation in both groups were kept. Aforementioned analysis was performed 1000 times to see how many times expression of the genes was found correlated with cytotoxicity information in randomly generated groups.

4.12. Statistical Analysis

Different groups were compared with each other by using t test. GraphPad Prism v8.0.1 (Graphpad Software INC.) was used to generate graphs and perform statistical tests. P-value less than 0.05 were considered as statistically significant.



Chapter 5

Results

5.1. Characterization of resistance mechanisms for 5-FU in colon cancer

To characterize drug resistance mechanisms for 5-FU in colon cancer cells, analyses were performed with GSEA, NetworkAnalyst, miRNet and MixOmics. In order to identify 5-FU sensitive and –resistant cell lines, drug response profiles were analyzed by using PharmocoDB web-application that integrates multiple high-throughput cancer pharmacogenomics datasets (See Methods section). Gene expression data of defined subgroups were used to identify altered molecular mechanisms by using GSEA tool. In 5-FU sensitive colon cancer cell lines, gene sets related to “MYC Targets” were enriched, indicating the cell proliferation and cell cycle (Figure 5.1).

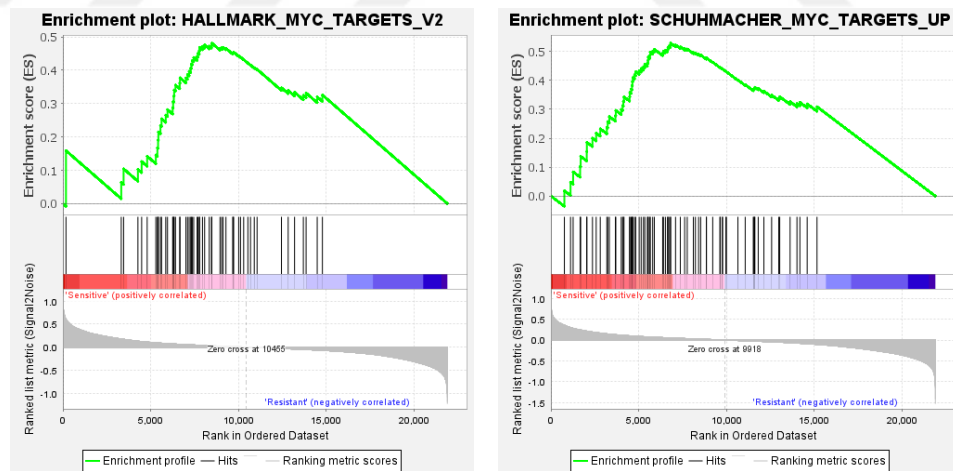


Figure 5. 1: Enriched gene sets in colon cancer cell lines sensitive to 5-FU. Enrichment plots indicates the enrichments of gene sets related to increased cell proliferation and cell cycle.

In 5-FU resistant phenotype, enriched gene sets included those related to epithelial to mesenchymal (EMT) phenotype, invasiveness, LEF1 and TGF β that are known to induce EMT (Figure 5.2). These findings showed that colon cancer cell lines sensitive to 5-FU is

more epithelial and proliferative in contrast to resistant cell lines which show mainly EMT phenotype.

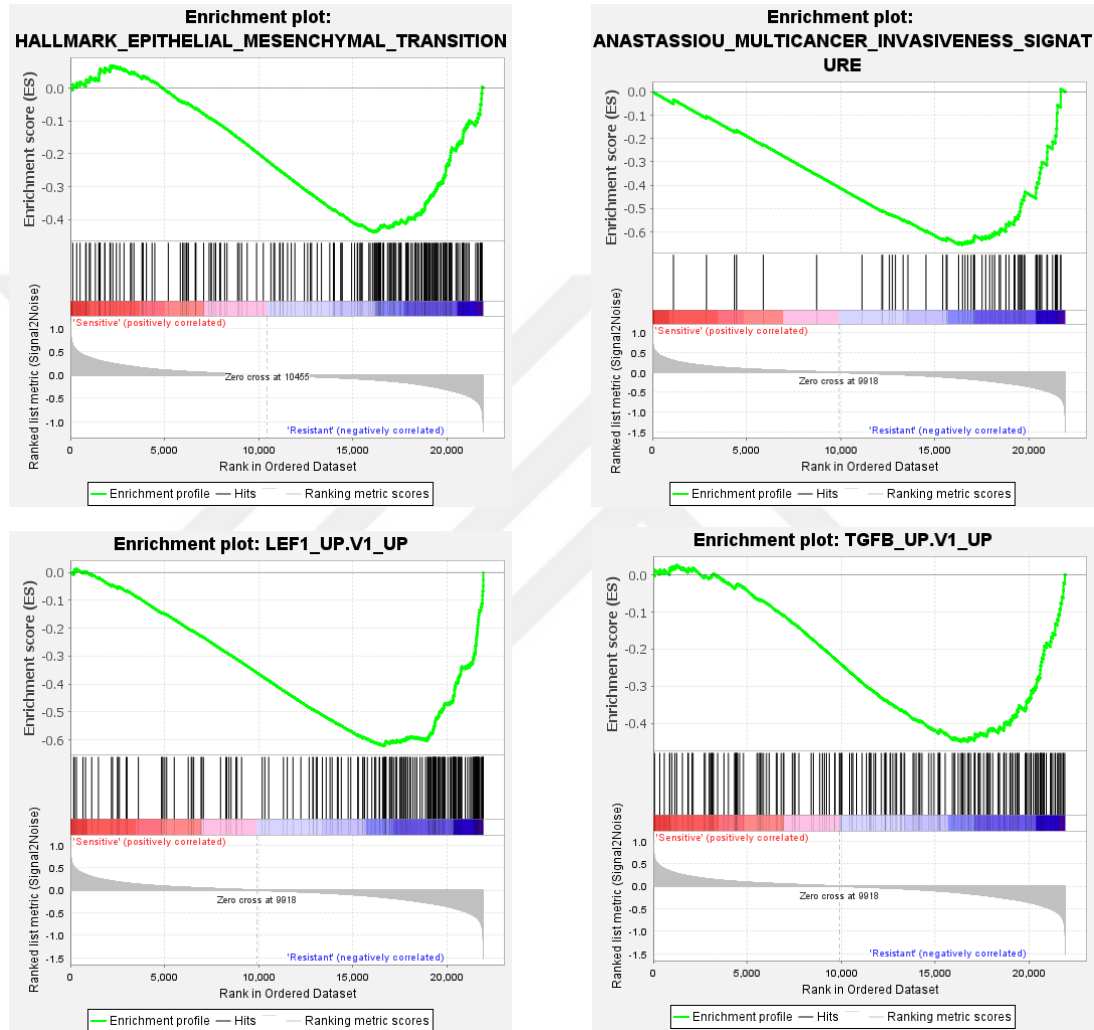


Figure 5. 2: Enriched gene sets in colon cancer cell lines resistant to 5-FU. Genes associated with epithelial to mesenchymal transition and invasiveness signature were enriched in colon cancer cell lines resistant to 5-FU.

To further analyze 5-FU resistance mechanisms, we performed a network analysis by using sensitivity associated gene list (See Methods). Protein-protein interaction (PPI) network mapping were performed via STRING interactome database with the gene list by using NetworkAnalyst 3.0 tool. Functional enrichment analysis on PPI networks were then

performed via integrated KEGG and Reactome databases. Cell cycle, cell proliferation, transcriptional regulation and chromatin organization related genes were found to be highly enriched in PPI network created by 5-FU sensitivity associated gene list (Figure 5.3).

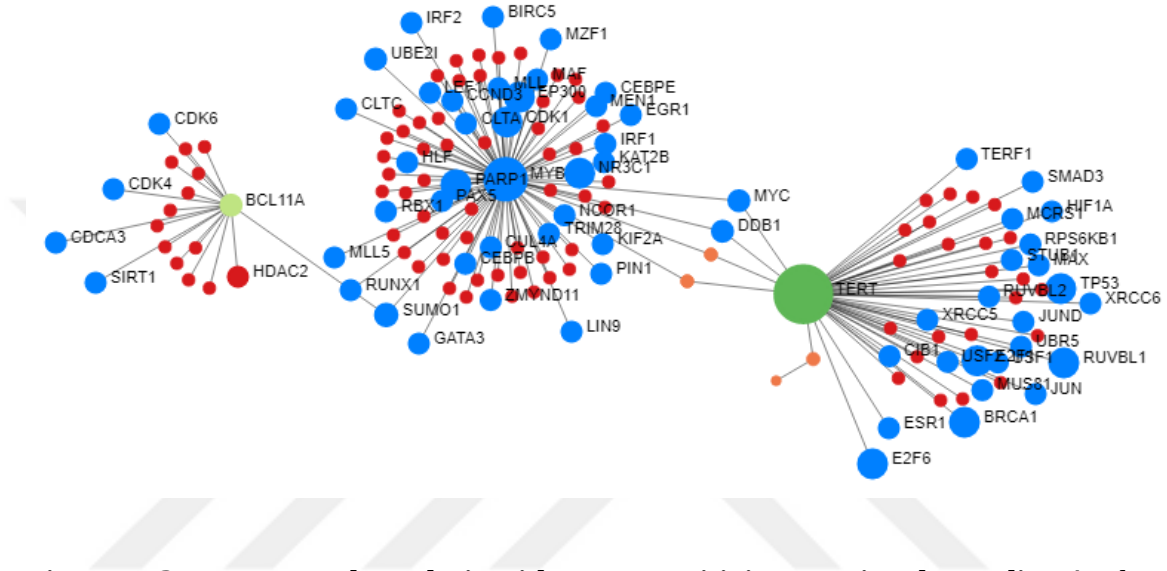


Figure 5. 3: PPI network analysis with 5-FU sensitivity associated gene list. Analysis resulted in enrichments of genes related to transcription, cell cycle, chromatin organization and cell proliferation. Blue nodes indicates genes associated with mentioned enrichments.

To investigate enrichments in resistance phenotype, protein-protein interaction (PPI) network mapping were performed via NetworkAnalyst by using resistance associated gene list. Genes related to PI3K/AKT signaling pathway, the mediator of EMT, were highly enriched in PPI network constructed by resistance associated gene list (Figure 5.4). These results confirms the phenotypes of 5-FU sensitive and –resistant subgroups of colon cancer cell lines, suggested by GSEA, as proliferative and mesenchymal, respectively.

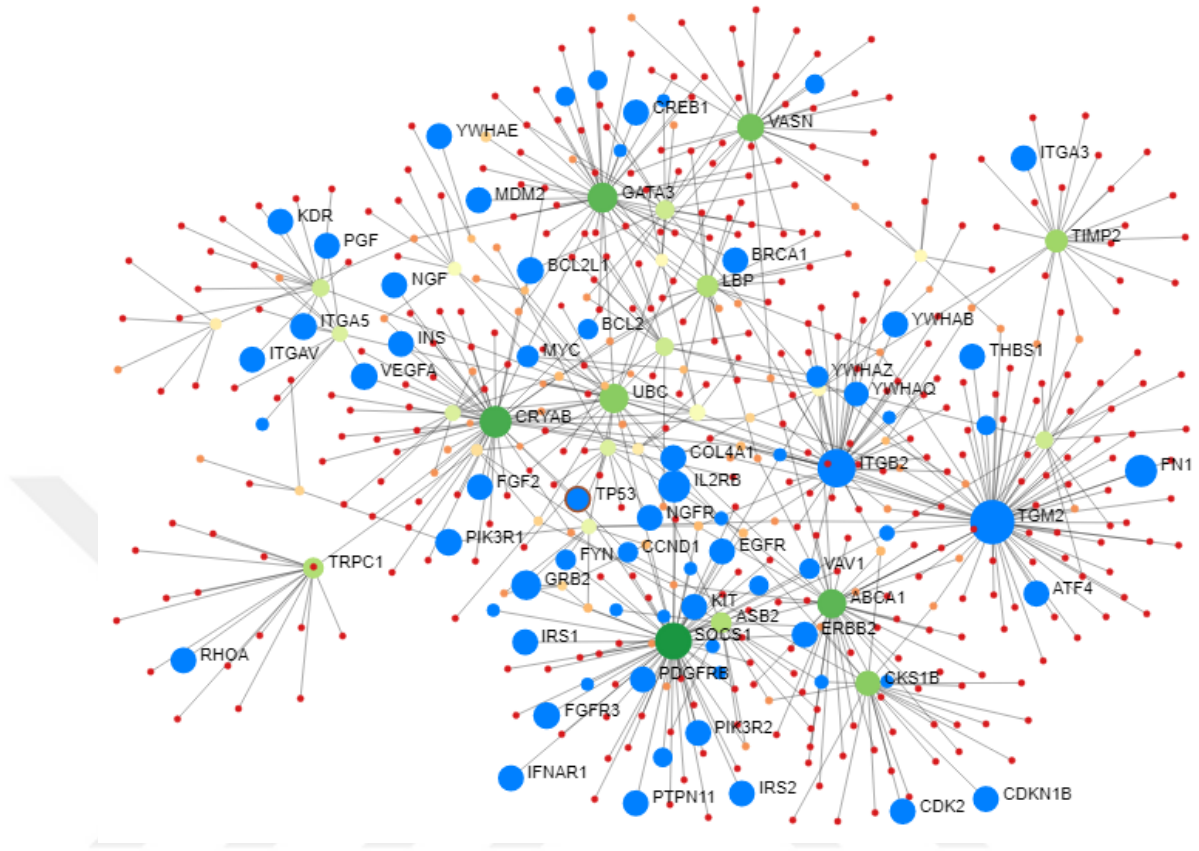


Figure 5. 4: PPI network analysis with 5-FU resistance associated gene list. Analysis resulted in enrichments of genes related to PI3K/AKT signaling pathway. Blue nodes indicates genes associated with PI3K/AKT pathway genes in the enrichment analysis.

To further identify the resistance mechanisms, miRNA functional analysis with miRNA-target interactions in a network context were performed by using a web-based platform, miRNet 2.0. We performed a network analysis in miRNet by mapping miRNAs and their targets. Additionally, PPIs were included to target networks. Functional enrichment analysis on PPI networks were then performed via integrated KEGG and Reactome databases. In a target network analysis performed with resistance associated miRNAs, genes associated with cell cycle were found to be highly enriched suggesting decreased proliferation in 5-FU resistant colon cancer cell lines (Figure 5.5).

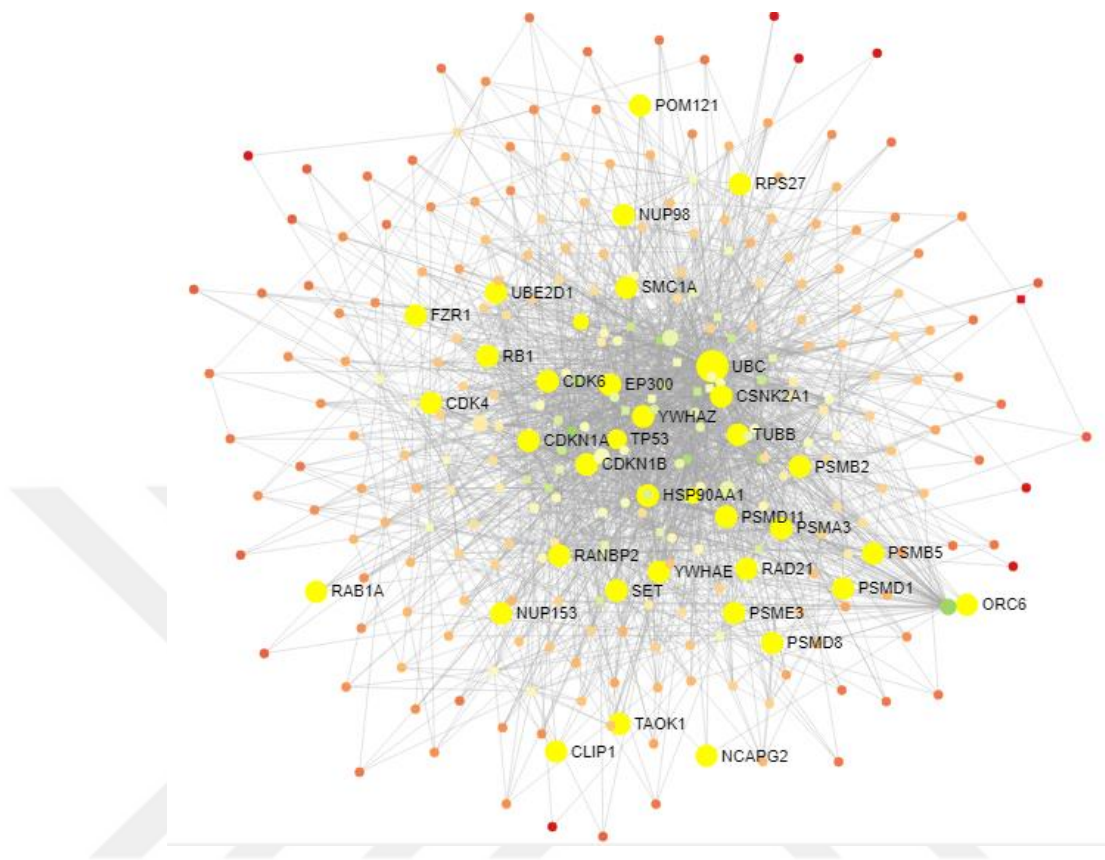


Figure 5. 5: 5-FU resistance associated miRNAs-target network analysis. Analysis resulted in enrichment in cell cycle related genes. Yellow nodes indicate cell cycle associated genes in the enrichment analysis.

To gain further insight into differential drug response profiles, we identified most discriminant subset of biological features by using mixOmics. In the context of supervised analysis, transcriptomic, mirnomic, proteomic and methylomic data were subjected to multi-omics integration, in turn, a highly correlated multi-omics signature that discriminates the sensitive and resistant cell lines was identified. This signature then further assessed to understand mechanisms underlying drug resistance. To do this, -omics data for same sensitive and resistant subgroups of cell lines from GSEA were subjected to integration. Feature selection process identified a multi-omics signature of 10 mRNA features, 11 miRNA features, 5 proteomic features and 6 methylation features. Figure 5.6 showed that selected features of each omics were highly correlated between each other's. Sample scatterplot (upper diagonal plot) shows that the selected features were able to discriminate sensitive and resistant cell lines. Lower diagonal showed the pearson

correlation coefficients of selected features of each omics, suggesting a good correlation between each other (Figure 5.6).

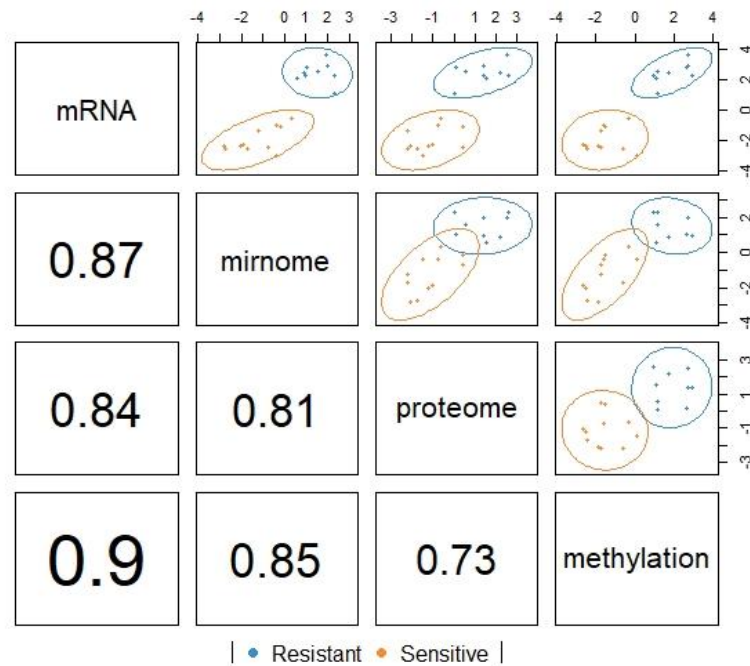


Figure 5. 6: Sample scatter plot and pearson correlation coefficients of mixOmics analysis based on 5-FU sensitivity profiles of colon cancer cell lines. Components of different omics data sets shows the discriminative power of selected features (upper triangular panel) and the high correlation between components (lower triangular panel). Blue color indicates resistant cell lines, orange color indicates sensitive cell lines.

Figure 5.7 shows the loading weights of each selected features on the component in transcriptomic data. TGM2 was most important feature for the component and it is highly expressed in 5-FU resistant cell lines. DNAH6 is the only feature associated with sensitivity, that shows upregulation in 5-FU sensitive cell lines (Figure 5.7).

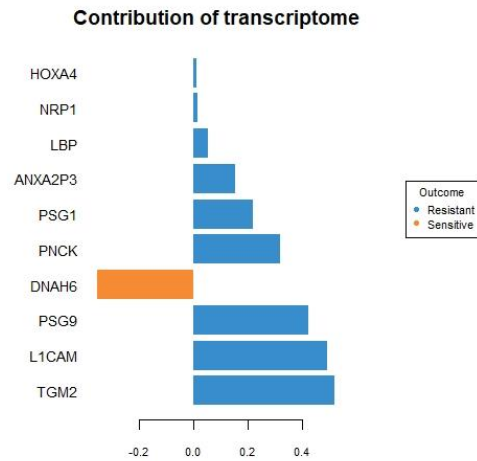


Figure 5. 7: List of selected mRNA variables and their loading weights associated with 5-FU sensitivity. Blue bars correspond to the 5-FU resistant subgroup in which the variable is most abundant. Orange bars correspond to the 5-FU sensitive subgroup in which the variable is most abundant.

Figure 5.8 shows the loading weights of selected miRNAs on the component from mirnomic data. Members of miR-200 family, suppressors of EMT, miR-200a, Mir-200b and Mir-429 were among the selected features associated with 5-FU sensitivity (Figure 5.8).

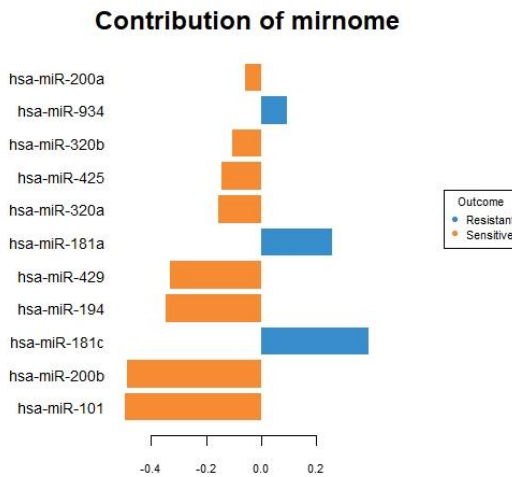


Figure 5. 8: List of selected miRNA variables and their loading weights associated with 5-FU sensitivity. Blue bars correspond to the 5-FU resistant subgroup in which the variable is most abundant. Orange bars correspond to the 5-FU sensitive subgroup in which the variable is most abundant.

Figure 5.9 shows the selected proteins on the component from proteomic data. Interestingly, protein expression of transglutaminase also showed abundance in 5-FU resistant cell lines, confirming mRNA levels. MAPK signaling pathway proteins were also associated with increased sensitivity against 5-FU (Figure 5.9).

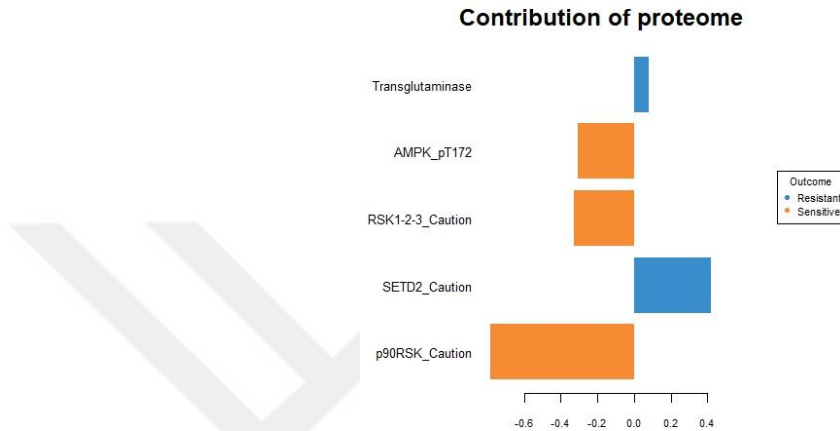


Figure 5. 9: List of selected protein variables and their loading weights associated with 5-FU sensitivity. Blue bars correspond to the 5-FU resistant subgroup in which the variable is most abundant. Orange bars correspond to the 5-FU sensitive subgroup in which the variable is most abundant.

Figure 5.10 shows the selected methylation events on the component from methylation data. Increased methylation levels in TSS region of PRICKLE2 was most important feature for the component in 5-FU resistant cell lines.

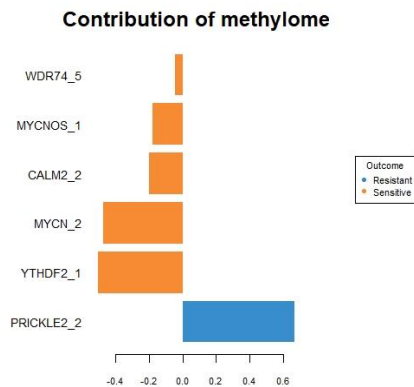


Figure 5. 10: List of selected methylation events and their loading weights associated with 5-FU sensitivity. Blue bars correspond to the 5-FU resistant subgroup in which the variable is most abundant. Orange bars correspond to the 5-FU sensitive subgroup in which the variable is most abundant.

Collectively, mixOmics analysis revealed the highly important variables that may explain the resistance mechanisms for 5-FU. Apart from the increased expressions of miR-200 family miRNAs in sensitive cell lines, the correlation between mRNA and protein levels of transglutaminase (Figure 5.11), as a regulator of EMT, can be potential target to overcome drug resistance against 5-FU [160].

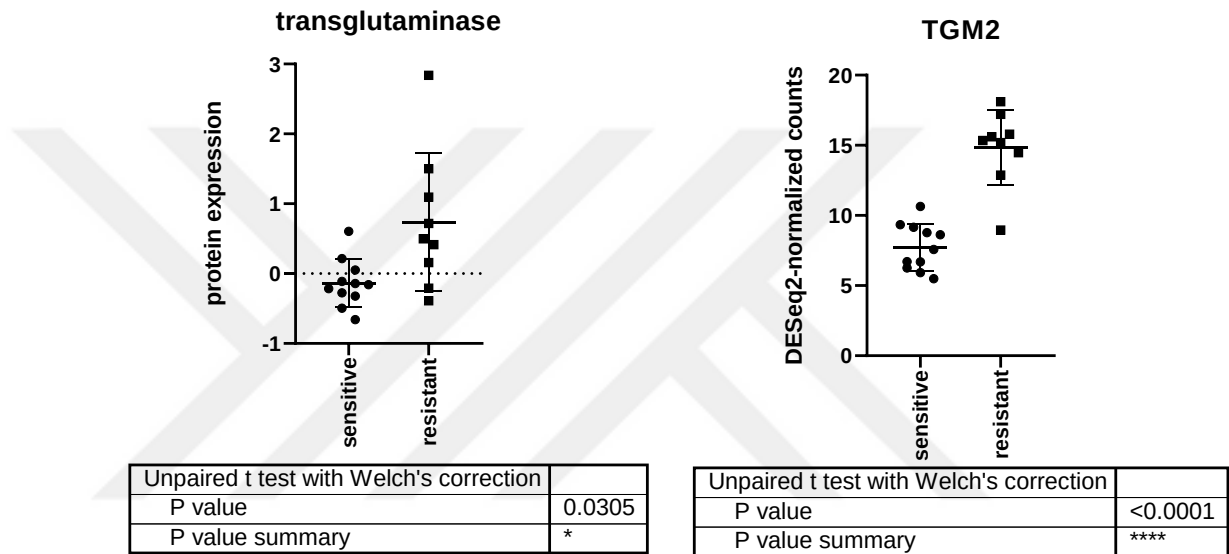


Figure 5. 11: Differential transglutaminase mRNA and protein levels in colon cancer cells. Protein (left) and mRNA (right) expression levels of transglutaminase were found to be upregulated in 5-FU resistant cell lines. t-test with Welch's correction were performed in GraphPad Prism software.

5.2. Characterization of resistance mechanisms for irinotecan in colon cancer

To characterize irinotecan drug resistance mechanisms in colon cancer cells, analyses were performed with GSEA, NetworkAnalyst, miRNet and MixOmics. Gene expression data were used to identify altered molecular mechanisms by using GSEA tool. In irinotecan sensitive colon cancer cell lines, gene sets related to “MYC Targets” were enriched, indicating the increased cell growth and cell proliferation (Figure 5.12).

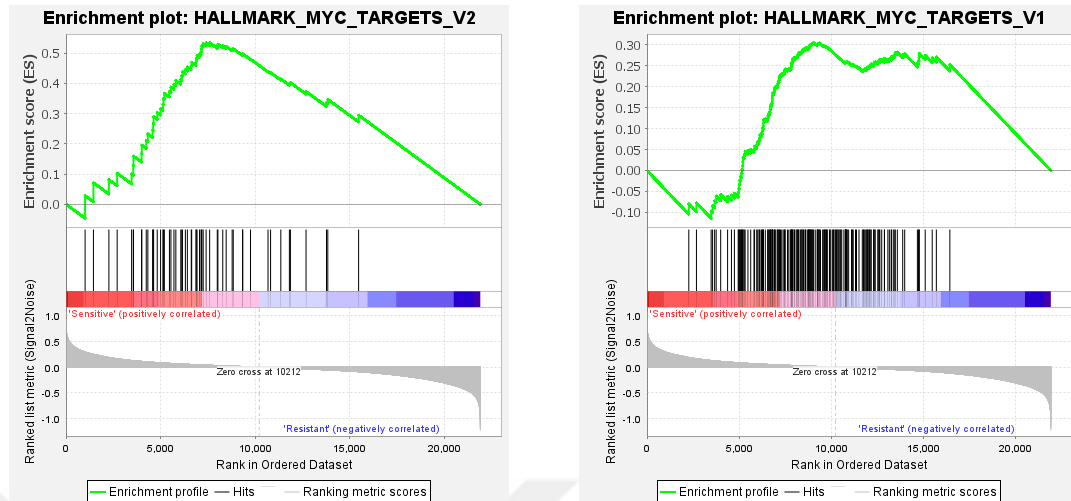


Figure 5. 12: Enriched gene sets in colon cancer cell lines sensitive to irinotecan. Enrichment plots of gene sets in irinotecan-sensitive colon cancer cell lines indicates significant enrichments of gene sets related to increased cell proliferation and cellular growth.

In irinotecan resistant subtype, enriched gene sets included those related to epithelial to mesenchymal (EMT) phenotype and stem cell phenotype indicating highly undifferentiated state of resistant cells (Figure 5.13). These findings showed that colon cancer cell lines sensitive to irinotecan is proliferative in contrast to resistant cell lines that show mainly EMT/stem cell phenotype.

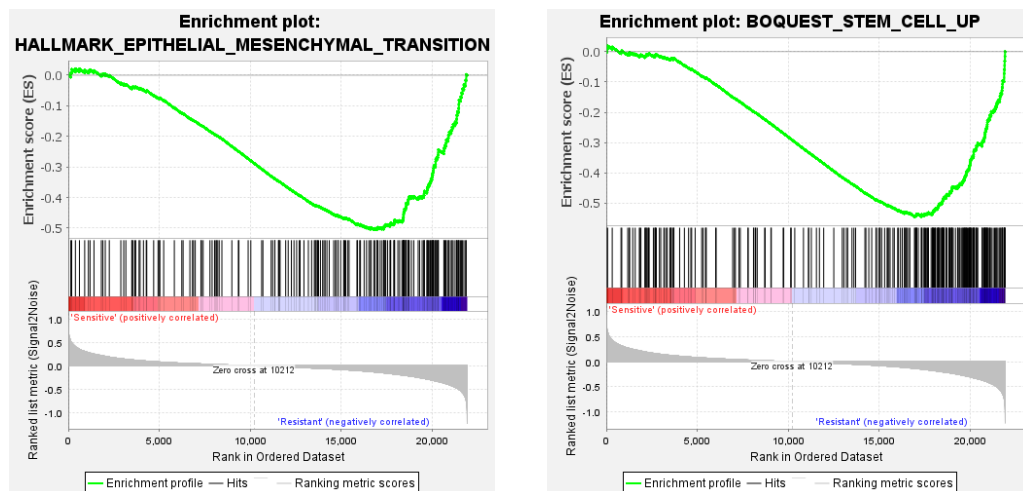


Figure 5. 13: Enriched gene sets in colon cancer cell lines resistant to irinotecan. Genes associated with epithelial to mesenchymal transition and stem cell signature were enriched in colon cancer cell lines resistant to irinotecan.

To further investigate the mechanisms of resistance to irinotecan in colon cancer cells, I performed PPI network analysis on Network Analyst tool by using resistance associated gene list. Functional enrichment analysis showed that genes related to EGFR tyrosine kinase inhibitor resistance and PI3K-AKT signaling pathway (Figure 5.14). These results suggest the role of EMT in drug resistance since both PI3K-AKT signaling pathway and EGFR tyrosine kinase inhibitor resistance are associated with epithelial to mesenchymal transition.

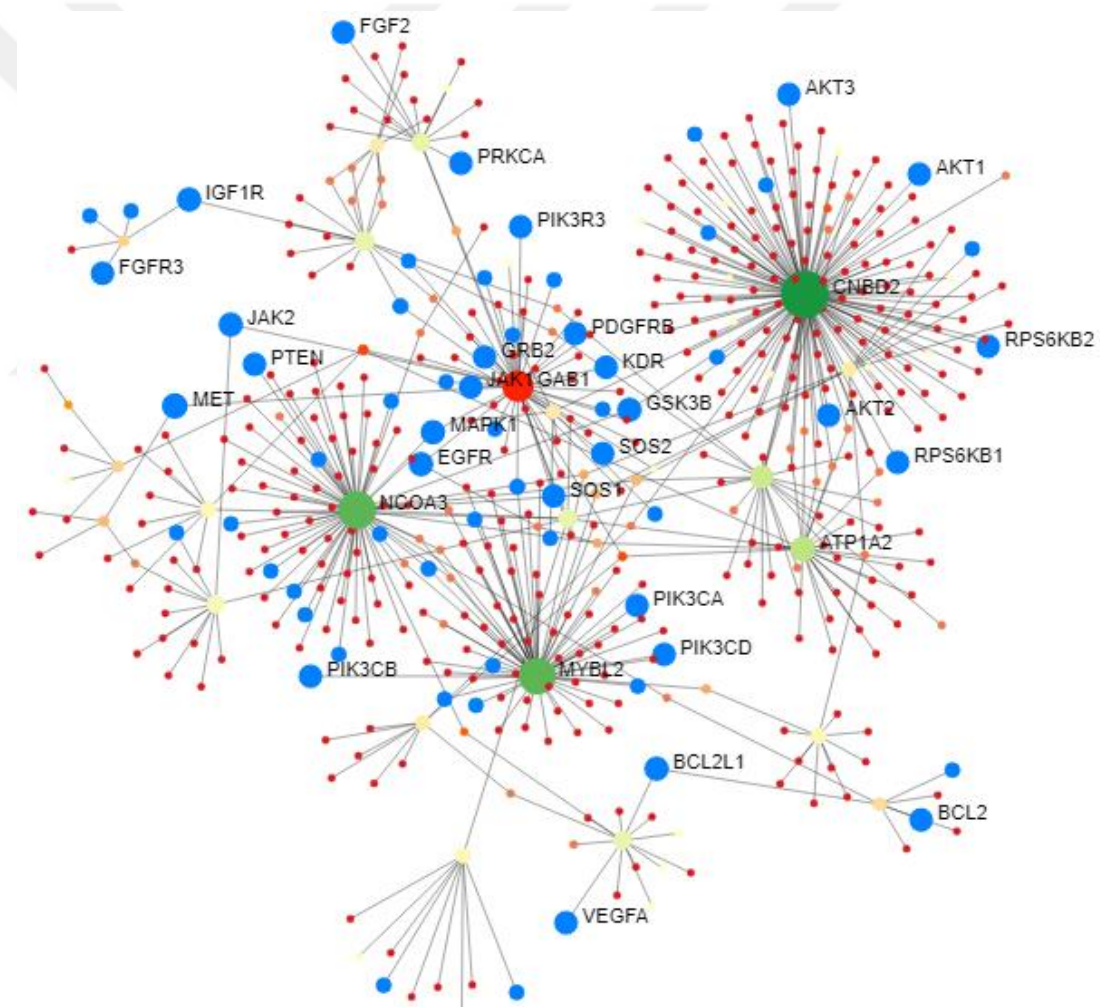


Figure 5. 14: PPI network analysis with irinotecan resistance associated gene list. Functional enrichment analysis of PPI network mapping resulted in enrichments of genes related to EGFR tyrosine kinase inhibitor resistance and PI3K-Akt signaling pathway. Blue nodes indicate genes associated with the aforementioned enrichments.

Analysis of resistance associated miRNAs-target networks including PPIs with miRNet showed the functional enrichments of cell cycle related proteins (Figure 5.15). This suggests that decreased cell proliferation may be regulated with resistance associated miRNAs in irinotecan resistant colon cancer cells.

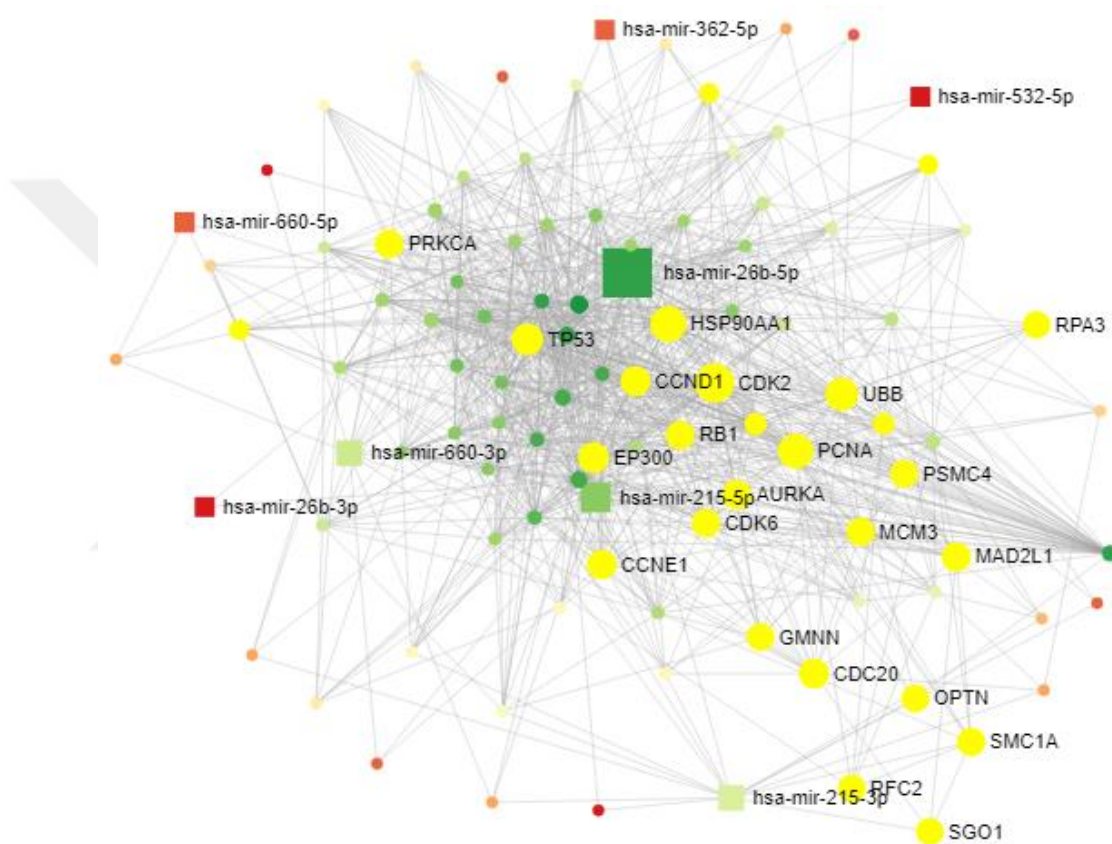


Figure 5. 15: miRNAs-target network analysis with irinotecan resistance associated miRNAs. Functional enrichment analysis of irinotecan-resistance associated miRNAs-target network mapping with PPIs resulted the enrichments of cell cycle related proteins. Yellow nodes indicate cell cycle related genes, square nodes show irinotecan-resistance associated miRNAs.

To gain further insight into differential drug response profiles, we identified most discriminant subset of biological features by using mixOmics. In the context of supervised analysis, transcriptomic, mirnomic, proteomic and methylomic data were subjected to multi-omics integration, in turn, a highly correlated multi-omics signature that discriminates the irinotecan-sensitive and -resistant cell lines was identified. Feature selection process identified a multi-omics signature of 7 mRNA features, 7 miRNA

features, 7 proteomic features and 6 methylation features. Figure 5.16 showed that selected features of each omics were highly correlated between each other's. Sample scatterplot (upper diagonal plot) shows that the selected features were able to discriminate irinotecan-sensitive and -resistant colon cancer cell lines. Lower diagonal showed the Pearson correlation coefficients of selected features of each omics, suggesting a good correlation between each other. For the selected features, highest correlation were observed between the components of mRNA and methylome (Figure 5.16).

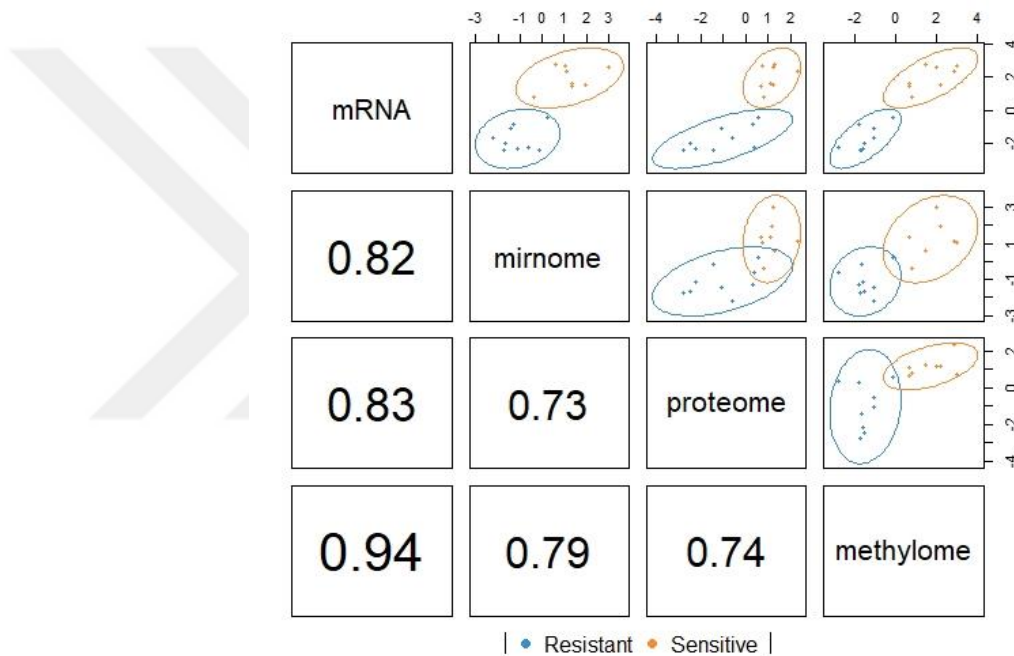


Figure 5. 16: Sample scatter plot and pearson correlation coefficients of mixOmics analysis based on irinotecan sensitivity profiles of colon cancer cell lines. Components of different omics data sets shows the discriminative power of selected features (upper triangular panel) and the high correlation between components (lower triangular panel). Blue color indicates irinotecan-resistant cell lines, orange color indicates irinotecan-sensitive cell lines.

Figure 5.17 shows the loading weights of each selected features on the component in transcriptomic data. Higher expressions of C8orf4, EGFL6, SLC6A4 and FOSL1 genes were associated with irinotecan resistance while SLC17A4, TMEM158 and AKAP12 expressions were abundant in irinotecan sensitive colon cancer cell lines (Figure 5.17).

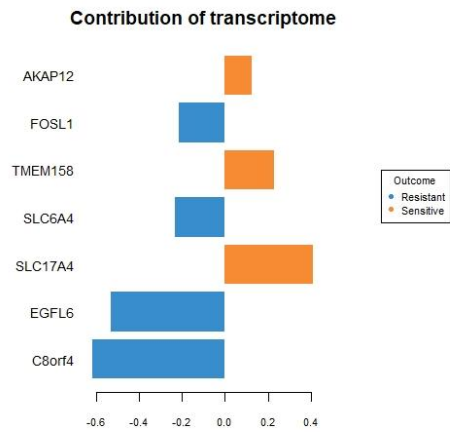


Figure 5. 17: List of selected mRNA variables and their loading weights associated with irinotecan sensitivity. Blue bars correspond to the irinotecan resistant subgroup in which the variable is most abundant. Orange bars correspond to the irinotecan sensitive subgroup in which the variable is most abundant.

Figure 5.18 shows the loading weights of selected miRNAs on the component from mirnomic data. Similar to 5-FU sensitivity profile, one of the miR-200 family members, miR-200a, was found to be associated with increased sensitivity to irinotecan (Figure 5.18).

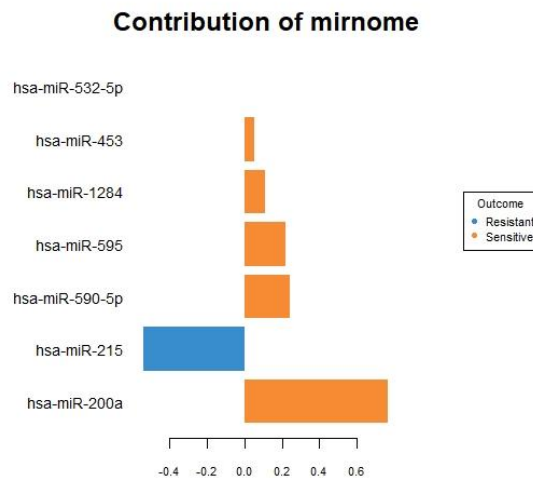


Figure 5. 18: List of selected miRNA variables and their loading weights associated with irinotecan sensitivity. Blue bars correspond to the irinotecan resistant subgroup in which the variable is most abundant. Orange bars correspond to the irinotecan sensitive subgroup in which the variable is most abundant.

Figure 5.19 shows the selected proteins on the component from proteomic data. Interestingly, protein expression of FRA1 (FOSL1) also showed abundance in irinotecan resistant cell lines, confirming mRNA levels. RB1 pS807_S811 and Caveolin-1 protein expression levels showed correlation with sensitivity to irinotecan (Figure 5.19). Additionally, five methylation events were selected by the mixOmics providing discrimination of cell lines based on sensitivity to irinotecan (Figure 5.20).

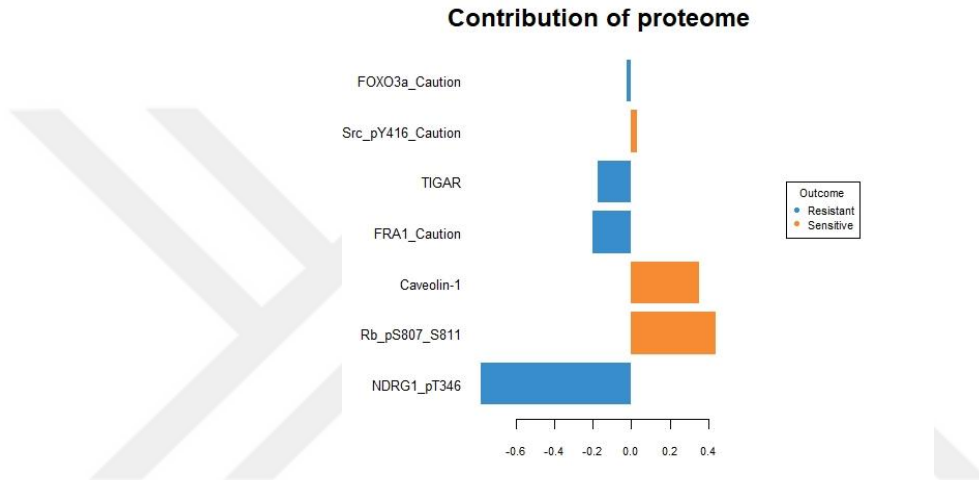


Figure 5. 19: List of selected protein variables and their loading weights associated with irinotecan sensitivity. Blue bars correspond to the irinotecan resistant subgroup in which the variable is most abundant. Orange bars correspond to the irinotecan sensitive subgroup in which the variable is most abundant.

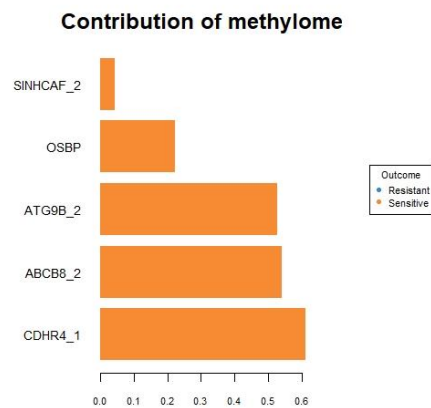


Figure 5. 20: List of selected methylation events and their loading weights associated with irinotecan sensitivity. Blue bars correspond to the irinotecan resistant subgroup in which the variable is most abundant. Orange bars correspond to the irinotecan sensitive subgroup in which the variable is most abundant.

Collectively, mixOmics analysis revealed the highly important variables that may explain the resistance mechanisms for irinotecan. FOSL1 (Fra1 protein) is one of the key variables in the selected features, having a role in cancer aggressiveness and EMT activated by Wnt/ β -catenin signaling pathway [161]. Figure 5.21 shows increased expression of FOSL1 in both mRNA and protein level.

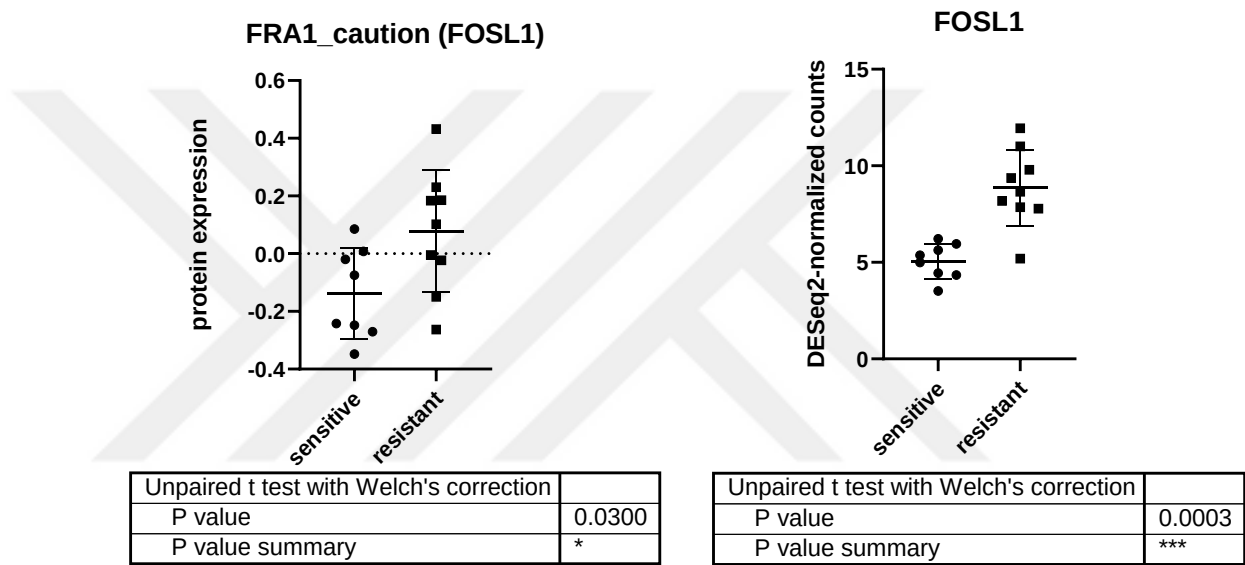


Figure 5. 21: Protein expression levels and mRNA expression levels of FOSL1 in irinotecan-sensitive and –resistant cell lines. Expression levels are significantly higher in resistant cell lines ($p < 0.05$). t-test with Welch's correction were performed in GraphPad Prism software.

5.3. Characterization of resistance mechanisms for irinotecan in pancreatic cancer

To characterize irinotecan drug resistance mechanisms in pancreatic cancer cell lines, analyses were performed with GSEA, NetworkAnalyst, miRNet and MixOmics. Gene expression data of defined subgroups were used to identify altered molecular mechanisms by using GSEA tool. In irinotecan sensitive pancreatic cancer cell lines, gene sets related to “epithelial differentiation” and genes upregulated after knockdown of EMT-activator ZEB1 were enriched, indicating the increased epithelial phenotype of sensitive cells (Figure 5.22).

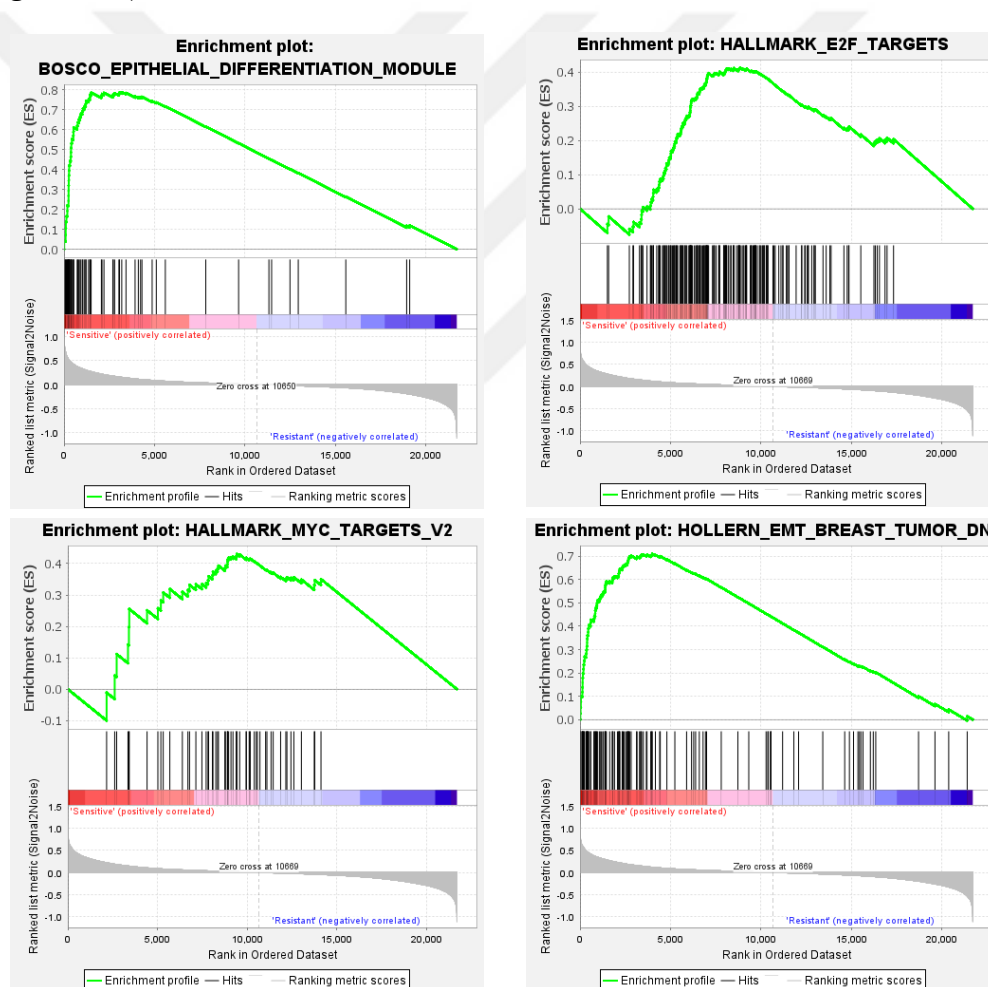


Figure 5. 22: Enriched gene sets in pancreatic cancer cell lines sensitive to irinotecan. Enrichment plots of highly enriched gene sets in irinotecan sensitive pancreatic cancer cell lines, indicating enrichments of gene sets related to epithelial phenotype.

In irinotecan resistant cell lines, enriched gene sets included those related to epithelial to mesenchymal (EMT) phenotype and multicancer invasiveness signature (Figure 5.23). These findings showed that pancreatic cancer cell lines sensitive to irinotecan more epithelial in contrast to resistant cell lines that show mainly EMT/invasive phenotype.

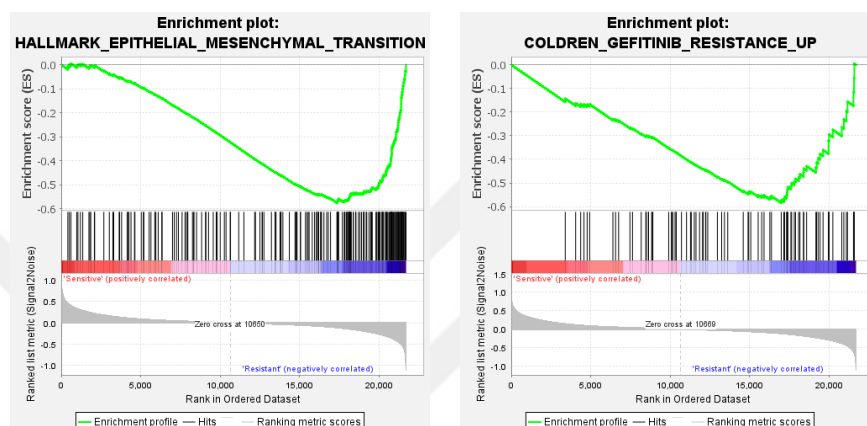


Figure 5. 23: Enriched gene sets in pancreatic cancer cell lines resistant to irinotecan. Enrichment plots of highly enriched gene sets in irinotecan resistant pancreatic cancer cell lines, indicating enrichments of gene sets related to EMT and invasive phenotype.

To further investigate the mechanisms of resistance to irinotecan in pancreatic cancer cells, I performed PPI network analysis on Network Analyst tool by using resistance associated gene list. Functional enrichment analysis showed that genes related to FGFR tyrosine kinase signaling were highly enriched in the network (Figure 5.24).

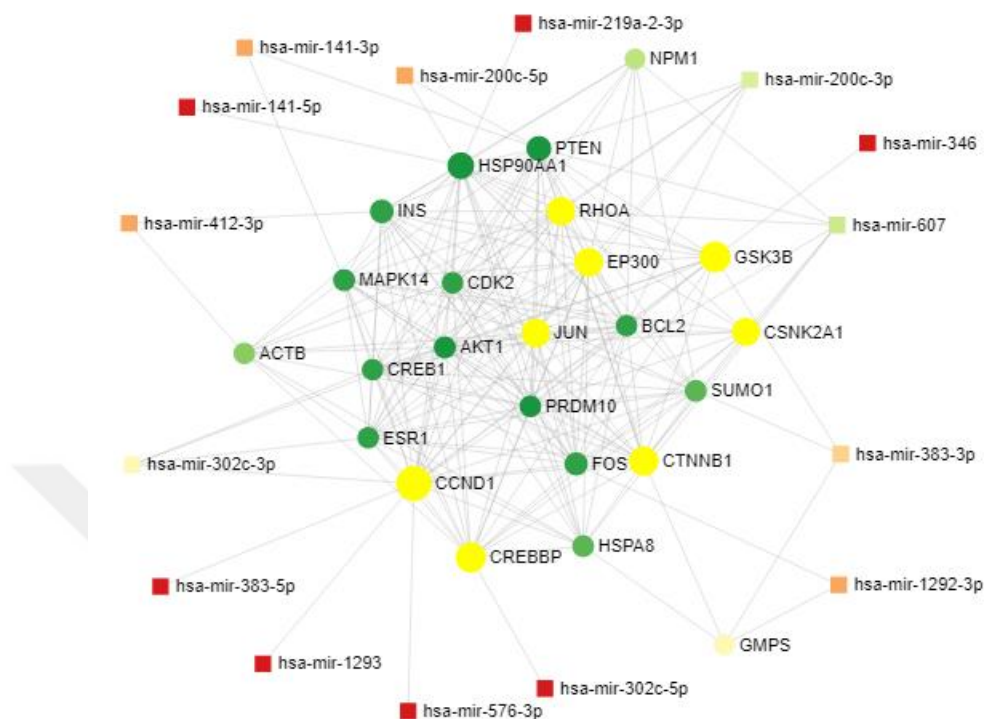


Figure 5. 25: PPI network analysis with irinotecan sensitivity associated miRNA list. Functional enrichment analysis of irinotecan-sensitivity associated miRNAs-target network mapping with PPIs resulted the enrichments of Wnt signaling proteins. Yellow nodes indicate Wnt signaling pathway related genes, square nodes show irinotecan-sensitivity associated miRNAs.

To gain further insight into differential drug response profiles, we identified most discriminant subset of biological features by using mixOmics. In the context of supervised analysis, transcriptomic, mirnomic, proteomic and methylomic data were subjected to multi-omics integration, in turn, a highly correlated multi-omics signature that discriminates the irinotecan-sensitive and -resistant cell lines was identified. Feature selection process identified a multi-omics signature of 11 mRNA variables, 12 miRNA features, 8 proteomic features and 7 methylation features. Figure 5.26 showed that selected features of each omics were highly correlated between each other's. Sample scatterplot (upper diagonal plot) shows that the selected features were able to discriminate irinotecan-sensitive and -resistant pancreatic cancer cell lines. Lower diagonal showed the Pearson correlation coefficients of selected features of each omics, suggesting a good correlation

between each other. For the selected features, highest correlation were observed between the components of mRNA and methylome (Figure 5.26).

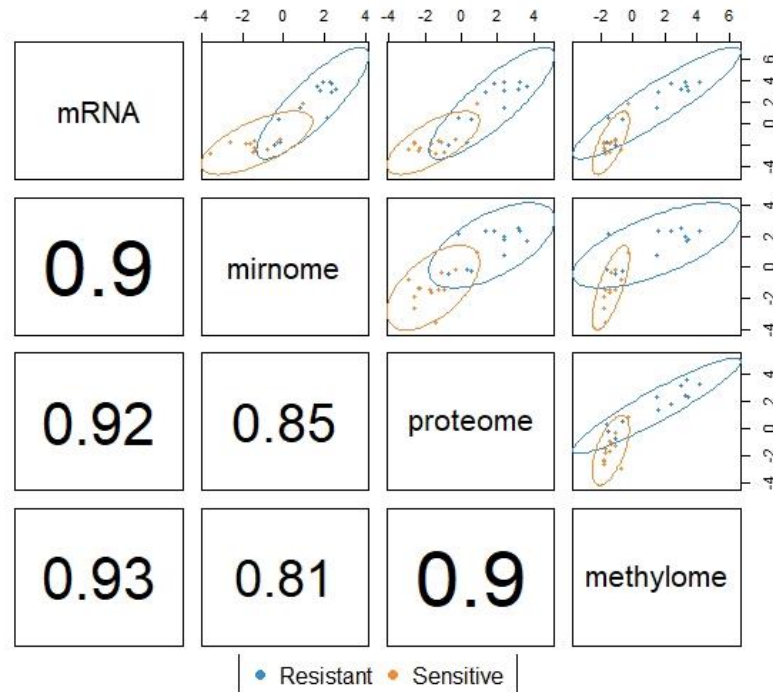


Figure 5. 26: Sample scatter plot and pearson correlation coefficients of mixOmics analysis based on irinotecan sensitivity profiles of pancreatic cancer cell lines. Components of different omics data sets shows the discriminative power of selected features (upper triangular panel) and the high correlation between components (lower triangular panel). Blue color indicates irinotecan-resistant cell lines, orange color indicates irinotecan-sensitive cell lines.

Figure 5.27 shows the loading weights of each selected features on the component in transcriptomic data. Higher expressions of ENPP1, NECAB1, CHST10, SLC26A10, GOLGA8R and TRPC1 genes were associated with irinotecan resistance while TNNT2, SYT8, UPK2 and SCEL expressions were abundant in irinotecan sensitive pancreatic cancer cell lines (Figure 5.27).

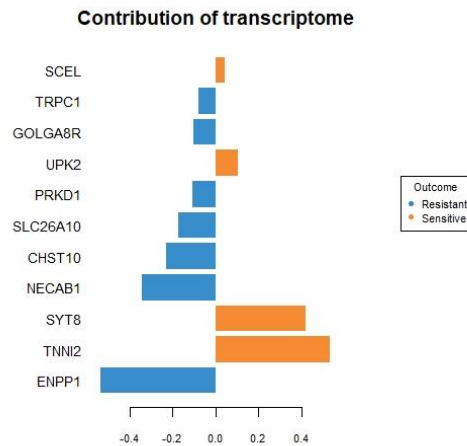


Figure 5. 27: List of selected mRNA variables and their loading weights associated with irinotecan sensitivity. Blue bars correspond to the irinotecan resistant subgroup in which the variable is most abundant. Orange bars correspond to the irinotecan sensitive subgroup in which the variable is most abundant.

Figure 5.28 shows the loading weights of selected miRNAs on the component from mirnomic data. miR-302c, miR-576-3p, miR-219-2-3p, miR-508-3p and miR-1288 were found to be associated with increased sensitivity to irinotecan. On the other hand, miR-140-5p and miR-96 were associated with irinotecan resistance (Figure 5.28).

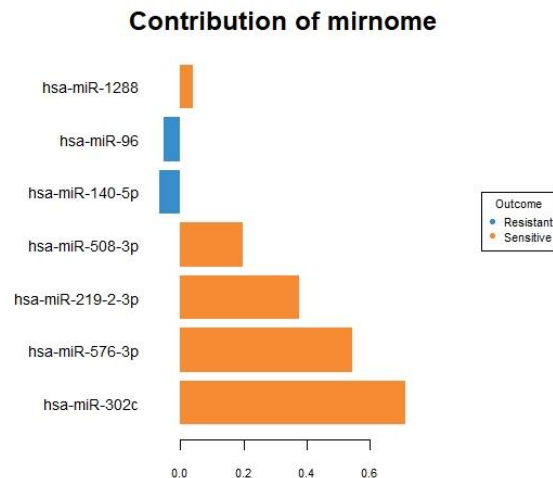


Figure 5. 28: List of selected miRNA variables and their loading weights associated with irinotecan sensitivity. Blue bars correspond to the irinotecan resistant subgroup in which the variable is most abundant. Orange bars correspond to the irinotecan sensitive subgroup in which the variable is most abundant.

Figure 5.29 shows the selected proteins on the component from proteomic data. Interestingly, protein expressions of beta-catenin, porin, bid, alpha-catenin and bcl-xl showed abundance in irinotecan sensitive cell lines. DJ-1 and PI3K-p110-alpha showed abundance in irinotecan resistant cell lines (Figure 5.29). Additionally, five methylation events were selected by the mixOmics providing discrimination of cell lines based on sensitivity to irinotecan (Figure 5.30).

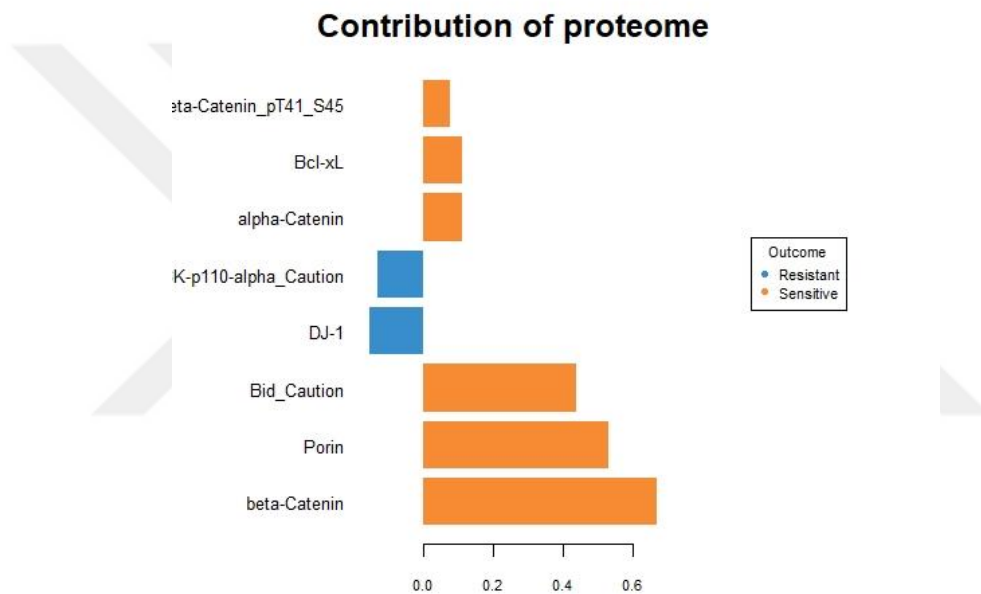


Figure 5. 29: List of selected protein variables and their loading weights associated with irinotecan sensitivity. Blue bars correspond to the irinotecan resistant subgroup in which the variable is most abundant. Orange bars correspond to the irinotecan sensitive subgroup in which the variable is most abundant.

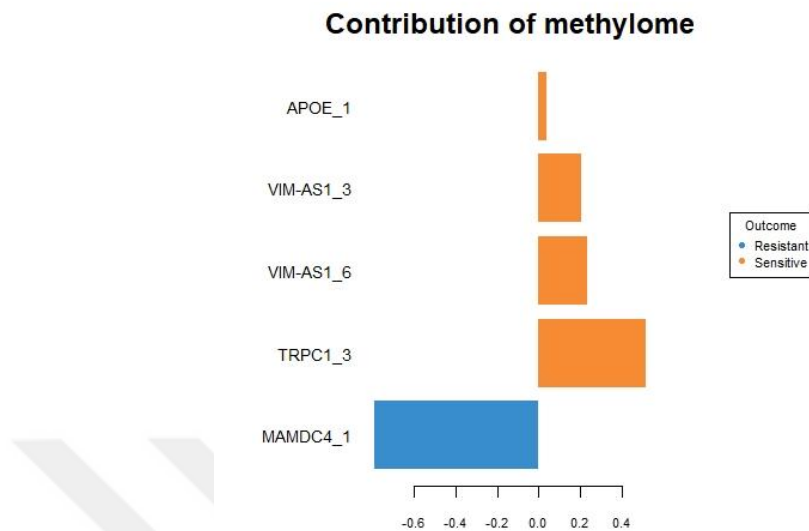


Figure 5. 30: List of selected methylation events and their loading weights associated with irinotecan sensitivity. Blue bars correspond to the irinotecan resistant subgroup in which the variable is most abundant. Orange bars correspond to the irinotecan sensitive subgroup in which the variable is most abundant.

5.4. Characterization of resistance mechanisms for gemcitabine in pancreatic cancer

To characterize gemcitabine drug resistance mechanisms in pancreatic cancer cell lines, analyses were performed with GSEA, NetworkAnalyst, miRNet and MixOmics. Gene expression data of defined subgroups were used to identify altered molecular mechanisms by using GSEA tool. In gemcitabine sensitive pancreatic cancer cell lines, genes associated with epithelial differentiation and gene set including genes upregulated in basal-like breast cancer cell lines as compared to the mesenchymal-like ones were enriched, indicating the increased epithelial phenotype of sensitive cells (Figure 5.31).

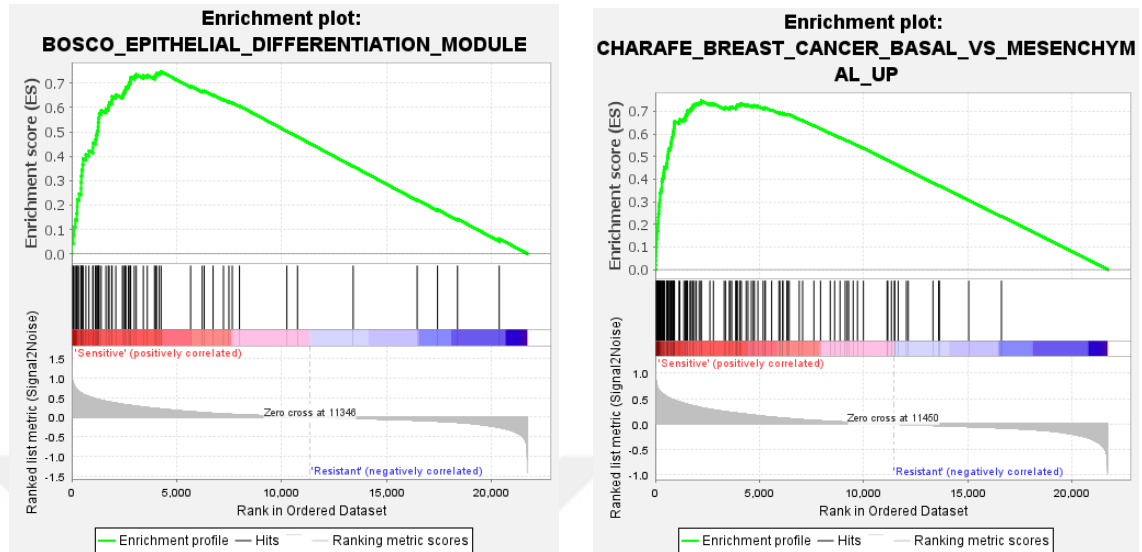


Figure 5. 31: Enriched gene sets in pancreatic cancer cell lines sensitive to gemcitabine. Enrichment plots of gene sets in gemcitabine-sensitive pancreatic cancer cell lines show significant enrichments of gene sets contain genes that are upregulated after ZEB1 knockdown and in basal-like breast cancer cell lines as compared to the mesenchymal-like ones.

In gemcitabine resistant cell lines, enriched gene sets included those related to epithelial to mesenchymal (EMT) and “MYC Targets” (Figure 5.32). These findings showed that pancreatic cancer cell lines sensitive to gemcitabine are more epithelial in contrast to resistant cell lines that show mainly EMT phenotype.

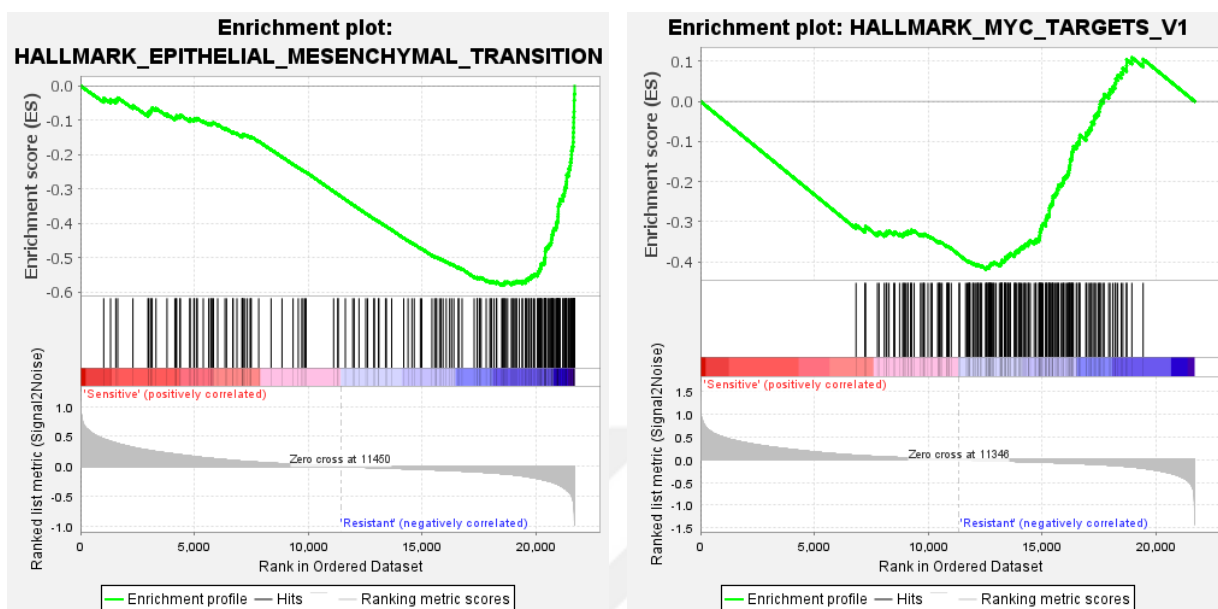


Figure 5. 32: Enriched gene sets in pancreatic cancer cell lines resistant to gemcitabine. Enrichment plots of highly enriched gene sets in gemcitabine resistant pancreatic cancer cell lines, indicating enrichments of gene sets related to EMT phenotype.

To further investigate the mechanisms of resistance to gemcitabine in pancreatic cancer cells, I performed PPI network analysis on Network Analyst tool by using resistance associated gene list. Functional enrichment analysis showed that genes related to FGFR tyrosine kinase signaling and PI3K-Akt signaling were highly enriched in the network (Figure 5.33).

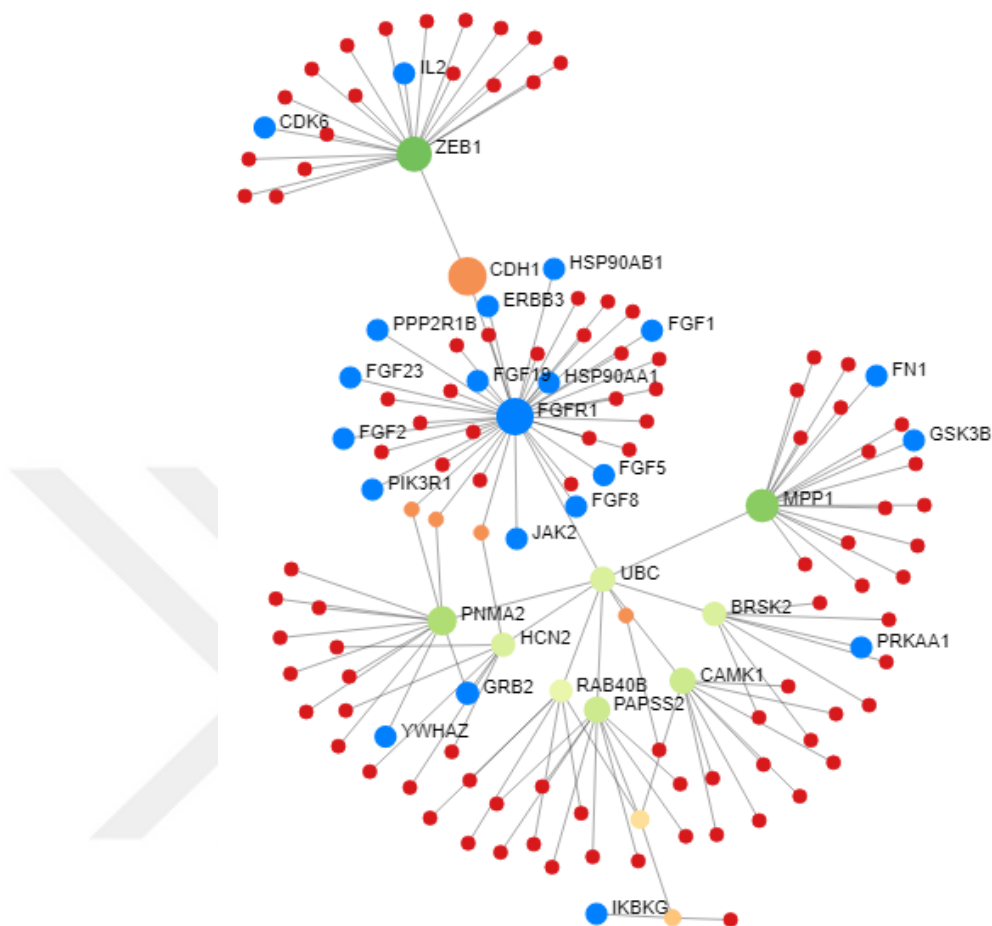


Figure 5. 33: PPI network analysis with gemcitabine resistance associated gene list.

Functional enrichment analysis of PPI network mapping resulted in enrichments of genes related to FGFR tyrosine kinase signaling and PI3K-Akt signaling. Blue nodes indicate genes associated with the aforementioned enrichments.

To gain further insight into differential drug response profiles, we identified most discriminant subset of biological features by using mixOmics. Feature selection process identified a multi-omics signature of 7 mRNA variables, 7 miRNA features, 7 proteomic features and 5 methylation features. Figure 5.34 showed that selected features of each omics were highly correlated between each other's. Sample scatterplot (upper diagonal plot) shows that the selected features were able to discriminate gemcitabine-sensitive and –resistant pancreatic cancer cell lines. Lower diagonal showed the Pearson correlation coefficients of selected features of each omics, suggesting a good correlation between each other. For the selected features, highest correlation were observed between the components of mRNA, mirnome and proteome (Figure 5.34).

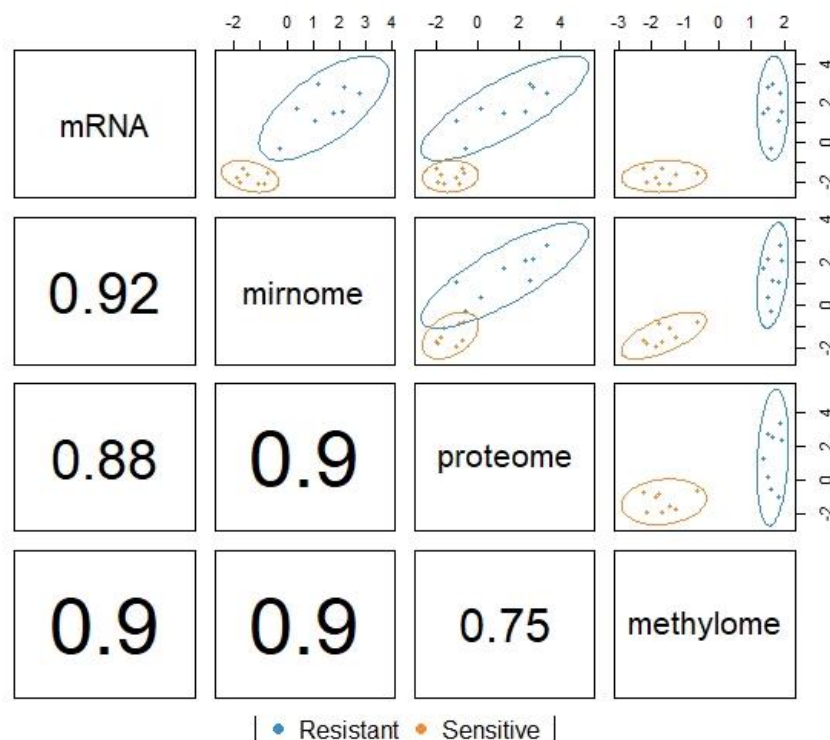


Figure 5. 34: Sample scatter plot and pearson correlation coefficients of mixOmics analysis based on irinotecan sensitivity profiles of pancreatic cancer cell lines. Components of different omics data sets shows the discriminative power of selected features (upper triangular panel) and the high correlation between components (lower triangular panel). Blue color indicates gemcitabine-resistant cell lines, orange color indicates irinotecan-sensitive cell lines.

Figure 5.35 shows the loading weights of each selected features on the component in transcriptomic data. Higher expressions of CAMK1 gene were associated with gemcitabine resistance while ALDH3B2, CLEC7A, ATP2C2, FAM83H-AS1, LIPM, and SYT7 expressions were abundant in gemcitabine sensitive pancreatic cancer cell lines (Figure 5.35).

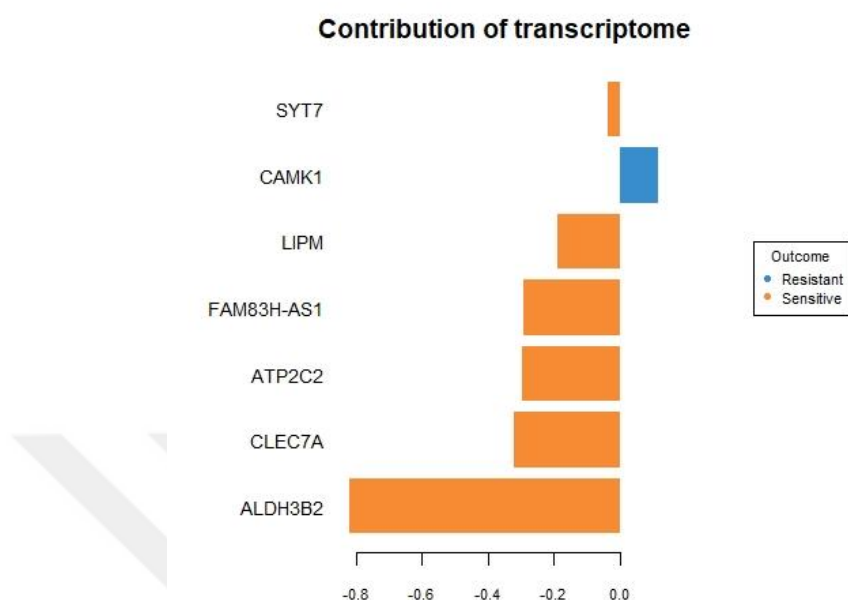


Figure 5. 35: List of selected mRNA variables and their loading weights associated with gemcitabine sensitivity. Blue bars correspond to the gemcitabine resistant subgroup in which the variable is most abundant. Orange bars correspond to the gemcitabine sensitive subgroup in which the variable is most abundant.

Figure 5.36 shows the loading weights of selected miRNAs on the component from mirnomic data. Similar to irinotecan sensitivity profile in pancreatic cancer cell lines, miR-140-5p was found to be associated with increased resistance to gemcitabine. Additionally, let-7g, miR-128, miR-29a, and miR-425 were associated with gemcitabine resistance. Sensitivity associated miRNAs included miR-876-3p and miR-141 (Figure 5.36).

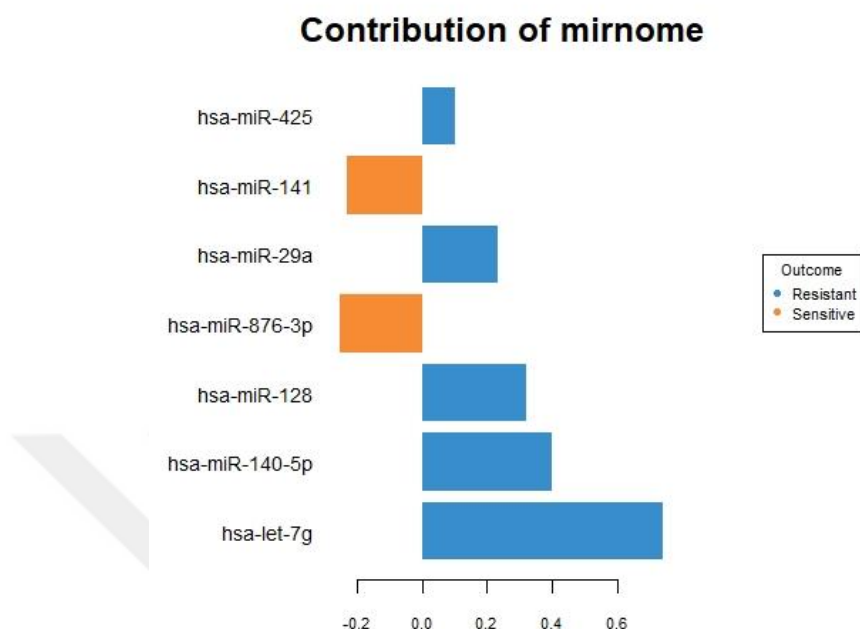


Figure 5. 36: List of selected miRNA variables and their loading weights associated with gemcitabine sensitivity. Blue bars correspond to the gemcitabine resistant subgroup in which the variable is most abundant. Orange bars correspond to the gemcitabine sensitive subgroup in which the variable is most abundant.

Figure 5.37 shows the selected proteins on the component from proteomic data. Protein expression E-Cadherin, claudin-7 of RAB25 also showed abundance in gemcitabine sensitive cell lines, confirming enrichment analysis results. TSC1 and STAT5-alpha expressions were associated with resistance to gemcitabine (Figure 5.37).

Additionally, five methylation events were selected by the mixOmics providing discrimination of cell lines based on sensitivity to gemcitabine (Figure 5.38). Two regions of TJP2 were found to be hypermethylated in sensitive cells. Nevertheless, mRNA levels were not correlated with methylation levels in TJP2 gene (data not shown).

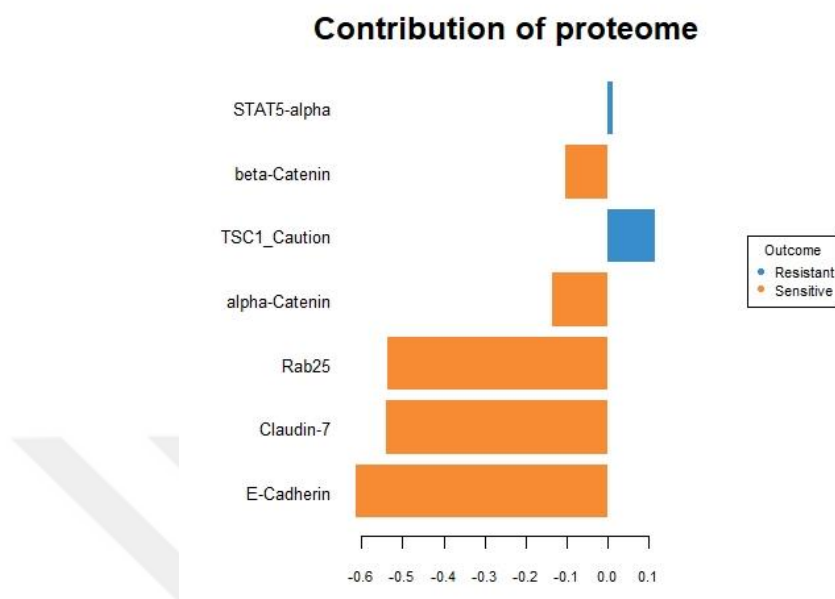


Figure 5. 37: List of selected protein variables and their loading weights associated with gemcitabine sensitivity. Blue bars correspond to the gemcitabine resistant subgroup in which the variable is most abundant. Orange bars correspond to the gemcitabine sensitive subgroup in which the variable is most abundant.

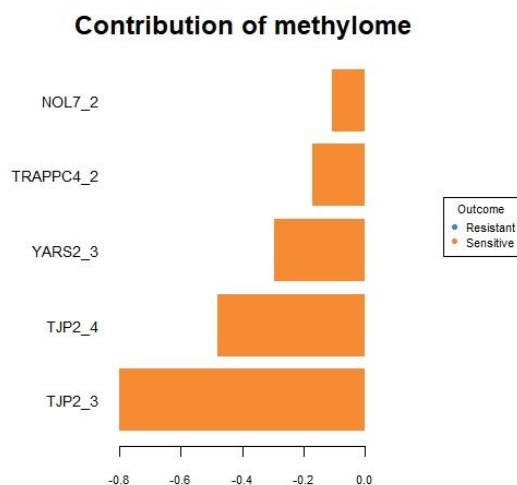
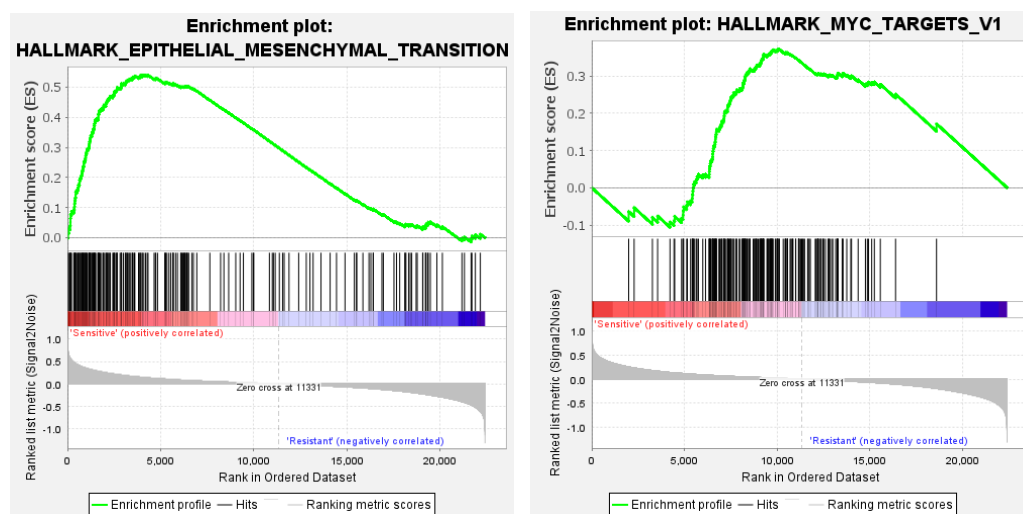


Figure 5. 38: List of selected methylation events and their loading weights associated with irinotecan sensitivity. Orange bars correspond to the gemcitabine sensitive subgroup in which the variable is most abundant.

5.5. Characterization of resistance mechanisms for doxorubicin in breast cancer

To characterize drug resistance mechanisms for doxorubicin in breast cancer cells, analyses were performed with GSEA, NetworkAnalyst, miRNet and MixOmics. In order to identify doxorubicin sensitive and –resistant cell lines, drug response profiles were analyzed by using PharmocoDB web-application that integrates multiple high-throughput cancer pharmacogenomics datasets. Gene expression data of defined subgroups were used to identify altered molecular mechanisms by using GSEA tool. In contrast to identified resistance mechanisms in colon and pancreatic cancer cells for 5-FU, irinotecan and gemcitabine, mechanisms for doxorubicin drug sensitivity in breast cancer cells showed clear distinction. In doxorubicin sensitive breast cancer cell lines, gene sets related to “Epithelial-Mesenchymal Transition” and “MYC Targets” were found to be enriched. Additionally, genes upregulated in invasive ductal carcinoma and in response to TGFB1 were enriched in doxorubicin sensitive cells (Figure 5.39).



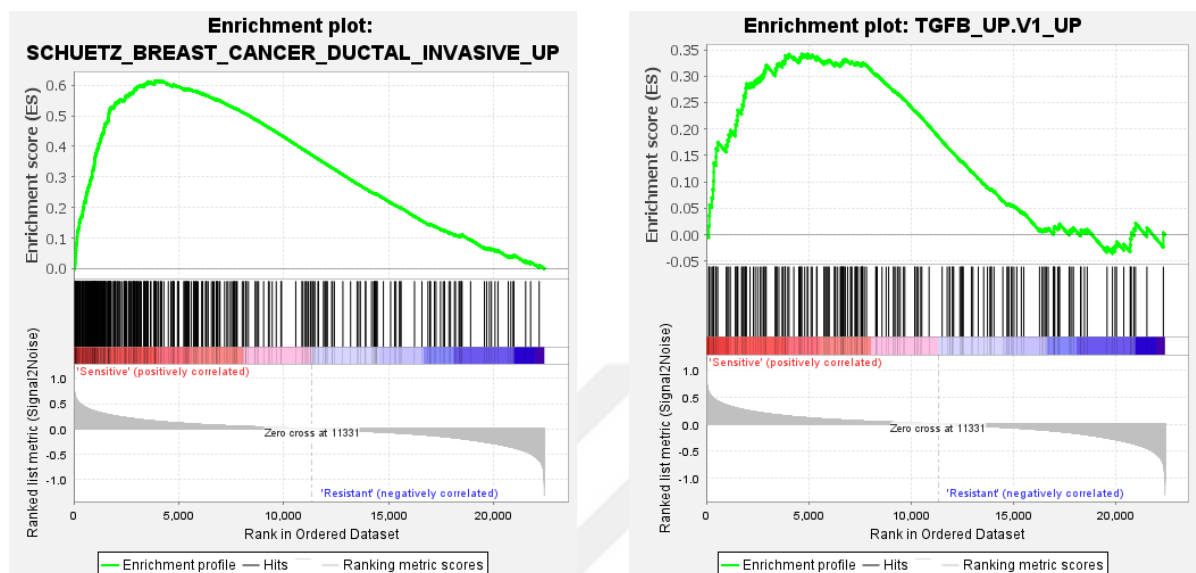


Figure 5. 39: Enriched gene sets in pancreatic cancer cell lines sensitive to doxorubicin. Enrichment plots of highly enriched gene sets in doxorubicin sensitive breast cancer cell lines, indicating enrichments of gene sets related to mesenchymal/invasive phenotype.

In doxorubicin resistant subgroup, GSEA analysis showed the enrichments in gene sets related to early and late estrogen response and upregulated genes in ESR1 positive cells (Figure 5.40).

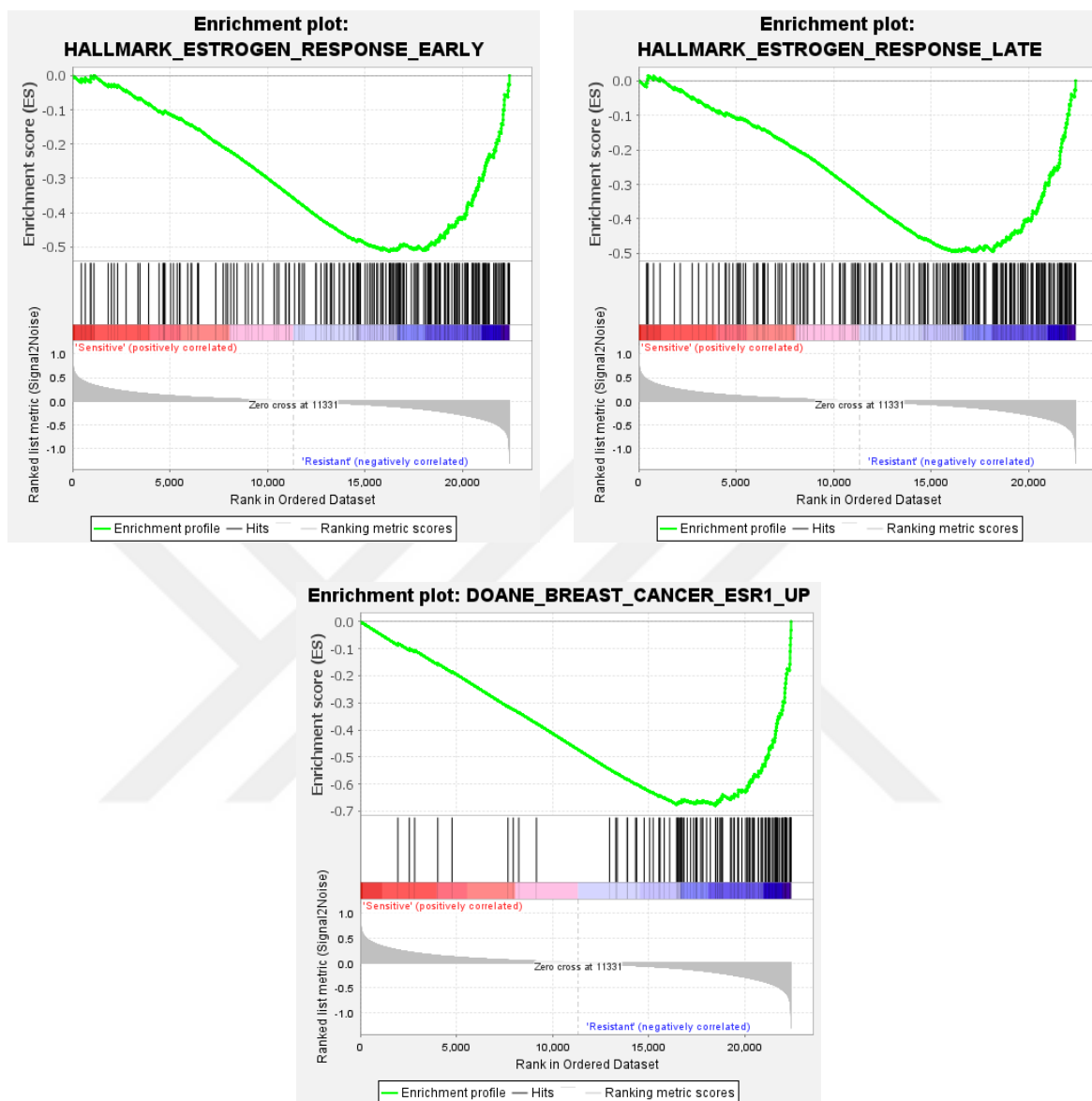


Figure 5. 40: Enriched gene sets in pancreatic cancer cell lines resistant to doxorubicin. Enrichment plots of highly enriched gene sets in doxorubicin resistant breast cancer cell lines, indicating enrichments of gene sets related to estrogen receptor positive phenotype.

To further investigate the mechanisms of sensitivity to doxorubicin in breast cancer cells, we performed PPI network analysis on Network Analyst tool by using sensitivity associated gene list. Functional enrichment analysis showed that genes related to TGF-beta receptor signaling that activates SMADs were highly enriched in the network (Figure 5.41).

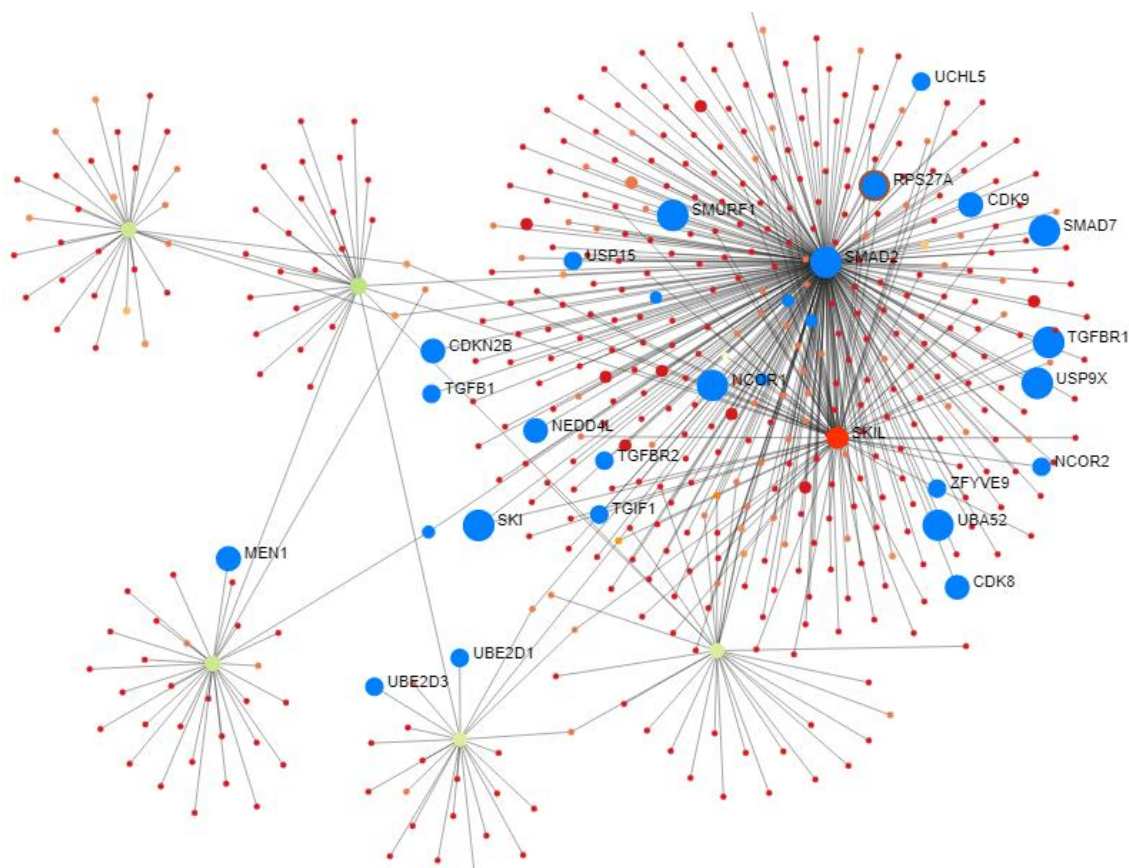


Figure 5. 41: PPI network analysis with doxorubicin sensitivity associated gene list. Functional enrichment analysis of PPI network mapping resulted in enrichments of genes related to TGF-beta receptor signaling activates SMADs. Blue nodes indicate genes associated with the aforementioned enrichments.

We performed PPI network analysis on Network Analyst tool by using resistance associated gene list. Functional enrichment analysis showed that genes related to ERBB signaling pathway were highly enriched in the network (Figure 5.42).

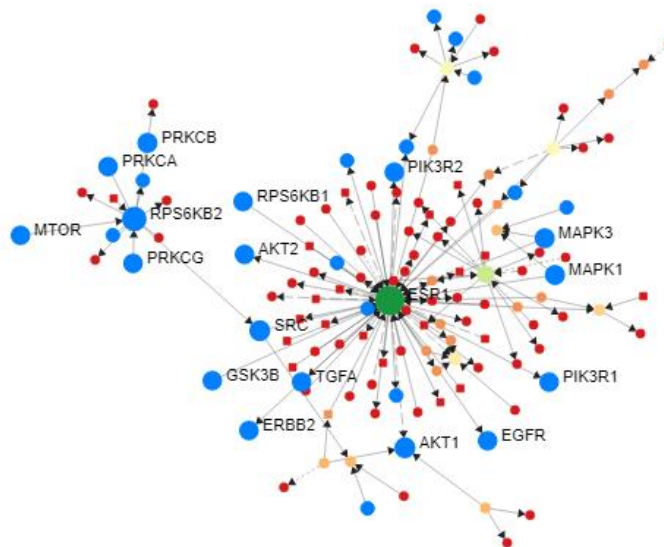


Figure 5. 42: PPI network analysis with doxorubicin resistance associated gene list. Functional enrichment analysis of PPI network mapping resulted in enrichments of genes related to ERBB signaling pathway. Blue nodes indicate genes associated with the aforementioned enrichments.

Analysis of resistance associated miRNAs-target networks including PPIs with miRNet showed the functional enrichments of PI3K-Akt signaling pathway proteins (Figure 5.43).

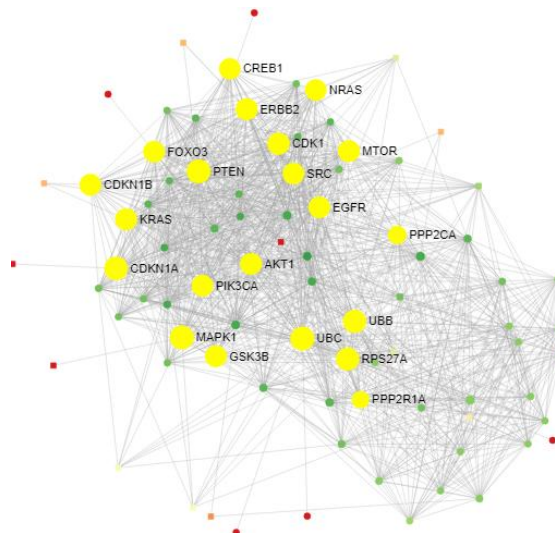


Figure 5. 43: PPI network analysis with doxorubicin resistance associated miRNA list. Functional enrichment analysis of doxorubicin-resistance associated miRNAs-target network mapping with PPIs resulted the enrichments of PI3K-AKT signaling proteins. Yellow nodes indicate PI3K-AKT signaling related genes, square nodes show doxorubicin-resistance associated miRNAs.

To gain further insight into differential drug response profiles, we identified most discriminant subset of biological features by using mixOmics. Feature selection process identified a multi-omics signature of 7 mRNA variables, 7 miRNA features, 7 proteomic features and 5 methylation features. Figure 5. showed that selected features of each omics were highly correlated between each other's. Sample scatterplot (upper diagonal plot) shows that the selected features were able to discriminate doxorubicin-sensitive and – resistant cell lines. Lower diagonal showed the Pearson correlation coefficients of selected features of each omics, suggesting a good correlation between each other. For the selected features, highest correlation were observed between the components of mRNA, mirnome and proteome (Figure 5.44).

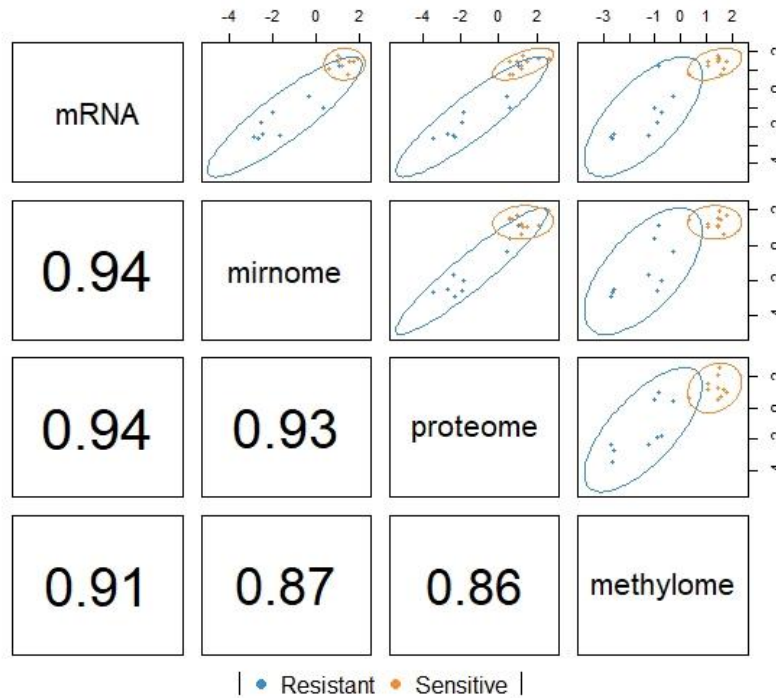


Figure 5. 44: Sample scatter plot and pearson correlation coefficients of mixOmics analysis based on doxorubicin sensitivity profiles of breast cancer cell lines. components of different omics data sets shows the discriminative power of selected features (upper triangular panel) and the high correlation between components (lower triangular panel). Blue color indicates doxorubicin-resistant cell lines, orange color indicates doxorubicin-sensitive cell lines.

Figure 5.45 shows the loading weights of each selected features on the component in transcriptomic data. Higher expressions of ALG1L, REEP6, TFF1 and EPX genes were associated with doxorubicin resistance while RANGRF, TRIB2 and ZSCAN23 expressions were abundant in doxorubicin sensitive breast cancer cell lines (Figure 5.45).

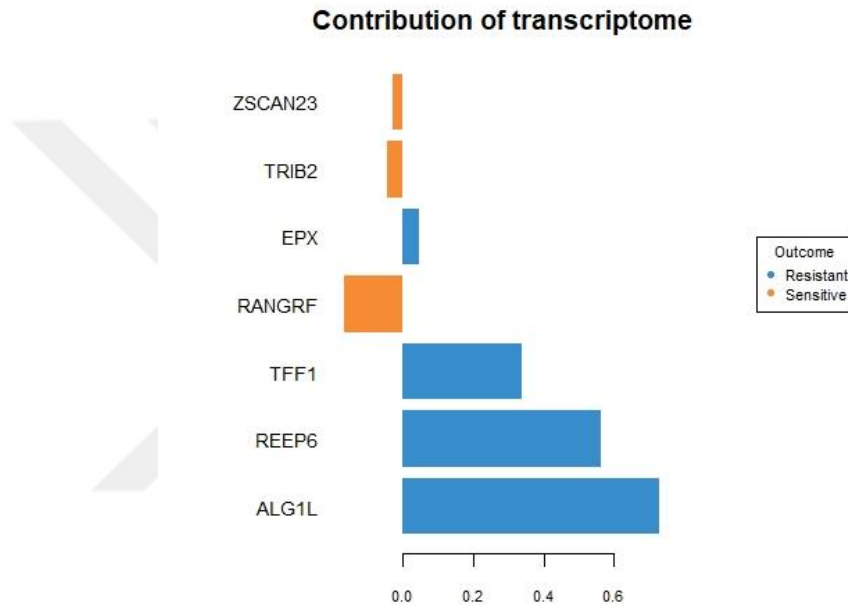


Figure 5. 45: List of selected mRNA variables and their loading weights associated with doxorubicin sensitivity. Blue bars correspond to the doxorubicin resistant subgroup in which the variable is most abundant. Orange bars correspond to the doxorubicin sensitive subgroup in which the variable is most abundant.

Figure 5.46 shows the loading weights of selected miRNAs on the component from mirnomic data. All selected miRNAs showed correlation with increased resistance to doxorubicin. miRNAs included miR-301a, miR-454, miR-16, miR-339-3p, miR-203, miR-193b and miR-26a (Figure 5.46).

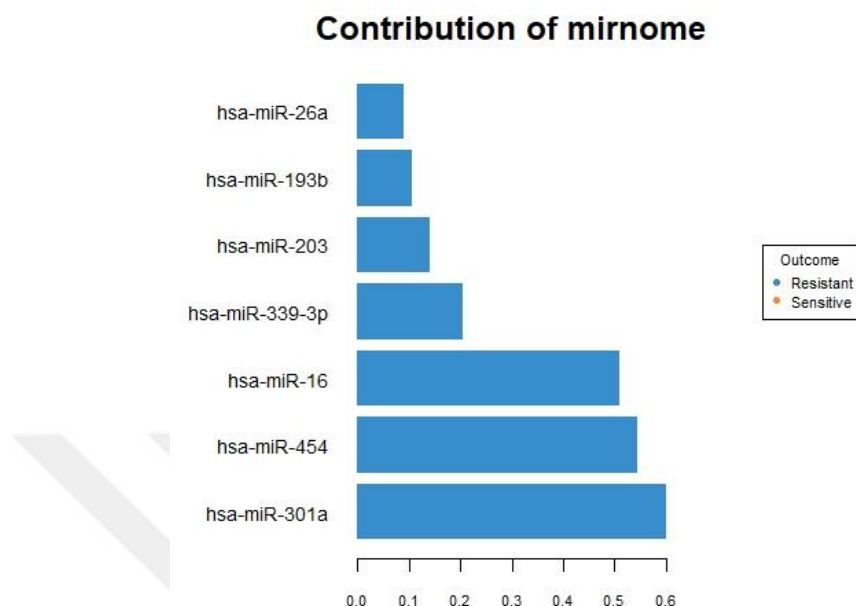


Figure 5. 46: List of selected miRNA variables and their loading weights associated with doxorubicin sensitivity. Blue bars correspond to the doxorubicin resistant subgroup in which the variable is most abundant.

Figure 5.47 shows the selected proteins on the component from proteomic data. Protein expression of GATA3, ER-Alpha, AR, HER3 and E-Cadherin showed abundance in doxorubicin resistant cell lines. On the other hand, cyclin E1 and ERK2 proteins were associated with doxorubicin sensitivity (Figure 5.47).

Additionally, five methylation events were selected by the mixOmics providing discrimination of cell lines based on sensitivity to doxorubicin (Figure 5.48).

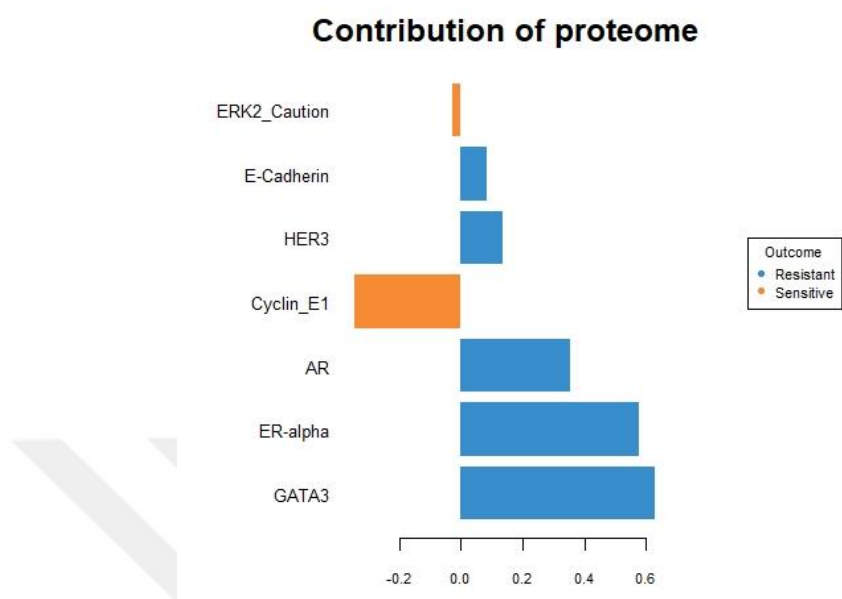


Figure 5. 47: List of selected protein variables and their loading weights associated with doxorubicin sensitivity. Blue bars correspond to the doxorubicin resistant subgroup in which the variable is most abundant. Orange bars correspond to the doxorubicin sensitive subgroup in which the variable is most abundant.

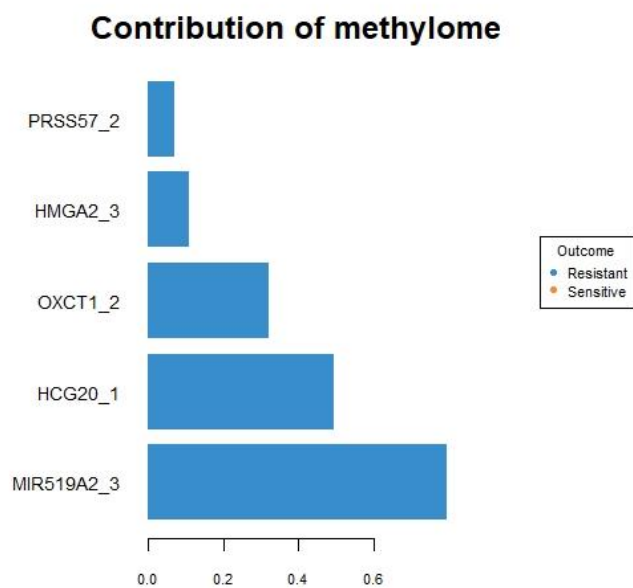


Figure 5. 48: List of selected methylation events and their loading weights associated with irinotecan sensitivity. Blue bars correspond to the irinotecan resistant subgroup in which the variable is most abundant.

5.6. Reversal of EMT by natural compounds to overcome EMT-driven drug resistance

Our *in silico* analyses revealed that EMT is a main player in chemotherapy resistance in different types of cancer. Therefore, we hypothesized that reversal of EMT with natural compounds can be effective strategy to overcome EMT-mediated drug resistance. To investigate the role of natural compounds in the modulation of epithelial/mesenchymal phenotypes of cancer cells, we used six cell lines from three different cancer types to explore whether treating these cancer cell lines with natural products results in switching of their epithelial/mesenchymal phenotype. First, we measured their EMT marker gene levels in cancer cell lines. These six cell lines displayed distinct characteristics based on expression of epithelial and mesenchymal marker genes, CDH1 and VIM respectively (Figure 5.49). Caco-2 exhibited high levels of CDH1 expression. KM12, LoVo and WiDr colon cancer cells had intermediate expression levels of CDH1 while MDA-MB-157 breast cancer cells and MIA PaCa-2 pancreatic cancer cells had highly low levels of CDH1 expression. On the other hand, VIM expression was high in MDA-MB-157 and MIA PaCa-2 cells while KM12 exhibited intermediate levels of expression and Caco-2, WiDr, LoVo cells had highly low levels of VIM expression. These data suggest that Caco2-, LoVo and WiDr have more epithelial characteristics compared to intermediary phenotype of KM12 and mesenchymal phenotypes of MDA-MB-157 and MIA PaCa-2 cells (Figure 5.49).

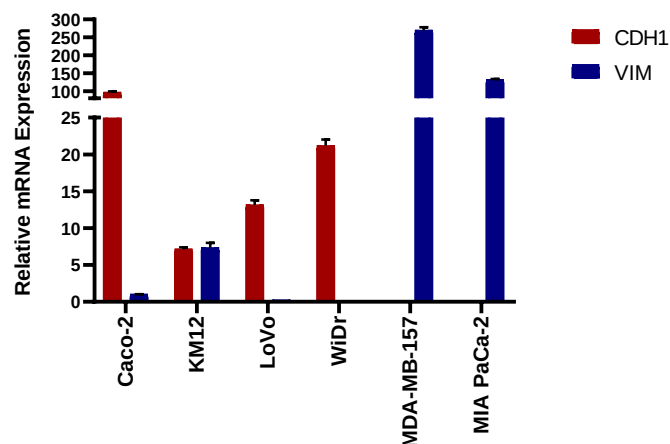


Figure 5. 49: Expression levels of EMT marker genes in cancer cell lines. Expression levels of epithelial marker CDH1 gene and mesenchymal marker VIM gene in Caco-2, KM12, LoVo and WiDr colon cancer cells, MDA-MB-157 breast cancer cell line and MIA PaCa-2 pancreatic cancer cell line was measured with qRT-PCR. GAPDH was used as reference gene to normalize expression levels; relative quantification is based on Caco-2 VIM expression that was set to 1.

To measure effects of natural compounds on epithelial/mesenchymal phenotype of cancer cell lines, we treated these cells with the eight natural compounds, Fulvestrant and Metformin at multiple doses and quantified the expression of CDH1 and VIM via qRT-PCR upon 48-hour treatment. An increase in CDH1, and a decrease in VIM expression upon treatment compared to the untreated control cells were considered as indicators of MET induction, and vice versa for EMT induction. In Caco-2 cells, MET induction was achieved with none of the treatments. On the contrary, treatments with 5, 20 μ M of Apigenin, 5, 20 μ M of Fisetin, 20 μ M of Resveratrol and 1, 5 μ M of Metformin resulted in upregulation of VIM. Forskolin and Fulvestrant treatments resulted in downregulation of both CDH1 and VIM. Furthermore, none of treatments were observed to upregulate CDH1 expression (Figure 5.50).

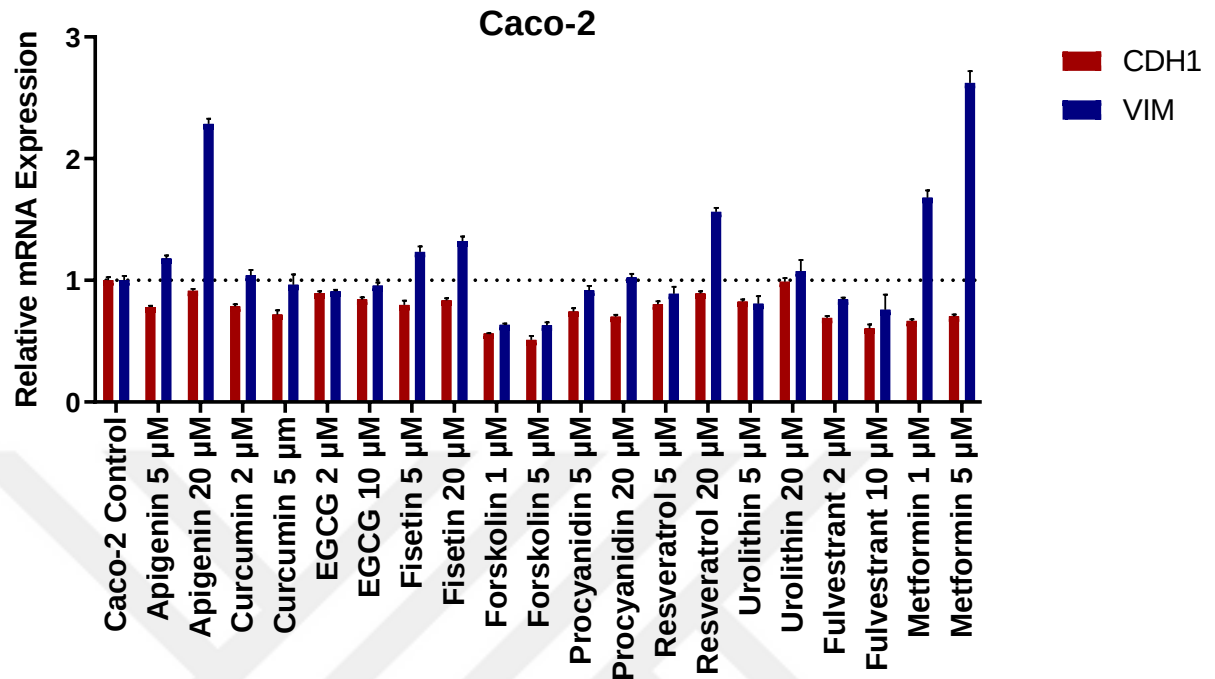


Figure 5. 50: Effect of natural compounds and repurposed agents on the expression of EMT marker genes in Caco-2. The cells were treated with indicated concentration for 48 h. Gene expression levels were evaluated by qRT-PCR and normalized according to GAPDH expression levels. Relative expressions of CDH1 and VIM were calculated by comparing to untreated Caco-2 cells.

In KM12 cells, MET induction were highly selective. Treatment with 5 μM of apigenin resulted in downregulation of VIM but had no effect on CDH1 expression levels. 20 μM of apigenin increased the downregulation of VIM but still had no effect on CDH1. Both doses of curcumin treatment downregulated CDH1 but only 10 μM of curcumin was shown to downregulate VIM. 20 μM of fisetin showed most promising induction of MET by upregulating CDH1 expression levels 100% and downregulating VIM expression levels almost 80%. Similarly, forskolin treatments resulted in 80% downregulation of VIM expression levels while CDH1 upregulation was limited. Urolithin treatments did not affect the expression levels of CDH1 and VIM while 1 μM of fulvestrant were shown to downregulate both genes and treatment with 5 μM of fulvestrant downregulated VIM expression slightly (Figure 5.51).

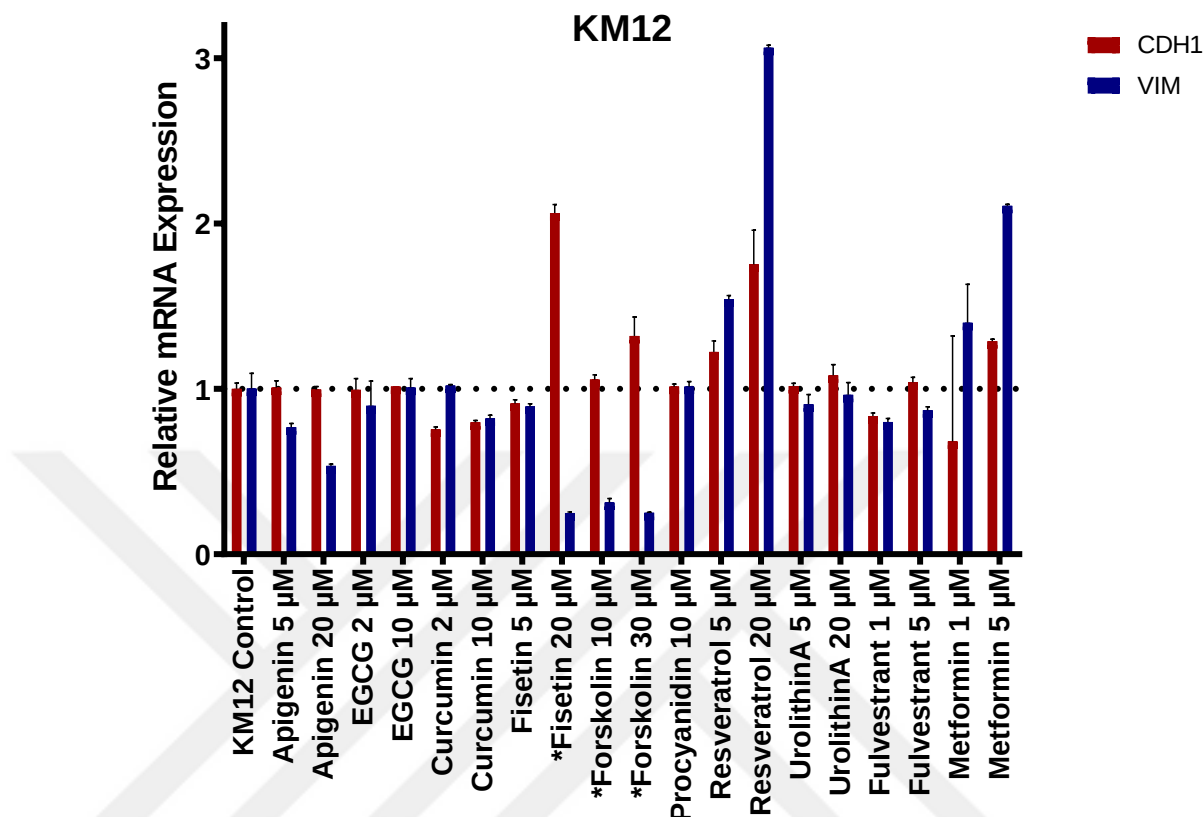


Figure 5. 51: Effect of natural compounds and repurposed agents on the expression of EMT marker genes in KM12. The cells were treated with indicated concentration for 48 h. Gene expression levels were evaluated by qRT-PCR and normalized according to GAPDH expression levels. Relative expressions of CDH1 and VIM were calculated by comparing to untreated KM12 cells.

In LoVo colon cancer cell line, 5 μ M of apigenin was shown to upregulate CDH1 and downregulate VIM. On the other hand, treatment with 20 μ M of apigenin resulted in downregulation of both genes. Curcumin did not induce the change in expression levels. Treatment with 5 μ M and 20 μ M of fisetin was shown to downregulate VIM levels similarly, only 20 μ M of fisetin was observed to downregulate CDH1 levels. 10 μ M of forskolin was shown to downregulate VIM, but higher dose did not exhibit similar effect. Only slight upregulation of CDH1 was observed with 30 μ M of forskolin. Low dose of urolithin A downregulated VIM slightly with no effect on CDH1 while higher dose was shown to downregulate both. Both doses of fulvestrant achieved upregulation of CDH1 but only 5 μ M of fulvestrant downregulated VIM expression (Figure 5.52).

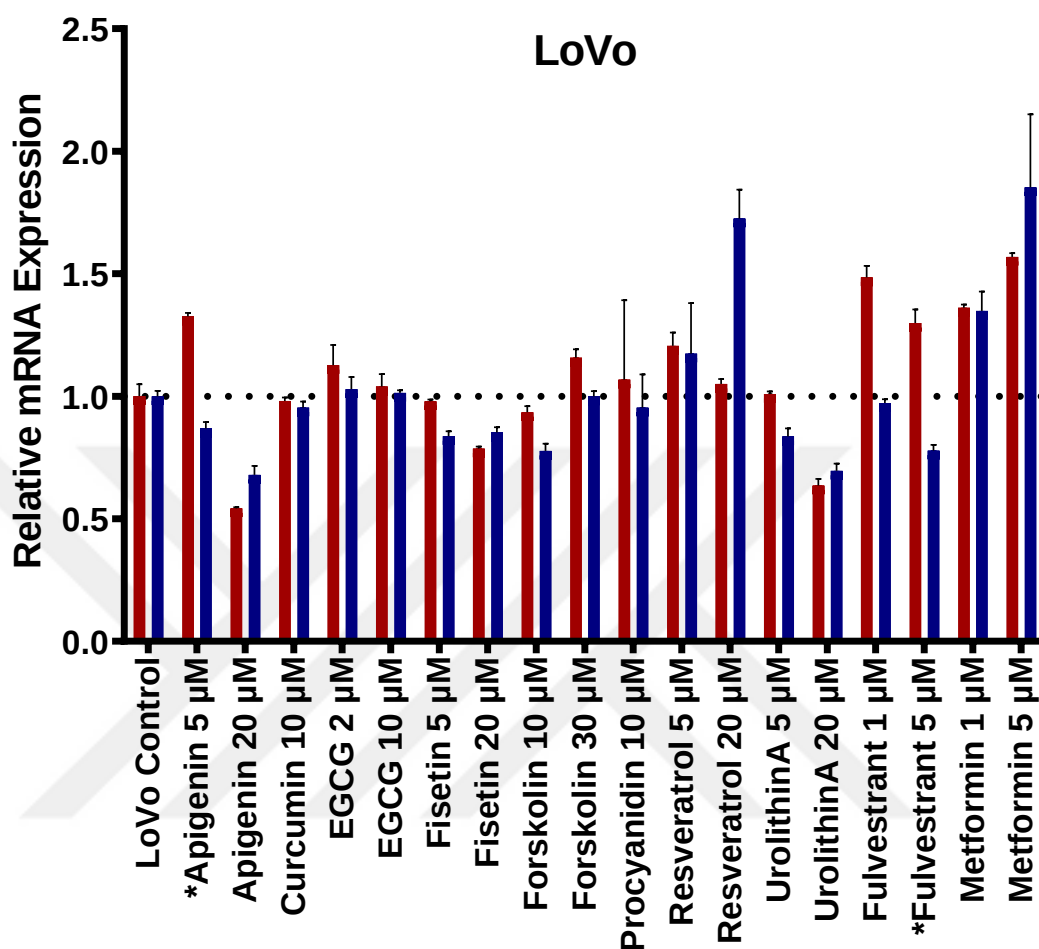


Figure 5. 52: Effect of natural compounds and repurposed agents on the expression of EMT marker genes in LoVo. The cells were treated with indicated concentration for 48 h. Gene expression levels were evaluated by qRT-PCR and normalized according to GAPDH expression levels. Relative expressions of CDH1 and VIM were calculated by comparing to untreated LoVo cells.

In WiDr cells, 5 µM of apigenin downregulated VIM expression, however, higher dose reversed this effect by upregulating VIM and downregulating CDH1. 2 µM of curcumin upregulated both CDH1 and VIM while high dose of curcumin only upregulated of CDH1 with no effect on VIM. Only 20 µM of fisetin affected the expression levels of CDH1 and VIM by downregulating. Treatments with forskolin were shown to downregulate both genes with more than 50% reduction in VIM levels. Slight upregulation in CDH1 with 5 µM urolithin A treatment were observed but 20 µM urolithin A treatment resulted in

downregulation of CDH1. 1 μ M of fulvestrant was shown to downregulate both genes while 5 μ M of fulvestrant had no effect on gene expression of these genes (Figure 5.53).

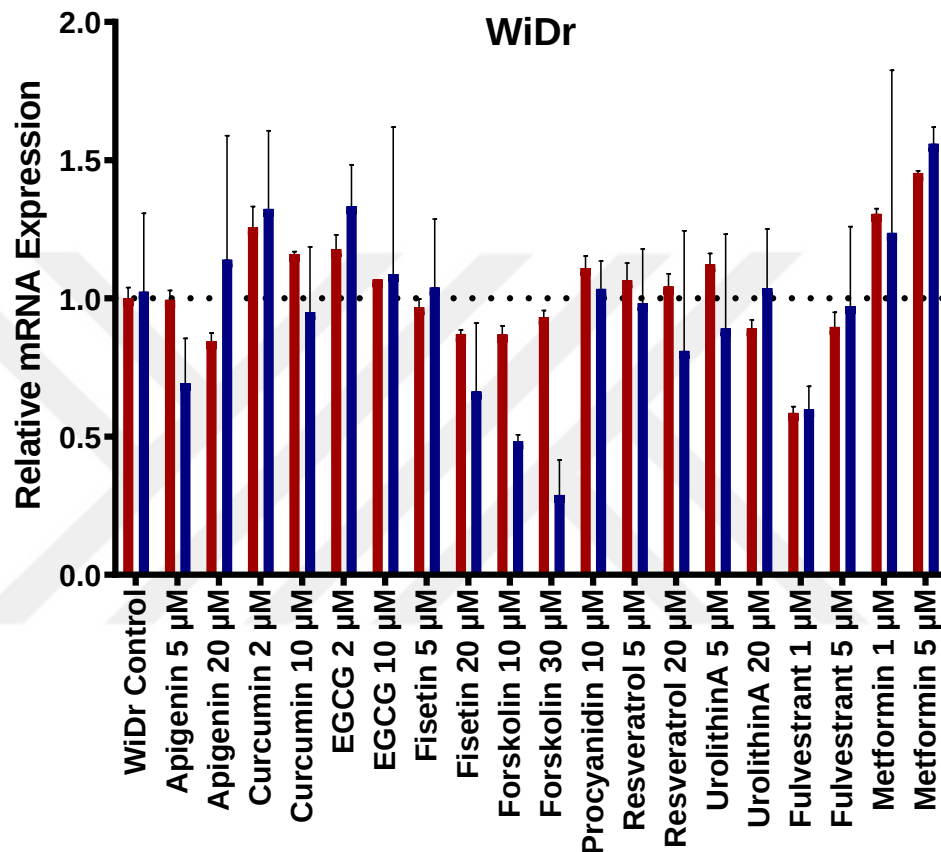


Figure 5. 53: Effect of natural compounds and repurposed agents on the expression of EMT marker genes in WiDr. The cells were treated with indicated concentration for 48 h. Gene expression levels were evaluated by qRT-PCR and normalized according to GAPDH expression levels. Relative expressions of CDH1 and VIM were calculated by comparing to untreated WiDr cells.

In MDA-MB-157 breast cancer cell line, only treatment with 20 μ M resveratrol resulted in CDH1 upregulation with slight decrease in VIM expression. All other treatments had no effect on CDH1 expression or resulted in 50% downregulation of CDH1 expression. Indeed, 2 μ M EGCG, 20 μ M procyanidin B2, 5 μ M resveratrol, fulvestrant and metformin treatments resulted in slight upregulation of VIM expression. Slight decreases

in VIM expression were observed in 20 μ M apigenin, fisetin and forskolin treatments (Figure 5.54).

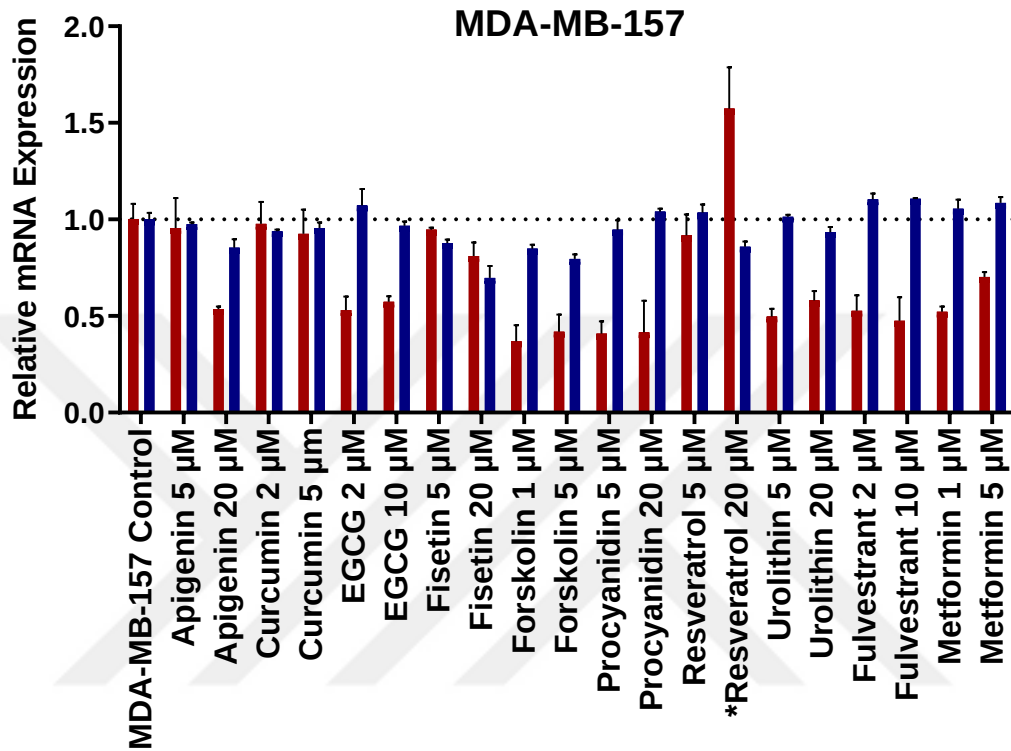


Figure 5. 54: Effect of natural compounds and repurposed agents on the expression of EMT marker genes in MDA-MB-157. The cells were treated with indicated concentration for 48 h. Gene expression levels were evaluated by qRT-PCR and normalized according to GAPDH expression levels. Relative expressions of CDH1 and VIM were calculated by comparing to MDA-MB-157 untreated cells.

In MIA PaCa-2 pancreatic cancer cell line, treatments were performed at single dose. All drug treatments resulted in upregulation of CDH1 expression. However, treatments downregulated VIM expression levels slightly except curcumin treatment which resulted in upregulation of VIM (Figure 5.55). Nevertheless, this increase in CDH1 expression may be considered as mild phenotypic switch since CDH1 levels were already very low even after the treatments when compared to CDH1 expression levels in colon cancer cell lines (Figure 5.55).

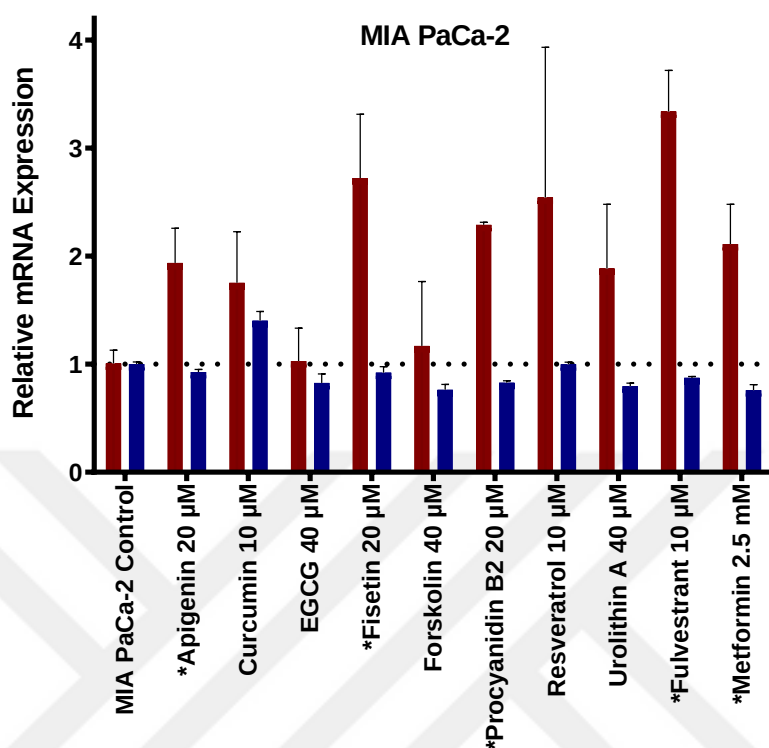


Figure 5. 55: Effect of natural compounds and repurposed agents on the expression of EMT marker genes in MIA PaCa-2. The cells were treated with indicated concentration for 48 h. Gene expression levels were evaluated by qRT-PCR and normalized according to GAPDH expression levels. Relative expressions of CDH1 and VIM were calculated by comparing to MIA PaCa-2 untreated cells.

Overall, only a few of natural compound treatments were able to induce MET in specific cancer cell lines in our experiments, suggesting a highly selective cell-line specific action (Table 5.1). Interestingly, none of the compounds were able to induce MET in the WiDr and Caco-2 cell lines. In LoVo cell line, apigenin and fulvestrant treatments resulted in MET induction. In KM12 cell line, Fisetin and Forskolin treatments resulted in MET induction. Resveratrol was the only natural compound that induces MET in MDA-MB-157. In MIA PaCa-2 cell line, partial EMT was observed almost all treatments.

Table 5. 1: Summary table of MET induction experiments.

	Induction of MET					
	LoVo	WiDr	Caco2	KM12	MDA-MB-157	MIA PaCa-2
Apigenin	complete	no	no	no	no	partial
Curcumin	no	no	no	no	no	no
EGCG	no	no	no	no	no	partial
Fisetin	no	no	no	complete	no	partial
Forskolin	no	no	no	complete	no	partial
Procyanidin B2	no	no	no	no	no	partial
Resveratrol	no	no	no	no	complete	partial
Urolithin A	no	no	no	no	no	partial
Fulvestrant	complete	no	no	no	no	partial
Metformin(mM)	no	no	no	no	no	partial
*complete: it indicates upregulation in CDH1 and downregulation in VIM expression. * partial: it suggests that treatments resulted in mild inductions, only upregulation in CDH1.						

5.6.1. Use of natural products to enhance 5-FU and Oxaliplatin sensitivity in KM12 colon cancer cells

Since MET induction were highly selective in different cell lines, we first sought to characterize the ability of natural products in enhancing sensitivity to conventional chemotherapeutics, 5-FU and oxaliplatin. We selected KM12 colon cancer cell line to perform combinational treatments, since most obvious MET inductions were observed with 20 μ M fisetin and 30 μ M forskolin treatments. KM12 cells were treated with 5-FU at six different concentrations (5-FU:100 μ M -0.01 μ M) alone or in combination with single dose of natural compounds. With the lowest dose of 5-FU, we observed that most of natural compounds affected cell viability of KM12. Curcumin, apigenin, fisetin and forskolin decreased cell viability more than 60%. With these doses, it was difficult to understand efficacy of natural compounds and fulvestrant in combination with 5-FU (Figure 5.56).

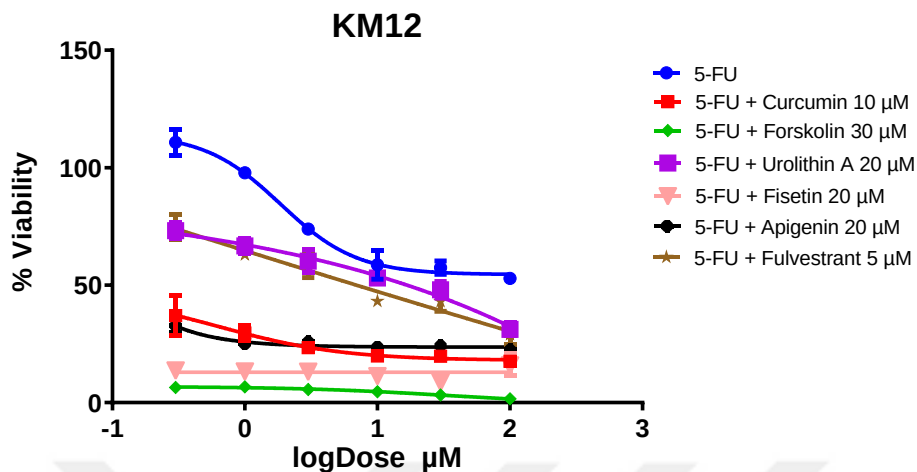


Figure 5. 56: Combination treatment of KM12 cells with 5-FU and natural compounds. KM12 cells were treated with six different doses of 5-FU (100 μM -0.5 μM) alone or in combination with natural compounds and fulvestrant.

KM12 cells were treated with oxaliplatin at six different concentrations (50 μM -0.01 μM) alone or in combination with single dose of natural compounds. Similarly, we could not understand the efficacy of natural compounds and fulvestrant in combination with oxaliplatin since Curcumin, apigenin, fisetin and forskolin decreased cell viability more than 60% (Figure 5.57). These results prompted us to measure effects of natural compounds on cell viability.

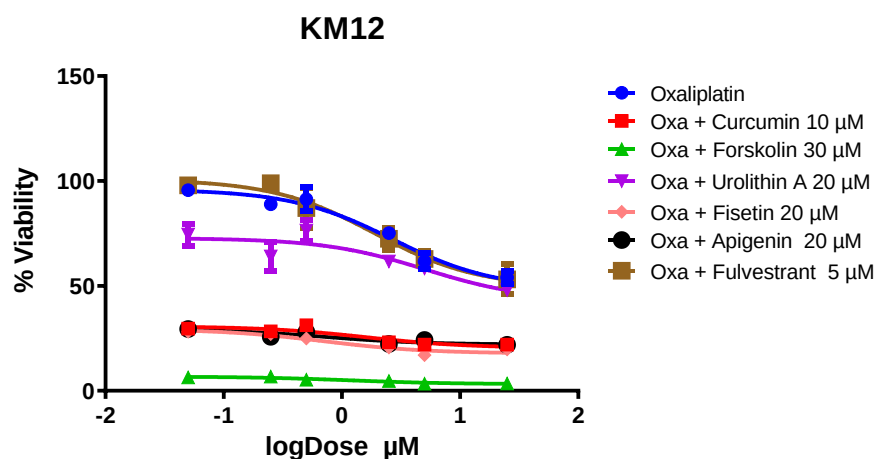


Figure 5. 57: Combination treatment of KM12 cells with oxaliplatin and natural compounds. KM12 cells were treated with six different doses of oxaliplatin (25 μM -0.05 μM) alone or in combination with natural compounds and fulvestrant.

5.6.2. Effects of natural products on cancer cell viability

We sought to characterize the inhibitory effects of natural products and re-purposed agents on various breast, colon and pancreatic cancer cell lines. We observed that breast cancer cell lines were highly resistant to most of the compounds screened, since IC₅₀ was not reached in 28 out of 51 compound-cell line combinations (Table 5.2). On the other hand, curcumin exhibited a remarkable dose-dependent inhibition of cell viability with IC₅₀ ranging from 12 to 40 μ M. Additionally, apigenin and resveratrol showed variance in the inhibitory activity on cell viability whereas fisetin and forskolin exhibit highly selective cytotoxicity on cell lines. MDA-MB-453 was the only breast cancer cell line that is sensitive to fulvestrant which may be a result of HER2-overexpression in this cell line while other cell lines were triple-negative. On the other hand, EGCG, procyanidin B2, urolithin A and metformin in micromolar concentrations do not have an effect on cell viability in breast cancer cell lines at all.

Table 5. 2: In vitro cytotoxicity analyses of natural compounds and repurposed agents in breast cancer cell lines.

IC ₅₀ (μ M)	CAL- 51	MDA-MB- 157	MDA-MB- 231	MDA-MB- 436	MDA-MB- 453	MDA-MB- 468
Apigenin	34	78	106	52	36	43
Curcumin	24	23	40	12	14	30
EGCG	NS	NS	RES	RES	RES	RES
Fisetin	41	39	RES	86	72	RES
Forskolin	48	RES	RES	RES	38	RES
Procyanidin B2	NS	NS	RES	NS	RES	RES
Resveratrol	NS	NS	33	54	64	30
Urolithin A	RES	RES	RES	RES	RES	RES
Fulvestrant	RES	RES	RES	RES	29	RES
Metformin	NS	NS	RES	RES	RES	RES

Similar to our findings in breast cancer, curcumin was the most effective compound for inhibition of viability in colon cancer cell lines with IC₅₀ ranging from 6 to 15 μ M. We observed moderate inhibition of cell viability with apigenin, EGCG, fisetin, resveratrol and urolithin A in micromolar levels. In addition, metformin in millimolar levels showed cytotoxic activity against colon cancer cell lines. The fact that KM12 is the only cancer

cell line sensitive to forskolin among our colon cancer cell line panel, is also evident in NCI-60 drug sensitivity data (Figure 5.58). On the other hand, procyanidin B2 and fulvestrant did not exhibit an inhibitory effect on colon cancer cell viability (Table 5.3).

Table 5. 3: In vitro cytotoxicity analyses of natural compounds and repurposed agents in colon cancer cell lines.

IC50 (μ M)	HCT8	KM12	LS513	LoVo	SW837	WiDr
Apigenin	19	26	12	28	50	40
Curcumin	12	10	6	9	15	8
EGCG	14	17	17	23	15	20
Fisetin	35	16	26	24	38	66
Forskolin	RES	4	RES	RES	RES	RES
Procyanidin B2	RES	RES	RES	RES	RES	RES
Resveratrol	35	47	24	27	25	39
Urolithin A	40	RES	30	28	40	52
Fulvestrant	RES	RES	RES	RES	RES	RES
Metformin(mM)	2	13	2	10	23	10

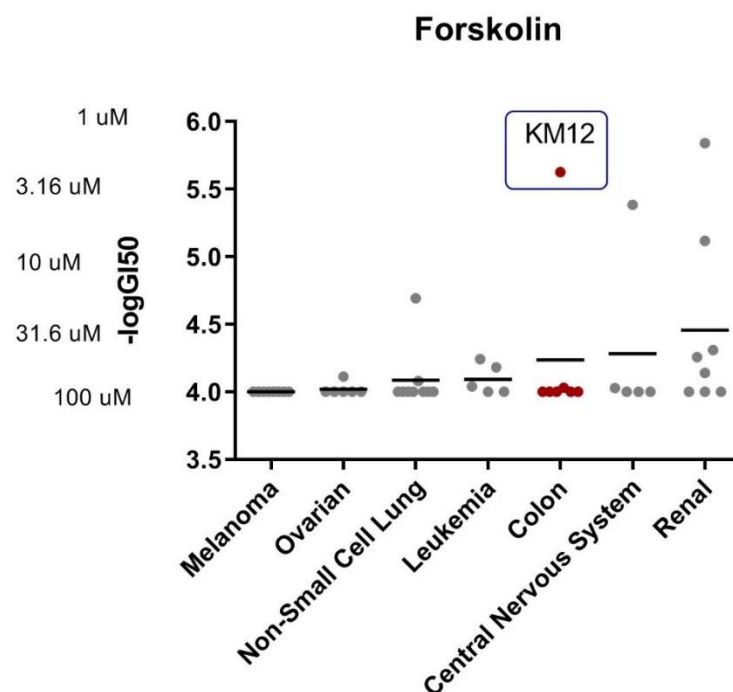


Figure 5. 58: Inhibitory effects of forskolin on different types of cancer cell lines. KM12 is the only colon cancer cell line that show sensitivity against forskolin in NCI-60 drug sensitivity data.

Pancreatic ductal adenocarcinoma (PDAC) is among most lethal cancer types, and a number of naturally occurring molecules in preclinical studies exhibit promising pharmacological features. We, next, examined the effects of natural compounds on viability of four pancreatic cancer cell lines (Table 5.4). As seen in breast and colon cancer cells, curcumin was the most effective natural compound on pancreatic cancer cell viability. We observed moderate cytotoxicity with apigenin EGCG, fisetin and resveratrol. Also, forskolin and urolithin A exhibited selective and mild effects on pancreatic cancer cell lines. Millimolar levels of metformin had also growth inhibitory effects in these cells. Similar to colon cancer cell lines, procyanidin B2 and fulvestrant had no effect on cell viability (Table 5.4).

Table 5. 4: In vitro cytotoxicity analyses of natural compounds and repurposed agents in pancreatic cancer cell lines.

IC50 (μ M)	AsPC-1	BxPC-3	MIA PaCa-2	SU8686
Apigenin	34	18	40	21
Curcumin	29	3	11	17
EGCG	35	26	87	22
Fisetin	90	25	38	45
Forskolin	RES	45	RES	RES
Procyanidin B2	RES	RES	RES	RES
Resveratrol	65	23	23	40
Urolithin A	RES	92	96	RES
Fulvestrant	RES	RES	RES	RES
Metformin(mM)	40	7	27	4

Collectively, 12, 13 and 15 cancer cell lines (out of 16) were resistant (IC50 has not reached) to forskolin, procyanidin B2 and fulvestrant, respectively, suggesting these compounds were not much effective in inhibition of cancer cell viability. Apigenin, curcumin and resveratrol were the only three compounds for which IC50 values could be generated for all cell lines tested. Among these compounds, lowest mean IC50 was obtained for curcumin in all three cancer types, 23.8 μ M, 10 μ M, 15 μ M for breast, colon and pancreatic cancer cells, respectively.

5.6.3. Combinatory effects of natural products and chemotherapy drugs on colon cancer cells

We next asked whether the treatment with natural compounds will result in chemosensitization. For this purpose, KM12 and WiDr colon cancer cell lines were treated with various doses of natural products alone or in combination with two doses (1 μ M and 10 μ M) of 5-FU after twenty-four hours of cell seeding. Cell viability assays were then performed 72 hours after treatments. In KM12, combination index analysis showed that none of the combinations of apigenin and 5-FU is synergistic; even we observed antagonisms in all treatments (Table 5.5).

Table 5. 5: Effects of apigenin treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.

	0 μ M		0.2 μ M		2 μ M		5 μ M		10 μ M		Apigenin
FU 0 μ M	99.3	100.7	102.4	103.5	99.3	99.2	99.9	99.3	90.7	86.1	
FU 1 μ M	95.0	94.2	97.6	95.1	94.8	96.7	96.5	102.2	87.7	80.3	
FU 10 μ M	57.9	58.5	59.1	59.2	60.5	58.1	65.9	62.3	61.5	61.6	

5-FU Dose	Apigenin Dose	Effect	CI
1	2	0.04	1.53
1	5	0.01	5.94
1	10	0.16	1.17
10	2	0.41	1.15
10	5	0.36	1.54
10	10	0.39	1.69

Further, combination index analysis showed that none of the combinations of curcumin and 5-FU is synergistic; even we observed antagonisms in all treatments (Table 5.6).

Table 5. 6: Effects of curcumin treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.

	0 μ M		0.1 μ M		0.5 μ M		2 μ M		5 μ M		Curcumin
FU 0 μ M	98.7	101.3	102.2	102.6	102.6	105.5	99.8	99.8	97.2	98.3	
FU 1 μ M	94.4	93.1	99.7	99.8	98.6	99.4	98.4	94.6	92.5	91.5	
FU 10 μ M	53.5	55.2	56.5	56.4	57.5	59.9	52.4	58.9	63.7	61.0	

5-FU Dose	Curcumin Dose	Effect	CI
1	2	0.04	2.23
1	5	0.08	1.54
10	2	0.44	1.14
10	5	0.38	1.61

Combination index analysis showed that none of the combinations of EGCG and 5-FU is synergistic; even we observed antagonisms in all treatments (Table 5.7).

Table 5. 7: Effects of EGCG treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.

	0 μ M	0.2 μ M	1 μ M	6 μ M	12 μ M	EGCG
FU 0 μ M	100.2 99.8	100.4 103.8	100.5 106.2	99.5 98.8	94.4 89.8	
FU 1 μ M	102.1 105.4	109.8 109.0	110.7 108.4	103.8 105.4	93.6 88.8	
FU 10 μ M	63.7 66.5	66.3 69.0	66.7 65.5	65.1 65.4	69.9 65.5	

5-FU Dose	EGCG Dose	Effect	CI
1	6	0.00	52.96
1	12	0.09	1.56
10	6	0.35	1.31
10	12	0.32	1.75

Fisetin is one of the natural compounds that induced MET in KM12 cell line. Combination index analysis showed that 10 μ M of fisetin and 1 μ M 5-FU treatment resulted in a slight synergism. On the other hand, we observed antagonisms in all other combinations (Table 5.8).

Table 5. 8: Effects of Fisetin treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.

	0 μ M	0.2 μ M	2 μ M	10 μ M	20 μ M	Fisetin
FU 0 μ M	99.5 100.5	102.6 100.0	101.1 98.5	78.8 77.1	50.5 48.6	
FU 1 μ M	86.8 88.8	91.7 91.4	93.1 91.0	70.3 71.0	49.9 47.9	
FU 10 μ M	55.8 58.3	57.3 56.3	57.0 57.0	57.5 56.4	49.0 50.4	

5-FU Dose	Fisetin Dose	Effect	CI
1	2	0.08	2.16
1	10	0.29	0.98
1	20	0.56	1.10
10	2	0.43	1.12
10	10	0.43	1.61
10	20	0.55	1.57

Forskolin is one of the natural compounds that induced MET in KM12 cell line. Except lowest doses of forskolin and 5-FU, all combinations resulted in synergy. Most synergistic effects were observed in combinations of 10 μ M 5-FU with 1, 4, and 10 μ M of forskolin (Table 5.9).

Table 5. 9: Effects of forskolin treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.

	0 μ M	0.1 μ M	1 μ M	4 μ M	10 μ M	Forskolin
FU 0 μ M	97.1 102.9	94.9 96.0	77.5 77.2	51.5 47.3	26.1 20.0	
FU 1 μ M	85.9 87.5	86.7 88.3	70.2 64.2	40.9 34.9	27.5 25.5	
FU 10 μ M	51.2 56.5	50.6 52.2	26.3 32.0	20.4 18.2	12.6 12.8	

5-FU Dose	Forskolin Dose	Effect	CI
1	0.1	0.13	1.34
1	1	0.33	0.91
1	4	0.62	0.75
1	10	0.74	0.99
10	0.1	0.49	0.91
10	1	0.71	0.37
10	4	0.81	0.37
10	10	0.87	0.41

In procyanidin B2 combinations, we observed that increasing doses of procyanidin induced cell viability. This prevent the calculations of combination index. However, based on percentages of viable cells we may say that 10 μ M of procyanidin B2 and 10 μ M of 5-FU combination indicated a synergy (Table 5.10).

Table 5. 10: Effects of procyanidin B2 treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.

	0 μ M	0.2 μ M	2 μ M	5 μ M	10 μ M	Procyanidin B2
FU 0 μ M	98.2 101.8	105.2 102.5	102.6 106.0	113.1 111.1	111.3 111.1	
FU 1 μ M	93.4 101.4	101.0 101.7	99.7 100.8	101.6 101.4	98.9 103.0	
FU 10 μ M	63.0 65.3	64.0 64.3	64.0 61.7	63.3 62.5	61.6 60.7	

Combination index analysis showed that none of the combinations of resveratrol and 5-FU is synergistic; even we observed antagonisms in all treatments (Table 5.11).

Table 5. 11: Effects of resveratrol treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.

	0 μ M	0.2 μ M	1 μ M	5 μ M	10 μ M	Resveratrol
FU 0 μ M	99.3 100.7	100.2 99.0	99.6 102.0	100.3 98.0	86.0 98.6	
FU 1 μ M	91.6 95.9	99.2 98.1	99.0 96.3	93.0 93.0	86.0 85.7	
FU 10 μ M	59.3 58.8	63.9 60.9	64.0 63.0	64.6 68.4	64.6 63.5	

5-FU Dose	Resveratrol Dose	Effect	CI
1	5	0.07	1.40
1	10	0.14	1.21
10	5	0.34	1.65
10	10	0.36	1.77

Urolithin A is one of the natural compounds that show no effect on EMT marker gene expressions in KM12 cell line. On the other hand, combination index analysis showed that some synergistic and additive effects of urolithin A and 5-FU combinations. Lower doses of 5-FU and urolithin A showed antagonisms. High doses of urolithin A with 1 μ M of 5-FU showed additive effects. Similarly, high doses of 5-FU with high doses of urolithin A showed additive effects. On the other hand, 10 μ M 5-FU and urolithin A combinations showed slight synergy with the CI ranging from 0.95 to 0.77, even though there was no significant MET induction with urolithin A in this cell line (Table 5.12).

Table 5. 12: Effects of urolithin A treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.

	0 μ M		0.2 μ M		2 μ M		10 μ M		20 μ M		Urolithin A
FU 0 μ M	100.5	99.5	97.9	95.6	92.7	96.4	99.9	97.4	98.3	98.5	
FU 1 μ M	91.3	92.1	91.3	91.5	90.3	92.9	87.5	90.5	88.9	89.0	
FU 10 μ M	55.4	54.6	51.6	49.6	49.4	49.1	51.8	53.6	55.0	52.8	

5-FU Dose	Urolithin A Dose	Effect	CI
1	0.2	0.09	1.21
1	2	0.09	1.18
1	10	0.11	0.89
1	20	0.11	0.90
10	0.2	0.49	0.82
10	2	0.51	0.77
10	10	0.47	0.90
10	20	0.46	0.95

Fulvestrant is one of the ineffective agent in MET induction with KM12 cell line. On the other hand, we observed synergistic and additive effects in combinations with 5-FU. Only lowest doses of fulvestrant and 5-FU showed antagonism (Table 5.13).

Table 5. 13: Effects of fulvestrant treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.

	0 μ M		0.2 μ M		1 μ M		2 μ M		5 μ M		Fulvestrant
FU 0 μ M	99.6	100.4	89.1	99.0	107.9	103.4	102.5	99.5	93.1	91.3	
FU 1 μ M	96.9	100.5	99.4	99.4	100.0	94.2	89.7	90.0	75.8	73.5	
FU 10 μ M	55.6	55.8	57.6	55.9	54.0	55.4	52.5	53.5	45.6	44.8	

5-FU Dose	Fulvestrant Dose	Effect	CI
1	0.2	0.01	1.96
1	5	0.25	0.24
10	0.2	0.43	1.02
10	5	0.55	0.80

Interestingly, we observed additive and slight synergy in combinations with metformin that upregulates both CDH1 and VIM in KM12 cell line (Table 5.14).

Table 5. 14: Effects of metformin treatment alone or in combinations with 5-FU on cell viability of KM12 and analysis of combination index of treatments.

	0 μ M		0.1 mM		0.5 mM		1 mM		4 mM		Metformin
FU 0 μ M	100.2	99.8	100.0	109.5	102.7	103.2	88.4	92.5	71.9	71.5	
FU 1 μ M	99.0	99.3	109.4	105.5	103.3	104.2	92.0	85.1	75.1	70.3	
FU 10 μ M	61.9	63.9	57.7	58.6	56.6	55.5	51.2	48.8	51.0	43.8	

5-FU Dose	Metformin Dose	Effect	CI
1	1	0.11	0.88
1	4	0.27	1.05
10	1	0.50	0.83
10	4	0.53	1.02

Natural compound treatments in WiDr showed no MET induction. On the other hand, combinations of 5 μ M and 10 μ M of apigenin with 1 μ M of 5-FU resulted in synergy. With 10 μ M of 5-FU, we observed slight antagonisms in combinations (Table 5.15).

Table 5. 15: Effects of apigenin treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.

	0 μ M		0.2 μ M		2 μ M		5 μ M		10 μ M		Apigenin
FU 0 μ M	98.1	101.9	98.1	99.1	89.3	96.5	92.0	90.5	90.7	90.1	
FU 1 μ M	87.6	88.2	84.9	86.2	89.6	85.2	86.6	83.8	77.9	77.4	
FU 10 μ M	43.5	45.8	45.3	45.2	47.0	46.8	45.8	48.2	47.8	46.3	

5-FU Dose	Apigenin Dose	Effect	CI
1	5	0.15	0.78
1	10	0.22	0.46
10	5	0.53	1.09
10	10	0.53	1.09

In curcumin combinations, we observed that increasing doses of curcumin induced cell viability. This prevent the calculations of combination index (Table 5.16).

Table 5. 16: Effects of curcumin treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.

	0 μ M		0.1 μ M		0.5 μ M		2 μ M		5 μ M		Curcumin
FU 0 μ M	94.3	105.7	105.1	97.2	102.3	104.2	103.1	103.8	95.6	96.6	
FU 1 μ M	89.9	88.6	92.2	90.2	92.8	93.2	90.0	90.0	83.0	82.7	
FU 10 μ M	46.5	47.5	51.2	49.7	54.4	49.9	54.2	57.2	51.9	49.3	

Synergisms were observed with combination of 1 μ M and 10 μ M of 5FU with various 12 μ M of EGCG. We observed antagonisms in combinations of 10 μ M of 5-FU with 1 and 6 μ M of EGCG. Other combinations resulted in additive effects (Table 5.17).

Table 5. 17: Effects of EGCG treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.

	0 μ M		0.2 μ M		1 μ M		6 μ M		12 μ M		EGCG
FU 0 μ M	100.4	99.6	94.8	100.7	98.0	98.8	90.0	79.8	91.8	83.4	
FU 1 μ M	84.2	83.4	91.4	88.3	82.5	82.6	78.5	74.4	58.9	58.7	
FU 10 μ M	53.0	61.0	62.1	57.3	65.7	62.6	59.1	60.6	48.6	44.4	

5-FU Dose	EGCG Dose	Effect	CI
1		1	0.18 0.94
1		6	0.24 0.88
1		12	0.41 0.52
10		1	0.36 1.70
10		6	0.40 1.44
10		12	0.54 0.75

In fisetin combinations, we observed slight synergy with lower doses of 5-FU. Higher doses of 5-FU combinations resulted in additive effects (Table 5.18).

Table 5. 18: Effects of fisetin treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.

	0 μ M		0.2 μ M		2 μ M		10 μ M		15 μ M		Fisetin
FU 0 μ M	100.4	99.6	96.0	92.8	91.5	93.7	92.6	91.1	93.3	89.9	
FU 1 μ M	79.2	78.5	76.3	77.9	78.3	76.7	76.6	77.7	75.9	76.1	
FU 10 μ M	45.5	40.7	43.6	44.4	46.6	44.8	44.6	44.9	43.7	42.6	

5-FU Dose	Fisetin Dose	Effect	CI
1		10	0.23 0.85
1		15	0.24 0.78
10		10	0.55 1.12
10		15	0.57 1.00

Combination index analysis showed that none of the combinations of forskolin and 5-FU is synergistic; even we observed antagonisms in all treatments (Table 5.19).

Table 5. 19: Effects of apigenin treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.

	0 μ M		0.1 μ M		1 μ M		4 μ M		10 μ M		Forskolin
FU 0 μ M	100.1	99.9	94.6	93.6	83.6	93.0	92.5	93.0	92.0	86.0	
FU 1 μ M	64.4	63.5	60.2	63.7	64.6	60.8	79.2	80.6	79.2	75.8	
FU 10 μ M	42.9	41.0	43.1	36.9	44.0	43.5	44.0	40.7	43.5	41.4	

5-FU Dose	Forskolin Dose	Effect	CI
1		5	0.20 10.16
1		10	0.23 6.97
10		5	0.58 1.02
10		10	0.58 1.09

In curcumin combinations, we observed that increasing doses of procyanidin B2 induced cell viability. This prevent the calculations of combination index (Table 5.20).

Table 5. 20: Effects of procyanidin B2 treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.

	0 μ M	0.2 μ M	2 μ M	5 μ M	10 μ M	Procyanidin B2
FU 0 μ M	101.3 98.7	108.6 101.2	102.4 102.2	98.8 100.6	105.3 99.2	
FU 1 μ M	84.2 87.9	88.6 88.4	89.7 90.4	87.6 89.5	87.1 85.4	
FU 10 μ M	52.2 52.2	54.8 54.4	54.3 54.8	54.2 52.9	53.9 48.6	

Combination index analysis showed that none of the combinations of resveratrol and 5-FU is synergistic; even we observed antagonisms in all treatments (Table 5.21).

Table 5. 21: Effects of resveratrol treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.

	0 μ M	0.2 μ M	1 μ M	5 μ M	10 μ M	Resveratrol
FU 0 μ M	98.1 101.9	101.2 103.4	105.3 103.0	101.5 96.6	95.2 92.8	
FU 1 μ M	82.4 83.6	85.7 96.7	97.9 98.5	97.2 94.6	92.9 95.4	
FU 10 μ M	47.1 47.0	45.2 46.7	47.8 45.2	46.5 46.0	43.1 42.1	

5-FU Dose	Resveratrol Dose	Effect	CI
1	5	0.04	9.17
1	10	0.06	5.83
10	5	0.54	1.13
10	10	0.57	1.12

In WiDr cell line, combination index analysis showed that none of the combinations of urolithin A and 5-FU is synergistic; even we observed antagonisms in all treatments (Table 5.22).

Table 5. 22: Effects of urolithin A treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.

	0 μ M	0.2 μ M	2 μ M	10 μ M	20 μ M	Urolithin A
FU 0 μ M	99.6 100.4	102.0 101.9	99.9 101.7	98.7 100.8	95.2 94.1	
FU 1 μ M	77.7 79.8	81.9 83.0	86.1 87.8	83.2 81.6	80.4 76.1	
FU 10 μ M	33.9 36.1	37.5 38.0	35.3 38.8	37.8 37.7	36.6 35.7	

5-FU Dose	Urolithin A Dose	Effect	CI
1	10	0.18	1.67
1	20	0.22	1.63
10	10	0.62	1.41
10	20	0.64	1.53

5-FU and fulvestrant combinations showed synergy with the CI ranging from 0.95 to 0.54, even though there was no significant MET induction with fulvestrant in this cell line (Table 5.23).

Table 5. 23: Effects of fulvestrant treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.

	0 μ M	0.2 μ M	1 μ M	2 μ M	5 μ M	Fulvestrant
FU 0 μ M	98.3 101.7	99.6 99.9	100.0 99.0	94.9 91.5	87.1 81.2	
FU 1 μ M	90.9 89.8	87.8 93.1	90.8 92.7	88.5 81.6	73.8 73.0	
FU 10 μ M	42.0 40.1	44.7 42.4	48.5 39.1	36.3 38.6	32.7 32.2	

5-FU Dose	Fulvestrant Dose	Effect	CI
1	2	0.15	0.95
1	5	0.27	0.58
10	2	0.63	0.88
10	5	0.68	0.74

Interestingly, we observed slight synergy in combinations with metformin that upregulates both CDH1 and VIM in WiDr cell line. (Table 5.24)

Table 5. 24: Effects of metformin treatment alone or in combinations with 5-FU on cell viability of WiDr and analysis of combination index of treatments.

	0 mM	0.1 mM	0.5 mM	1 mM	4 mM	Metformin
FU 0 μ M	99.1 100.9	105.2 111.5	107.6 101.6	96.2 102.4	89.5 84.3	
FU 1 μ M	77.5 82.7	85.8 81.0	77.5 76.0	75.5 74.9	75.3 73.4	
FU 10 μ M	36.7 39.0	38.8 37.2	36.4 36.7	34.8 34.7	32.4 29.7	

5-FU Dose	Metformin Dose	Effect	CI
1	1	0.25	0.87
1	4	0.26	1.32
10	1	0.65	0.91
10	4	0.69	0.94

Additionally, we used rectal cancer cell line SW837 in our analysis to examine the ability of natural products and repurposed agents as a chemotherapy enhancer by combining irinotecan, which is a component of FOLFIRI that is routinely used in colorectal cancer, mainly as a second line treatment. Combination of 2 μ M of irinotecan and 1 μ M of apigenin resulted in synergy. Other combinations showed additive effect and antagonisms (Table 5.25).

Table 5. 25: Effects of apigenin treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.

	0 μ M		1 μ M				10 μ M				Apigenin
Irinotecan 0 μ M	97.2	102.1	99.6	101.3	97.2	98.9	77.6	81.2	83.6	89.1	
Irinotecan 2 μ M	88.8	94.4	92.5	90.5	85.8	84.7	68.2	78.6	78.2	82.1	
Irinotecan 40 μ M	49.8	53.0	48.9	50.9	51.5	51.4	47.6	50.6	51.5	52.2	

Irinotecan Dose	Apigenin Dose	Effect	CI
2	1	0.12	0.74
2	10	0.23	0.96
40	1	0.49	1.01
40	10	0.49	1.29

In SW837 cell line, combination index analysis showed that none of the combinations of curcumin and irinotecan is synergistic; even we observed antagonisms in all treatments (Table 5.26).

Table 5. 26: Effects of curcumin treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.

	0 μ M		1 μ M				10 μ M				Curcumin
Irinotecan 0 μ M	98.8	101.2	98.7	97.5	100.0	103.5	61.5	59.2	55.7	55.7	
Irinotecan 2 μ M	94.6	94.9	100.3	106.9	104.6	100.8	62.5	63.3	60.4	59.3	
Irinotecan 40 μ M	54.8	55.5	63.3	62.9	58.0	57.1	28.1	33.2	31.7	29.3	

Irinotecan Dose	Curcumin Dose	Effect	CI
2	1	0.00	76.65
2	10	0.39	1.11
40	1	0.40	1.35
40	10	0.69	1.01

Combination index analysis showed that none of the combinations of EGCG and irinotecan is synergistic; even we observed antagonisms in all treatments (Table 5.27).

Table 5. 27: Effects of treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.

	0 μ M		2 μ M				20 μ M				EGCG
Irinotecan 0 μ M	99.6	101.2	92.5	92.4	100.7	93.7	6.3	1.8	2.0	1.3	
Irinotecan 2 μ M	91.7	94.1	86.9	92.8	89.7	85.5	7.4	8.4	8.3	7.3	
Irinotecan 40 μ M	54.8	48.1	57.7	52.4	46.3	46.2	2.5	3.3	2.0	2.4	

Irinotecan Dose	EGCG Dose	Effect	CI
2	2	0.11	1.29
2	20	0.92	1.45
40	2	0.49	1.33
40	20	0.97	1.02

Combination index analysis showed that none of the combinations of fisetin and irinotecan is synergistic; only 15 μ M Fisetin and irinotecan combinations resulted in additive effect. (Table 5.28).

Table 5. 28: Effects of treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.

	0 μ M		1.5 μ M				15 μ M				Fisetin
Irinotecan 0 μ M	97.2	102.8	97.4	103.4	96.3	102.6	79.7	81.6	83.5	82.1	
Irinotecan 2 μ M	91.9	94.9	97.1	95.4	98.3	95.3	73.4	73.9	73.9	73.6	
Irinotecan 40 μ M	49.1	56.0	58.8	58.2	53.6	55.1	40.2	41.3	39.2	40.5	

Irinotecan Dose	Fisetin Dose		Effect	CI
2	1.5		0.04	2.42
2	15		0.26	0.97
40	1.5		0.44	1.21
40	15		0.60	0.99

Combination index analysis showed that none of the combinations of Forskolin and irinotecan is synergistic; even we observed antagonisms in all treatments (Table 5.29).

Table 5. 29: Effects of forskolin treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.

	0 μ M		1 μ M				10 μ M				Forskolin
Irinotecan 0 μ M	99.9	100.8	102.4	100.9	98.9	96.2	100.7	99.7	103.1	93.5	
Irinotecan 2 μ M	92.8	93.6	94.1	96.1	94.7	95.2	93.6	88.4	99.2	92.7	
Irinotecan 40 μ M	48.2	52.1	58.3	60.4	57.6	57.0	56.3	54.8	55.8	51.5	

Irinotecan Dose	Forskolin Dose		Effect	CI
2	1		0.05	1.74
2	10		0.07	1.69
40	1		0.42	1.44
40	10		0.45	1.22

Procyanidin B2 which has an effect on neither inhibition of cellular viability, nor induction of MET in colon cancer cell lines, exhibited synergism with the CI ranging from 0.89 to 0.35 in SW837 (Table 5.30).

Table 5. 30: Effects of procyanidin B2 treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.

	0 μ M		2 μ M				20 μ M				Procyanidin B2
Irinotecan 0 μ M	103.2	96.1	93.4	90.3	94.3	92.8	87.8	90.3	87.9	87.1	
Irinotecan 2 μ M	91.5	86.4	85.8	81.9	85.9	84.7	80.0	77.0	79.7	73.7	
Irinotecan 40 μ M	52.5	46.8	48.5	45.0	48.1	49.4	45.0	45.3	43.0	41.0	

Irinotecan Dose	Procyanidin B2 Dose		Effect	CI
2	2		0.15	0.64
2	20		0.22	0.35
40	2		0.52	0.89
40	20		0.56	0.71

Combination index analysis showed the combinations of resveratrol and irinotecan is slightly additive (Table 5.31).

Table 5. 31: Effects of resveratrol treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.

	0 μ M		1 μ M				10 μ M				Resveratrol
Irinotecan 0 μ M	101.4	98.6	94.3	94.5	100.3	95.1	77.7	75.6	72.7	76.4	
Irinotecan 2 μ M	84.6	91.1	90.3	91.9	95.5	91.7	77.4	73.7	75.6	71.7	
Irinotecan 40 μ M	50.5	50.4	53.9	52.6	55.9	51.8	46.7	44.8	46.2	44.6	

Irinotecan Dose	Resveratrol Dose	Effect	CI
2	1	0.08	1.08
2	10	0.25	1.07
40	1	0.46	0.99
40	10	0.54	0.91

In SW837 cell line, urolithin A combinations with 40 μ M of irinotecan resulted in synergy. 2 μ M of irinotecan and 20 μ M of urolithin A combination resulted in a slight additive effect (Table 5.32).

Table 5. 32: Effects of urolithin A treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.

	0 μ M		2 μ M				20 μ M				Urolithin A
Irinotecan 0 μ M	98.8	100.8	100.0	96.0	99.4	96.9	78.9	78.8	77.2	77.2	
Irinotecan 2 μ M	87.7	94.3	87.5	92.0	92.2	90.8	77.3	76.2	70.3	67.1	
Irinotecan 40 μ M	51.8	57.4	57.6		51.5	59.6	49.1	44.7	44.1	41.5	

Irinotecan Dose	Urolithin A Dose	Effect	CI
2	2	0.09	1.17
2	20	0.27	0.94
40	2	0.58	0.50
40	20	0.55	0.84

Interestingly, 40 μ M of irinotecan and 5 μ M of fulvestrant combination resulted in synergy, other combinations also showed additive effect (Table 5.33).

Table 5. 33: Effects of fulvestrant treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.

	0 μ M		0.5 μ M				5 μ M				Fulvestrant
Irinotecan 0 μ M	98.6	101.4	96.0	90.5	97.8	94.2	72.9	72.6	73.5	67.7	
Irinotecan 2 μ M	86.7	86.2	84.5	94.2	92.0	88.7	66.2	69.1	65.2	63.3	
Irinotecan 40 μ M	52.3	49.8	48.7	49.3	53.2	50.8	35.7	39.1	34.9	34.7	

Irinotecan Dose	Fulvestrant Dose	Effect	CI
2	0.5	0.10	2.17
2	5	0.34	0.86
40	0.5	0.49	1.04
40	5	0.64	0.54

We observed that irinotecan and 4 μ M of metformin combinations resulted in synergy (Table 5.34).

Table 5. 34: Effects of metformin treatment alone or in combinations with irinotecan on cell viability of SW837 and analysis of combination index of treatments.

	0 mM		0.4 mM				4 mM				Metformin
Irinotecan 0 μ M	99.6	100.4	98.3	100.0	97.0	101.9	87.0	84.6	82.6	78.7	
Irinotecan 2 μ M	85.9	93.0	83.9	91.7	91.0	89.9	72.2	74.2	73.6	73.9	
Irinotecan 40 μ M	54.5	49.3	50.7	54.7	51.5	50.7	44.0	40.7	43.1	41.2	

Irinotecan Dose	Metformin Dose	Effect	CI
2	0.4	0.11	1.26
2	4	0.26	0.46
40	0.4	0.48	1.00
40	4	0.58	0.57

5.6.4. Combination of curcumin and resveratrol with irinotecan in pancreatic cancer cell lines

We next investigated the combinatory effects of natural products and chemotherapy drugs using constant-ratio combinations of irinotecan, commonly used chemotherapeutic in pancreatic cancer, with widely studied natural compounds curcumin and resveratrol in pancreatic cancer cell lines, AsPC-1, BxPC-3, MIA PaCa-2 and SU8686 (Figure 5.59 and Figure 5.60). Most of the curcumin/irinotecan combinations resulted in antagonistic effect while 15 μ M irinotecan and 20 μ M curcumin combination in AsPC-1 and SU8686 cell lines showed synergy. Only the highest dose combinations of irinotecan and resveratrol resulted in slight synergy in BxPC-3, MIA PaCa-2 and SU8686. Although we observed mild induction of MET by natural products in MIA PaCa-2 cell line, this did not enhance irinotecan sensitivity.

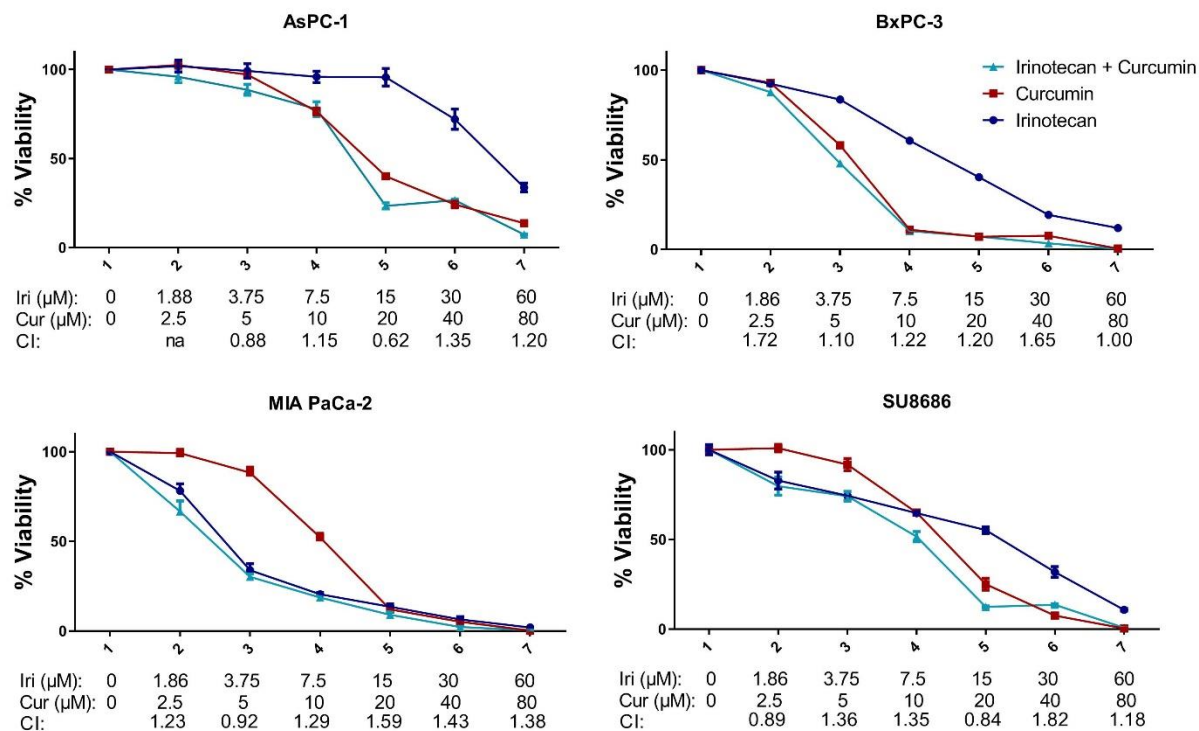


Figure 5. 59: Combination treatment of curcumin and irinotecan in pancreatic cancer cell lines. The cells were treated with indicated concentrations for 72 h. Data are presented as the mean $\pm$ SD of 4 replicates. CI=Combination Index Value, CI<1 indicates synergy.

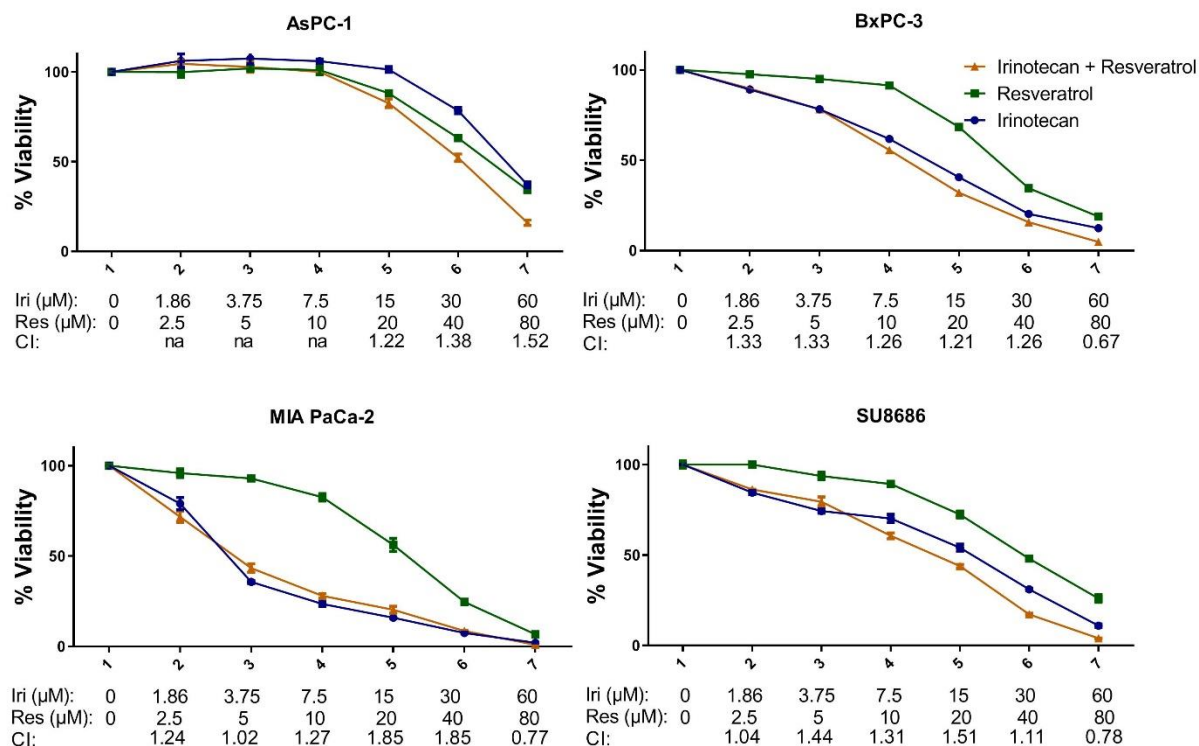


Figure 5.60: Combination treatment of resveratrol and irinotecan in pancreatic cancer cell lines. The cells were treated with indicated concentrations for 72 h. Data are presented as the mean $\pm$ SD of 4 replicates. CI=Combination Index Value, CI<1 indicates synergy.

5.6.5. Combinatory effects of natural compounds and gemcitabine in MIA PaCa-2 cells.

Gemcitabine is considered the standard agent for the treatment of pancreatic cancer either alone or in combination. Therefore, we evaluated the combinatory effects of natural products in MIA PaCa-2 cell line, with gemcitabine (Figure 5.61). Six different combinations of gemcitabine were combined with the single dose of natural products. Synergy was observed in only 10 μ M of resveratrol and two doses of Gemcitabine combinations. On the other hand, combinations of low dose gemcitabine showed additive effects in MIA PaCa-2, however, the effect evolved from additive into antagonistic at higher doses. The fact that synergy was observed with only gemcitabine among three conventional drugs (irinotecan, gemcitabine, gefitinib) combined with resveratrol shows that synergistic effects of a specific natural compound cannot be generalized even within

one cell line, instead the specific molecular cascades targeted by natural compound-drug pair are most likely critical factors. Overall, these data indicated that there is no perfect overlap between MET inducing and chemo-sensitizing compounds, which is likely to stem from the existence of more complex mechanisms underlying synergy patterns.

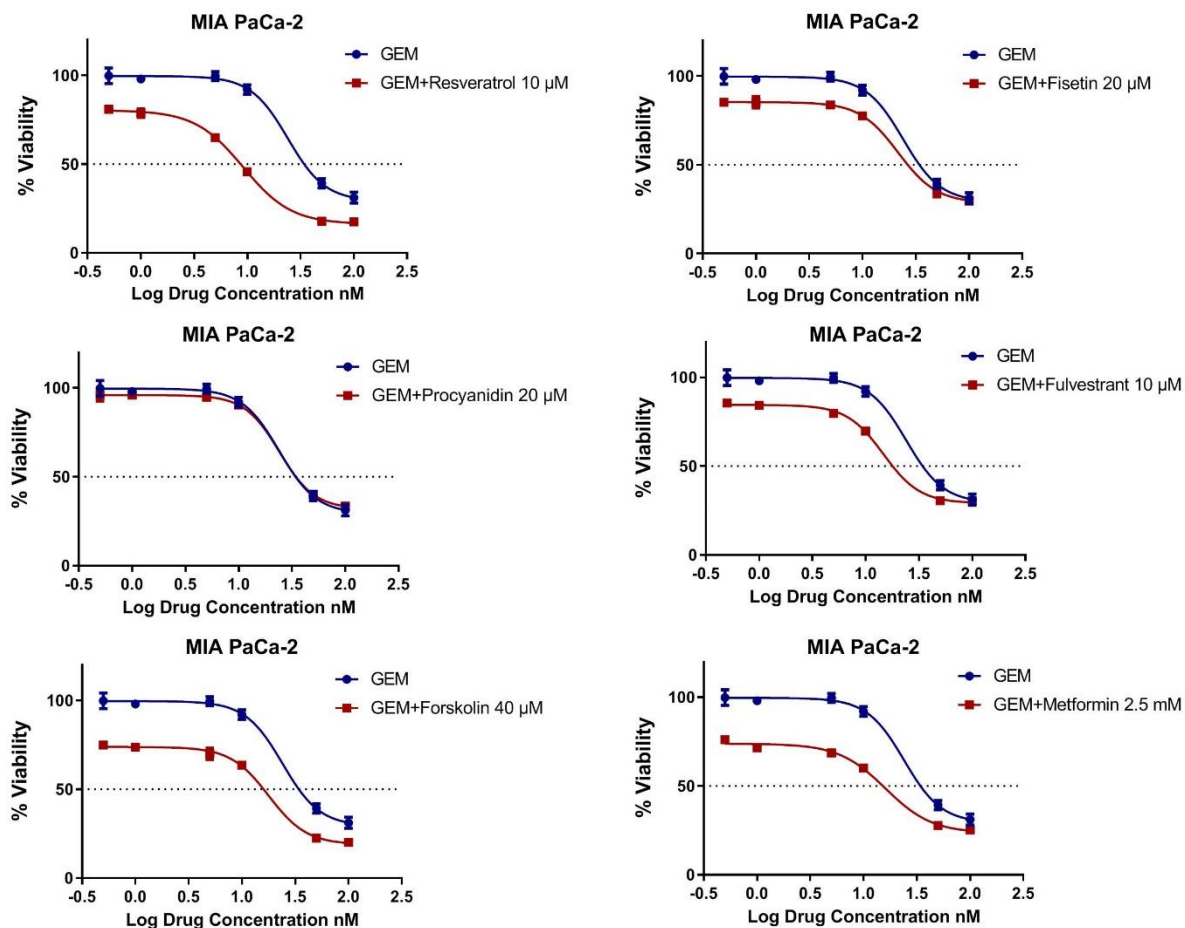


Figure 5. 61: Combination treatment of natural compounds and gemcitabine in MIA PaCa-2 pancreatic cancer cell line. The cells were treated with indicated concentrations for 72 h. Data were presented as the mean $\pm$ SD of 4 replicates.

5.6.6. Combination treatment of natural products and targeted agent Gefitinib in MIA PaCa-2 cells

We then evaluated the combinatory effects of natural products in MIA PaCa-2 cell line and the targeted agent gefitinib, an EGFR inhibitor; since EGFR inhibition has shown promising activity in pancreatic cancer (Figure 5.62). Six different combinations gefitinib were combined with the single dose of natural products. No synergistic effect was noted for gefitinib. On the other hand, combinations of low dose gefitinib, showed additive effects in MIA PaCa-2, however, the effect evolved from additive into antagonistic at higher doses. The fact that synergy was observed with only gemcitabine among three conventional drugs (irinotecan, gemcitabine, gefitinib) combined with resveratrol shows that synergistic effects of a specific natural compound cannot be generalized even within one cell line, instead the specific molecular cascades targeted by natural compound-drug pair are most likely critical factors. Overall, these data indicated that there is no perfect overlap between MET inducing and chemo-sensitizing compounds, which is likely to stem from the existence of more complex mechanisms underlying synergy patterns.

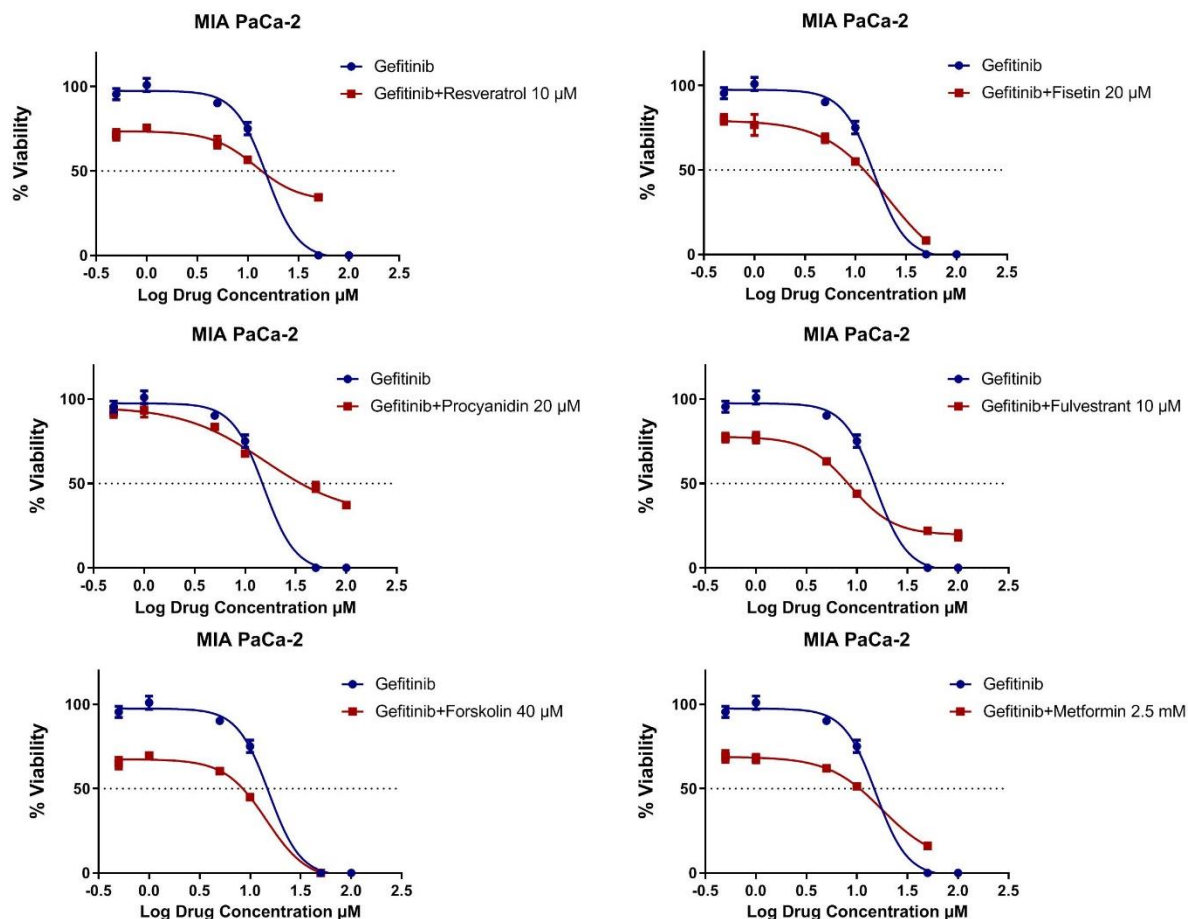


Figure 5. 62: Combination treatment of natural compounds and gefitinib in MIA PaCa-2 pancreatic cancer cell line. The cells were treated with indicated concentrations for 72 h. Data are presented as the mean $\pm$ SD of 4 replicates.

5.6.7. Combination treatment of natural products and targeted agent Selumetinib in MIA PaCa-2 cells

We then evaluated the combinatory effects of natural products in MIA PaCa-2 cell line and the targeted agent selumetinib, MEK kinase inhibitor (Figure 5.63). Six different combinations selumetinib were combined with the single dose of natural products. No synergistic effect was noted for selumetinib.

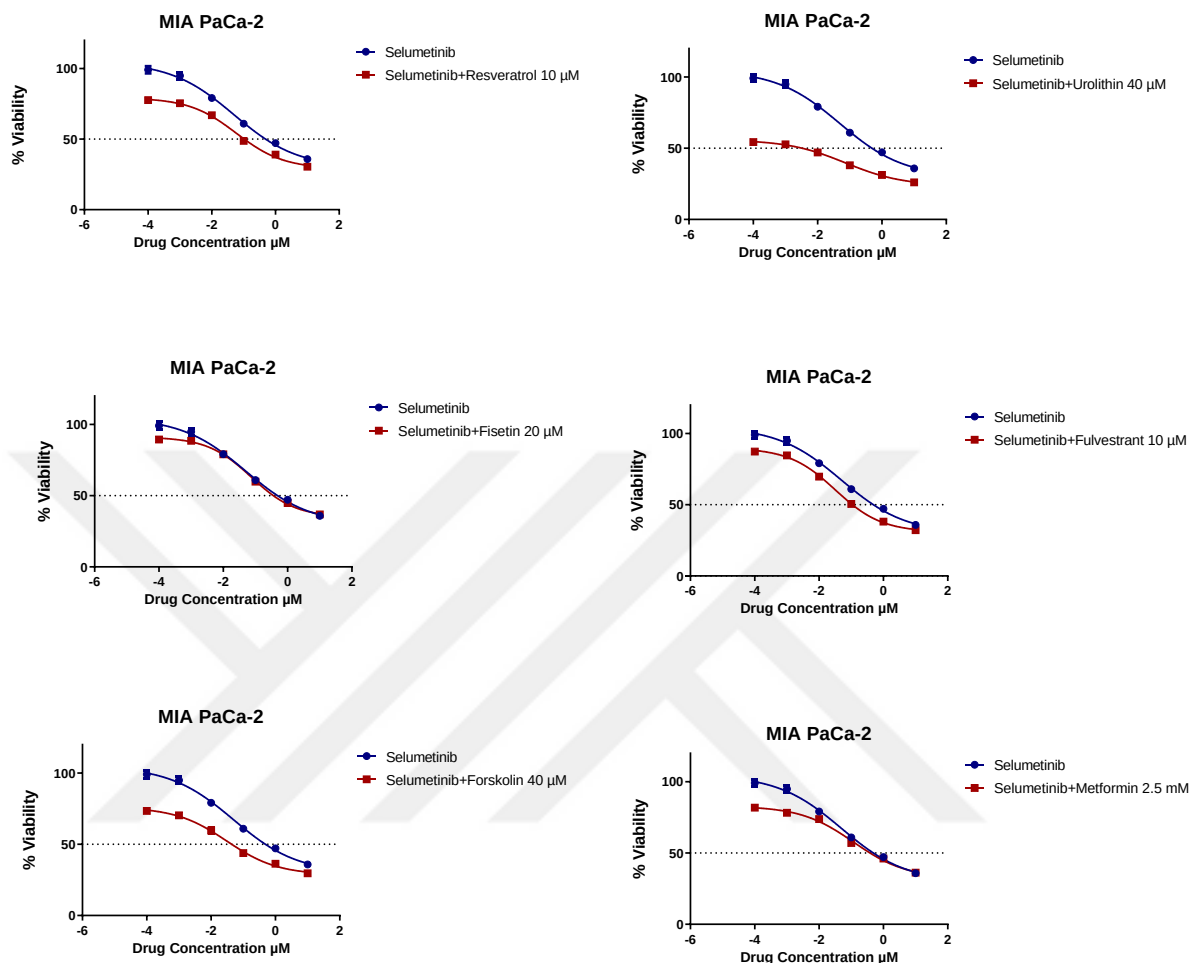


Figure 5. 63: Combination treatment of natural compounds and Selumetinib in MIA PaCa-2 pancreatic cancer cell line. The cells were treated with indicated concentrations for 72 h. Data are presented as the mean $\pm$ SD of 4 replicates.

Among the compounds we examined in this study, almost none were found to both trigger MET and induce chemosensitization. (Table 5. 35) In KM12, although fisetin and forskolin induced MET, the fisetin and 5-FU combination did not show a synergistic effect. On the other hand, Urolithin A synergized with 5-FU in KM12, in the absence of MET induction. Furthermore, slight MET induction in MIA PaCa-2 with natural compounds did not lead to improved sensitivity to irinotecan, gemcitabine and gefitinib in almost all combinations.

Table 5. 35: Summary table of combinational treatments.

Chemotherapeutic	Synergy with chemotherapeutics								
	5-FU	5-FU	Iritonecan	Iritonecan	Iritonecan	Iritonecan	Gemcitabine	Gefitinib	Iritonecan
	WiDr	KM12	SW837	AsPC-1	BxPC-3	MIA PaCa-2	MIA PaCa-2	MIA PaCa-2	SU8686
Apigenin	strong	no	no	-	-	-	-	-	-
Curcumin	no	no	no	strong	no	no	-	-	no
EGCG	strong	no	no	-	-	-	-	-	-
Fisetin	weak	no	no	-	-	-	no	no	-
Forskolin	no	strong	no	-	-	-	no	no	-
Procyanidin B2	no	no	strong	-	-	-	no	no	-
Resveratrol	no	no	no	no	weak	weak	strong	no	weak
Urolithin A	no	weak	strong	-	-	-	-	-	-
Fulvestrant	weak	strong	weak	-	-	-	no	no	-
Metformin(mM)	weak	weak	strong	-	-	-	no	no	-
* -: no data									
*no: CI>1									
*strong: CI<0.7									
*weak: 1<CI<0.7									

5.7. Intrinsic resistance against c-Src inhibitor in melanoma and strategies for overcoming

5.7.1. Inhibitory effects of c-Src inhibitor 10a on 15 primary melanoma cells

To investigate the efficacy of c-Src inhibitor 10a, cytotoxic responses to 10a tested on 15 melanoma cells. After seeding and incubation of melanoma cells for 24 hours, cells were treated with 10 using 8 different concentrations ranging from 0.01 μ M to 50 μ M. Drug sensitivity against 10a occurred over a remarkably wide range of doses with IC₅₀ values ranging from 0.09 to 9.01 μ M (Table 5.36).

Table 5. 36: The half-maximal inhibitory concentration (IC₅₀) values for c-Src inhibitor 10a in melanoma cells

Melanoma cells	IC ₅₀ (μ M)
M307	0.09
M133	0.17
M189	0.23
SHM20A2	0.36
M187	0.72
M144	1.88
M24	2.10
M34	2.43
M77	4.32
M7	4.86
M1	5.90
M433	5.90
M2025	7.75
M221	8.95
M193	9.01

5.7.2. mRNA based biomarkers to predict chemosensitivity against 10a

The variation in drug response to 10a strongly encourage us to reveal underlying molecular differences among these cells. Thus, gene signature of chemosensitivity against 10a were investigated by using gene expression data from these melanoma cells. For this purpose, the gene expression data were correlated with IC₅₀ values using 2-fold cross validation with a 1000 iteration method. Briefly, in each iteration, cells were randomly divided into two groups, and a Pearson's r value between 10a IC₅₀ and gene expression data for each gene was calculated for these groups. This was repeated 1000 times. We then determined whether the correlations obtained for a given gene were consistently in the same direction (either positively or negatively correlated in both groups) where the results were statistically significant in all groups. This analysis showed that expression of 9 genes was consistently and significantly correlated with IC₅₀ values in more than 900 iterations in the same direction. In more than 800 iterations, expression levels of 24 genes showed consistent and significant correlations with IC₅₀ values in the same directions. We chose 46 genes whose expression profile showed significant concordance with chemosensitivity of 10a in more than 700 iterations for classifying melanoma cells (Table 5.37). Gene list with their significant iteration numbers in 1000 iterations can be shown in Table 5.38.

Table 5. 37: Number of genes with a frequency of significance in more than 900, 800 and 700 iterations.

	>900		>800		>700	
	# of genes	Opposite Direction	# of genes	Opposite Direction	# of genes	Opposite Direction
10a	9	0	24	0	46	0

Table 5. 38: List of 46 genes that were significantly associated with 10a cytotoxicity values.

Gene	Same Direction Positive r	Same Direction Negative r	Opposite Direction
PDLIM4	982	0	0
GNAS	949	0	0
IL13RA1	945	0	0
PLOD2	934	0	0
TMED7	926	0	0
ERLIN2	918	0	0

CREG1	909	0	0
PROCR	894	0	0
C5orf15	847	0	0
LAMP2	835	0	0
SGCB	804	0	0
PTGES	794	0	0
PGK2	768	0	0
C1GALT1C1	743	0	0
TBC1D9B	717	0	0
CAST	716	0	0
ING4	715	0	0
CCNG2	711	0	0
PTBP1	0	972	0
DDX19A	0	923	0
PARVB	0	889	0
NCL	0	878	0
NSUN5	0	871	0
RRP1B	0	868	0
FUBP1	0	859	0
MDC1	0	857	0
SMCR7L	0	843	0
EXOSC7	0	834	0
RRP1	0	822	0
KATNB1	0	812	0
HUWE1	0	802	0
UBAP2	0	798	0
DUS1L	0	792	0
CAD	0	783	0
AZIN1	0	782	0
USP13	0	776	0
RBM12	0	767	0
MTERFD2	0	765	0
TTC15	0	749	0
GNL3L	0	746	0
C14orf169	0	744	0
TBRG4	0	744	0
PSME3	0	730	0
XRCC5	0	726	0
HIRIP3	0	718	0
SAP30BP	0	716	0

Hierarchical clustering analysis using gene expression data of chemosensitivity associated 46 genes revealed two subgroups of cells which are relatively sensitive and resistant to c-Src inhibitor 10a (Figure 5.64) According to hierarchical clustering, these subgroups of melanoma cells and their IC50 distribution were depicted in Figure 5.65.

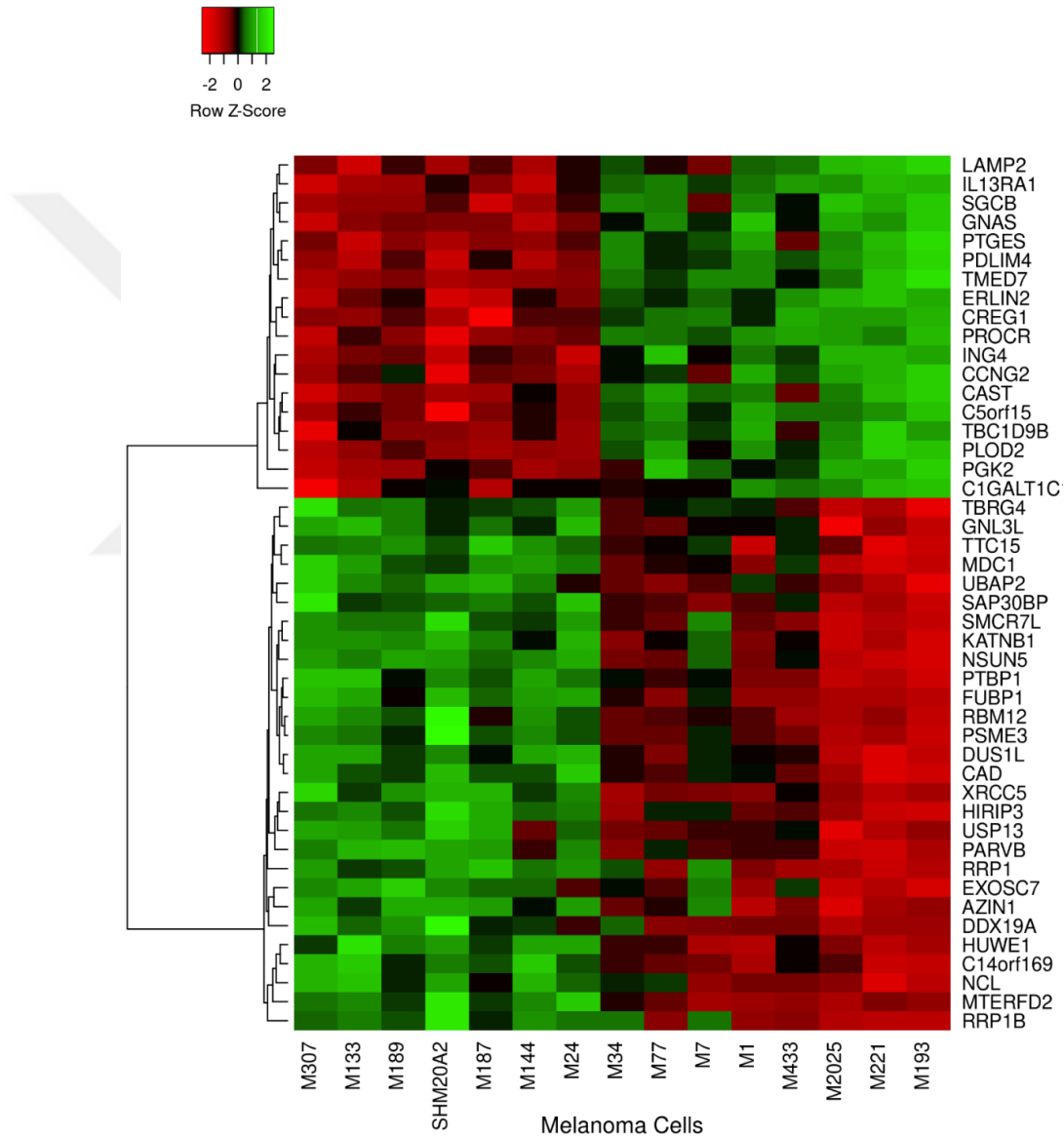


Figure 5. 64: Hierarchical clustering of cell lines according to 10a chemosensitivity biomarker genes.

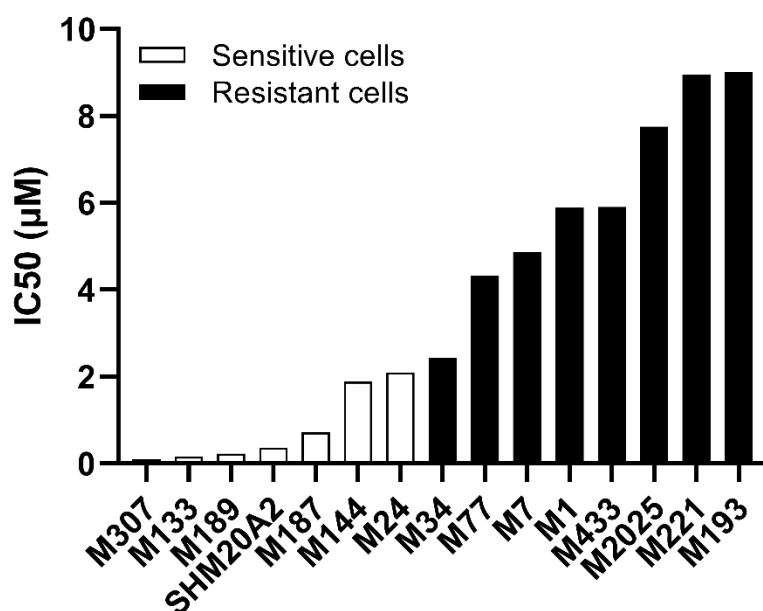


Figure 5. 65: Sensitivity profiles of fifteen melanoma cells. Cytotoxicity is expressed in terms of IC50 (y-axis).

5.7.3. Biological characteristics of 10a-sensitive and resistant subgroups

In order to perceive the biological differences between 10a-sensitive and resistant subgroups of melanoma cells, gene set enrichment analysis (GSEA) were performed. GSEA revealed a significant enrichment (FDR q value<0.25) in gene sets, including E2F target genes associated with cell cycle and DNA strand elongation related genes in the sensitive group, suggesting a proliferative phenotype in these melanoma cells (Figure 5.66).

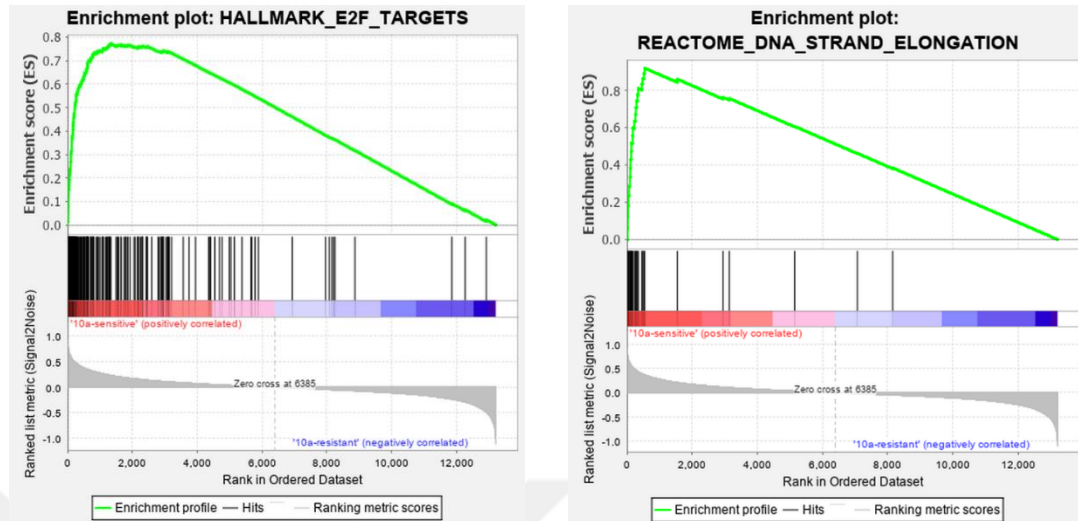


Figure 5.66: Comparative analysis of differentially enriched molecular gene sets among sensitive and resistant cells by GSEA. Gene sets including cell cycle related targets of E2F transcription factor and DNA strand elongation related genes were enriched in 10a-sensitive melanoma cells.

On the other hand, resistant melanoma cells showed significant enrichments of gene sets associated with epithelial-to-mesenchymal transition and multicancer invasiveness signatures, indicating an invasive/differentiation phenotype in resistant melanoma cells (Figure 5.67).

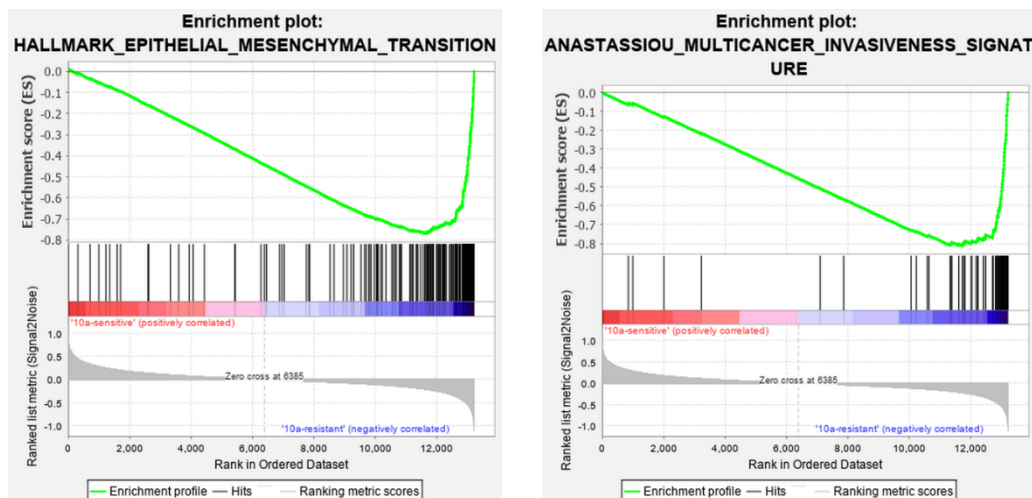


Figure 5.67: Comparative analysis of differentially enriched molecular gene sets among sensitive and resistant cells by GSEA. Gene sets defining EMT as in wound healing, fibrosis, and metastasis and multicancer invasion associated genes showed enrichment in 10a-resistant melanoma cells.

Significant gene sets (FDR q value<0.001) enriched in either sensitive and resistant melanoma cells by using “Hallmark”, “Curated” and “Gene Ontology (GO)” databases of Broad Institute were also listed in Table 5.39.

Table 5. 39: Enriched gene sets in 10a-sensitive and –resistant melanoma cells.

Enriched Gene Sets in 10a-Sensitive Cell Lines							
	Gene Set	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val
Hallmark	HALLMARK_E2F_TARGETS	187	0.77	3.29	<0.001	<0.001	<0.001
	HALLMARK_G2M_CHECKPOINT	195	0.71	3.02	<0.001	<0.001	<0.001
	HALLMARK_MYC_TARGETS_V1	188	0.59	2.52	<0.001	<0.001	<0.001
	HALLMARK_MYC_TARGETS_V2	56	0.65	2.26	<0.001	<0.001	<0.001
	HALLMARK_MITOTIC_SPINDLE	174	0.48	2.02	<0.001	<0.001	<0.001
Curated	ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER	136	0.85	3.49	<0.001	<0.001	<0.001
	SOTIRIOU_BREAST_CANCER_GRADE_1_VS_3_UP	145	0.82	3.43	<0.001	<0.001	<0.001
	CROONQUIST_IL6_DEPRIVATION_DN	89	0.88	3.33	<0.001	<0.001	<0.001
	DUTERTRE ESTRADIOL_RESPONSE_24HR_UP	253	0.74	3.26	<0.001	<0.001	<0.001
	MANALO_HYPOXIA_DN	277	0.72	3.24	<0.001	<0.001	<0.001
GO	GO_DNA_DEPENDENT_DNA_REPLICATION	117	0.71	2.82	<0.001	<0.001	<0.001
	GO_SISTER_CHROMATID_SEGREGATION	141	0.68	2.81	<0.001	<0.001	<0.001
	GO_DNA_REPLICATION	215	0.64	2.8	<0.001	<0.001	<0.001
	GO_NUCLEAR_CHROMOSOME_SEGREGATION	181	0.64	2.75	<0.001	<0.001	<0.001
	GO_CHROMOSOME_SEGREGATION	225	0.63	2.75	<0.001	<0.001	<0.001
Enriched Gene Sets in 10a-Resistant Cell Lines							
	Gene Set	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val
Hallmark	HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	196	-0.77	-3.21	<0.001	<0.001	<0.001
	HALLMARK_HYPOXIA	189	-0.6	-2.52	<0.001	<0.001	<0.001
	HALLMARK_UV_RESPONSE_DN	143	-0.6	-2.4	<0.001	<0.001	<0.001
	HALLMARK_TNFA_SIGNALING_VIA_NFKB	197	-0.57	-2.38	<0.001	<0.001	<0.001
	HALLMARK_ANGIOGENESIS	34	-0.68	-2.12	<0.001	<0.001	<0.001
Curated	REN_ALVEOLAR_RHABDOMYOSARCOMA_DN	393	-0.71	-3.2	<0.001	<0.001	<0.001
	BOQUEST_STEM_CELL_UP	252	-0.71	-3.05	<0.001	<0.001	<0.001
	SCHUETZ_BREAST_CANCER_DUCTAL_INVASIVE_UP	347	-0.67	-2.97	<0.001	<0.001	<0.001
	ONDER_CDH1_TARGETS_2_UP	251	-0.68	-2.91	<0.001	<0.001	<0.001
	ANASTASSIOU_MULTICANCER_INVASIVENESS_SIGNATU	64	-0.81	-2.9	<0.001	<0.001	<0.001
GO	GO_EXTRACELLULAR_MATRIX_STRUCTURAL_CONSTITUE	134	-0.7	-2.78	<0.001	<0.001	<0.001
	GO_EXTRACELLULAR_MATRIX	422	-0.58	-2.64	<0.001	<0.001	<0.001
	GO_BASEMENT_MEMBRANE	74	-0.72	-2.59	<0.001	<0.001	<0.001
	GO_COLLAGEN_CONTAINING_EXTRACELLULAR_MATRIX	334	-0.57	-2.53	<0.001	<0.001	<0.001
	GO_EXTRACELLULAR_STRUCTURE_ORGANIZATION	368	-0.56	-2.51	<0.001	<0.001	<0.001

5.7.4. Protein–chemical interaction network analysis to further investigate resistance mechanism

To further investigate mechanisms behind resistance to 10a, we performed protein–chemical interaction network analysis with 46 genes using the NetworkAnalyst online tool. This revealed that, out of 18 resistance associated proteins, 15 extensively interact with a pyrazolopyrimidine derivative and three histone deacetylase (HDAC) inhibitors, valproic acid, trichostatin A, and entinostat (Figure 5.68). Expectedly, our analysis revealed (6-(4-(2-piperidin-1-ylethoxy) phenyl))-3- pyridin-4-ylpyrazolo(1,5-a) pyrimidine, as a hub chemical, which is structurally similar to pyrazolopyrimidine derivative 10a, showing interactions with 12 resistance related proteins. Interestingly, three HDAC inhibitors have interactions mostly with resistance-associated genes, and are also known as differentiating agents. Valproic acid was a hub chemical with the highest number of interactions.

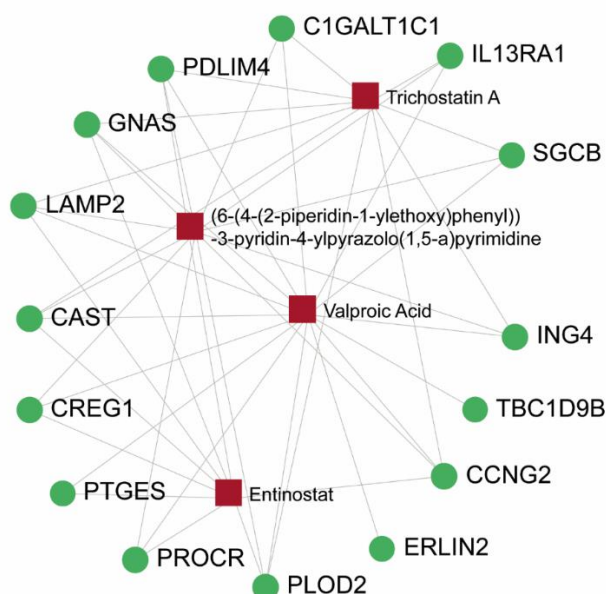


Figure 5. 68: Protein-chemical interaction network with chemosensitivity associated genes. Network was constructed using the NetworkAnalyst online tool to identify chemical interacts with 10a-sensitivity associated proteins. Red squares represent the chemicals, green nodes represent proteins interacting with chemicals and edges connect proteins to interacting chemicals. Betweenness filter was applied to obtain hub chemicals. Valproic acid interacts with 15 proteins represented in the network while (6-(4-(2-piperidin-1-ylethoxy) phenyl))-3-pyridin-4-ylpyrazolo (1,5-a) pyrimidine interacts with 12 proteins, Trichostatin a interacts with 10, Entinostat interacts with 9.

5.7.5. Use of valproic acid to enhance chemotherapy against 10a

Because our previous findings validated proliferative and invasive phenotypes in chemosensitive and resistant cells, respectively, protein chemical network analysis prompted us to use valproic acid to induce differentiation to enhance sensitivity of resistant cells to 10a. Cell viability was assessed 72 h after treatments. In M221 cells, 20 μ M 10a treatment resulted in a 55% reduction in viability, whereas little or no decrease in viability was observed with 50, 200, and 800 nM of sodium valproate treatment alone. However, combining 50 nM sodium valproate with 20 μ M 10a resulted in a 75% decrease in viability. This effect increased to 84% when 200 nM sodium valproate was used. The most significant effect was observed at 20 μ M 10a and 0.8 mM sodium valproate treatment, where a 92% decrease in cell viability was seen (Figure 5.69).

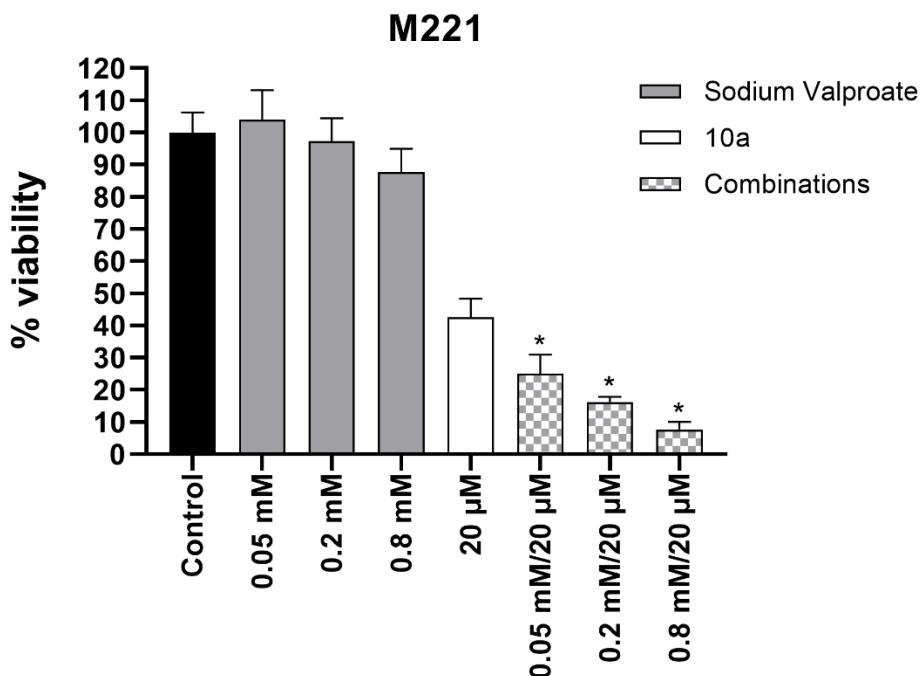


Figure 5. 69: Combination treatment of 10a-resistant M221 melanoma cells with 10a and sodium valproate. After 24 hours of cell seeding, cells were treated with different doses (0.05 mM to 0.8 mM) of Sodium Valproate in the absence or presence of 10a. Viable cells were quantified using Cell-Titer Glo assay after 72 hours. *p <0.05 (t-test).

To quantify the degree of synergism, the combination index (CI) was calculated by the Chou–Talalay method in which $CI < 1$ shows synergistic effects, $CI = 1$ shows additive effects, and $CI > 1$ shows antagonistic effects.¹⁹ Combined 10a and sodium valproate treatment showed synergistic effects for all combinations with CI values ranging from 0.766 to 0.603 (Table 5.40).

Table 5. 40: Combination Index values of combinations in M221 melanoma cells.

<u>Dose (μM)</u> 10a	<u>Dose (mM)</u> Sodium Valproate	<u>Effect</u>	<u>Combination Index (CI)</u>
20.0	0.05	0.75	0.766
20.0	0.2	0.84	0.681
20.0	0.8	0.924	0.603

Similarly, the sodium valproate–10a combination showed synergy in the more resistant M193 cells as well (Figure 5.70). Twenty micromolar 10a treatment alone, decreased viability to 47% in M193 cells, whereas 0.8, 4, and 6 mM of sodium valproate showed little or no decrease in viability. Adding 0.8 mM sodium valproate to 20 μM of 10a resulted in a 75% decrease in viability. Combining 20 μM 10a and 4 mM sodium valproate treatment resulted in an 84% reduction in viability. Twenty micromolar 10a and 6 mM sodium valproate combination resulted in 89% decrease in viability. CI values ranging from 0.835 to 0.698 suggested synergism in all combinations (Table 5.41).

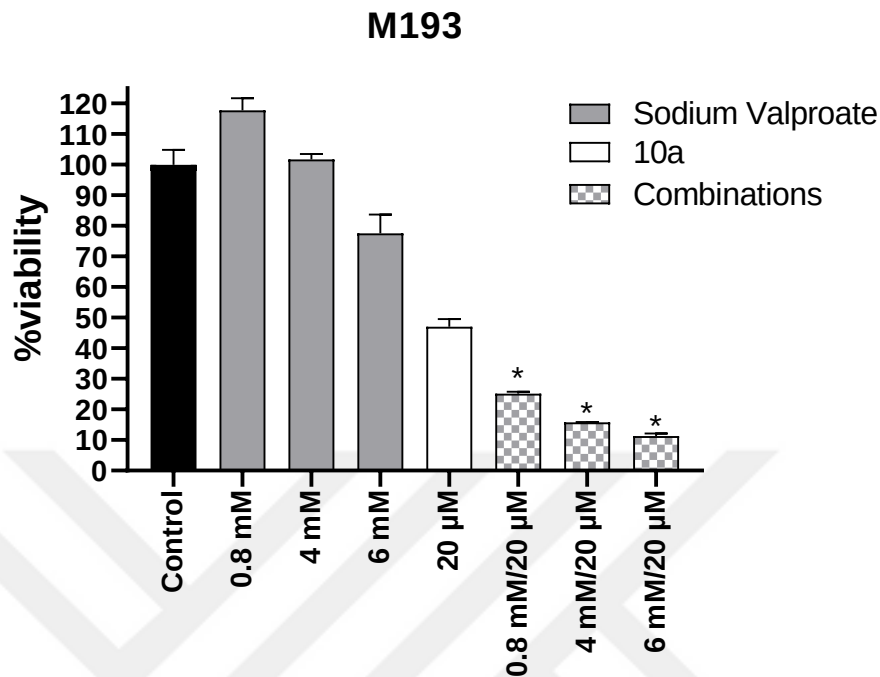


Figure 5. 70: Combination treatment of 10a-resistant M193 melanoma cells with 10a and sodium valproate. After 24 hours of cell seeding, cells were treated with different doses (0.05 mM to 0.8 mM) of sodium valproate in the absence or presence of 10a. Viable cells were quantified using Cell-Titer Glo assay after 72 hours. *p < 0.05 (t-test).

Table 5. 41: Combination Index values of combinations in M193 melanoma cells.

<u>Dose (μM)</u> 10a	<u>Dose (mM)</u> Sodium Valproate	<u>Effect</u>	<u>Combination Index (CI)</u>
20.0	0.8	0.749	0.835
20.0	4	0.842	0.760
20.0	6	0.89	0.698

5.7.6. Minimal number of predictive biomarkers of 10a sensitivity

To identify a minimal number of biomarkers that could help classify melanoma subtypes which show differential 10a sensitivity, we selected 4 genes out of the 46, based on highest fold-change ($\log_2FC > 0.2$ and < -0.2) in both directions and significance (p-value < 0.001) between sensitive and resistant cells (highlighted in Figure 5.65). TMED7 and PLOD2,

whose increased expression correlates with resistance to 10a, along with XRCC5 and NSUN5 whose increased expression is indicative of sensitivity were chosen to be validated in vitro. Expression of these four genes were quantified by RT-qPCR analysis in four sensitive cells (M307, M189, SHM20A2 and M24) and five resistant cells (M34, M433, M2025, M221, and M193). Quantification of gene expression levels of four genes revealed that TMED7 and PLOD2 were upregulated in 10a-resistant cells, whereas XRCC5 and NSUN5 were relatively downregulated, as expected (Figure 5.71).

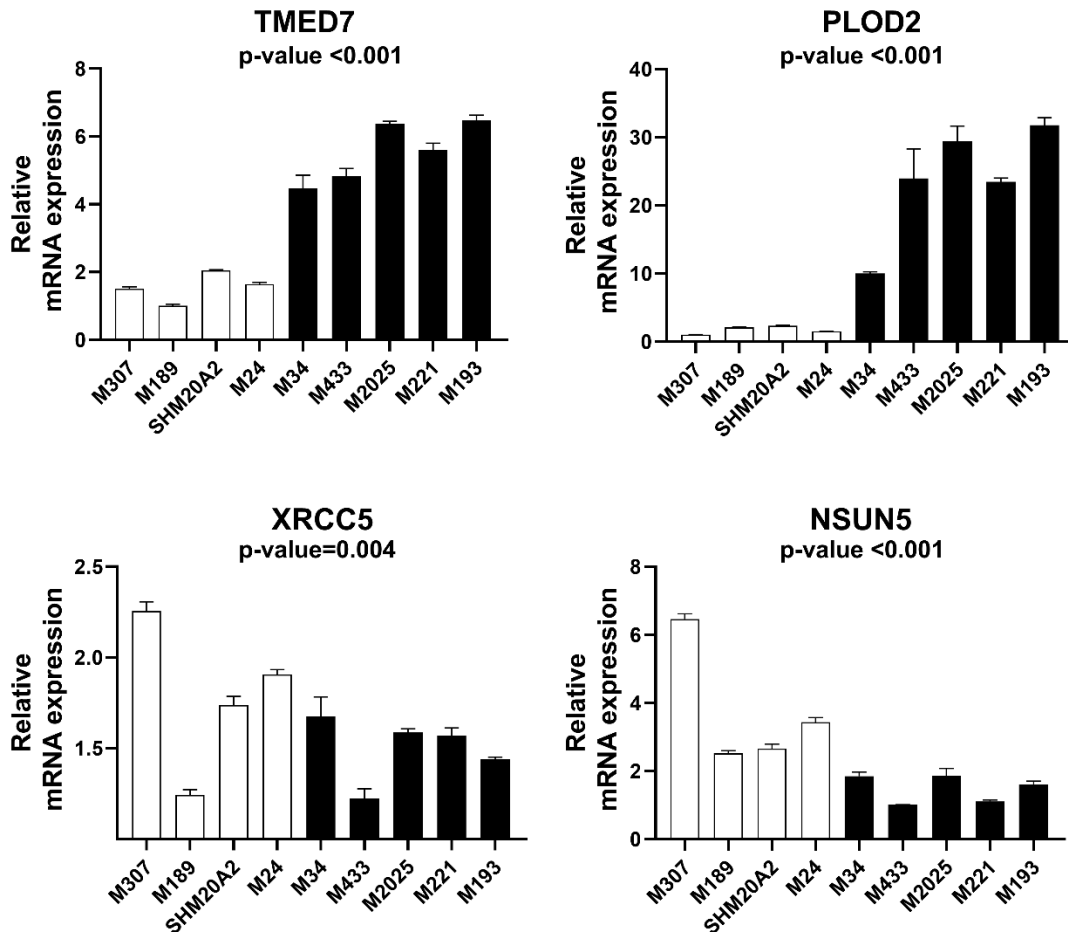


Figure 5. 71: Gene expression analysis of candidate biomarkers for 10a chemo-sensitivity. TMED7, PLOD2, XRCC5 and NSUN5 expressions were quantified by real-time qPCR. GAPDH was used as endogenous control. M307 and SHM20A2 represent sensitive cells and M2025 is a 10a-resistant cell. p-values indicate t test comparison of expression levels between sensitive and resistant cells.

Chapter 6

Discussion

6.1. Characterization of chemotherapy resistance mechanisms

Cancer continues to be leading cause of mortality because many of therapeutic resistance mechanisms remains incompletely understood. While genetic mechanisms undoubtedly play a role in mediating resistance to conventional chemotherapeutics and some targeted agents, non-genetic mechanisms are also gaining greater recognition in the absence of a clear genetic cause. [36] We therefore wanted to characterize resistance mechanisms in a comprehensive manner by using multiple omics data sets and bioinformatics tools that help understanding of complex biological processes.

In recent years, detecting biological molecules with high-throughput technologies is giving researchers more data to understand various biological processes and complex human diseases, such as cancer. Large-scale projects like Cancer Cell Line Encyclopedia (CCLE) provide comprehensive genetic and chemical characterization of cancer cell lines. [162] Here, we revealed important mechanisms that have role in differential responsiveness of cancer cell lines to chemotherapeutics. We performed GSEA to interpret gene expression data of cancer cells that show differential response to chemotherapeutics. By using differentially expressed genes and miRNA, we constructed PPI networks and miRNA-target networks to assess the potential drug resistance mechanisms. Furthermore, we performed multi-omics data integration to reveal key features (mRNA, microRNA, protein and methylation event) that discriminates drug-sensitive and –resistant cell lines. Our findings from *in silico* analyses showed the role of EMT and EMT-associated signaling pathways in drug resistance. On the other hand, sensitivity to chemotherapeutics was found to be associated with increased cell proliferation and suppression of EMT-phenotype. Interestingly, we observed a distinct sensitivity profile in breast cancer cell lines in which EMT was associated with increased sensitivity to doxorubicin.

6.2. Resistance mechanisms to 5-FU in colon cancer cells

Our *in silico* analyses to characterize 5-FU resistance mechanisms in colon cancer cells revealed that key molecular mechanisms and molecules are associated with drug response. GSEA showed that gene sets related to “MYC Targets” are enriched in sensitive cells, suggesting a proliferative phenotype. It is expected since conventional chemotherapies mainly target proliferating cancer cells [163].

In 5-FU resistant cell lines, enriched gene sets include those related to EMT, multi-cancer invasiveness signature, LEF1 and TGFB-mediated gene signature. Several studies have been reported that EMT is associated with 5-FU resistance in colon cancer cells [65, 164, 165]. It is also known that multi-cancer invasion associated gene signature includes many EMT markers [166]. LEF1, a stemness regulator and an EMT inducer, was shown to have a role in 5-FU resistance. Targeting LEF1-DCLK1 axis by niclosamide led to sensitization of colon cancer cells to 5-FU [167, 168]. TGFB is also known as a major mediator of EMT [169]. Altogether, GSEA suggested a resistance profile associated with EMT, invasive and stemness phenotypes.

Further analyzing sensitivity and resistance-associated gene list in network-based tools can improve understanding of how these genes contribute to chemotherapy resistance. Functional enrichment analysis on PPI network constructed with sensitivity associated gene list indicated the enrichments of gene sets related to cell cycle, cell proliferation, transcriptional regulation and chromatin organization. This confirms GSEA analysis results that show enrichments in “MYC targets”. On the other hand, functional enrichment analysis on PPI network constructed with resistance associated gene list suggested the role of PI3K-AKT signaling pathway on 5-FU resistance. It is not surprising since studies relate over-activation of AKT pathway and decreased E-cadherin expression with increased 5-FU resistance [170]. This suggests that PI3K-AKT pathway-mediated EMT can be main player in mechanisms underlying 5-FU resistance in colon cancer cells.

We then constructed miRNA-target networks combined with PPIs to understand the role of miRNAs in 5-FU resistance. Functional enrichment analysis suggested the role of resistance-

associated miRNAs in the regulation of cell cycle-related genes, suggesting inhibition of cell proliferation.

We then performed multi-omics integration analysis to identify multi-omics signature that discriminates the sensitive and resistant cell lines. 10 mRNA variables were identified in feature selection method. Among these, TGM2 was associated with resistance and had the highest importance with its discriminative power. TGM2 is a member of transglutaminase family, which catalyzes protein cross-linking. In several studies, TGM2 was shown to have a role in the development, progression and metastasis of cancer. Also, an implication of TGM2 in EMT, stemness and invasion has long been discussed [171]. L1CAM is another resistance associated feature, it is also shown to associate with EMT [172]. These findings suggest the importance of EMT in resistance, even if they don't directly promote 5-FU resistance. They can be just biomarkers of resistance. Furthermore, multi-omics integration analysis with mixOmics provided 11 miRNA variables, including three members of the miR-200 family. These miRNAs function as tumor suppressors, they are commonly downregulated in cancer cells. They have a pivotal role in the suppression of EMT by inhibiting ZEB1 and ZEB2 transcription factors [173]. Here, we found an association between 5-FU sensitivity and increased expressions of miR-200a, miR-200b and miR-429. Additionally, miR-181a and miR-181c, that are associated with metastasis, invasion and recurrence, are found to be associated with 5-FU resistance [174, 175]. Features selected in proteomic data included TGM2 protein. This confirms the mRNA levels and raises the importance of TGM2 as a candidate molecule in 5-FU resistance. Altogether, these show that TGM2 can be a candidate target to overcome 5-FU resistance in colon cancer.

6.3. Resistance mechanisms to irinotecan in colon cancer

Although irinotecan is a standard chemotherapeutic of the first-line treatments for metastatic colorectal cancer, irinotecan resistance appears to develop by some mechanisms [176]. Here, we aimed to further characterize drug resistance mechanisms via single-level and multi-omics approaches. GSEA analysis resulted in a similar profile with 5-FU resistance in colon cancer cells. Genes related to “MYC Targets” were highly enriched in sensitive cells, suggesting a proliferative phenotype. On the other hand, EMT and stem cell-related gene signature were enriched in resistant cell lines. So, distinction in sensitivity profile seems to be originated from EMT phenotype.

Construction of a PPI network with resistance-associated gene list uncovered the functional enrichments of gene signature related to PI3K-AKT signaling and EGFR tyrosine kinase inhibitor resistance. PI3K-AKT signaling was also at the forefront in 5-FU resistance. Here, we found that irinotecan resistance correlates with EGFR inhibitor resistance. Several studies suggest that EGFR inhibitor resistance is mediated by EMT [63, 177]. In this sense, irinotecan and EGFR inhibitors may share a common fate. Next, similar to miRNA profile in 5-FU sensitivity, we found that resistance-associated miRNAs-target network resulted in enrichments of cell cycle related genes.

Multi-omics integration analysis revealed a molecular signature consisting of 7 mRNAs, 7 miRNAs, 7 proteins and 5 methylation events. Among these mRNAs and proteins, both mRNA and protein levels of FOSL1 were associated with increased resistance to irinotecan. FOSL1 is a transcription factor that is highly expressed in colon cancer patients and associated with poor prognosis [161]. Recent studies revealed the role of FOSL1 in stem-like cell reprogramming processes and its contribution to EMT by binding TGFB1, ZEB1 and ZEB1 promoters [178, 179]. This finding suggests FOSL1 as a candidate target to overcome irinotecan resistance in colon cancer. Among the miRNA variables, miR-200a has the highest contribution for discrimination of sensitive and resistant cell lines. High expression was observed in the irinotecan-sensitive cell lines, similar to 5-FU sensitive cell lines. The only miRNA that is associated with resistance was miR-215. This can be related

to its role in cell proliferation. A study suggests that miR-215 reduces cell proliferation through the suppression of DTL expression by causing G2-arrest [180].

6.4. Resistance mechanisms to irinotecan in pancreatic cancer

Irinotecan is also second-line treatment option for patients with metastatic pancreatic cancer [181]. Similar to previous analyses, GSEA analysis with gene-expression data of sensitive and resistant subtypes revealed that cell cycle, cell proliferation and epithelial phenotypes related gene sets were enriched in sensitive cells. In irinotecan resistant pancreatic cancer cell lines, enriched gene sets included those related to EMT signature and gefitinib resistance profile. Since gefitinib is an EGFR inhibitor, these results confirm the similarities in irinotecan resistance profile in pancreatic and colon cancer cell lines [182].

PPI network analysis done with resistance associated gene list resulted in functional enrichment of FGFR signaling pathway. Studies suggested that mechanism of FGFR mediated resistance can be related to EMT [183]. miRNA-target network constructed with sensitivity associated miRNAs resulted in functional enrichment of WNT signaling pathway, known to induce EMT [184].

Multi-omics integration analysis revealed a molecular signature consisting of 11 mRNAs, 7 miRNAs, 8 proteins and 5 methylation events. Among these mRNAs and methylation events, correlation between expression of TRPC1 and methylation of its TSS region draws attention. Data suggest that demethylation of its TSS results in upregulation of TRPC1 in resistant cell lines. Although its role in cancer remains poorly understood, some researches support its role in EMT [185]. Additionally, ENPP1, another selected features, is associated with induction of EMT and stem cell features [186].

Among miRNA variables, the highest contribution to discrimination of sensitive and resistant cell lines was made by miR-302c which is shown to restrain AKT-mediated EMT in hepatocellular carcinoma cells [187]. miR-140-5p was one of the selected variables by MixOmics and associated with irinotecan resistance. This can be related to ability of miR-140-5p to inhibit cell proliferation [188]. Among selected proteins, DJ-1 is one the protein associated with resistance to irinotecan. Li et al. recently suggested that DJ-1 enhances FGF9,

in turn, resulting in induction of EMT. So, FGFR signaling mediated EMT may be a key player in irinotecan resistance in pancreatic cancer cell lines.

6.5. Resistance mechanisms to gemcitabine in pancreatic cancer

Despite the development of chemoresistance after initial response, gemcitabine is still the leading chemotherapeutic for the treatment of pancreatic cancer [189]. GSEA revealed enrichments of gene sets in relation to epithelial phenotype in gemcitabine sensitive pancreatic cancer cell lines. In gemcitabine resistant cell lines enriched gene sets included those related to EMT and “Myc Targets”, suggesting mesenchymal and proliferative phenotype. It is a controversial finding since gemcitabine works by killing proliferative cells. On the other hand, there are some studies showing that decreased cell proliferation results in reversal of gemcitabine resistance [190, 191].

Multi-omics integration analysis revealed a molecular signature consisting of 7 mRNAs, 7 miRNAs, 7 proteins and 5 methylation events. Among these mRNAs, CAMK1 is the only gene related to gemcitabine resistance. Azorsa et al. found that CAMK1 knockdown can be a way to sensitize gemcitabine resistant pancreatic cancer cells. They suggest the role of CAMK1 in preventing apoptosis as how it contributes to gemcitabine resistance [192]. Among miRNAs, miR-141 was found to be associated gemcitabine sensitivity. So, continuously we found different members of the miR-200 family in correlation to drug sensitivity. miR-29a was also in the list of miRNAs associated with gemcitabine resistance. Nagano et al. suggested the miR-29a induced Wnt/ β -catenin signaling pathway activation as a resistance mechanism for gemcitabine [36]. Among selected protein variables, E-Cadherin had the highest contribution to discrimination, confirming epithelial phenotype in sensitive cell lines as an epithelial marker. Additionally, Claudin-7 was one of the sensitivity-associated proteins, which shows also association with epithelial characteristics [193].

6.6. Resistance mechanisms to doxorubicin in breast cancer

Doxorubicin is one of the main chemotherapeutics applied for breast cancer treatment but doxorubicin resistance has been widely reported. Our findings in characterization of resistance mechanisms are highly controversial. Firstly, we identified an opposite pattern seen in 5-FU/irinotecan resistance in colon cancer and irinotecan/gemcitabine resistance in pancreatic cancer cell lines in which EMT has been shown as a main driver of resistance. Further, most of research supports the role of EMT in drug resistance [194]. However, we, in these analyses, found that EMT was associated with increased sensitivity to doxorubicin. In GSEA, gene sets related to EMT, invasion and TGFB signaling were found to be enriched in sensitive cells. As expected, cell proliferation-related gene set had shown enrichment. In resistant cell lines, we observed enrichments of estrogen receptor associated gene sets. In terms of molecular breast cancer subtypes, we realized that sensitive cell lines mainly consist of basal subtypes while resistant cell lines consist of luminal subtypes.

Further functional enrichment analysis on PPI network constructed with sensitivity associated gene list revealed that genes related to TFGF receptor signaling that activates SMADs were enriched. On the other hand, functional enrichment analysis resulted in enrichment ERBB signaling pathway associated genes in PPI network constructed with resistance associated gene list. When we map resistance-associated miRNA-targets network, functional enrichment refers the inhibition of PI3K-AKT signaling in doxorubicin-resistant breast cancer cell lines. Nevertheless, these findings are contradiction with literature.

Multi-omics integration analysis revealed a molecular signature consisting of 7 mRNAs, 7 miRNAs, 7 proteins and 5 methylation events. Among selected mRNA variables, TFF1 is one the genes associated with increased resistance to doxorubicin. Pelden et al. also suggested that it is upregulated by estrogen and involved in doxorubicin induced apoptosis resistance [195]. Among selected miRNA variables, there was no miRNA associated with sensitivity. miR-301a is one of these, and it is shown to promote doxorubicin resistance through targeting AMPKa1 in osteosarcoma cells [196]. Selected protein variables associated with resistant phenotype reflect epithelial and ER+ characteristics of cell lines.

Overall, these findings manifest that drug response data sets or discovered doxorubicin resistance mechanisms should be carefully reviewed. Different drug response data sets show good correlation in response profile of breast cancer cell lines to doxorubicin. This may suggest its reliability, but *in vitro* studies have also strong arguments. So, there can be more complex mechanisms underlying doxorubicin resistance which cannot be explained with these *in silico* approaches.

6.7. Targeting EMT to overcome drug resistance in cancer

EMT is a well-known phenomenon whereby tumor cells lose their epithelial characteristics and acquire mesenchymal characteristics. Emerging evidence suggests a molecular and phenotypic association between EMT and chemotherapy resistance in several cancer types [197]. Therefore, targeting EMT has been considered a promising strategy to overcome therapy resistance in cancer.

A wide range of natural products has been reported as being cytotoxic for cancer cells and therefore useful in cancer therapy. Natural products can act as therapeutic, preventive or chemosensitizer agents. Enhancement of chemotherapy sensitivity in tumor cells using naturally occurring biomolecules is a new and promising strategy aiming to promote the efficacy of conventional chemotherapeutics and targeted drugs [198]. Several studies suggest that inhibition or reversal of EMT by natural products results in resensitizing drug resistant cancer cells [80, 99, 101, 199].

In the present study, we used eight natural compounds and 2 repurposed agents that were previously shown to modulate EMT in different types of cancer cell lines, to test their capability to sensitize cancer cells to chemotherapy in a comprehensive manner. Although there are studies supporting the modulatory effects of all these compounds on EMT, only a few of them were able to induce MET in specific cancer cell lines in our experiments, suggesting a highly selective cell-line specific action. Interestingly, none of the compounds were able to induce MET in the Caco-2 cell line, which can easily be induced to undergo EMT and MET under various other conditions [200]. In fact, some treatments resulted in

upregulation of VIM suggesting the induction of EMT. Moreover, the MET induction with fisetin and forskolin seen in KM12 was not observed in other colon cancer cell lines. This suggests that MET induction with these natural compounds may vary according to cellular and molecular characteristics of cell lines even within the same cancer type. Similarly, the breast cancer cell line MDA-MB-157 exhibited MET only with resveratrol. Since various cellular mechanisms are affected by resveratrol, including mammalian target of rapamycin (mTOR) signaling, miRNA modulation, AMP-activated protein kinase (AMPK) activation, NF- κ B activation, nitric oxide production, histone deacetylase (HDAC) inhibition, and cytochrome P450 inhibition [201], it might be that MET induced in this cell line is managed through one or more of these mechanisms which may not have been targeted or effected enough by other compounds in our panel.

Induction of MET has been assessed based on reduction in expression of VIM, and increase in expression of CDH1 upon treatment throughout this study. Exceptionally, MIA PaCa-2 which had a very low basal expression of CDH1, had a very dramatic increase in CDH1 expression induced by multiple natural compounds. Almost no change was recorded for VIM expression, therefore we did not consider these changes as markers of MET in this cell line. Evaluation of expression changes of multiple mesenchymal markers may be needed to better identify if these compounds can induce MET in this cell line.

Among the compounds we examined in this study, almost none were found to both trigger MET and induce chemo-sensitization. In KM12, although fisetin and forskolin induced MET, the fisetin and 5FU combination did not show a synergistic effect. On the other hand, Urolithin A synergized with 5-FU in KM12, in the absence of MET induction. Furthermore, slight MET induction in MIA PaCa-2 with natural compounds did not lead to improved sensitivity to irinotecan, gemcitabine and gefitinib in almost all combinations.

These findings show that synergy between natural compounds and chemotherapeutics/targeted agents can be observed only for some cell lines and that the underlying mechanism is likely not the simple induction of MET but possibly a combination of several other mechanisms. The fact that synergistic effects were also quite variable across cancer types as well as in cell lines within a given cancer type suggests that the mechanisms underlying these observations are highly cell line specific.

6.8. Mesenchymal/invasive phenotype in melanoma as a mechanism for c-Src inhibitor resistance

Several Src inhibitors have been available for clinical assessment including dasatinib and saracatinib, which are utilized in clinical trials in melanoma [150, 151]. However, increased risk of toxicity and minimal effectiveness of these drugs uncover a need for more potent c-Src inhibitors and/or a better definition of melanoma subtypes that would be suitable for Src inhibitor-based treatment protocols.

The in vitro and in silico data we report here indicate that melanoma cells with proliferative or mesenchymal features clearly exhibit distinct sensitivity patterns for a novel c-Src inhibitor 10a. This is in agreement with our previous work, where a proliferative or invasive phenotype corresponded to 10a-sensitive and resistant cells, respectively [202]. Protein-chemical interaction network analysis with sensitivity associated gene list revealed that valproic acid is one of the hub chemicals in the network. This can be explained by phenotypic differences between sensitive and resistant melanoma cells. Resistant cells exhibited a mesenchymal/invasive phenotype, suggesting a dedifferentiated state. Several studies suggest valproic acid as a differentiating agent [203-205]. on the other hand, the role of valproic acid on EMT is highly controversial. Some studies suggest that valproic acid promotes EMT while some shows its role in inhibition of EMT [206-209]. Expecting the reversal of mesenchymal/resistant phenotype, we performed combination of 10a with the histone deacetylase inhibitor, valproic acid. Results indicated that valproic acid can be used to enhance 10a cytotoxicity in resistant cells. However, we did not check the expression levels of EMT markers after treatment with valproic acid.

Last, we want to identify a minimal number of biomarkers that could help classify melanoma subtypes which show differential 10a sensitivity. Our expression data show TMED7, PLOD2, XRCC5, and NSUN5 are capable of classifying in vitro responses to 10a. The role of TMED7 in cancer is poorly understood. Sheng et al. suggested that PLOD2 may promote cancer stem-cell like phenotype, in turn, induces drug resistance [210]. Sensitivity associated gene, XRCC5, was shown to promote cell proliferation through modulation of COX-2 in colon cancer cells [211]. Similarly, NSUN5 was also found to be associated with increased

cell proliferation [212]. Collectively, these biomarkers reflect the phenotypes that they are enriched. Further studies of 10a in vivo, combination treatments with valproic acid, and clinical validation of biomarker genes would yield further insights into mechanisms of drug action, degree of cotreatment synergy, and clinical reliability of sensitivity biomarkers.



Chapter 7

Bibliography

1. de Anda-Jauregui, G. and E. Hernandez-Lemus, Computational Oncology in the Multi-Omics Era: State of the Art. *Front Oncol*, 2020. **10**: p. 423.
2. Kusch, N. and A. Schuppert, Two-step multi-omics modelling of drug sensitivity in cancer cell lines to identify driving mechanisms. *PLoS One*, 2020. **15**(11): p. e0238961.
3. Oh, M., et al., DRIM: A Web-Based System for Investigating Drug Response at the Molecular Level by Condition-Specific Multi-Omics Data Integration. *Front Genet*, 2020. **11**: p. 564792.
4. Yildiz, G., Integrated multi-omics data analysis identifying novel drug sensitivity-associated molecular targets of hepatocellular carcinoma cells. *Oncol Lett*, 2018. **16**(1): p. 113-122.
5. Cancer Genome Atlas Research, N., et al., The Cancer Genome Atlas Pan-Cancer analysis project. *Nat Genet*, 2013. **45**(10): p. 1113-20.
6. Lawrence, M.S., et al., Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature*, 2014. **505**(7484): p. 495-501.
7. Ghandi, M., et al., Next-generation characterization of the Cancer Cell Line Encyclopedia. *Nature*, 2019. **569**(7757): p. 503-508.
8. Iorio, F., et al., A Landscape of Pharmacogenomic Interactions in Cancer. *Cell*, 2016. **166**(3): p. 740-754.
9. Subramanian, A., et al., Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*, 2005. **102**(43): p. 15545-50.
10. Reimand, J., et al., Pathway enrichment analysis and visualization of omics data using g:Profiler, GSEA, Cytoscape and EnrichmentMap. *Nat Protoc*, 2019. **14**(2): p. 482-517.
11. Xia, J., M.J. Benner, and R.E. Hancock, NetworkAnalyst--integrative approaches for protein-protein interaction network analysis and visual exploration. *Nucleic Acids Res*, 2014. **42**(Web Server issue): p. W167-74.
12. Zhou, G., et al., NetworkAnalyst 3.0: a visual analytics platform for comprehensive gene expression profiling and meta-analysis. *Nucleic Acids Res*, 2019. **47**(W1): p. W234-W241.
13. Chang, L., et al., miRNet 2.0: network-based visual analytics for miRNA functional analysis and systems biology. *Nucleic Acids Res*, 2020. **48**(W1): p. W244-W251.
14. Fan, Y., et al., miRNet - dissecting miRNA-target interactions and functional associations through network-based visual analysis. *Nucleic Acids Res*, 2016. **44**(W1): p. W135-41.
15. Rohart, F., et al., mixOmics: An R package for 'omics feature selection and multiple data integration. *PLoS Comput Biol*, 2017. **13**(11): p. e1005752.
16. Nguyen, D.V. and D.M. Rocke, Multi-class cancer classification via partial least squares with gene expression profiles. *Bioinformatics*, 2002. **18**(9): p. 1216-26.

17. Rohart, F., et al., MINT: a multivariate integrative method to identify reproducible molecular signatures across independent experiments and platforms. *BMC Bioinformatics*, 2017. **18**(1): p. 128.
18. Singh, A., et al., DIABLO: an integrative approach for identifying key molecular drivers from multi-omics assays. *Bioinformatics*, 2019. **35**(17): p. 3055-3062.
19. Sung, H., et al., Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*, 2021.
20. Hanahan, D. and R.A. Weinberg, Hallmarks of cancer: the next generation. *Cell*, 2011. **144**(5): p. 646-74.
21. Danaei, G., et al., Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. *Lancet*, 2005. **366**(9499): p. 1784-93.
22. Fares, J., et al., Molecular principles of metastasis: a hallmark of cancer revisited. *Signal Transduct Target Ther*, 2020. **5**(1): p. 28.
23. Vanneman, M. and G. Dranoff, Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer*, 2012. **12**(4): p. 237-51.
24. Mun, E.J., et al., Tumor-Treating Fields: A Fourth Modality in Cancer Treatment. *Clin Cancer Res*, 2018. **24**(2): p. 266-275.
25. Nikolaou, M., et al., The challenge of drug resistance in cancer treatment: a current overview. *Clin Exp Metastasis*, 2018. **35**(4): p. 309-318.
26. Garraway, L.A. and P.A. Janne, Circumventing cancer drug resistance in the era of personalized medicine. *Cancer Discov*, 2012. **2**(3): p. 214-26.
27. Ng, K.P., et al., A common BIM deletion polymorphism mediates intrinsic resistance and inferior responses to tyrosine kinase inhibitors in cancer. *Nat Med*, 2012. **18**(4): p. 521-8.
28. Konieczkowski, D.J., et al., A melanoma cell state distinction influences sensitivity to MAPK pathway inhibitors. *Cancer Discov*, 2014. **4**(7): p. 816-27.
29. Pao, W., et al., Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med*, 2005. **2**(3): p. e73.
30. Turke, A.B., et al., Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. *Cancer Cell*, 2010. **17**(1): p. 77-88.
31. Li, J., et al., Aberrant corticosteroid metabolism in tumor cells enables GR takeover in enzalutamide resistant prostate cancer. *Elife*, 2017. **6**.
32. Wang, J., et al., Epigenetic changes of EGFR have an important role in BRAF inhibitor-resistant cutaneous melanomas. *J Invest Dermatol*, 2015. **135**(2): p. 532-541.
33. Son, B., et al., The role of tumor microenvironment in therapeutic resistance. *Oncotarget*, 2017. **8**(3): p. 3933-3945.
34. Le Magnen, C., M.M. Shen, and C. Abate-Shen, Lineage Plasticity in Cancer Progression and Treatment. *Annu Rev Cancer Biol*, 2018. **2**: p. 271-289.
35. Chatterjee, N. and T.G. Bivona, Polytherapy and Targeted Cancer Drug Resistance. *Trends Cancer*, 2019. **5**(3): p. 170-182.
36. Marine, J.C., S.J. Dawson, and M.A. Dawson, Non-genetic mechanisms of therapeutic resistance in cancer. *Nat Rev Cancer*, 2020. **20**(12): p. 743-756.
37. Longley, D.B., D.P. Harkin, and P.G. Johnston, 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer*, 2003. **3**(5): p. 330-8.

38. Sasada, S., et al., Metabolomic analysis of dynamic response and drug resistance of gastric cancer cells to 5-fluorouracil. *Oncol Rep*, 2013. **29**(3): p. 925-31.
39. Li, P., et al., lncRNA HOTAIR Contributes to 5FU Resistance through Suppressing miR-218 and Activating NF-kappaB/TS Signaling in Colorectal Cancer. *Mol Ther Nucleic Acids*, 2020. **20**: p. 879-880.
40. Marin, J.J.G., et al., Cellular Mechanisms Accounting for the Refractoriness of Colorectal Carcinoma to Pharmacological Treatment. *Cancers (Basel)*, 2020. **12**(9).
41. Duong, H.Q., et al., Silencing of NRF2 Reduces the Expression of ALDH1A1 and ALDH3A1 and Sensitizes to 5-FU in Pancreatic Cancer Cells. *Antioxidants (Basel)*, 2017. **6**(3).
42. Wang, X., et al., TFAP2C promotes stemness and chemotherapeutic resistance in colorectal cancer via inactivating hippo signaling pathway. *J Exp Clin Cancer Res*, 2018. **37**(1): p. 27.
43. Xie, T., et al., FOXM1 evokes 5-fluorouracil resistance in colorectal cancer depending on ABCC10. *Oncotarget*, 2017. **8**(5): p. 8574-8589.
44. Das, D., et al., 1,3-Bis(2-chloroethyl)-1-nitrosourea enhances the inhibitory effect of resveratrol on 5-fluorouracil sensitive/resistant colon cancer cells. *World J Gastroenterol*, 2013. **19**(42): p. 7374-88.
45. Park, J.M., et al., MSH3 mismatch repair protein regulates sensitivity to cytotoxic drugs and a histone deacetylase inhibitor in human colon carcinoma cells. *PLoS One*, 2013. **8**(5): p. e65369.
46. Xu, Y. and M.A. Villalona-Calero, Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity. *Ann Oncol*, 2002. **13**(12): p. 1841-51.
47. Nygard, S.B., et al., Assessment of the topoisomerase I gene copy number as a predictive biomarker of objective response to irinotecan in metastatic colorectal cancer. *Scand J Gastroenterol*, 2014. **49**(1): p. 84-91.
48. Gongora, C., et al., New Topoisomerase I mutations are associated with resistance to camptothecin. *Mol Cancer*, 2011. **10**: p. 64.
49. Boyer, J., et al., Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin, and irinotecan. *Clin Cancer Res*, 2004. **10**(6): p. 2158-67.
50. Zhao, J., et al., Association of single nucleotide polymorphisms in MTHFR and ABCG2 with the different efficacy of first-line chemotherapy in metastatic colorectal cancer. *Med Oncol*, 2014. **31**(1): p. 802.
51. Burris, H.A., 3rd, et al., Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol*, 1997. **15**(6): p. 2403-13.
52. Amrutkar, M. and I.P. Gladhaug, Pancreatic Cancer Chemoresistance to Gemcitabine. *Cancers (Basel)*, 2017. **9**(11).
53. Bhutia, Y.D., et al., CNT1 expression influences proliferation and chemosensitivity in drug-resistant pancreatic cancer cells. *Cancer Res*, 2011. **71**(5): p. 1825-35.
54. Spratlin, J., et al., The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. *Clin Cancer Res*, 2004. **10**(20): p. 6956-61.
55. Saiki, Y., et al., DCK is frequently inactivated in acquired gemcitabine-resistant human cancer cells. *Biochem Biophys Res Commun*, 2012. **421**(1): p. 98-104.

56. Weizman, N., et al., Macrophages mediate gemcitabine resistance of pancreatic adenocarcinoma by upregulating cytidine deaminase. *Oncogene*, 2014. **33**(29): p. 3812-9.
57. Rejiba, S., et al., Gemcitabine-based chemogene therapy for pancreatic cancer using Ad-dCK::UMK GDEPT and TS/RR siRNA strategies. *Neoplasia*, 2009. **11**(7): p. 637-50.
58. Rose, M.G., M.P. Farrell, and J.C. Schmitz, Thymidylate synthase: a critical target for cancer chemotherapy. *Clin Colorectal Cancer*, 2002. **1**(4): p. 220-9.
59. Wang, C., et al., Establishment of human pancreatic cancer gemcitabine-resistant cell line with ribonucleotide reductase overexpression. *Oncol Rep*, 2015. **33**(1): p. 383-90.
60. Cox, J. and S. Weinman, Mechanisms of doxorubicin resistance in hepatocellular carcinoma. *Hepat Oncol*, 2016. **3**(1): p. 57-59.
61. Hu, T., et al., Mechanisms of drug resistance in colon cancer and its therapeutic strategies. *World J Gastroenterol*, 2016. **22**(30): p. 6876-89.
62. Oakman, C., et al., The role of topoisomerase IIalpha and HER-2 in predicting sensitivity to anthracyclines in breast cancer patients. *Cancer Treat Rev*, 2009. **35**(8): p. 662-7.
63. Tulchinsky, E., et al., EMT: A mechanism for escape from EGFR-targeted therapy in lung cancer. *Biochim Biophys Acta Rev Cancer*, 2019. **1871**(1): p. 29-39.
64. Nieto, M.A., et al., EMT: 2016. *Cell*, 2016. **166**(1): p. 21-45.
65. Kim, A.Y., et al., Epithelial-mesenchymal Transition is Associated with Acquired Resistance to 5-Fluorouracil in HT-29 Colon Cancer Cells. *Toxicol Res*, 2015. **31**(2): p. 151-6.
66. Yang, Y., et al., Epithelial-mesenchymal transition and cancer stem cell-like phenotype induced by Twist1 contribute to acquired resistance to irinotecan in colon cancer. *Int J Oncol*, 2017. **51**(2): p. 515-524.
67. Weadick, B., et al., EMT-Induced Gemcitabine Resistance in Pancreatic Cancer Involves the Functional Loss of Equilibrative Nucleoside Transporter 1. *Mol Cancer Ther*, 2021. **20**(2): p. 410-422.
68. Lee, A.F., et al., Reverse epithelial-mesenchymal transition contributes to the regain of drug sensitivity in tyrosine kinase inhibitor-resistant non-small cell lung cancer cells. *PLoS One*, 2017. **12**(7): p. e0180383.
69. Liu, J., et al., Niclosamide inhibits epithelial-mesenchymal transition and tumor growth in lapatinib-resistant human epidermal growth factor receptor 2-positive breast cancer. *Int J Biochem Cell Biol*, 2016. **71**: p. 12-23.
70. Kim, H.P., et al., Testican-1-mediated epithelial-mesenchymal transition signaling confers acquired resistance to lapatinib in HER2-positive gastric cancer. *Oncogene*, 2014. **33**(25): p. 3334-41.
71. Zipser, M.C., et al., A proliferative melanoma cell phenotype is responsive to RAF/MEK inhibition independent of BRAF mutation status. *Pigment Cell Melanoma Res*, 2011. **24**(2): p. 326-33.
72. Kitai, H., et al., Epithelial-to-Mesenchymal Transition Defines Feedback Activation of Receptor Tyrosine Kinase Signaling Induced by MEK Inhibition in KRAS-Mutant Lung Cancer. *Cancer Discov*, 2016. **6**(7): p. 754-69.
73. Du, B. and J.S. Shim, Targeting Epithelial-Mesenchymal Transition (EMT) to Overcome Drug Resistance in Cancer. *Molecules*, 2016. **21**(7).

74. Zhou, Y., et al., Salinomycin decreases doxorubicin resistance in hepatocellular carcinoma cells by inhibiting the beta-catenin/TCF complex association via FOXO3a activation. *Oncotarget*, 2015. **6**(12): p. 10350-65.
75. Meidhof, S., et al., ZEB1-associated drug resistance in cancer cells is reversed by the class I HDAC inhibitor mocetinostat. *EMBO Mol Med*, 2015. **7**(6): p. 831-47.
76. Namba, T., et al., Zidovudine, an anti-viral drug, resensitizes gemcitabine-resistant pancreatic cancer cells to gemcitabine by inhibition of the Akt-GSK3beta-Snail pathway. *Cell Death Dis*, 2015. **6**: p. e1795.
77. Chou, C.C., et al., AMPK reverses the mesenchymal phenotype of cancer cells by targeting the Akt-MDM2-Foxo3a signaling axis. *Cancer Res*, 2014. **74**(17): p. 4783-95.
78. Zhao, Z., et al., Metformin inhibits the IL-6-induced epithelial-mesenchymal transition and lung adenocarcinoma growth and metastasis. *PLoS One*, 2014. **9**(4): p. e95884.
79. Vazquez-Martin, A., et al., Metformin regulates breast cancer stem cell ontogeny by transcriptional regulation of the epithelial-mesenchymal transition (EMT) status. *Cell Cycle*, 2010. **9**(18): p. 3807-14.
80. Qu, C., et al., Metformin reverses multidrug resistance and epithelial-mesenchymal transition (EMT) via activating AMP-activated protein kinase (AMPK) in human breast cancer cells. *Mol Cell Biochem*, 2014. **386**(1-2): p. 63-71.
81. Hamilton, D.H., et al., Targeting Estrogen Receptor Signaling with Fulvestrant Enhances Immune and Chemotherapy-Mediated Cytotoxicity of Human Lung Cancer. *Clin Cancer Res*, 2016. **22**(24): p. 6204-6216.
82. Li, Y., et al., Dietary Natural Products for Prevention and Treatment of Breast Cancer. *Nutrients*, 2017. **9**(7).
83. Kim, C. and B. Kim, Anti-Cancer Natural Products and Their Bioactive Compounds Inducing ER Stress-Mediated Apoptosis: A Review. *Nutrients*, 2018. **10**(8).
84. Fabiani, R., Antitumoral Properties of Natural Products. *Molecules*, 2020. **25**(3).
85. Subramaniam, S., K.R. Selvaduray, and A.K. Radhakrishnan, Bioactive Compounds: Natural Defense Against Cancer? *Biomolecules*, 2019. **9**(12).
86. Guilford, J.M. and J.M. Pezzuto, Natural products as inhibitors of carcinogenesis. *Expert Opin Investig Drugs*, 2008. **17**(9): p. 1341-52.
87. Nurgali, K., R.T. Jagoe, and R. Abalo, Editorial: Adverse Effects of Cancer Chemotherapy: Anything New to Improve Tolerance and Reduce Sequelae? *Front Pharmacol*, 2018. **9**: p. 245.
88. Lin, S.R., et al., Natural compounds as potential adjuvants to cancer therapy: Preclinical evidence. *Br J Pharmacol*, 2020. **177**(6): p. 1409-1423.
89. Tai, C.J., et al., Aqueous Extract of Solanum nigrum Leaves Induces Autophagy and Enhances Cytotoxicity of Cisplatin, Doxorubicin, Docetaxel, and 5-Fluorouracil in Human Colorectal Carcinoma Cells. *Evid Based Complement Alternat Med*, 2013. **2013**: p. 514719.
90. Carranza-Torres, I.E., et al., Organotypic culture of breast tumor explants as a multicellular system for the screening of natural compounds with antineoplastic potential. *Biomed Res Int*, 2015. **2015**: p. 618021.

91. Nair, B., et al., Kaempferol-Mediated Sensitization Enhances Chemotherapeutic Efficacy of Sorafenib Against Hepatocellular Carcinoma: An In Silico and In Vitro Approach. *Adv Pharm Bull*, 2020. **10**(3): p. 472-476.
92. Sanchez, B.G., et al., Combination of the natural product capsaicin and docetaxel synergistically kills human prostate cancer cells through the metabolic regulator AMP-activated kinase. *Cancer Cell Int*, 2019. **19**: p. 54.
93. Bahrami, A., M. Majeed, and A. Sahebkar, Curcumin: a potent agent to reverse epithelial-to-mesenchymal transition. *Cell Oncol (Dordr)*, 2019. **42**(4): p. 405-421.
94. Toden, S., et al., Curcumin mediates chemosensitization to 5-fluorouracil through miRNA-induced suppression of epithelial-to-mesenchymal transition in chemoresistant colorectal cancer. *Carcinogenesis*, 2015. **36**(3): p. 355-67.
95. Meng, X., et al., Curcumin increases efficiency of gamma-irradiation in gliomas by inhibiting Hedgehog signaling pathway. *Cell Cycle*, 2017. **16**(12): p. 1181-1192.
96. Lu, Y., et al., Curcumin may reverse 5-fluorouracil resistance on colonic cancer cells by regulating TET1-NKD-Wnt signal pathway to inhibit the EMT progress. *Biomed Pharmacother*, 2020. **129**: p. 110381.
97. Burns, J., et al., Plant foods and herbal sources of resveratrol. *J Agric Food Chem*, 2002. **50**(11): p. 3337-40.
98. Yar Saglam, A.S., et al., Resveratrol enhances the sensitivity of FL118 in triple-negative breast cancer cell lines via suppressing epithelial to mesenchymal transition. *Mol Biol Rep*, 2021.
99. Xu, J., et al., Resveratrol reverses Doxorubicin resistance by inhibiting epithelial-mesenchymal transition (EMT) through modulating PTEN/Akt signaling pathway in gastric cancer. *J Exp Clin Cancer Res*, 2017. **36**(1): p. 19.
100. Baribeau, S., et al., Resveratrol inhibits cisplatin-induced epithelial-to-mesenchymal transition in ovarian cancer cell lines. *PLoS One*, 2014. **9**(1): p. e86987.
101. Jin, X., et al., Resveratrol promotes sensitization to Doxorubicin by inhibiting epithelial-mesenchymal transition and modulating SIRT1/beta-catenin signaling pathway in breast cancer. *Cancer Med*, 2019. **8**(3): p. 1246-1257.
102. Tong, J., et al., Apigenin inhibits epithelial-mesenchymal transition of human colon cancer cells through NF-kappaB/Snail signaling pathway. *Biosci Rep*, 2019. **39**(5).
103. Qin, Y., et al., Apigenin inhibits NF-kappaB and snail signaling, EMT and metastasis in human hepatocellular carcinoma. *Oncotarget*, 2016. **7**(27): p. 41421-41431.
104. Lee, H.H., et al., Antitumor and Anti-Invasive Effect of Apigenin on Human Breast Carcinoma through Suppression of IL-6 Expression. *Int J Mol Sci*, 2019. **20**(13).
105. Tang, S.N., et al., EGCG enhances the therapeutic potential of gemcitabine and CP690550 by inhibiting STAT3 signaling pathway in human pancreatic cancer. *PLoS One*, 2012. **7**(2): p. e31067.
106. Li, J., et al., Fisetin Inhibited Growth and Metastasis of Triple-Negative Breast Cancer by Reversing Epithelial-to-Mesenchymal Transition via PTEN/Akt/GSK3beta Signal Pathway. *Front Pharmacol*, 2018. **9**: p. 772.
107. Pattabiraman, D.R., et al., Activation of PKA leads to mesenchymal-to-epithelial transition and loss of tumor-initiating ability. *Science*, 2016. **351**(6277): p. aad3680.
108. Li, D., et al., Procyanidin B2 inhibits high glucose-induced epithelial-mesenchymal transition in HK2 human renal proximal tubular epithelial cells. *Mol Med Rep*, 2015. **12**(6): p. 8148-54.

109. Zhao, W., et al., Metabolite of ellagitannins, urolithin A induces autophagy and inhibits metastasis in human sw620 colorectal cancer cells. *Mol Carcinog*, 2018. **57**(2): p. 193-200.
110. Gonzalez-Sarrias, A., et al., The ellagic acid-derived gut microbiota metabolite, urolithin A, potentiates the anticancer effects of 5-fluorouracil chemotherapy on human colon cancer cells. *Food Funct*, 2015. **6**(5): p. 1460-9.
111. Cancer Genome Atlas, N., Genomic Classification of Cutaneous Melanoma. *Cell*, 2015. **161**(7): p. 1681-96.
112. Gandini, S., et al., Meta-analysis of risk factors for cutaneous melanoma: I. Common and atypical naevi. *Eur J Cancer*, 2005. **41**(1): p. 28-44.
113. Holly, E.A., et al., Number of melanocytic nevi as a major risk factor for malignant melanoma. *J Am Acad Dermatol*, 1987. **17**(3): p. 459-68.
114. Tsao, H. and K. Niendorf, Genetic testing in hereditary melanoma. *J Am Acad Dermatol*, 2004. **51**(5): p. 803-8.
115. Tagliabue, E., et al., MC1R variants as melanoma risk factors independent of at-risk phenotypic characteristics: a pooled analysis from the M-SKIP project. *Cancer Manag Res*, 2018. **10**: p. 1143-1154.
116. Markovic, S.N., et al., Malignant melanoma in the 21st century, part 1: epidemiology, risk factors, screening, prevention, and diagnosis. *Mayo Clin Proc*, 2007. **82**(3): p. 364-80.
117. Elder, D.E., et al., The 2018 World Health Organization Classification of Cutaneous, Mucosal, and Uveal Melanoma: Detailed Analysis of 9 Distinct Subtypes Defined by Their Evolutionary Pathway. *Arch Pathol Lab Med*, 2020. **144**(4): p. 500-522.
118. Krishnan, T., A.M. Menzies, and R. Roberts-Thomson, Recent advancements in melanoma management. *Intern Med J*, 2021. **51**(3): p. 327-333.
119. Domingues, B., et al., Melanoma treatment in review. *Immunotargets Ther*, 2018. **7**: p. 35-49.
120. Miller, K.D., et al., Cancer treatment and survivorship statistics, 2016. *CA Cancer J Clin*, 2016. **66**(4): p. 271-89.
121. van Zeijl, M.C., et al., (Neo)adjuvant systemic therapy for melanoma. *Eur J Surg Oncol*, 2017. **43**(3): p. 534-543.
122. Batus, M., et al., Optimal management of metastatic melanoma: current strategies and future directions. *Am J Clin Dermatol*, 2013. **14**(3): p. 179-94.
123. Garbe, C., et al., Diagnosis and treatment of melanoma. European consensus-based interdisciplinary guideline - Update 2016. *Eur J Cancer*, 2016. **63**: p. 201-17.
124. Henderson, M.A., et al., Adjuvant lymph-node field radiotherapy versus observation only in patients with melanoma at high risk of further lymph-node field relapse after lymphadenectomy (ANZMTG 01.02/TROG 02.01): 6-year follow-up of a phase 3, randomised controlled trial. *Lancet Oncol*, 2015. **16**(9): p. 1049-1060.
125. Hong, A.M., et al., Adjuvant Whole-Brain Radiation Therapy Compared With Observation After Local Treatment of Melanoma Brain Metastases: A Multicenter, Randomized Phase III Trial. *J Clin Oncol*, 2019. **37**(33): p. 3132-3141.
126. Liniker, E., et al., Activity and safety of radiotherapy with anti-PD-1 drug therapy in patients with metastatic melanoma. *Oncoimmunology*, 2016. **5**(9): p. e1214788.

127. Pires da Silva, I., et al., Incidence, features and management of radionecrosis in melanoma patients treated with cerebral radiotherapy and anti-PD-1 antibodies. *Pigment Cell Melanoma Res*, 2019. **32**(4): p. 553-563.
128. Kim, C., et al., Long-term survival in patients with metastatic melanoma treated with DTIC or temozolomide. *Oncologist*, 2010. **15**(7): p. 765-71.
129. Fonkem, E., et al., Melanoma brain metastasis: overview of current management and emerging targeted therapies. *Expert Rev Neurother*, 2012. **12**(10): p. 1207-15.
130. Tawbi, H.A., et al., Inhibition of DNA repair with MGMT pseudosubstrates: phase I study of lomeguatrib in combination with dacarbazine in patients with advanced melanoma and other solid tumours. *Br J Cancer*, 2011. **105**(6): p. 773-7.
131. Sinnberg, T., et al., Inhibition of PI3K-AKT-mTOR signaling sensitizes melanoma cells to cisplatin and temozolomide. *J Invest Dermatol*, 2009. **129**(6): p. 1500-15.
132. Dronca, R.S., et al., Phase II study of temozolomide (TMZ) and everolimus (RAD001) therapy for metastatic melanoma: a North Central Cancer Treatment Group study, N0675. *Am J Clin Oncol*, 2014. **37**(4): p. 369-76.
133. Chanda, M. and M.S. Cohen, Advances in the discovery and development of melanoma drug therapies. *Expert Opin Drug Discov*, 2021: p. 1-29.
134. Dutton-Regester, K. and N.K. Hayward, Reviewing the somatic genetics of melanoma: from current to future analytical approaches. *Pigment Cell Melanoma Res*, 2012. **25**(2): p. 144-54.
135. Chapman, P.B., et al., Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*, 2011. **364**(26): p. 2507-16.
136. Hauschild, A., et al., Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *Lancet*, 2012. **380**(9839): p. 358-65.
137. Dummer, R., et al., Overall survival in patients with BRAF-mutant melanoma receiving encorafenib plus binimetinib versus vemurafenib or encorafenib (COLUMBUS): a multicentre, open-label, randomised, phase 3 trial. *Lancet Oncol*, 2018. **19**(10): p. 1315-1327.
138. Long, G.V., et al., Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. *N Engl J Med*, 2014. **371**(20): p. 1877-88.
139. Hugo, W., et al., Non-genomic and Immune Evolution of Melanoma Acquiring MAPKi Resistance. *Cell*, 2015. **162**(6): p. 1271-85.
140. Long, G.V., et al., Increased MAPK reactivation in early resistance to dabrafenib/trametinib combination therapy of BRAF-mutant metastatic melanoma. *Nat Commun*, 2014. **5**: p. 5694.
141. Wagle, N., et al., MAP kinase pathway alterations in BRAF-mutant melanoma patients with acquired resistance to combined RAF/MEK inhibition. *Cancer Discov*, 2014. **4**(1): p. 61-8.
142. Larkin, J., et al., Five-Year Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. *N Engl J Med*, 2019. **381**(16): p. 1535-1546.
143. Callahan, M.K., et al., Nivolumab Plus Ipilimumab in Patients With Advanced Melanoma: Updated Survival, Response, and Safety Data in a Phase I Dose-Escalation Study. *J Clin Oncol*, 2018. **36**(4): p. 391-398.
144. Wolchok, J.D., et al., Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med*, 2013. **369**(2): p. 122-33.

145. Kumar, A., A.S. Jaggi, and N. Singh, Pharmacology of Src family kinases and therapeutic implications of their modulators. *Fundam Clin Pharmacol*, 2015. **29**(2): p. 115-30.
146. Irby, R.B. and T.J. Yeatman, Role of Src expression and activation in human cancer. *Oncogene*, 2000. **19**(49): p. 5636-42.
147. Simatou, A., et al., Historical retrospective of the SRC oncogene and new perspectives (Review). *Mol Clin Oncol*, 2020. **13**(4): p. 21.
148. Belli, S., et al., c-Src and EGFR Inhibition in Molecular Cancer Therapy: What Else Can We Improve? *Cancers (Basel)*, 2020. **12**(6).
149. Wu, J., et al., Dasatinib inhibits primary melanoma cell proliferation through morphology-dependent disruption of Src-ERK signaling. *Oncol Lett*, 2013. **5**(2): p. 527-532.
150. Kluger, H.M., et al., A phase 2 trial of dasatinib in advanced melanoma. *Cancer*, 2011. **117**(10): p. 2202-8.
151. Gangadhar, T.C., et al., Phase II study of the Src kinase inhibitor saracatinib (AZD0530) in metastatic melanoma. *Invest New Drugs*, 2013. **31**(3): p. 769-73.
152. Schenone, S., et al., Biologically driven synthesis of pyrazolo[3,4-d]pyrimidines as protein kinase inhibitors: an old scaffold as a new tool for medicinal chemistry and chemical biology studies. *Chem Rev*, 2014. **114**(14): p. 7189-238.
153. Hanke, J.H., et al., Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem*, 1996. **271**(2): p. 695-701.
154. Radi, M., et al., Identification of potent c-Src inhibitors strongly affecting the proliferation of human neuroblastoma cells. *Bioorg Med Chem Lett*, 2011. **21**(19): p. 5928-33.
155. Ye, J., et al., Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*, 2012. **13**: p. 134.
156. Livak, K.J. and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 2001. **25**(4): p. 402-8.
157. Smirnov, P., et al., PharmacoDB: an integrative database for mining in vitro anticancer drug screening studies. *Nucleic Acids Res*, 2018. **46**(D1): p. D994-D1002.
158. Babicki, S., et al., Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Res*, 2016. **44**(W1): p. W147-53.
159. Lotem, M., et al., Adjuvant Autologous Melanoma Vaccine for Macroscopic Stage III Disease: Survival, Biomarkers, and Improved Response to CTLA-4 Blockade. *J Immunol Res*, 2016. **2016**: p. 8121985.
160. Ayinde, O., Z. Wang, and M. Griffin, Tissue transglutaminase induces Epithelial-Mesenchymal-Transition and the acquisition of stem cell like characteristics in colorectal cancer cells. *Oncotarget*, 2017. **8**(12): p. 20025-20041.
161. Liu, Y., M. Yue, and Z. Li, FOSL1 promotes tumorigenesis in colorectal carcinoma by mediating the FBXL2/Wnt/beta-catenin axis via Smurf1. *Pharmacol Res*, 2021. **165**: p. 105405.
162. Barretina, J., et al., The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*, 2012. **483**(7391): p. 603-7.

163. Sethy, C. and C.N. Kundu, 5-Fluorouracil (5-FU) resistance and the new strategy to enhance the sensitivity against cancer: Implication of DNA repair inhibition. *Biomed Pharmacother*, 2021. **137**: p. 111285.
164. Findlay, V.J., et al., SNAI2 modulates colorectal cancer 5-fluorouracil sensitivity through miR145 repression. *Mol Cancer Ther*, 2014. **13**(11): p. 2713-26.
165. Wang, B., et al., GDF15 Repression Contributes to 5-Fluorouracil Resistance in Human Colon Cancer by Regulating Epithelial-Mesenchymal Transition and Apoptosis. *Biomed Res Int*, 2020. **2020**: p. 2826010.
166. Anastassiou, D., et al., Human cancer cells express Slug-based epithelial-mesenchymal transition gene expression signature obtained in vivo. *BMC Cancer*, 2011. **11**: p. 529.
167. Kobayashi, W. and M. Ozawa, The transcription factor LEF-1 induces an epithelial-mesenchymal transition in MDCK cells independent of beta-catenin. *Biochem Biophys Res Commun*, 2013. **442**(1-2): p. 133-8.
168. Park, S.Y., et al., Inhibition of LEF1-Mediated DCLK1 by Niclosamide Attenuates Colorectal Cancer Stemness. *Clin Cancer Res*, 2019. **25**(4): p. 1415-1429.
169. Willis, B.C. and Z. Borok, TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol*, 2007. **293**(3): p. L525-34.
170. Kim, E.J., et al., Over-activation of AKT signaling leading to 5-Fluorouracil resistance in SNU-C5/5-FU cells. *Oncotarget*, 2018. **9**(28): p. 19911-19928.
171. Malkomes, P., et al., Transglutaminase 2 promotes tumorigenicity of colon cancer cells by inactivation of the tumor suppressor p53. *Oncogene*, 2021. **40**(25): p. 4352-4367.
172. Chen, J., F. Gao, and N. Liu, L1CAM promotes epithelial to mesenchymal transition and formation of cancer initiating cells in human endometrial cancer. *Exp Ther Med*, 2018. **15**(3): p. 2792-2797.
173. Mongroo, P.S. and A.K. Rustgi, The role of the miR-200 family in epithelial-mesenchymal transition. *Cancer Biol Ther*, 2010. **10**(3): p. 219-22.
174. Wei, Z., et al., miR-181a mediates metabolic shift in colon cancer cells via the PTEN/AKT pathway. *FEBS Lett*, 2014. **588**(9): p. 1773-9.
175. Yamazaki, N., et al., High expression of miR-181c as a predictive marker of recurrence in stage II colorectal cancer. *Oncotarget*, 2017. **8**(4): p. 6970-6983.
176. Hammond, W.A., A. Swaika, and K. Mody, Pharmacologic resistance in colorectal cancer: a review. *Ther Adv Med Oncol*, 2016. **8**(1): p. 57-84.
177. Zhu, X., et al., EMT-Mediated Acquired EGFR-TKI Resistance in NSCLC: Mechanisms and Strategies. *Front Oncol*, 2019. **9**: p. 1044.
178. Bakiri, L., et al., Fra-1/AP-1 induces EMT in mammary epithelial cells by modulating Zeb1/2 and TGFbeta expression. *Cell Death Differ*, 2015. **22**(2): p. 336-50.
179. Pecce, V., et al., The role of FOSL1 in stem-like cell reprogramming processes. *Sci Rep*, 2021. **11**(1): p. 14677.
180. Song, B., et al., Molecular mechanism of chemoresistance by miR-215 in osteosarcoma and colon cancer cells. *Mol Cancer*, 2010. **9**: p. 96.
181. Yi, S.Y., et al., Irinotecan monotherapy as second-line treatment in advanced pancreatic cancer. *Cancer Chemother Pharmacol*, 2009. **63**(6): p. 1141-5.
182. Rahman, A.F., H.M. Korashy, and M.G. Kassem, Gefitinib. *Profiles Drug Subst Excip Relat Methodol*, 2014. **39**: p. 239-64.

183. Zhou, Y., et al., FGF/FGFR signaling pathway involved resistance in various cancer types. *J Cancer*, 2020. **11**(8): p. 2000-2007.
184. DiMeo, T.A., et al., A novel lung metastasis signature links Wnt signaling with cancer cell self-renewal and epithelial-mesenchymal transition in basal-like breast cancer. *Cancer Res*, 2009. **69**(13): p. 5364-73.
185. Azimi, I., et al., TRPC1 is a differential regulator of hypoxia-mediated events and Akt signalling in PTEN-deficient breast cancer cells. *J Cell Sci*, 2017. **130**(14): p. 2292-2305.
186. Hu, M., et al., Dysregulated ENPP1 increases the malignancy of human lung cancer by inducing epithelial-mesenchymal transition phenotypes and stem cell features. *Am J Cancer Res*, 2019. **9**(1): p. 134-144.
187. Yang, L., et al., The tumor suppressive miR-302c-3p inhibits migration and invasion of hepatocellular carcinoma cells by targeting TRAF4. *J Cancer*, 2018. **9**(15): p. 2693-2701.
188. Wu, D., et al., miR-140-5p inhibits the proliferation and enhances the efficacy of doxorubicin to breast cancer stem cells by targeting Wnt1. *Cancer Gene Ther*, 2019. **26**(3-4): p. 74-82.
189. Fryer, R.A., et al., Mechanisms underlying gemcitabine resistance in pancreatic cancer and sensitisation by the iMiD lenalidomide. *Anticancer Res*, 2011. **31**(11): p. 3747-56.
190. Cao, J., et al., TM4SF1 Promotes Gemcitabine Resistance of Pancreatic Cancer In Vitro and In Vivo. *PLoS One*, 2015. **10**(12): p. e0144969.
191. Sun, Q., et al., Role of hepatocyte nuclear factor 4 alpha in cell proliferation and gemcitabine resistance in pancreatic adenocarcinoma. *Cancer Cell Int*, 2019. **19**: p. 49.
192. Azorsa, D.O., et al., Synthetic lethal RNAi screening identifies sensitizing targets for gemcitabine therapy in pancreatic cancer. *J Transl Med*, 2009. **7**: p. 43.
193. Aiello, N.M., et al., Metastatic progression is associated with dynamic changes in the local microenvironment. *Nat Commun*, 2016. **7**: p. 12819.
194. Singh, A. and J. Settleman, EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene*, 2010. **29**(34): p. 4741-51.
195. Pelden, S., et al., The trefoil factor 1 (TFF1) protein involved in doxorubicin-induced apoptosis resistance is upregulated by estrogen in breast cancer cells. *Oncol Rep*, 2013. **30**(3): p. 1518-26.
196. Zhang, Y., G. Duan, and S. Feng, MicroRNA-301a modulates doxorubicin resistance in osteosarcoma cells by targeting AMP-activated protein kinase alpha 1. *Biochem Biophys Res Commun*, 2015. **459**(3): p. 367-73.
197. Smith, B.N. and N.A. Bhowmick, Role of EMT in Metastasis and Therapy Resistance. *J Clin Med*, 2016. **5**(2).
198. de Oliveira Junior, R.G., et al., Sensitization of tumor cells to chemotherapy by natural products: A systematic review of preclinical data and molecular mechanisms. *Fitoterapia*, 2018. **129**: p. 383-400.
199. Yar Saglam, A.S., et al., Resveratrol enhances the sensitivity of FL118 in triple-negative breast cancer cell lines via suppressing epithelial to mesenchymal transition. *Mol Biol Rep*, 2021. **48**(1): p. 475-489.

200. Yilmaz-Ozcan, S., et al., Epigenetic mechanisms underlying the dynamic expression of cancer-testis genes, PAGE2, -2B and SPANX-B, during mesenchymal-to-epithelial transition. *PLoS One*, 2014. **9**(9): p. e107905.
201. Britton, R.G., C. Kover, and K. Brown, Direct molecular targets of resveratrol: identifying key interactions to unlock complex mechanisms. *Ann N Y Acad Sci*, 2015. **1348**(1): p. 124-33.
202. Senses, K.M., et al., Phenotype-based variation as a biomarker of sensitivity to molecularly targeted therapy in melanoma. *Medchemcomm*, 2017. **8**(1): p. 88-95.
203. An, S.Y., et al., Valproic acid promotes differentiation of hepatocyte-like cells from whole human umbilical cord-derived mesenchymal stem cells. *Tissue Cell*, 2014. **46**(2): p. 127-35.
204. Kondo, Y., et al., Histone deacetylase inhibitor valproic acid promotes the differentiation of human induced pluripotent stem cells into hepatocyte-like cells. *PLoS One*, 2014. **9**(8): p. e104010.
205. Vukicevic, V., et al., Valproic acid enhances neuronal differentiation of sympathoadrenal progenitor cells. *Mol Psychiatry*, 2015. **20**(8): p. 941-50.
206. Jahani, M., H. Khanahmad, and P. Nikpour, Evaluation of the Effects of Valproic Acid Treatment on Cell Survival and Epithelial-Mesenchymal Transition-Related Features of Human Gastric Cancer Cells. *J Gastrointest Cancer*, 2021. **52**(2): p. 676-681.
207. Lan, X., et al., Valproic acid (VPA) inhibits the epithelial-mesenchymal transition in prostate carcinoma via the dual suppression of SMAD4. *J Cancer Res Clin Oncol*, 2016. **142**(1): p. 177-85.
208. Mao, S., et al., Valproic acid inhibits epithelialmesenchymal transition in renal cell carcinoma by decreasing SMAD4 expression. *Mol Med Rep*, 2017. **16**(5): p. 6190-6199.
209. Zhang, S., et al., Valproic acid promotes the epithelial-to-mesenchymal transition of breast cancer cells through stabilization of Snail and transcriptional upregulation of Zeb1. *Eur J Pharmacol*, 2019. **865**: p. 172745.
210. Sheng, X., et al., PLOD2 contributes to drug resistance in laryngeal cancer by promoting cancer stem cell-like characteristics. *BMC Cancer*, 2019. **19**(1): p. 840.
211. Zhang, Z., et al., XRCC5 cooperates with p300 to promote cyclooxygenase-2 expression and tumor growth in colon cancers. *PLoS One*, 2017. **12**(10): p. e0186900.
212. Jiang, Z., et al., High expression of NSUN5 promotes cell proliferation via cell cycle regulation in colorectal cancer. *Am J Transl Res*, 2020. **12**(7): p. 3858-3870.

Chapter 9

Publications

Copyright Permissions

1. ACS Medicinal Chemistry Letters
“Predictive Gene Signature for Pyrazolopyrimidine Derivative c-Src Inhibitor 10a Sensitivity in Melanoma Cells”



Predictive Gene Signature for Pyrazolopyrimidine Derivative c-Src Inhibitor 10a Sensitivity in Melanoma Cells
Author: Baris Kucukkaraduman, Can Turk, Anna L. Fallacara, et al
Publication: ACS Medicinal Chemistry Letters
Publisher: American Chemical Society
Date: May 1, 2020
Copyright © 2020, American Chemical Society

PERMISSION/LICENSE IS GRANTED FOR YOUR ORDER AT NO CHARGE

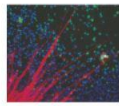
This type of permission/license, instead of the standard Terms and Conditions, is sent to you because no fee is being charged for your order. Please note the following:

- Permission is granted for your request in both print and electronic formats, and translations.
- If figures and/or tables were requested, they may be adapted or used in part.
- Please print this page for your records and send a copy of it to your publisher/graduate school.
- Appropriate credit for the requested material should be given as follows: "Reprinted (adapted) with permission from {COMPLETE REFERENCE CITATION}. Copyright (YEAR) American Chemical Society." Insert appropriate information in place of the capitalized words.
- One-time permission is granted only for the use specified in your RightsLink request. No additional uses are granted (such as derivative works or other editions). For any uses, please submit a new request.

If credit is given to another source for the material you requested from RightsLink, permission must be obtained from that source.

[BACK](#)[CLOSE WINDOW](#)

2. Brain Research
“Simultaneous miRNA and mRNA transcriptome profiling of glioblastoma samples reveals a novel set of OncomiR candidates and their target genes”



Simultaneous miRNA and mRNA transcriptome profiling of glioblastoma samples reveals a novel set of OncomiR candidates and their target genes
Author: Sukru Gulluoglu, Emre Can Tuysuz, Mesut Sahin, Aysegul Kuskucu, Cumhuri Kaan Yaltirik, Ugur Ture, Baris Kucukkaraduman, Muhammad Waqas Akbar, Ali Osmay Gure, Omer Faruk Bayrak, Altay Burak Dalan
Publication: Brain Research
Publisher: Elsevier
Date: 1 December 2018
© 2018 Published by Elsevier B.V.

Journal Author Rights

Please note that, as the author of this Elsevier article, you retain the right to include it in a thesis or dissertation, provided it is not published commercially. Permission is not required, but please ensure that you reference the journal as the original source. For more information on this and on your other retained rights, please visit: <https://www.elsevier.com/about/our-business/policies/copyright#Author-rights>

[BACK](#)[CLOSE WINDOW](#)

3. Journal of Cancer

“A Stemness and EMT Based Gene Expression Signature Identifies Phenotypic Plasticity and is A Predictive but Not Prognostic Biomarker for Breast Cancer”

Permission request

Request to reproduce figures or tables in other publications or journals: There is no need to obtain permission if copyrights of the figures or tables belong to Ivyspring International Publisher. Please acknowledge the original source of publication in our journal with full citation. If the copyrights of the figures or tables belong to other publishers or authors, please contact the original publishers or authors for permissions.

Authors or co-authors: As an author or co-author of the article, you are free to include or reuse any part of the materials in your thesis or other publications that you write, with citation of the original source in our journal. There is no need to obtain permission.

4. PloS One

“A novel 20-gene prognostic score in pancreatic adenocarcinoma”

Copyright: © 2020 Demirkol Canlı et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

5. PloS One

“Renin angiotensin system genes are biomarkers for personalized treatment of acute myeloid leukemia with Doxorubicin as well as etoposide”

Copyright: © 2020 Turk et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Predictive Gene Signature for Pyrazolopyrimidine Derivative c-Src Inhibitor 10a Sensitivity in Melanoma Cells

Baris Kucukkaraduman, Can Turk, Anna L. Fallacara, Murat Isbilen, Kerem M. Senses, Zeynep O. Ayyildiz, Muhammad W. Akbar, Michal Lotem, Maurizio Botta, and Ali O. Gure*

Cite This: *ACS Med. Chem. Lett.* 2020, 11, 928–932

Read Online

ACCESS |

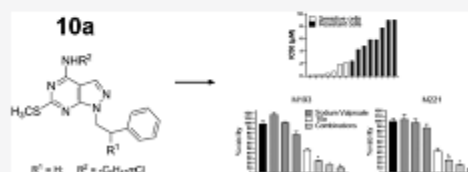
Metrics & More

Article Recommendations

Supporting Information

ABSTRACT: Melanoma is a highly aggressive cancer with poor prognosis. Although more than 80% of melanomas harbor an activating mutation in genes within the MAPK pathway, which are mutually exclusive, usefulness of therapies targeting MAPK pathway are impeded by innate and/or acquired resistance in most patients. In this study, using melanoma cells, we report the efficacy of a recently developed pyrazolo[3,4-d]pyrimidine derived c-Src inhibitor 10a and identify a molecular signature which is predictive of 10a chemosensitivity. We show that the expression of TMED7, PLOD2, XRCC5, and NSUN5 are candidate biomarkers for 10a sensitivity. Although an undifferentiated/mesenchymal/invasive status of melanoma cells is associated with resistance to 10a, we show here for the first time that melanoma cells can be sensitized to 10a via treatment with valproic acid, a histone deacetylase inhibitor.

KEYWORDS: melanoma, c-Src, drug resistance, valproic acid, sensitivity biomarkers



Melanoma is one of the most aggressive types of cancers with heterogeneous features.¹ Treatment strategies and success rates vary depending on many factors, including feasibility of surgery, stage of the melanoma, presence of specific genetic mutations, and the rate of tumor growth. While surgery is the standard approach for localized and early stage melanoma, advanced stages require lymph node dissection, radiation therapy, and medication assisted therapies, including immunotherapy, chemotherapy, and targeted therapy.^{2,3} However, long-term efficacy of such treatments is limited by their side effects and innate or acquired resistance. Therefore, new treatment strategies might help improve patient's quality of life and increase survival time.

The c-Src proto-oncogene is a nonreceptor protein kinase which is activated by different classes of cellular receptors, resulting in a diverse spectrum of biological activities including cell cycle progression, apoptosis, cell adhesion, migration, transformation, and various immune responses.^{4,5} Advanced stages of cancer, tumor growth, progression, and metastasis are associated with increased expression and activity of c-Src in melanoma and other solid tumors.^{4,6} Hence, c-Src represents a promising therapeutic target for the treatment of melanoma.

In this line, several Src inhibitors have been available for clinical assessment, including dasatinib and saracatinib, which are utilized in clinical trials in melanoma. Dasatinib is known to inhibit Src and other tyrosine kinases, including c-KIT, PDGFR, c-FMS, EphA receptor, and BCR-ABL fusion.⁷ Nevertheless, a clinical trial has suggested that daily dasatinib treatment has only a minimal benefit in unresectable

melanoma patients, the 6-month progression free survival (PFS) rate being only 13% among these patients and with high rates of toxicity.⁸ Similarly, saracatinib was also minimally effective in a phase II study.⁹ Hence, there is a need for more potent c-Src inhibitors and/or a better definition of melanoma subtypes that would be suitable for Src inhibitor-based treatment protocols.

Pyrazolo[3,4-d]pyrimidine derivatives show antitumor activity by inhibiting several serine/threonine or tyrosine kinases.¹⁰ 10a is the 6-methylthio derivative of pyrazolo[3,4-d]pyrimidine, which was shown to inhibit c-Src selectively.¹¹ In this regard, we investigated the efficacy and molecular mechanisms of response to 10a in human melanoma cells. In this study, based on cytotoxic activity of 10a in 15 melanoma cells, we report the identification of a new gene signature which can predict chemosensitivity to 10a. Two distinct phenotypes of cells, defined as sensitive and resistant, were further analyzed to reveal an underlying mechanism for this differential response to 10a. We found that proliferative or mesenchymal features of the cells are associated with distinct sensitivity of 10a. Through a protein–chemical interaction network analysis, we identified that three histone deacetylase

Special Issue: In Memory of Maurizio Botta: His Vision of Medicinal Chemistry

Received: December 31, 2019

Accepted: February 18, 2020

Published: February 18, 2020



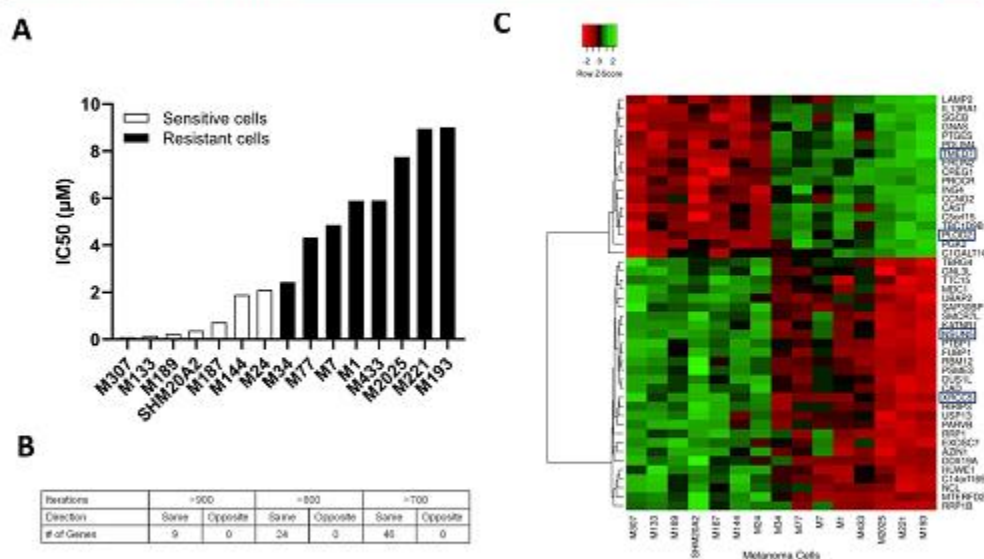


Figure 1. *In vitro* cytotoxicity analyses of c-Sec inhibitor 10a on melanoma cells and hierarchical clustering of cell lines according to 10a cytotoxicity biomarker genes. (A) Fifteen melanoma cells were analyzed for sensitivity against 10a. Cytotoxicity is expressed in terms of IC₅₀ (y-axis). (B) 10a chemosensitivity biomarker genes were determined by 2-fold cross validation with 1000 iterations, and 9, 24, and 46 genes had a frequency of significance in more than 900, 800, and 700 iterations, respectively. (C) Melanoma cells were hierarchically clustered with 46 genes that were significantly correlated with cytotoxicity values against 10a for more than 700 iterations. Two clusters were defined as sensitive (M307, M133, M189, SHM20A2, M187, M144, M24) and resistant (M34, M77, M7, M1, M433, M2025, M221, M193). Standardized expression values were used for clustering, and clustering was performed with the complete linkage method on the heatmap online tool.¹⁵

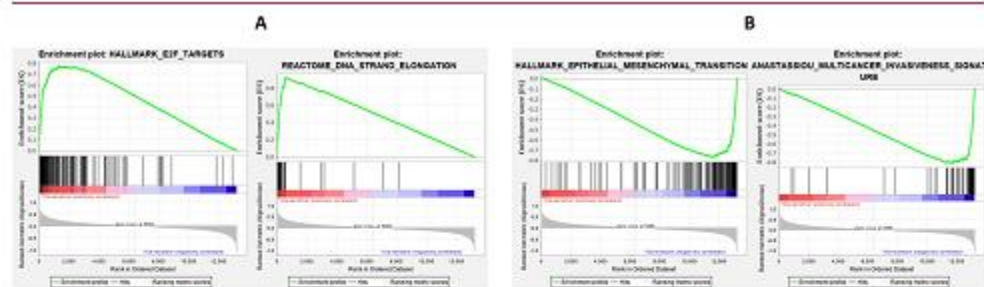


Figure 2. Comparative analysis of differentially enriched molecular gene sets among sensitive and resistant cells by GSEA. (A) Cells were grouped as sensitive and resistant based on clustering with the 104 chemosensitivity associated gene list seen in Figure 1C. Gene sets including cell cycle related targets of E2F transcription factor (left) and DNA strand elongation related genes (right) were enriched in 104-sensitive melanoma cells. (B) Gene sets defining epithelial to mesenchymal transition as in wound healing, fibrosis, and metastasis (left) and multicancer invasion associated genes (right) showed enrichment in 104-resistant melanoma cells. FDR values for each enrichment plot were less than 0.001.

inhibitors, valproic acid, entinostat, and trichostatin A, were predicted to synergize with 10a. The synergizing effect of valproic acid was validated *in vitro*. We also aimed to define a minimal number of genes that could be used as biomarkers of 10a sensitivity. We show that the expression level of four genes can be used to predict drug sensitivity against 10a.

When tested on 15 melanoma cells, cytotoxic responses to 10a occurred over a remarkably wide range of doses with IC_{50} values ranging from 0.09 to 9.01 μ M (Figure 1A, Table S1). This variation strongly suggests underlying molecular differ-

ences among these cells and thus prompted us to identify a gene signature of chemosensitivity for 10a. For this purpose, we correlated gene expression data from melanoma cells obtained by C-MAP analysis with IC₅₀ values using a 2-fold cross validation with a 1000 iteration method (see [Supporting Information](#)).¹² Briefly, in each iteration, cells were randomly divided into two groups, and a Pearson's *r* value between 10a IC₅₀ and gene expression data for each gene was calculated for these groups. This was repeated 1000 times. We then determined whether the correlations obtained for a given

gene were consistently in the same direction (either positively or negatively correlated in both groups) where the results were statistically significant in all groups. This analysis showed that expression of 9 genes was consistently and significantly correlated with IC₅₀ values in more than 900 iterations in the same direction. In more than 800 iterations, expression levels of 24 genes showed consistent and significant correlations with IC₅₀ values in the same directions. We chose 46 genes whose expression profile showed significant concordance with chemosensitivity of 10a in more than 700 iterations for classifying melanoma cells (Figure 1B). Hierarchical clustering analysis with these 46 genes revealed two subgroups of cells which are relatively sensitive and resistant to 10a (Figure 1C).

To investigate molecular mechanisms underlying the differential response to 10a, we performed gene set enrichment analysis (GSEA)¹³ with sensitive and resistant subgroups defined with the 46-gene list. M307, M133, M189, SHM20A2, M187, M144, and M24 cells constituted the sensitive subgroup while M34, M77, M7, M1, M433, M202S, M221, and M193 constituted the resistant subgroup. GSEA revealed a significant enrichment for gene sets, including E2F target genes related to cell cycle and DNA strand elongation related genes in the sensitive subgroup, suggesting a proliferative phenotype in these cells (Figure 2A, Table S2). On the other hand, resistant subgroups showed significant enrichments of gene sets related to epithelial-mesenchymal transition and multicancer invasiveness signatures, indicating an invasive/mesenchymal phenotype in resistant cells (Figure 2B). These results are concordant with previous findings where melanoma cells were classified into invasive, proliferative, and intermediate categories.¹⁴

To further investigate mechanisms behind resistance to 10a, we performed protein–chemical interaction network analysis with 46 genes using the NetworkAnalyst¹⁵ online tool. This revealed that, out of 18 resistance associated proteins, 15 extensively interact with a pyrazolopyrimidine derivative and three histone deacetylase (HDAC) inhibitors, valproic acid, trichostatin A, and entinostat (Figure 3). Expectedly, our analysis revealed (6-(4-(2-piperidin-1-ylethoxy)phenyl))-3-pyridin-4-ylpyrazolo(1,5-a)pyrimidine, as a hub chemical, which is structurally similar to pyrazolopyrimidine derivative 10a, showing interactions with 12 resistance related proteins. Interestingly, three HDAC inhibitors have interactions mostly with resistance-associated genes, and are also known as differentiating agents.^{17,18} Valproic acid was a hub chemical with the highest number of interactions. Because our previous findings validated proliferative and invasive phenotypes in chemosensitive and resistant cells, respectively, protein chemical network analysis prompted us to use valproic acid to induce differentiation to enhance sensitivity of resistant cells to 10a.

For this purpose, the 10a resistant M221 and M193 cells were treated with various doses of valproic acid (as sodium valproate) alone or in combination with 10a after 24 h of cell seeding. Cell viability was assessed 72 h after treatments. In M221 cells, 20 μ M 10a treatment resulted in a 55% reduction in viability, whereas little or no decrease in viability was observed with 50, 200, and 800 nM of sodium valproate treatment alone. However, combining 50 nM sodium valproate with 20 μ M 10a resulted in a 75% decrease in viability. This effect increased to 84% when 200 nM sodium valproate was used. The most significant effect was observed at 20 μ M 10a

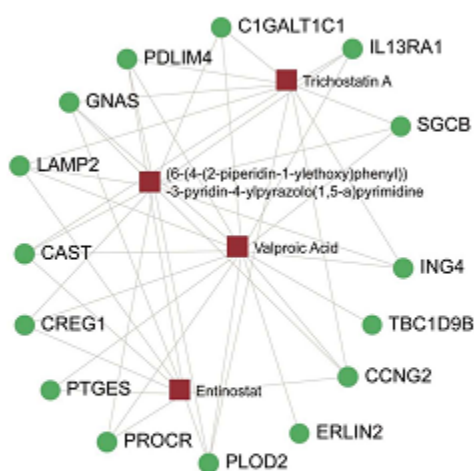


Figure 3. Protein–chemical interaction network with chemosensitivity associated genes. A protein–chemical interaction network was constructed using the NetworkAnalyst online tool to identify chemicals that interact with 10a-sensitivity associated proteins. Red squares represent the chemicals; green nodes represent proteins interacting with chemicals, and edges connect proteins to interacting chemicals. Betweenness filter (cutoff = 11.0) was applied to obtain hub chemicals. Valproic acid interacts with 15 proteins represented in the network, while (6-(4-(2-piperidin-1-ylethoxy)phenyl))-3-pyridin-4-ylpyrazolo(1,5-a)pyrimidine interacts with 12 proteins, trichostatin A interacts with 10, and entinostat interacts with 9.

and 0.8 mM sodium valproate treatment, where a 92% decrease in cell viability was seen (Figure 4A). To quantify the degree of synergism, the combination index (CI) was calculated by the Chou–Talalay method in which CI < 1 shows synergistic effects, CI = 1 shows additive effects, and CI > 1 shows antagonistic effects.¹⁹ Combined 10a and sodium valproate treatment showed synergistic effects for all combinations with CI values ranging from 0.766 to 0.603 (Figure 4A). Similarly, the sodium valproate–10a combination showed synergy in the more resistant M193 cells as well (Figure 4B). Twenty micromolar 10a treatment alone, decreased viability to 47% in M193 cells, whereas 0.8, 4, and 6 mM of sodium valproate showed little or no decrease in viability. Adding 0.8 mM sodium valproate to 20 μ M of 10a resulted in a 75% decrease in viability. Combining 20 μ M 10a and 4 mM sodium valproate treatment resulted in an 84% reduction in viability. Twenty micromolar 10a and 6 mM sodium valproate combination resulted in 89% decrease in viability. CI values ranging from 0.835 to 0.698 suggested synergism in all combinations (Figure 4B). These results show that sodium valproate acts as a potent sensitizer for 10a in melanoma.

To identify a minimal number of biomarkers that could help classify melanoma subtypes which show differential 10a sensitivity, we selected 4 genes out of the 46, based on highest fold-change ($\log_2\text{FC} > 0.2$ and < -0.2) in both directions and significance (p -value < 0.001) between sensitive and resistant cells (highlighted in Figure 1C). TMED7 and PLOD2, whose increased expression correlates with resistance to 10a, along

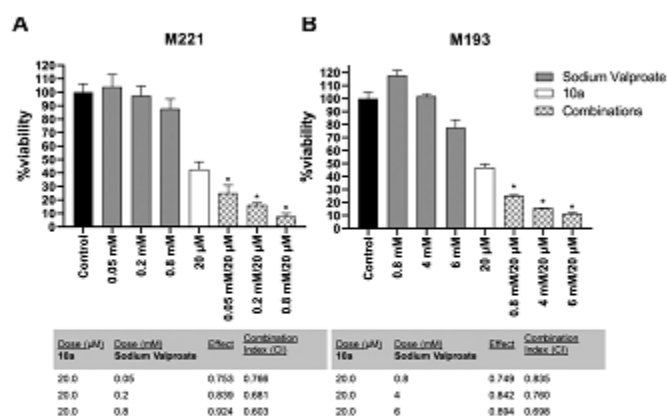


Figure 4. Combination treatment of 10a-resistant M221 and M193 melanoma cells with 10a and sodium valproate. (A) After 24 h of cell seeding, M221 cells were treated with different doses (0.05–0.8 mM) of sodium valproate in the absence or presence of 10a. Viable cells were quantified using Cell-Titer Glo assay after 72 h. Combination index values of these combinations suggests synergistic effects in all combinations where CI < 1 indicates synergy. (B) After 24 h of seeding, M193 cells were treated with different doses (0.8–6 mM) of sodium valproate in the absence or presence of 10a. Viable cells were quantified using Cell-Titer Glo assay after 72 h. Combination index values of these combinations suggests synergistic effects in all combinations. * $p < 0.05$ (t test).

with XRCC5 and NSUN5 whose increased expression is indicative of sensitivity were chosen to be validated *in vitro*. Expression of these four genes were quantified by RT-qPCR analysis in four sensitive cells (M307, M189, SHM20A2 and M24) and five resistant cells (M34, M433, M2025, M221, and M193). Quantification of gene expression levels of four genes revealed that TMED7 and PLOD2 were upregulated in 10a-resistant cells, whereas XRCC5 and NSUN5 were relatively downregulated, as expected (Figure 5).

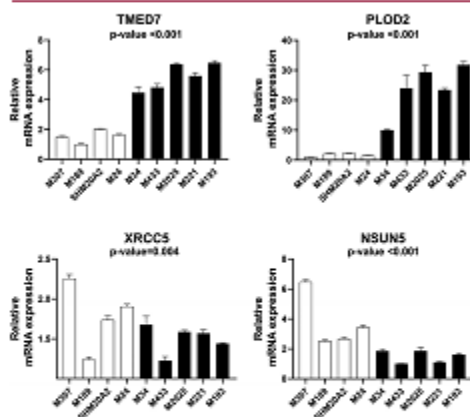


Figure 5. Gene expression analysis of candidate biomarkers for 10a chemosensitivity. TMED7, PLOD2, XRCC5, and NSUN5 expressions are quantified by real-time qPCR. GAPDH is used as endogenous control. M307, M189, SHM20A2, and M24 represent sensitive cells and M34, M433, M2025, M221, and M193 are 10a-resistant cells. p -Values indicate t test comparison of expression levels between sensitive and resistant cells.

The *in vitro* and *in silico* data we report here indicate that melanoma cells with proliferative or mesenchymal features clearly exhibit distinct sensitivity patterns for the c-Src inhibitor 10a. This is in agreement with our previous work, where a proliferative or invasive phenotype corresponded to 10a-sensitive and resistant cells, respectively.¹⁴ Additionally, combination of 10a with the histone deacetylase inhibitor, valproic acid, indicates that valproic acid can be used to enhance 10a cytotoxicity in these cells. Our expression data show TMED7, PLOD2, XRCC5, and NSUN5 are capable of classifying *in vitro* responses to 10a (Figure S1). Further studies of 10a *in vivo*, combination treatments with valproic acid, and clinical validation of biomarker genes would yield further insights into mechanisms of drug action, degree of cotreatment synergy, and clinical reliability of sensitivity biomarkers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmmedchemlett.9b00679>.

Experimental details for cell culture, bioinformatics analyses, *in vitro* biological assays, combination index analysis, quantification of gene expression levels, hierarchical clustering with *in vitro* data and tables containing *in silico* data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Ali O. Gure — Department of Molecular Biology and Genetics, Bilkent University, Ankara 06800, Turkey; orcid.org/0000-0002-4064-8608; Email: agure@bilkent.edu.tr

Authors

Baris Kucukkaraduman — Department of Molecular Biology and Genetics, Bilkent University, Ankara 06800, Turkey

Can Turk – Faculty of Medicine, Department of Medical Microbiology, Lokman Hekim University, Ankara 06510, Turkey

Anna L. Fallacara – Department of Biotechnology, Chemistry and Pharmacy, "Department of Excellence 2018-2022", University of Siena, Siena 53100, Italy

Murat Isbilen – DNAFect Genetics Consulting R&D and Biotechnology Inc., Kocaeli 41470, Turkey

Kerem M. Senses – Department of Molecular Biology and Genetics, Zonguldak Bulent Ecevit University, Zonguldak 67100, Turkey

Zeynep O. Ayyildiz – Department of Genome Sciences and Molecular Biotechnology, Izmir International Biomedicine and Genome Institute, Dokuz Eylul University, Izmir 35340, Turkey

Muhammad W. Akbar – Department of Molecular Biology and Genetics, Bilkent University, Ankara 06800, Turkey

Michal Lotem – Sharet Institute of Oncology, Hadassah Hebrew Hospital, Jerusalem 91120, Israel

Maurizio Botta – Department of Biotechnology, Chemistry and Pharmacy, "Department of Excellence 2018-2022", University of Siena, Siena 53100, Italy; orcid.org/0000-0003-0456-6995

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acsmmedchemlett.9b00679>

Notes

†M.B.: Deceased on August 2, 2019.

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

B.K. was funded by the TUBITAK-BIDEB 2211 scholarship program. This work was supported by the Scientific and Technological Research Council of Turkey (112S023) and COST action CM1106.

ABBREVIATIONS

MAPK, mitogen-activated protein kinase; TMED7, transmembrane P24 trafficking protein 7; PLOD2, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2; XRCC5, X-ray repair cross complementing 5; NSUN5, NOP2/Sun RNA methyltransferase 5; PDGFR, platelet-derived growth factor receptor; EPHA, ephrin A; C-MAP, connectivity map; GSEA, gene set enrichment analysis

REFERENCES

- (1) Cancer Genome Atlas, N. Genomic Classification of Cutaneous Melanoma. *Cell* **2015**, 161 (7), 1681–96.
- (2) Strojjan, P. Role of radiotherapy in melanoma management. *Radiol. Oncol.* **2010**, 44 (1), 1–12.
- (3) Domingues, B.; Lopes, J. M.; Soares, P.; Populo, H. Melanoma treatment in review. *Immunotargets Ther.* **2018**, 7, 35–49.
- (4) Irby, R. B.; Yeatman, T. J. Role of Src expression and activation in human cancer. *Oncogene* **2000**, 19 (49), 5636–42.
- (5) Slukla, D.; Meng, Y.; Roux, B.; Pande, V. S. Activation pathway of Src kinase reveals intermediate states as targets for drug design. *Nat. Commun.* **2014**, 5, 3397.
- (6) Lee, J. H.; Pyon, J. K.; Kim, D. W.; Lee, S. H.; Nam, H. S.; Kim, C. H.; Kang, S. G.; Lee, Y. J.; Park, M. Y.; Jeong, D. J.; Cho, M. K. Elevated c-Src and c-Yes expression in malignant skin cancers. *J. Exp. Clin. Cancer Res.* **2010**, 29, 116.
- (7) Araujo, J.; Logothetis, C. Dasatinib: a potent SRC inhibitor in clinical development for the treatment of solid tumors. *Cancer Treat. Rev.* **2010**, 36 (6), 492–500.

(8) Klager, H. M.; Dudek, A. Z.; McCann, C.; Ritacco, J.; Southard, N.; Jilaveanu, L. B.; Molinaro, A.; Senol, M. A phase 2 trial of dasatinib in advanced melanoma. *Cancer* **2011**, 117 (10), 2202–8.

(9) Gangadhar, T. C.; Clark, J. I.; Karrison, T.; Gajewski, T. F. Phase II study of the Src kinase inhibitor saracatinib (AZD0530) in metastatic melanoma. *Invest. New Drugs* **2013**, 31 (3), 769–73.

(10) Schenone, S.; Radi, M.; Musumeci, F.; Brullo, C.; Botta, M. Biologically driven synthesis of pyrazolo[3,4-d]pyrimidines as protein kinase inhibitors: an old scaffold as a new tool for medicinal chemistry and chemical biology studies. *Chem. Rev.* **2014**, 114 (14), 7189–238.

(11) Radi, M.; Brullo, C.; Crespan, E.; Tintori, C.; Musumeci, F.; Biava, M.; Schenone, S.; Dreassi, E.; Zamperini, C.; Maga, G.; Pagano, D.; Angelucci, A.; Bologna, M.; Botta, M. Identification of potent c-Src inhibitors strongly affecting the proliferation of human neuroblastoma cells. *Bioorg. Med. Chem. Lett.* **2011**, 21 (19), 5928–33.

(12) Lotem, M.; Merims, S.; Frank, S.; Hamburger, T.; Nissan, A.; Kadouri, L.; Cohen, J.; Straussman, R.; Eisenberg, G.; Frankenburg, S.; Carmon, E.; Alaiyan, B.; Shneibbaum, S.; Oge Ayyildiz, Z.; Isbilen, M.; Miert Senses, K.; Ron, L.; Steinberg, H.; Smith, Y.; Shiloni, E.; Gure, A. O. Peretz, T. Adjuvant Autologous Melanoma Vaccine for Macroscopic Stage III Disease: Survival, Biomarkers, and Improved Response to CTLA-4 Blockade. *J. Immunol. Res.* **2016**, 2016, 8121985.

(13) Subramanian, A.; Tamayo, P.; Mootha, V. K.; Mukherjee, S.; Ebert, B. L.; Gillette, M. A.; Paulovich, A.; Pomeroy, S. L.; Golub, T. R.; Lander, E. S.; Mesirov, J. P. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, 102 (43), 15545–50.

(14) Senses, K. M.; Ghasemi, M.; Akbar, M. W.; Isbilen, M.; Fallacara, A. L.; Frankenburg, S.; Schenone, S.; Lotem, M.; Botta, M.; Gure, A. O. Phenotype-based variation as a biomarker of sensitivity to molecularly targeted therapy in melanoma. *MedChemComm* **2017**, 8 (1), 88–95.

(15) Babicki, S.; Arndt, D.; Marcu, A.; Liang, Y.; Grant, J. R.; Maciejewski, A.; Wishart, D. S. Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Res.* **2016**, 44 (W1), W147–53.

(16) Zhou, G.; Soufan, O.; Ewald, J.; Hancock, R. E. W.; Basu, N.; Xia, J. NetworkAnalyst 3.0: a visual analytics platform for comprehensive gene expression profiling and meta-analysis. *Nucleic Acids Res.* **2019**, 47 (W1), W234–W241.

(17) Gottlicher, M.; Minucci, S.; Zhu, P.; Kramer, O. H.; Schimpf, A.; Giavara, S.; Sleeman, J. P.; Lo Coco, F.; Nervi, C.; Pelicci, P. G.; Heinzel, T. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J.* **2001**, 20 (24), 6969–78.

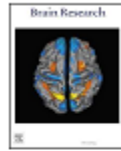
(18) Marks, P. A.; Richon, V. M.; Rifkind, R. A. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. *J. Natl. Cancer Inst.* **2000**, 92 (15), 1210–6.

(19) Chou, T. C.; Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **1984**, 22, 27–55.



Contents lists available at ScienceDirect

Brain Research

journal homepage: www.elsevier.com/locate/brainres

Research report

Simultaneous miRNA and mRNA transcriptome profiling of glioblastoma samples reveals a novel set of OncomiR candidates and their target genes



Sukru Gulluoglu^{a,b}, Emre Can Tuysuz^{a,b}, Mesut Sahin^c, Aysegul Kuskucu^a, Cumhuri Kaan Yaltirik^d, Ugur Ture^d, Baris Kucukkaraduman^e, Muhammad Waqas Akbar^e, Ali Osmay Gure^e, Omer Faruk Bayrak^a, Altay Burak Dalan^{f,*}

^a Department of Medical Genetics, Yeditepe University Medical School, Istanbul, Turkey

^b Department of Biotechnology, Institute of Science, Yeditepe University, Istanbul, Turkey

^c Department of Neuroscience and Neuroengineering, Institute of Science Ataturk University, Erzurum, Turkey

^d Department of Neurosurgery, Yeditepe University Medical School, Yeditepe University, Istanbul, Turkey

^e Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

^f Department of Biochemistry, Yeditepe University Medical School, Istanbul, Turkey

HIGHLIGHTS

- miR-21 and miR-339 affect migration, invasion and apoptosis of GBM cells.
- TUSC3 and NEGR1 is correlated with young age at diagnosis in GBM.
- miR-21 expression level was correlated with older age at diagnosis in GBM.
- TUSC3 expression is associated with higher tumor volume in GBM patients.
- Low miR-92b, miR-182, NEFM and UNC13C is indicative of shorter overall survival.

ARTICLE INFO

Keywords:
Glioblastoma
Survival
Microarray
MiR-21-5p
MiRNA
Prognosis

ABSTRACT

Although glioblastomas are common, there remains a need to elucidate the underlying mechanisms behind their initiation and progression and identify molecular pathways for improving treatment. In this study, sixteen fresh-frozen glioblastoma samples and seven samples of healthy brain tissues were analyzed with miRNA and whole transcriptome microarray chips. Candidate miRNAs and mRNAs were selected to validate expression in fifty patient samples in total with the criteria of abundance, relevance and prediction scores. miRNA and target mRNA relationships were assessed by inhibiting selected miRNAs in glioblastoma cells. Functional tests have been conducted in order to see the effects of miRNAs on invasion, migration and apoptosis of GBM cells. Analyses were carried out to determine correlations between selected molecules and clinicopathological features. 1332 genes and 319 miRNAs were found to be dysregulated by the microarrays. The results were combined and analyzed with Transcriptome Analysis Console 3 software and the DAVID online database. Primary differential pathways included Ras, HIF-1, MAPK signaling and cell adhesion. OncomiR candidates 21-5p, 92b-3p, 182-5p and 339-5p for glioblastoma negatively correlated with notable mRNA targets both in tissues and in *in vitro* experiments. miR-21-5p and miR-339-5p significantly affected migration, invasion and apoptosis of GBM cells *in vitro*. Significant correlations with overall survival, tumor volume, recurrence and age at diagnosis were discovered. In this article we present valuable integrated microarray analysis of glioblastoma samples regarding miRNA and gene-expression levels. Notable biomarkers and miRNA-mRNA interactions have been identified, some of which correlated with clinicopathological features in our cohort.

Abbreviations: WHO, World health organization; miRNA, micro RNA; PCR, Polymerase Chain Reaction; ANOVA, Analysis of variance; RPKM, Reads per Kilobase Million; DAVID, Database for Annotation Visualization and Integrated Discovery

* Corresponding author at: İçerenköy Mahallesi Hastane Sokak No: 4, 4/1 Atagehir, Istanbul, Turkey.

E-mail addresses: aysegul.kuskucu@yeditepe.edu.tr (A. Kuskucu), kucukkaraduman@ug.bilkent.edu.tr (B. Kucukkaraduman), muhammadakbar@bilkent.edu.tr (M.W. Akbar), agure@bilkent.edu.tr (A.O. Gure), ofbayrak@yeditepe.edu.tr (O.F. Bayrak), abdalan@yeditepe.edu.tr, haskanlik@yeditepe.edu.tr (A.B. Dalan).

<https://doi.org/10.1016/j.brainres.2018.08.035>

Received 3 May 2018; Received in revised form 7 August 2018; Accepted 31 August 2018

Available online 31 August 2018

0006-8993/© 2018 Published by Elsevier B.V.

1. Introduction

Glioblastoma is the most frequently occurring primary brain tumor in adults, comprising half of all diffuse gliomas. It is a poorly differentiated astrocytic tumor, with high cell density, nuclear atypia, a high Ki-67 index, invasiveness and resistance to chemotherapy (Louis et al., 2007; Ricard et al., 2012). Despite new approaches to therapy, the prognosis of the disease is still poor with a median survival time of 12–15 months. Thus, new treatment strategies are needed (Vogelbaum and Aghi, 2015).

microRNAs (miRNAs) are small, non-coding RNA molecules that are about 22 nucleotides long. There is growing interest in the role of miRNAs in the initiation and progression of cancer. Several studies have been conducted to discover the signature miRNA expression patterns of glioblastoma, and functional experiments have been undertaken on a number of molecules and their downstream targets. One study determined that miR-26a regulates PTEN in high-grade gliomas and plays a role in tumorigenesis (Huse et al., 2009). In another study, miR-10b was upregulated in the disease and associated with a high grade and, potentially, invasion (Sasayama et al., 2009). In other studies, miR-21, miR-7, miR-34a and miR-101 were presented as key miRNAs for the disease (Chen et al., 2008; Kefas et al., 2008; Li et al., 2011; Smits et al., 2010).

Recently, the World Health Organization (WHO) announced a new classification of glioblastomas in which IDH-wildtype and IDH-mutant tumors were classified as different groups. IDH-wildtype is observed in 90% of glioblastoma cases with a vast majority in patients over the age of 55. The IDH-mutant type is observed in 10% of all cases, mostly in younger patients (Cloughesy et al., 2014; Louis et al., 2016; Ohgaki and Kleihues, 2013).

A better understanding of molecular mechanisms behind the initiation and progression of glioblastoma is needed. In this study, we sought to uncover data about miRNA and mRNA relationships in glioblastomas by combining the mRNA and miRNA expression profiles of 16 glioblastoma samples, four of which were IDH mutants, seven healthy brain tissue samples, and two healthy cell lines. Here, we present an analysis of integrated data with respect to glioblastoma versus healthy and IDH-wildtype versus IDH-mutant subtypes. Notable miRNAs and mRNAs were individually observed for targeting, along with their correlation with clinicopathological features in our patient cohort.

2. Results

2.1. Differentially expressed miRNAs and mRNAs in our GBM cohort

16 glioblastoma samples, 7 samples of healthy brain tissue, and 2 healthy astrocyte cell lines were used to profile miRNA and gene expression. For additional tests with patient samples 50 patient samples were used (Supp. Table 1). 1332 genes were found to be upregulated and 1149 were found to be downregulated as a result of whole transcriptome microarray (see Supp. Fig. 1, which shows the array study report for the gene expression microarray). With the miRNA microarray 319 miRNAs were found to be upregulated and 214 were found to be downregulated (see Supp. Fig. 2, which shows the array study report for the miRNA microarray). To increase confidence levels for the selection of candidate genes and miRNAs, a fold change (linear) of less than -4 or greater than 4 and an ANOVA p value (condition pair) of < 0.01 were chosen as cutoff value in both arrays. The results from both microarrays were combined and analyzed by using TAC software (Affymetrix) and DAVID online databases. Dysregulated mRNAs were analyzed for their collective effect on important molecular and cellular events (Table 1).

Additionally, further comparisons of microarray profiles were conducted by comparing IDH-wildtype samples with IDH-mutant samples. Twelve IDH-wildtype and four IDH-mutant samples were compared. Between the two groups, 34 miRNAs and 641 mRNAs were

differentially regulated. Table 2 lists the affected pathways and notable deregulated genes.

Candidate mRNAs and miRNAs were chosen according to pathways and networks relevant to glioblastoma, as well as well-matched expression levels of miRNAs and their potential mRNA targets. In order to eliminate nonconforming miRNA-target gene pairs in terms of expression, miRNAs and their relevant predicted and validated targets have been compiled from databases and filtered according to consistent expression in miRNA-target gene pairs. miRNA-target gene pairs which had nonconforming expression levels, were left out and, only pairs with high miRNA expression and low target gene expression were selected in order to ensure the targeting is phenotypically in our current cohort. Next, web-based databases: mirdb.org/mirDB/, mirtarbase.mbc.ntu.edu.tw, http://targetscan.org/, pictar.mdc-berlin.de, http://www.microrna.org have been used to evaluate which of the gene-miRNA pairs are relevant to GBM and cancer in general. Predicted targets were further selected to balance prediction-match scores, expression level and the relevancy and novelty of the genes in glioblastoma research literature. The target prediction scores from the above mentioned databases were taken into consideration in order to increase the reliability of the selection (Supp. Table 2). The data discussed in this publication have been deposited into the NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE90604 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE90604>).

2.2. Microarray data was confirmed with real-time PCR in a greater number of patient samples

The levels of selected miRNAs and mRNAs in patient samples were verified through real-time PCR. The levels of miR-21-5p, miR-92b-3p, miR-182-5p, and miR-339-5p were in synchrony with the microarray data as their levels were significantly higher in tumor samples than in healthy samples (Fig. 1). NEGR1, ANKS1B, NEFM, NEFL, UNC13C, NPTX1 and TUSC3 levels were also confirmed to be coherent with the microarray data. All results were statistically significant (Fig. 1).

2.3. Anti-miR mimics were successfully transfected into GBM cells

The transfection capability of the X-tremeGENE siRNA Transfection Reagent, and the ability of the anti-miR mimics to suppress their target miRNAs in two glioblastoma cell lines were assessed. Cy3-labeled anti-miR mimics were visualized after transfection. Fluorescence microscopy showed that the Cy3-labeled miRNA constructs were successfully transfected into cells (Supp. Fig. 3A). No fluorescence was detected in the control groups. Upon transfection of anti-miR mimics, corresponding miRNA levels significantly decreased in the glioblastoma cell lines after 48 h (Supp. Fig. 3B).

2.4. Correlation between miRNAs and corresponding targets in glioblastoma cells

We checked whether miRNAs and their target mRNAs are negatively correlated in glioblastoma samples 48 h after transfection of anti-miRs into cell lines by directly measuring the miRNA levels and the levels of their corresponding mRNA targets. In cell lines, UNC13C levels could not be detected. The remaining anti-miRs successfully increased the corresponding target mRNA level in at least one of the cell lines (Fig. 2).

To evaluate the potential regulation of mRNA targets by selected miRNAs, we checked, if there was a correlation between their levels in patient samples. miR-21-5p expression was negatively correlated with NEGR1 and ANKS1B expression, and miR-92b-3p expression was negatively correlated with NEFL expression, whereas miR-339-5p expression was negatively correlated with TUSC3 expression (Fig. 3).

Table 1
Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, functions and notable molecules of glioblastoma versus healthy samples.

Functional Analysis					
	Gene Count	%	p-value	Benjamini	
Cell Junction	52	15.0	6.2E-21	9.8E-19	
Cell Adhesion	25	7.2	8.7E-7	1.4E-5	
EGF-like domain	15	4.3	3.1E-5	4.0E-4	
cAMP	5	1.4	2.3E-3	1.0E-2	
Kinase	21	6.1	1.5E-2	9.6E-2	
Inflammatory response	7	2.0	3.5E-2	1.8E-1	
Notch signaling	4	1.2	4.8E-2	2.3E-1	
KEGG PATHWAY Analysis					
Cell adhesion molecules	12	3.5	1.4E-4	3.1E-3	
Ras signaling	13	3.8	2.1E-3	2.5E-2	
HPV-1 signaling	7	2.0	1.5E-2	1.2E-1	
MAPK signaling	10	2.9	7.6E-2	3.6E-1	
Notable Molecules					
Symbol	Exp Fold Change	Location	Molecule Type	Biomarker Application	Drug(s)
CD68	4.14	Plasma Membrane	other	diagnosis, efficacy	
CDKN2A	4.16	Nucleus	transcription regulator	diagnosis, disease progression, efficacy, prognosis	
RNASE2	4.25	Cytoplasm	enzyme	diagnosis	
VIM	4.61	Cytoplasm	other	diagnosis, efficacy, prognosis	
JAG1	4.7	Extracellular Space	growth factor	efficacy	
HILPDA	5.07	Cytoplasm	other	diagnosis	
NAMPT	5.49	Extracellular Space	cytokine	diagnosis, prognosis	RPT-9274
EZH2	6.29	Nucleus	transcription regulator	diagnosis, disease progression, efficacy	EPZ-6438, GSK2816126, CPI-1205
IL6R	6.48	Plasma Membrane	transmembrane receptor	efficacy	tocilizumab
NMP14	8.08	Extracellular Space	peptidase	diagnosis, prognosis	rebinostat, marimastat, prinomastat
TIMP4	8.09	Extracellular Space	other	diagnosis, prognosis	
WFS1	8.26	Cytoplasm	enzyme	diagnosis	
ANGPT2	9.87	Extracellular Space	growth factor	diagnosis, efficacy, prognosis	trebananib, CVX-060
ABCC3	12.16	Plasma Membrane	transporter	diagnosis, prognosis	
TOP2A	13.38	Nucleus	enzyme	diagnosis, efficacy, prognosis, response to therapy	etoposide, cytarabine
CD44	13.53	Plasma Membrane	enzyme	diagnosis, disease progression, prognosis	
CHIL3	22.38	Extracellular Space	enzyme	diagnosis, efficacy, prognosis	

For the GBM vs. healthy sample comparison in our cohort, number of genes, percentage of genes in the function or pathway in comparison to the total number of filtered genes for analysis, p-value and Benjamini-Hochberg value for false discovery rates are listed. Additionally relevant molecules are listed with fold change, location, type of molecule, their present role as a biomarker and drugs that act against them.

2.5. miR-21 and miR-339 inhibition decreases migration and invasion in GBM cell lines

Downregulation of miR-21-5p and miR-339-5p significantly decreased invasion and migration capacity of A172 and U-87 cells. anti-miR-182-5p transfection decreased invasion and migration capacity of A172, but it had no effect on invasion and migration in U-87. miR-92b had no observable effect on migration and invasion of GBM cell lines (Fig. 4A,B).

2.6. miR-21 and miR-339 might play a role in apoptosis evasion of GBM cells

Reduction of miR-21-5p and miR-339-5p levels stimulates apoptosis in GBM. anti-miR-21-5p transfection increased Annexin V + 7AAD⁺ cell population, namely the early apoptotic cells after 48 h by %12 in A172 and %32 in A172. anti-miR-339-5p transfection also increased apoptosis by %21 and %62 in A172 and U87, respectively. Anti-miR-92b-3p had no effect on apoptosis in U-87, whereas, it induced apoptosis by %52 in A172 when compared with anti-scr control (Fig. 4C).

2.7. A Number of miRNAs in the dataset correlated with drug sensitivity in RNA-Seq data obtained for GBM

5 miRNAs (MIR155HG, MIR4325, MIR181B2, MIR1234, MIR193A) were identified. 4 of these miRNAs showed a positive and one showed a

negative correlation with irinotecan IC50 values (Fig. 5A). As the result of the second test, we found that increased MIR339 expression correlated with decreased sensitivity to 17AAG (HSP90 inhibitor) in both CCL6 and CGP. MIR339 level was also found to be inversely correlated with Belinostat resistance (HDAC inhibitor) in CGP. Increased MIR92B level was related to resistance to WZ3105 (multi kinase inhibitor, including SRC, ROCK2, NTRK2, FLT3, IRAK1, others). miR-21 level was correlated with ponatinib, and miR-182 level was correlated with Talazoparib resistance (PARP inhibitor) in CGP (Fig. 5B).

2.8. Correlation between the level of dysregulated miRNAs and mRNAs with clinicopathological features of glioblastoma patients

We evaluated the relationships between miRNA and mRNA levels and the clinicopathological features of glioblastoma patients, including age at diagnosis, WHO grade, age at diagnosis, tumor volume, p53, p16, MGMT, Ki-67, Olig-2 and survival. For the chi-square tests, patient samples were separated into two groups of low and high levels of the corresponding miRNA or mRNA level by using the median value in each group.

Among the clinical characteristics evaluated, TUSC3 and NEGR1 expressions were correlated with young age at diagnosis while miR-21-5p expression level was correlated with older age at diagnosis. TUSC3 was also associated with higher tumor volume (Fig. 6). As a result of chi-square tests, TUSC3 was found to be inversely correlated with Olig-2 staining. miR-21-5p was inversely correlated with MGMT status.

Table 2
Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, functions and notable molecules of IDH-wildtype versus IDH-mutant glioblastoma samples.

IDH- vs IDH + Functional Analysis					
	Gene Count	%	p-value	Benjamini	
Cell Adhesion	33	6.4	3.4E-9	3.7E-7	
Cell Junction	31	6.0	3.5E-5	1.3E-3	
EGF-like Domain	13	2.5	3.3E-3	4.6E-2	
KEGG PATHWAY Analysis					
Focal adhesion	17	3.3	8.8E-6	1.7E-3	
Proteoglycans in cancer	16	3.1	2.6E-5	2.4E-3	
MAPK signaling	15	2.9	1.3E-3	5.7E-2	
PI3K-Akt signaling	17	3.3	3.2E-3	9.6E-2	
Ras signaling	11	2.1	2.5E-2	2.9E-1	
cAMP signaling	9	1.8	6.6E-2	4.8E-1	
Notable Molecules					
Symbol	Exp Fold Change	Location	Molecule Type	Biomarker Application	Drug(s)
PCSK2	-8.33	Extracellular Space	peptidase	diagnosis	
CD38	-4.7	Plasma Membrane	enzyme	efficacy, prognosis,	Daratumumab
ERBB3	-4.2	Plasma Membrane	kinase	efficacy, prognosis, safety	afatinib, sapitinib, cecostimab
ERBB4	-3.84	Plasma Membrane	kinase	efficacy, safety	afatinib, osimertinib, piroquinib
PLAT	3.48	Extracellular Space	peptidase	diagnosis, efficacy, prognosis, safety	6-aminocaproic acid
MMP14	3.37	Extracellular Space	peptidase	diagnosis, prognosis	rebinastat, marimastat, prinomastat
PDPN	3.68	Plasma Membrane	other	diagnosis, disease progression, prognosis	
FLNC	3.81	Cytoplasm	other	diagnosis	
MMP9	3.91	Extracellular Space	peptidase	diagnosis, disease progression, efficacy, prognosis	MMP2 MMP9 inhibitor, GS-5745, rebinastat, marimastat, prinomastat, glucosamine
MMP2	2.69	Extracellular Space	peptidase	diagnosis, disease progression, efficacy, prognosis, response to therapy	MMP2 MMP9 inhibitor, GS-5745, rebinastat, marimastat, prinomastat, glucosamine
SOCS3	4.04	Cytoplasm	phosphatase	diagnosis	marimastat, prinomastat
EMP3	4.23	Plasma Membrane	other	diagnosis	
TIMP1	4.34	Extracellular Space	cytokine	diagnosis, disease progression, efficacy, prognosis	
TNC	4.62	Extracellular Space	other	diagnosis, response to therapy	
TNFAIP6	4.78	Extracellular Space	Inflammation		
ADAM12	5.28	Plasma Membrane	peptidase	diagnosis	
ADM	5.37	Extracellular Space	other	prognosis	
miR-21	5.51	Cytoplasm	microRNA	diagnosis	
ADAMTS9	7.46	Extracellular Space	peptidase	diagnosis, disease progression	
SERPINE1	8.9	Extracellular Space	other	diagnosis, disease progression, efficacy, prognosis	drotrecogin alfa
IGFBP2	9.04	Extracellular Space	other	diagnosis, efficacy, prognosis	
VEGFA	9.19	Extracellular Space	growth factor	diagnosis, efficacy, prognosis, safety, unspecified application	Dalteparin, bevacizumab, ranibizumab
IBSP	16.29	Extracellular Space	other	efficacy	
ABCC3	21.58	Plasma Membrane	transporter	diagnosis, prognosis	

For the comparison of IDH wild-type and IDH mutant samples in our cohort, number of genes, percentage of genes in the function or pathway in comparison to the total number of filtered genes for analysis, p-value and Benjamini-Hochberg value for false discovery rates are listed. Additionally relevant molecules are listed with fold change, location, type of molecule, their present role as a biomarker and drugs that act against them.

Additionally low NEGR1 level was associated with recurrence in our samples. Finally, NEGR1 was positively correlated with p16 (Table 3). As a result of Kaplan-Meier survival analysis low levels of miR-92b-3p, miR-182-5p, NEFM and UNC13C were associated with shorter overall survival (Fig. 7). Bioinformatics survival analysis showed that expression of miR-182, NEFM and UNC13C were positively correlated with increased overall survival time in GBM. Although miR-92b expression was associated with good prognosis in our patient cohort, we could not detect any significance for miR-92b on survival in survival analysis with The Cancer Genome Atlas (TCGA) data (Supp. Fig. 4).

3. Discussion

Glioblastoma is the most aggressive primary malignant tumor of the brain that can arise in the cerebral cortex, cerebellum, brain stem or spinal cord. These tumors appear most commonly after the seventh decade of life. Glioblastomas are poorly differentiated astrocytomas that exhibit low apoptosis, high mitosis and rich neo-angiogenesis. Although the origins and genetic features of this tumor are highly heterogeneous, common amplifications of EGFR and PDGFRA, and

losses of NF1, PTEN and CDKN2A/B, have been observed (Brennan et al., 2013; Patel et al., 2012; Ramkissoon et al., 2015). Recently, WHO defined a criterion of IDH mutation to determine the glioblastoma grade. Mutations in IDH1 were detected in an integrated genomic analysis study (Parsons et al., 2008) and tumors with an IDH-mutated genotype are associated with double the overall median survival rate compared with those having an IDH-wildtype genotype (Molenaar et al., 2014). With only a median survival of 14 months under combined therapy, glioblastoma continues to be one of the most devastating tumors of the central nervous system despite extensive studies to determine molecular markers and new surgical and treatment methods (Shirazi et al., 2011; Veliz et al., 2015). Therefore, efforts to identify biomarkers that are related to tumor grade, survival and clinicopathological features are crucial to improve treatment options for patients with the disease. In this study, we present a combined miRNA-mRNA analysis of a glioblastoma cohort with the latest versions of two microarrays using the same set of patient samples for both. Although there is a considerable number of studies about miRNA and gene expression profiles in GBM tissues, combining the data from miRNA and mRNA profiles of the same patients in our study provides novel and

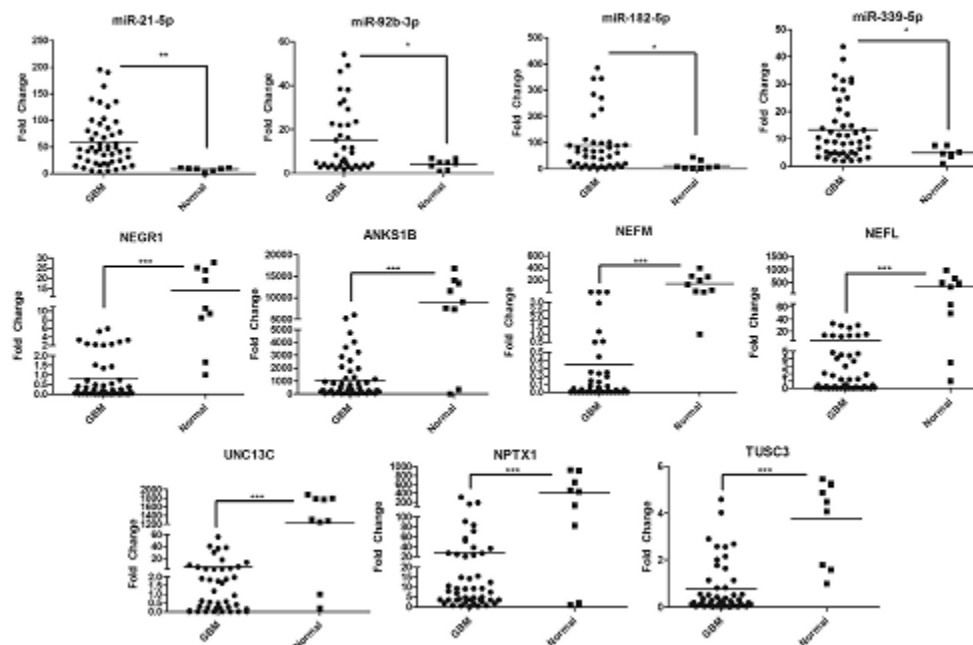


Fig. 1. The relative expression levels of selected miRNAs and mRNAs in patient samples as determined by real-time PCR analysis. Each point represents a patient sample or healthy control cell line. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

valuable information about glioblastoma pathways and gene networks, since this is the most reliable approach to observe how the level of a miRNA can influence the level of its target genes in the cell.

In both glioblastoma versus healthy and IDH-wildtype versus IDH-mutated groups, we observed activation of similar pathways and functions as a result of analysis using the DAVID database. In the IDH-wildtype group, the major tumor-driving pathways in glioblastoma, such as MAPK, Ras and cAMP signaling, were more active than in the IDH-mutated group. Furthermore, ITGB3, ITGB5, MMP2, MMP9 and PLAU, which have been associated with glioma invasiveness, were upregulated in the GBM vs healthy comparison. This pattern might be one of the driving factors of a worse prognosis (Zheng et al., 2014). We also observed that, in the IDH-wildtype versus IDH-mutated comparison, PI3K-Akt signaling was active which is in synchrony with previous studies (Birner et al., 2014; Wakimoto et al., 2014). This relation was absent in the tumor-versus-healthy comparison. Such differences can be put to use to elucidate the different mechanisms behind tumorigenesis in IDH-wildtype and IDH-mutated tumors. Additionally, as a result of *in silico* drug sensitivity correlation study, we have presented further miRNAs; miR-155, miR-4325, miR-182b, miR-1234 and miR-192a as potentially important genes for GBM as they significantly correlate with resistance to irinotecan which is a cytotoxic drug. Moreover, as a result of a linear correlation analysis between miRNA RPKM values and IC50 data RNA-Seq analysis, using E-MTAB-2706 data, miR-339, miR-92b, miR-21 and miR-182 were found to be correlated with resistance to 17AAG, WZ3105, AP24534 and Talazoparib respectively.

In this study, miRNAs 339-5p, 21-5p, 92b-3p, and 182-5p were found to be significantly upregulated in glioblastoma samples. The potential oncogenic role of miR-339 in glioblastomas and its relationship with ICAM-1 has been mentioned previously, in which downregulation of the miRNA, increases ICAM-1 expression and promotes

CTL-mediated cytotoxicity (Ueda et al., 2009). Previous studies have pointed out the overexpression of miR-21 in glioblastoma and other cancers as a predictive marker for overall survival (Yang et al., 2014). In glioblastomas, overexpressed miR-92b-3p is associated with invasion, migration and apoptotic evasion (Song et al., 2016). Additionally, miR-182-5p has been reported to be upregulated by STAT3 and to promote glioma tumorigenesis (Xue et al., 2016). In this study, miR-21-5p is associated with decreased MGMT expression. It has been reported that low expression of MGMT is associated with longer overall survival and response to radiotherapy and chemotherapy in glioblastoma (Hegi et al., 2005; Rivera et al., 2010). In the current study, low miR-92b-3p and miR-182-5p levels were correlated with a shorter overall survival. miR-182-5p downregulation was also found to be significantly correlated with overall survival in the TCGA analysis. Additionally, miR-21-5p and miR-339-5p were both associated with migration, invasion and apoptosis in both GBM cell lines used in this study. Upregulation of miR-21 has been associated with increased invasion and migration capability in U-87 and A-172 previously (Luo et al., 2017). Our study confirms these results, as downregulation of the miRNA has decreased these abilities. Our results also confirm the anti-apoptotic role of miR-21 in gliomas that has been reported previously (Chai et al., 2018). Taken together, these three miRNAs can be driving factors of the rapid proliferation, and invasive character of glioblastoma cells.

After transfecting glioblastoma cell lines with the corresponding anti-miR molecules, we have observed that NEGR1, ANKSB, NEFM, NEFL, NPTX1 and TUSC3 levels significantly increased in at least one of the glioblastoma cell lines. NEGR1 is a newly identified tumor suppressor that has been shown to be downregulated in colon, liver, lung, ovary, stomach and pancreatic cancers. Additionally, downregulation of the gene caused an increase in cell migration and invasion in these cells (Kim et al., 2014). We discovered that this gene is also

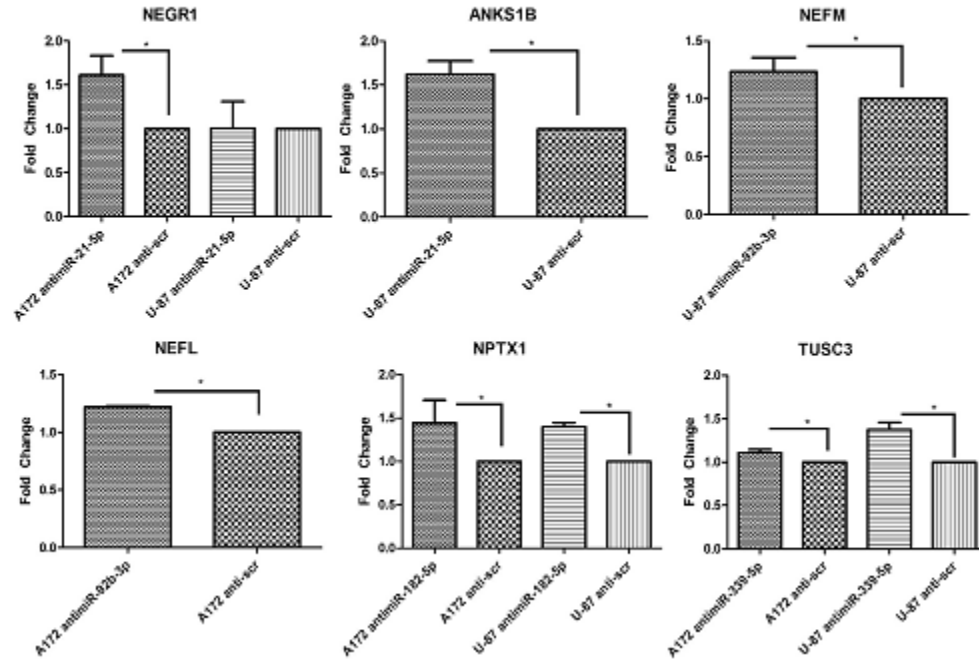


Fig. 2. mRNA expression levels after targeting with corresponding anti-miRNA molecules. Anti-miR-21-5p, Anti-miR-93-3p, Anti-miR-182-5p, and Anti-miR-339-5p were administered to A-172 and U-87 cells, and target mRNA levels were determined after 48 h. No UNL13C level could be detected in the cell lines; therefore, they were excluded from the results. '*' indicates $p < 0.05$.

downregulated in glioblastoma samples. Additionally, NEGR1 expression is positively correlated with the p16 level in our cohort. Several studies have reported that p16 dominantly suppresses glioblastoma growth and is a well-known tumor suppressor for the disease (Hung

et al., 2000; Simon et al., 2002). These findings suggest that NEGR1 might be executing a tumor suppressor role in GBM.

NEFM and NEFL cooperate to form neurofilaments in mature neurons. DNA methylation-associated silencing of neurofilament genes,

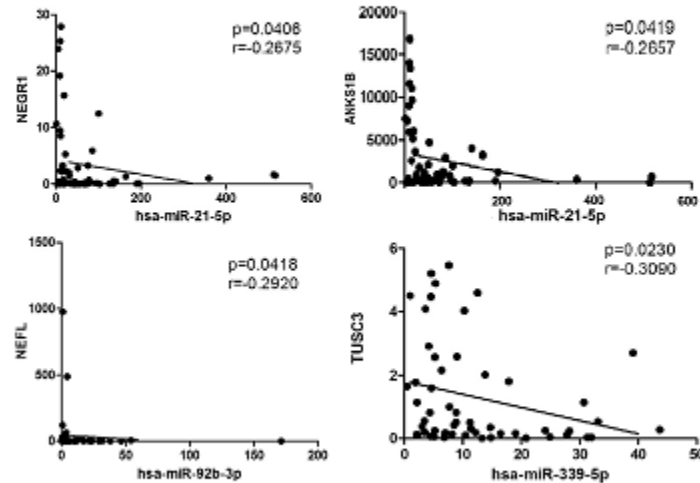


Fig. 3. Spearman correlation of miRNAs and their corresponding mRNA targets in patient samples. 'p' indicates significance and 'r' is the correlation coefficient.

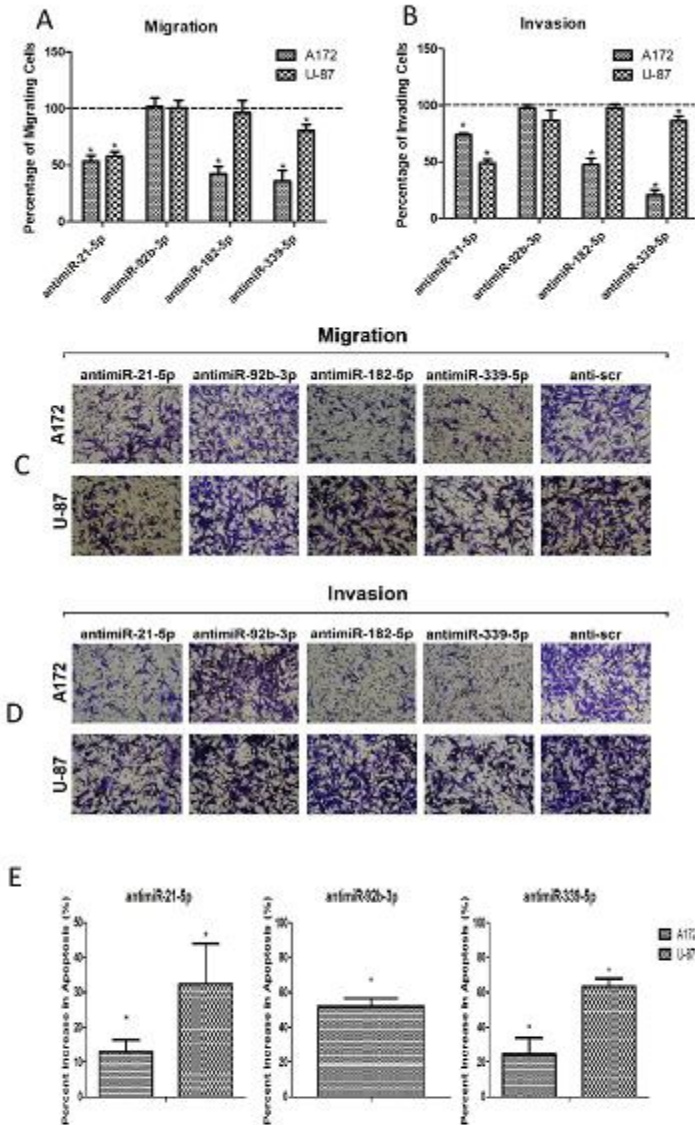


Fig. 4. Functional effects of selected miRNAs on GBM cell lines. A) Columns indicating relative migration capability after anti-miR transfection of A172 and U-87 cell lines. B) Columns indicating relative invasion capability after anti-miR transfection of A172 and U-87 cell lines. C, D) Representative images of the transwell chambers under light microscope. E) Columns indicate percent increase in relative number of apoptotic cells after anti-miRNA transfection of A172 and U-87 cell lines in comparison to anti-miRNA mimic controls. * indicates $p < 0.05$.

including NEFM and NEFL, promotes an aggressive phenotype in breast, pancreatic, gastric and colon cancers. Restoring neurofilament production through this gene network reduces cell proliferation and growth of breast cancer cells (Culmon et al., 2015). Here, we show that these two genes are also downregulated in glioblastoma, and regulated with miR-92b-3p. Moreover, low level NEFM is associated with a shorter overall survival in our samples as well as the TCGA database

analysis. Furthermore, lower NEFL levels are associated with a higher risk of recurrence in our cohort. In one study, an abundance of neurofilament-protein expression was associated with a distinct clinical and anatomical behavior as well as higher overall survival and progression-free survival in patients with glioblastoma (Pallud et al., 2012). Taken together, neurofilament-related genes and regulators such as miR-92b-3p, NEFM and NEFL might be decisive biomarkers for glioblastoma

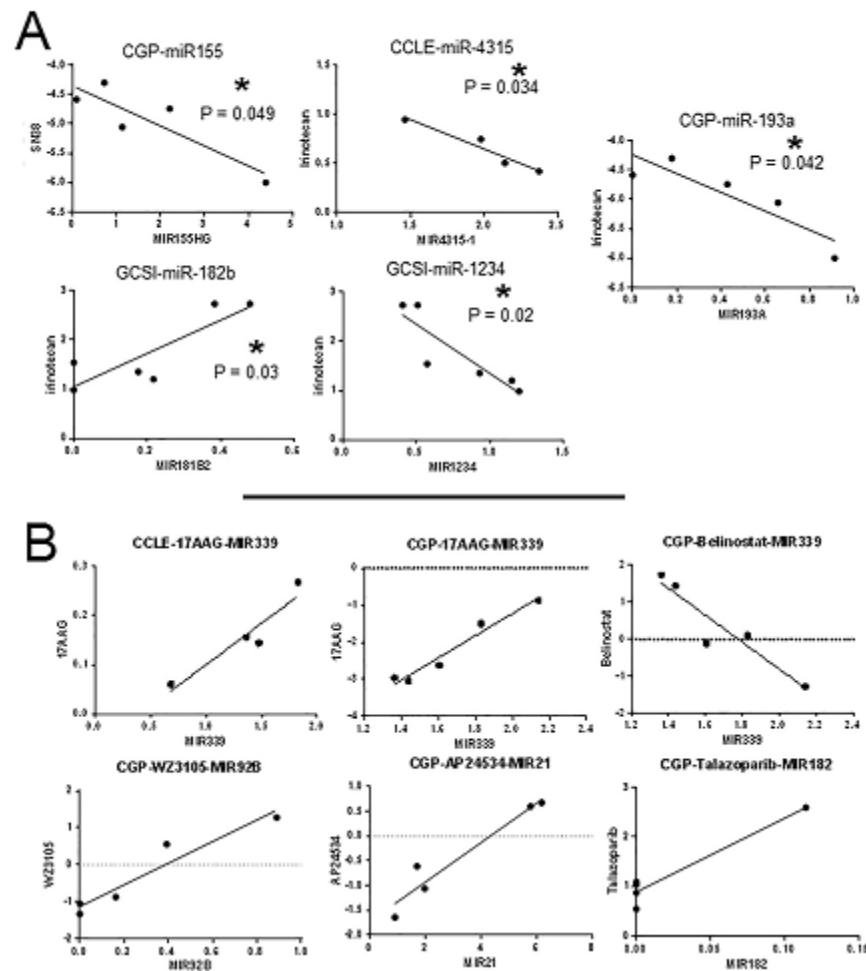


Fig. 5. In silico drug sensitivity analysis of miRNAs. A) IC50 value of Irinotecan is positively correlated with MIR155HG, MIR4325, MIR1234, MIR193A and negatively correlated MIR181B2. B) Selected miRNAs were also correlated with 17AAG (HSP90 inhibitor), Belinostat resistance (HDAC inhibitor), WZ3105 (multi kinase inhibitor), AP24534 resistance (multi-target inhibitor of Abl, PDGFR α , VEGFR2, FGFR1 and Src) and Talazoparib resistance (PARP inhibitor). “*” indicates $p < 0.05$.

progression.

UNC13C was identified as one of the most frequently mutated genes in gingivo-buccal oral squamous cell carcinoma (India Project Team of the International Cancer Genome, 2013). Both in the current study and the TCGA, this gene was correlated with overall survival which is another hint that it might be an important cancer-associated gene. TUSC3 is a potential tumor suppressor in cancers, and in ovarian cancer it is associated with progression-free and overall survival (Pils et al., 2013). Our results reveal that this gene is also a potential tumor suppressor in glioblastomas as it is significantly downregulated in glioblastoma samples. In the current study TUSC3 is inversely correlated with Olig2 expression in patient samples, which is a specific GBM cancer stem cell marker (Trepant et al., 2015). Nevertheless, TUSC3 had a positive correlation with tumor size, which is a contradictory result.

4. Conclusions

Our study presents valuable integrated data about mRNA and miRNA expression in glioblastoma samples. Markers that can play a role in glioblastoma pathophysiology have been determined, and the results reveal a relationship between miRNAs and novel potential target molecules in glioblastoma. Further studies of these gene networks can produce translational information, leading to a better understanding of the initiation and progression of glioblastomas and perhaps introducing alternative treatment approaches for the disease.

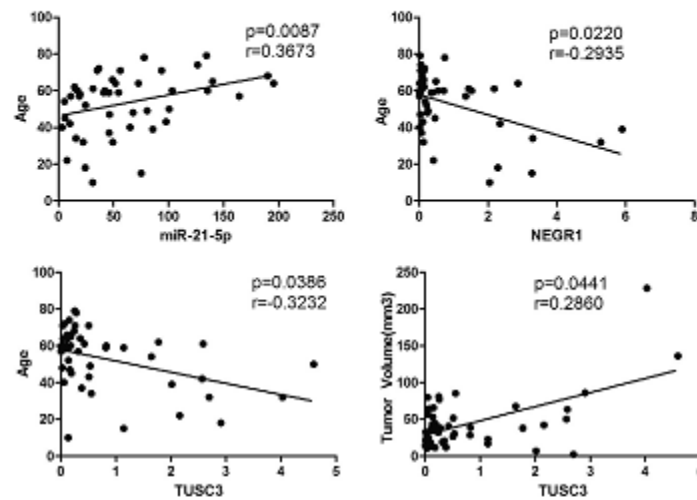


Fig. 6. Spearman correlation between clinicopathological features and the level of selected miRNAs and genes: miR-25p with Age, NEGR1 with Age, TUSC3 with Age and TUSC3 with Tumor volume. 'p' indicates significance and 'r' is the correlation coefficient.

Table 3
Correlation between the expression level of selected miRNAs and mRNAs with clinicopathological features.

Clinicopathological Features	Molecule Name	Low	High	Total	p-Value
Olig2	TUSC3				
	Positive	25	21		0.0371
	Negative	0	4		
MGMT Status	miR-21-5p				
	Positive	23	12	35	0.0308
	Negative	2	13	15	
Recurrence	NEFL				
	Recurrence	6	1	7	0.0416
	No Recurrence	19	24	43	
P16 Status	NEGR1				
	Positive	3	11	14	0.0117
	Negative	22	14	36	

Selected miRNAs and mRNAs were analyzed with the chi-square test for Gender, Olig2, MGMT status, Recurrence, WHO grade, p16 status. Significant results are shown here.

5. Experimental procedure

5.1. Ethics statement

This study was approved by the Institutional Review Board of Yeditepe University Hospital (No: 000274/2013), and written informed consent was obtained from all participants.

5.2. Sample collection

GBM tissue samples and healthy tissue samples were obtained from Department of Neurosurgery, Yeditepe University Medical School, Yeditepe University, Istanbul, Turkey from July 2013 to February 2015. Fifty GBM tissue samples and seven healthy tissue samples were used. Five of the seven healthy tissues were autologous tissues taken from corresponding patients in our tumor sample group. The other two were healthy brain tissues obtained from other brain surgery. Tissues were fresh frozen and preserved at -80°C until RNA extraction. The

clinicopathological data was collected retrospectively. The follow-up time was calculated from the date of surgery to death or last check date (May 2016). GBM tissue samples that were pathologically defined as GBM and contained at least 50% tumor were included to the study. Samples lacking sufficient clinicopathological information or sufficient follow-up time were excluded from the study.

Clinical information was collected for each sample, including demographic data, tumor location, and prognosis. (Supp. Table 1).

5.3. Cell cultures

A-172 and U-87 cell lines, available at ATCC: The Global Bioresource Center, were kindly provided by Koc University (Istanbul, Turkey). Cells were cultured in culture flasks or well plates in DMEM and EMEM, respectively, containing 10% fetal bovine serum and 1% antibiotics. Normal Human Astrocytes were obtained from Lonza (Allendale, NJ) and grown with AGM BulletKit (Lonza). Human Fetal Astrocytes were obtained from ABM (Richmond, British Columbia, Canada), and grown in collagen-coated plates according to the manufacturer's protocol.

Cell lines were checked routinely for surface markers and molecular markers by using Sanger sequencing for short tandem repeats. Polymerase chain reaction-based mycoplasma kit was routinely used to screen for contamination.

5.4. Microarray analysis

Tissues were ground with liquid nitrogen, followed by the TRIzol (ThermoFisher Scientific) method, according to the manufacturer's protocol, to obtain total RNA. Cell lines were harvested through the direct addition of TRIzol to proceed to total RNA isolation. Whole transcriptome expression profiling was done using Affymetrix Human Gene 2.1 ST Array Strip (Cat no: 902114, Affymetrix, Santa Clara, CA). miRNA microarray analysis was done with the Affymetrix miRNA 4.1 Array Strip (Cat no: 902404). The GeneAtlas system (Affymetrix) was used to hybridize, wash, stain and visualize the chips. The acquired data was analyzed with Transcriptome Analysis Console (TAC) 3 software (Affymetrix) and the DAVID online database.

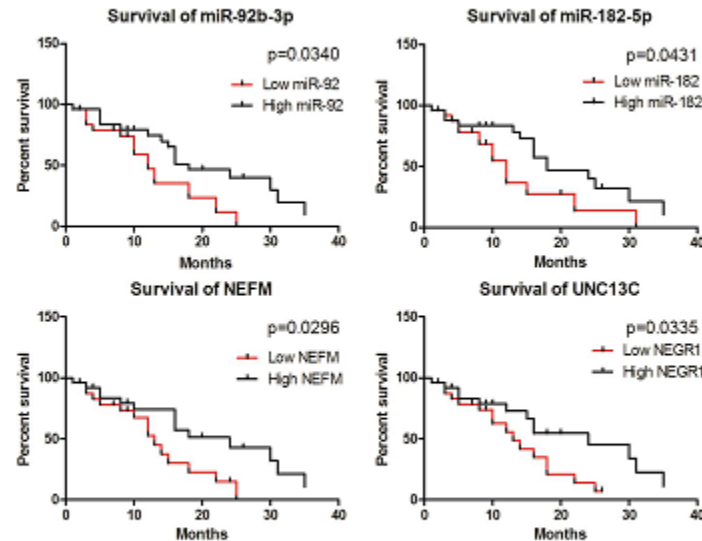


Fig. 7. Kaplan-Meier survival plots of our patient cohort. High and low miR-92b-3p, miR-182-5p, NEFM and UNC13C groups were defined according to the relative expression levels, separated according to the median values.

5.5. miRNA and mRNA expression levels

Levels of miRNAs of interest in glioblastoma tissues, control tissues, and cell lines were evaluated with real-time polymerase chain reaction (PCR) using miRNA primers obtained from Exiqon (Vedhæk, Denmark) after cDNA synthesis from total RNA samples, according to the manufacturer's protocol (Exiqon, Cat. No.: 203300). mRNA levels were measured with Taqman primers (ThermoFisher Scientific) after cDNA synthesis. Data was analyzed with the $2^{-\Delta\Delta Ct}$ method. 5S RNA and GAPDH were used for normalization.

5.6. Anti-miRNA transfections

Cy3 Dye-Labeled anti-miR Negative Control #1 (Cat: AM17011, ThermoFisher Scientific) was used to test the ability of X-tremeGENE siRNA Transfection Reagent (Roche, Germany) to transfect glioblastoma cell lines after 48 h. After validation, cells were transfected with anti-miRNA mimics (Ambion anti-miR miRNA Precursors PM: 114065) followed by total RNA isolation, miRNA reverse transcription and real-time PCR. The targeted miRNA levels were measured 48 h after transfection. Scrambled anti-miRNA molecules were used as the control group.

5.7. Apoptosis assay

BD Apoptosis Detection Kit (BD Pharmingen, San Diego, California) was used to detect early apoptotic cells (Annexin V + 7-AAD-) 48 h after transfection of anti-miR-21-5p, anti-miR-92b-3p, anti-miR-182-5p and anti-miR-339-5p according to the manufacturer's protocol by using BD FACS Aria III (BD Biosciences).

5.8. Trans-well invasion and migration assay

Transwell membrane inserts of 8.0 μ m (Millipore, Burlington, Massachusetts) were used to evaluate changes in the invasion and migration of transfected cells. Chambers were coated with 100 μ l of 250 μ g/ml Basement Membrane Matrigel (BD Biosciences) and

incubated overnight at 37 °C for the invasion assay. A172 and U-87 cells were transfected with anti-miR-21-5p, anti-miR-92b-3p, anti-miR-182-5p and anti-miR-339-5p. After 24 h of transfection, cells were de-attached with trypsin and 5x10⁴ cells were transferred into the upper chambers in 100 μ l serum-free DMEM. The lower chambers filled with 1300 μ l DMEM supplemented with 20% FBS and incubated for two days. After that, Inserts were fixed with 3.7% formaldehyde, permeabilized with methanol and stained with crystal violet. Inverted microscope was used to score.

5.9. In silico correlation analysis of miRNA expression and drug sensitivity

To identify miRNAs whose expression correlates with drugs sensitivity, we utilized RNA-seq data obtained for glioblastoma cell lines from the E-MTAB-2706 (Klijn et al., 2015). We first performed a linear correlation analysis between miRNA RPKM values and IC50 data generated for irinotecan in 3 different cytotoxicity databases, CCLE (Barretina et al., 2012), CGP (Garnett et al., 2012) and gCSI (Haverty et al., 2016). miRNAs that showed a correlation (Pearson's) of either greater than 0.6 or < -0.6 with IC50 values in all three databases, and which had a p value (Pearson's) lower than 0.05 in at least one database were selected. We next tested whether miRNAs 21, 92b, 182 OR 339 expression in glioblastoma correlated with sensitivity to any of the drugs in the three databases mentioned above.

5.10. Statistical analysis

The one-way between-subjects ANOVA (unpaired) method was used to evaluate the microarray results. Real-time PCR data was analyzed by the $2^{-\Delta\Delta Ct}$ method. Spearman's two-tailed correlation test was used to determine correlations between tumor size and mRNA-miRNA levels, and miRNA and mRNA target levels. For other clinicopathological features, the two-tailed chi-square test was used. The calculation and interpretation of p values for functions and gene networks at Tables 1 and 2 was done with a right-tailed Fisher's exact test. The overall survival graphs were prepared by using Kaplan-Meier survival analysis with the Mantel-Cox test. All other statistical analyses were done using

student's *t*-test. Differences with *p* values of < 0.05 were considered statistically significant. The high and low miRNA or mRNA expression groups of patients were separated according to the median expression level value. Significant outliers were detected and excluded from analysis. For confirmation of survival analyses, miRNAs and mRNAs which are significantly associated with overall survival time in our cohort were also analyzed in online biomarker validation tools (Aguirre-Gamboa et al., 2013; Aguirre-Gamboa and Trevino, 2014). Glioblastoma data obtained from TCGA (The Cancer Genome Atlas) were analyzed using bioinformatics tools (TCGA data version: 2016_01_28).

Acknowledgements

We would like to thank Julie Yamamoto for her editorial assistance.

Funding

This work was supported by Yeditepe University Hospital, Istanbul.

Availability of data and materials

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE90604.

Authors' Contributions

SG and MS carried out microarrays. Cell culturing and flow cytometry was done by ECT. Tumor samples and patient data were acquired by CKY and UT. SG and OFB performed the statistical analysis. SG, AK and ABD were involved in writing and editing the manuscript. BK, MWA and AOG performed *in silico* correlation analysis of miRNA expression and drug sensitivity. SG, ABD and OFB designed the study and provided financial support for this work. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.brainres.2018.08.035>.

References

- Aguirre-Gamboa, R., Gomez-Rueda, H., Martinez-Ledesma, E., Martinez-Torteyo, A., Chacolla-Huaringa, R., Rodriguez-Barrionuevo, A., Tamez-Pena, J.G., Trevino, V., 2013. SurvExpress: an online biomarker validation tool and database for cancer gene expression data using survival analysis. *PLoS One* 8, e74250.
- Aguirre-Gamboa, R., Trevino, V., 2014. SurvMicro: assessment of miRNA-based prognostic signatures for cancer clinical outcomes by multivariate survival analysis. *Bioinformatics* 30, 1630–1632.
- Barretina, J., Caporaso, G., Cawthon, N., Kottmann, K., Margolin, A.A., Kim, S., Wilson, C.J., Lehkar, J., Kryukov, G.V., Sonkin, D., Reddy, A., Liu, M., Murray, L., Berger, M.F., Monahan, J.E., Morris, P., Meltzer, J., Korejwa, A., Jano-Valbuena, J., Mapa, P.A., Thibault, J., Reio-Purton, E., Ramon, P., Shipway, A., Engels, L.H., Cheng, J., Yu, G.K., Yu, J., Apsol Jr., P., de Silva, M., Jagtap, K., Jones, M.D., Wang, L., Hattori, C., Palanciano, F., Gupta, S., Mahan, S., Sotgiu, C., Onofrio, R.C., Irfeld, Y., MacConaill, L., Winckler, W., Rosch, M., Li, N., Mesirov, J.P., Gabriel, S.B., Getz, G., Ardlie, K., Chan, V., Myer, V.E., Weber, B.L., Porter, J., Warmuth, M., Finan, P., Harris, J.L., Meyerson, M., Golub, T.R., Morrissey, M.P., Sellers, W.R., Schlegel, R., Garraway, L.A., 2012. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* 483, 603–607.
- Birner, P., Pasch, S., Christov, C., Mihaylova, S., Tzoumangelou-Utzir, K., Natchev, S., Schoppmann, S.F., Tchorbanov, A., Streubel, B., Tuetzenberg, J., Guentchev, M., 2014. Mutant IDH1 inhibits PI3K/Akt signaling in human glioma. *Cancer* 120, 2445–2447.
- Brennan, C.W., Verhaak, R.G., McKenna, A., Campos, B., Nourbakhsh, H., Salama, S.R., Zheng, S., Chakravarty, D., Sanborn, J.Z., Berman, S.H., Beroukhi, R., Bernard, B., Wu, C.J., Getz, G., Shmulevich, I., Bachholtz-Sloan, J., Zou, L., Vegnna, R., Shukla, S.A., Ciriello, G., Yang, W.K., Zhang, W., Sougnez, C., Miklosden, T., Aldape, K., Bogen, D.D., Van Meir, E.G., Prasad, M., Sloan, A., Black, K.L., Eberhart, J., Finocchiaro, G., Friedman, W., Andrews, D.W., Guha, A., Innocenti, M., O'Neill, B.P., Folz, G., Myers, J., Weisenberger, D.J., Penny, R., Kuchelapati, R., Perou, C.M., Hayes, D.N., Gibbs, R., Marra, M., Mills, G.B., Lander, E., Spellman, P., Wilson, R., Sander, C., Weinstein, J., Meyerson, M., Gabriel, S., Laird, P.W., Haussler, D., Getz, G., Chin, L., Network, T.R., 2013. The somatic genomic landscape of glioblastoma. *Cell* 155, 463–477.
- Calmon, M.F., Jeschke, J., Zhang, W., Dhir, M., Sichenko, C., Herrera, A., Tsai, H.C., O'Hagan, H.M., Pappou, E.P., Hooker, C.M., Fu, T., Scherbel, K.E., Gubrichson, E., Rahal, P., Herman, J.G., Baylin, S.B., Ahuja, N., 2015. Epigenetic silencing of neurofilament genes promotes an aggressive phenotype in breast cancer. *Epigenetics* 10, 622–632.
- Chai, C., Song, L.J., Han, S.Y., Li, X.Q., Li, M., 2018. MicroRNA-21 promotes glioma cell proliferation and inhibits senescence and apoptosis by targeting SPRY1 via the PI3K/AKT signaling pathway. *CNS Neurosci. Ther.*
- Chen, Y., Liu, W., Chao, T., Zhang, Y., Yan, X., Gong, Y., Qiang, B., Yuan, J., Sun, M., Peng, X., 2008. MicroRNA-21 down-regulates the expression of tumor suppressor PDCD4 in human glioblastoma cell T98G. *Cancer Lett.* 272, 197–205.
- Cloughesy, T.F., Cavenee, W.K., Mischel, P.S., 2014. Glioblastoma: from molecular pathology to targeted treatment. *Annu. Rev. Pathol.* 9, 1–25.
- Edgar, R., Domrachev, M., Lash, A.E., 2002. Gene Expression Omnibus: NCM gene expression and hybridization array data repository. *Nucleic Acids Res.* 30, 207–210.
- Genetti, M.J., Edelman, E.J., Heidorn, S.J., Greenman, C.D., Dastur, A., Liu, K.W., Greening, P., Thompson, I.R., Luo, X., Soares, J., Liu, Q., Iorio, F., Sardex, D., Chen, L., Milano, R.J., Bignell, G.R., Tan, A.T., Davies, H., Stevenson, J.A., Barthorpe, S., Lutz, S.B., Kogera, F., Lawrence, K., McLaren-Douglas, A., Mitropoulos, X., Mironenko, T., Thi, H., Richardson, L., Zhou, W., Jewett, F., Zhang, T., O'Brien, P., Boivert, J.L., Price, S., Har, W., Yang, W., Deng, X., Butler, A., Choi, H.G., Chang, J.W., Basella, J., Stamenkovic, I., Engelman, J.A., Sharma, S.V., Delattre, O., Saez-Rodriguez, J., Gray, N.S., Settleman, J., Futreal, P.A., Haber, D.A., Stratton, M.R., Ramaswamy, S., McDermott, U., Benes, C.H., 2012. Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature* 483, 570–575.
- Haverly, P.M., Lin, E., Tan, J., Yu, Y., Lam, B., Lianoglou, S., Neve, R.M., Martin, S., Settleman, J., Yanch, R.L., Bourgon, R., 2016. Reproducible pharmacogenomic profiling of cancer cell line panels. *Nature* 533, 333–337.
- Hegi, M.E., Diserens, A.C., Gorlia, T., Hamou, M.F., de Tilly, M., Weller, M., Kros, J.M., Hainfellner, J.A., Mason, W., Mariani, L., Bromberg, J.E., Hau, P., Miralmon, R.O., Cairncross, J.G., Janzer, R.C., Stupp, R., 2005. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N. Engl. J. Med.* 352, 997–1003.
- Hong, K.S., Hong, C.Y., Lee, J., Lin, S.K., Huang, S.C., Wang, T.M., Tse, V., Silverberg, G.D., Wong, S.C., Hsiao, M., 2009. Expression of p16INK4A induces dominant suppression of glioblastoma growth in situ through necrosis and cell cycle arrest. *Biochem. Biophys. Res. Commun.* 269, 718–725.
- Hsu, J.T., Brennan, C., Hamberdumyan, D., Wei, B., Pero, J., Rouhanifard, S.H., Sohr, Lee, C., le Sage, C., Agami, R., Tuschl, T., Holland, E.C., 2009. The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo. *Genes Dev.* 23, 1327–1337.
- India Project Team of the International Cancer Genome, C., 2013. Mutational landscape of gingivo-buccal oral squamous cell carcinoma reveals new recurrently-mutated genes and molecular subgroups. *Nat. Commun.* 4, 2873.
- Kefau, B., Godlewski, J., Comau, L., Li, Y., Abouadler, R., Hawkinson, M., Lee, J., Pine, H., Chioara, E.A., Lawler, S., Purcell, B., 2008. microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. *Cancer Res.* 68, 3566–3572.
- Kim, H., Huang, J.S., Lee, B., Hong, J., Lee, S., 2014. Newly identified cancer-associated role of human neuronal growth regulator 1 (NEGR1). *J. Cancer* 5, 598–608.
- Klika, C., Durinck, S., Stawski, E.W., Haverly, P.M., Jiang, Z., Liu, H., Depeshard, J., Mayba, O., Gnad, F., Liu, J., Pan, G., Reeder, J., Cao, Y., Mukhyala, K., Selvaraj, S.K., Yu, M., Zynda, G.J., Brauer, M.J., Wu, T.D., Gentileman, R.C., Manning, G., Yanch, R.L., Bourgon, R., Stokoe, D., Medrussa, Z., Neve, R.M., de Sauvage, F.J., Settleman, J., Seshagiri, S., Zhang, Z., 2015. A comprehensive transcriptional portrait of human cancer cell lines. *Nat. Biotechnol.* 33, 306–312.
- Li, W.B., Ma, M.W., Dong, L.J., Wang, F., Chen, L.X., Li, X.R., 2011. MicroRNA-34a targets notch1 and inhibits cell proliferation in glioblastoma multiforme. *Cancer Biol. Ther.* 12, 477–483.
- Louis, D.N., Ohgaki, H., Wiestler, O.D., Cavenee, W.K., Burger, P.C., Jouvet, A., Scheithauer, B.W., Kleihues, P., 2007. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* 114, 97–109.
- Louis, D.N., Perry, A., Reifenberger, G., von Deimling, A., Figarella-Branger, D., Cavenee, W.K., Ohgaki, H., Wiestler, O.D., Kleihues, P., Ellison, D.W., 2016. The 2016 world health organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol.* 131, 803–820.
- Luo, G., Luo, W., Sun, X., Lin, J., Wang, M., Zhang, Y., Luo, W., Zhang, Y., 2017. MicroRNA21 promotes migration and invasion of glioma cells via activation of Sox2 and beta-catenin signaling. *Mol. Med. Rep.* 15, 187–193.
- Molenaar, R.J., Vorbaan, D., Lamba, S., Zanon, C., Jeuken, J.W., Boots-Sprenger, S.H., Wesseling, P., Hulsebos, T.J., Troost, D., van Tilburg, A.A., Levenstra, S., Vandertop, W.P., Bardelli, A., von Noorden, C.J., Bleeker, F.E., 2014. The combination of IDH1 mutations and MGMT methylation status predicts survival in glioblastoma better than either IDH1 or MGMT alone. *Neuro Oncol.* 16, 1263–1273.
- Ohgaki, H., Kleihues, P., 2013. The definition of primary and secondary glioblastoma. *Clin. Cancer Res.* 19, 764–772.
- Palhaj, J., Diczmis, E., Audureau, E., Devaux, B., Souillard-Sommar, R., Sami, N., Page, P., Bravon, F., Kotziak, M., Oppenheim, C., Dhermain, F., Schlimper, M., Meder, J.F.,

- Roux, F.K., Varlet, P., 2012. Neuronal immunorepression and a distinct subtype of adult primary supratentorial glioblastoma with a better prognosis. *J. Neurosurg.* 117, 476–485.
- Parsons, D.W., Jones, S., Zhang, X., Lin, J.C., Leary, R.J., Angerer, P., Mankou, P., Carter, H., Siu, I.M., Gallia, G.L., Olivi, A., McLendon, R., Rasheed, B.A., Keir, S., Nikolskaya, T., Nikolsky, Y., Buzam, D.A., Tokleah, H., Diaz Jr., L.A., Hartigan, J., Smith, D.R., Strimberg, R.L., Marie, S.K., Shiro, S.M., Yan, H., Riggins, G.J., Bigner, D.D., Kachin, R., Papadopoulos, N., Parmigiani, G., Vogelstein, B., Velculescu, V.E., Kinzler, K.W., 2008. An integrated genomic analysis of human glioblastoma multiforme. *Science* 321, 1807–1812.
- Patel, M., Vogelbaum, M.A., Barnett, G.H., Jalali, R., Ahlawalia, M.S., 2012. Molecular targeted therapy in recurrent glioblastoma: current challenges and future directions. *Exp. Opin. Investig. Drugs* 21, 1247–1266.
- Pills, D., Horak, P., Vashara, P., Ance, M., Petz, M., Alfanz, A., Gugerell, A., Wittinger, M., Gless, A., Auner, V., Tong, D., Zellinger, R., Bratcu, E.I., Schouli, J., Krainer, M., 2013. Methylation status of TUSC3 is a prognostic factor in ovarian cancer. *Cancer* 119, 946–954.
- Ramkissoon, S.H., Bi, W.L., Schumacher, S.E., Ramkissoon, L.A., Haider, S., Kioff, D., Dubuc, A., Brown, L., Burns, M., Gyan, J.B., Abedalthagafi, M., Kang, Y.J., Schultz, N., Beardon, D.A., Lee, E.Q., Rinne, M.L., Norden, A.D., Nayak, L., Ruland, S., Doherty, L.M., LaFrankie, D.C., Horvath, M., Aizer, A.A., Russo, A., Arvold, N.D., Claus, E.B., Al-Mefty, O., Johnson, M.D., Golby, A.J., Dunn, I.F., Chiocca, E.A., Tripp, L., Santiago, S., Folkerth, R.D., Karsner, P., Rollins, B.J., Lindeman, N.J., Wen, P.Y., Ugon, A.H., Beroukhi, R., Alexander, B.M., Ugon, K.L., 2015. Clinical implementation of integrated whole-genome copy number and mutation profiling for glioblastoma. *Neuro Oncol.* 17, 1344–1355.
- Ricard, D., Mhail, A., Ducray, F., Lohut, M., Hoang-Xuan, K., Delattre, J.Y., 2012. Primary brain tumours in adults. *Lancet* 379, 1996–1996.
- Rivera, A.L., Pellozzi, C.E., Gilbert, M.R., Colman, H., De La Cruz, C., Salinas, E.P., Bekele, B.N., Aldape, K.D., 2010. MGMT promoter methylation is predictive of response to radiotherapy and prognostic in the absence of adjuvant alkylating chemotherapy for glioblastoma. *Neuro Oncol.* 12, 116–121.
- Suzayama, T., Nishihara, M., Kondoh, T., Hosoda, K., Kohmura, E., 2009. MicroRNA-10b is overexpressed in malignant glioma and associated with tumor invasive factors, uPAR and RhoC. *Int. J. Cancer* 125, 1407–1413.
- Shinozaki, H.A., Grimm, S., Raizer, J., Mehta, M.P., 2011. Combined modality approaches in the management of adult glioblastoma. *Front. Oncol.* 1, 36.
- Simon, M., Simon, C., Kester, G., Hans, V.H., Schramm, J., 2002. Conditional expression of the tumor suppressor p16 in a heterotopic glioblastoma model results in loss of p16 expression. *J. Neurooncol.* 60, 3–12.
- Smith, M., Nilsson, J., Mir, S.E., van der Stoep, F.M., Hulleman, E., Niers, J.M., de Wit, Hamer, P.C., Marquet, V.E., Cloos, J., Kirschovsky, A.M., Noske, D.P., Tannous, B.A., Wurdinger, T., 2010. miR-101 is down-regulated in glioblastoma resulting in E2F2-induced proliferation, migration, and angiogenesis. *Oncotarget* 1, 710–720.
- Song, H., Zhang, Y., Liu, N., Wan, C., Zhang, D., Zhao, S., Kong, Y., Yuan, L., 2016. miR-92b regulates glioma cells proliferation, migration, invasion, and apoptosis via PTEN/Akt signaling pathway. *J. Physiol. Biochem.* 72, 205–211.
- Trepant, A.L., Bouchart, C., Riviere, S., Sauvage, S., Decaestecker, C., Demetter, P., Salmen, I., 2015. Identification of OLIG2 as the most specific glioblastoma stem cell marker starting from comparative analysis of data from similar DNA chip microarray platforms. *Tumour Biol.* 36, 1943–1953.
- Ueda, R., Kohanbash, G., Sasaki, K., Fujita, M., Zhu, X., Kastnerhuber, E.R., McDonald, H.A., Potter, D.M., Hamilton, R.L., Lotze, M.T., Khan, S.A., Sobol, R.W., Okada, H., 2009. Dicer-regulated microRNAs 222 and 339 promote resistance of cancer cells to cytotoxic T-lymphocytes by down-regulation of ICAM-1. *Proc. Natl. Acad. Sci. U.S.A.* 106, 10746–10751.
- Veliz, I., Loo, Y., Carillo, O., Karachaliou, N., Nigro, O., Rosell, R., 2015. Advances and challenges in the molecular biology and treatment of glioblastoma—is there any hope for the future? *Ann. Transl. Med.* 3, 7.
- Vogelbaum, M.A., Agbi, M.K., 2015. Correction-enhanced delivery for the treatment of glioblastoma. *Neuro. Oncol.* 172 (Suppl), i13–i18.
- Wakimoto, H., Tanaka, S., Curry, W.T., Loebel, F., Zhao, D., Tateishi, K., Chen, J., Klefke, I.K., Lelic, N., Kim, J.C., Diaz-Santagata, D., Ellison, L.W., Berger, D.R., Fendt, S.M., Vander Heiden, M.G., Batchelor, T.T., Irfate, A.J., Cahill, D.P., Chi, A.S., 2014. Targetable signaling pathway mutations are associated with malignant phenotype in IDH-mutant gliomas. *Clin. Cancer Res.* 20, 2898–2909.
- Xue, J., Zhou, A., Wu, Y., Morris, S.A., Lin, K., Arzin, S., Verhaak, R., Fuller, G., Xie, K., Heimberger, A.B., Huang, S., 2016. miR-182-5p induced by STAT3 activation promotes glioma tumorigenesis. *Cancer Res.* 76, 4293–4304.
- Yang, C.H., Yue, J., Pfeiffer, S.R., Fan, M., Paulas, E., Hosni-Ahmed, A., Sims, M., Qayyum, S., Davidoff, A.M., Handorf, C.B., Pfeiffer, L.M., 2014. MicroRNA-21 promotes glioblastoma tumorigenesis by down-regulating insulin-like growth factor-binding protein-3 (IGFBP3). *J. Biol. Chem.* 289, 25079–25087.
- Zheng, Q., Han, L., Dong, Y., Tian, J., Huang, W., Liu, Z., Jia, X., Jiang, T., Zhang, J., Li, X., Kang, C., Ren, H., 2014. JAK2/STAT3 targeted therapy suppresses tumor invasion via disruption of the EGFRvIII/JAK2/STAT3 axis and associated focal adhesion in EGFRvIII-expressing glioblastoma. *Neuro Oncol.* 16, 1229–1243.

Research Paper

A Stemness and EMT Based Gene Expression Signature Identifies Phenotypic Plasticity and is A Predictive but Not Prognostic Biomarker for Breast Cancer

Muhammad Waqas Akbar¹, Murat Isbilen^{1,2}, Nevin Belder¹, Secil Demirkol Canli^{1,3}, Baris Kucukkaraduman¹, Can Turk¹, Ozgur Sahin¹, Ali Osmay Gure¹✉

1. Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey.
2. DNAFect Genetics Consulting R&D and Biotechnology Inc., Kocaeli, Turkey.
3. Molecular Pathology Application and Research Center, Hacettepe University, Ankara, Turkey.

✉ Corresponding author: agure@bilkent.edu.tr; Bilkent University, Department of Molecular Biology and Genetics, SB-228; Cankaya, Ankara, Turkey 06800.

© The author(s). This is an open access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0/>). See <http://vyspring.com/terms> for full terms and conditions.

Received: 2019.03.05; Accepted: 2019.11.03; Published: 2020.01.01

Abstract

Aims: Molecular heterogeneity of breast cancer results in variation in morphology, metastatic potential and response to therapy. We previously showed that breast cancer cell line sub-groups obtained by a clustering approach using highly variable genes overlapped almost completely with sub-groups generated by a drug cytotoxicity-profile based approach. Two distinct cell populations thus identified were CSC(cancer stem cell)-like and non-CSC-like. In this study we asked whether an mRNA based gene signature identifying these two cell types would explain variation in stemness, EMT, drug sensitivity, and prognosis *in silico* and *in vitro*. **Main methods:** *In silico* analyses were performed using publicly available cell line and patient tumor datasets. *In vitro* analyses of phenotypic plasticity and drug responsiveness were obtained using human breast cancer cell lines. **Key findings:** We find a novel gene list (CNCL) that can generate both categorical and continuous variables corresponding to the stemness/EMT (epithelial to mesenchymal transition) state of tumors. We are presenting a novel robust gene signature that unites previous observations related either to EMT or stemness in breast cancer. We show *in silico*, that this signature perfectly predicts behavior of tumor cells tested *in vitro*, and can reflect tumor plasticity. We thus demonstrate for the first time, that breast cancer subtypes are sensitive to either Lapatinib or Midostaurin. The same gene list is not capable of predicting prognosis in most cohorts, except for one that includes patients receiving neo-adjuvant taxane therapy. **Significance:** CNCL is a robust gene list that can identify both stemness and the EMT state of cell lines and tumors. It can be used to trace tumor cells during the course of phenotypic changes they undergo, that result in altered responses to therapeutic agents. The fact that such a list cannot be used to identify prognosis in most patient cohorts suggests that presence of factors other than stemness and EMT affect mortality.

Key words: Breast cancer, predictive biomarkers, tumor plasticity, transcriptomics.

Introduction

Breast cancer (BC) is the second leading cause of mortality after lung cancer in women¹. Currently the molecular classification of BC is based on expression of Estrogen Receptor (ER), Progesterone Receptor (PR) and Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2). Another classification, which is also based on gene expression differences identifies luminal A, luminal B, Her2 enriched, basal, and normal like subgroups, and provides a better prediction of prognosis and drug

response^{2,3}. Some studies identified a cancer stem cell (CSC)-like subpopulation in BC, which was suggested as being responsible for metastasis and relapse of disease^{4,5}. In general, CSCs are generally defined as CD44⁺/CD24⁻ cells that possess the capacity of self-renewal⁶. The CSC hypothesis suggests - by definition - that non-CSC cells would be generated from CSC cells. On the other hand, a related but distinct mechanism, i.e. epithelial to mesenchymal

transition (EMT), describes a reversible process through which epithelial cells transform into a mesenchymal state with the associated loss of epithelial features such as cell to cell contact and intracellular tight junctions, and the gain of mesenchymal features such as fibroblastoid morphology, increased motility and metastatic capability⁷. Cells which undergo EMT show similar features to CSCs such as tumorigenesis, lack of differentiation, mammosphere formation and resistance to anti-cancer therapies, suggesting that these two might be defining the same phenotype⁸. Mesenchymal cells can switch to an epithelial state (MET) under certain conditions^{9,10}. The presence of CSCs has been conclusively shown *in vivo*¹¹ and *in vitro*^{12,13}. It is known that CSC-like cells can be generated or enriched via mammosphere formation¹⁴ or the ectopic expression of Twist or Snail¹⁵. However, both of these are also known to induce EMT. It is not yet clear if cells defined as CSCs in BC are identical to mesenchymal cells, and whether if via EMT, epithelial cell populations in BC can revert back to a CSC-like state.

An important distinction between CSC-like and non-CSC-like cells (or epithelial and mesenchymal cells) is their differential response to anti-cancer therapy. Commonly used drugs like Paclitaxel and Doxorubicin have been shown to affect primarily non-CSC cells, thereby enriching CSC-like populations in tumors^{16,17}. Therefore, for successful tumor therapy, it seems drugs which specifically target CSC-like cells are also required. Ultimately, by the use of two drugs alternately or in parallel, both CSC- and non-CSC like cells could be eliminated.

In this study, using cells classified according to a novel gene-list (CNCL), we were able to identify candidate drugs for both CSC- and non-CSC cell types in breast cancer and validated our findings in several datasets *in silico*, as well as *in vitro*. However, we were not able to observe a consistent difference in clinical outcome in the majority of BC patient cohorts whose tumors had been thus classified.

Methods

In silico analyses

For *in silico* gene expression analysis, microarray datasets were downloaded from genomic data hosting websites, ArrayExpress (<https://www.ebi.ac.uk/arrayexpress/>) and Gene expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>). Each dataset was RMA normalized using BRB array tools¹⁸. FPKM values were used from GSE73526 which is a next generation RNA-sequencing dataset. Cluster tree 3.0 program¹⁹ was used to hierarchically cluster data

and heatmaps were generated using Java Treeview²⁰. For both genes and samples, Euclidean distances were calculated using complete linkage. Datasets generated using Affymetrix U133 A, B or U133 plus2 or Illumina platforms were used. Details of the datasets used are given in **Supplementary Table 1**. For *in silico* drug cytotoxicity analysis, IC50 or normalized activity area (AA) values were downloaded from CCLE²¹ and CGP²². We did not select patients in any of the cohorts utilizing any filters as we intended to test if CNCL could identify prognosis, independent of clinical characteristics.

Cell culture conditions

Breast cancer cell lines, MDA-MB-157, MDA-MB-231, MDA-MB-453, MDA-MB-468, MDA-MB-436, ZR751, JIMT1, BT474, BT20, CAL51 and MCF7 were cultured in DMEM media, while HCC202, HCC1954, HCC70, HCC1143, HCC38, T47D and HCC1937 cell lines were cultured in RPMI media. Respective media was supplemented with 10% FBS, 1% 200mM L-Glutamine (Lonza) and 1% 10K/10K Penicillin - Streptomycin (Lonza) of the total volume. All cell lines were incubated at 5% CO₂ and 37 °C in humidified incubator.

Mammosphere culture (3D culture)

MDA-MB-157 cells were cultured in 75cm² low attachment flasks (Corning) to generate mammospheres (3D culture) in 3 separate experiments according to a previously published protocol²³. Briefly, to generate mammosphere media, serum free DMEM media was enriched with 1X B27 (Invitrogen), 10 ng/ml EGF (Sigma Aldrich), FGF 20 ng/ml (Sigma Aldrich), 2 µg/ml heparin (Sigma Aldrich), L-Glutamine (Lonza) and Penicillin - Streptomycin (Lonza). To initiate mammosphere cultures, cells grown in monolayers were detached and resuspended in mammosphere media. Cells were counted and 2x10⁵ cells were cultured in 75cm² low attachment flasks. After 3 days of culture, cells formed mammospheres. These mammospheres were separated from suspension using a 40 µm cell strainer (BD). Spheres were retrieved from the strainer by inverting it over petri dish and washing with PBS. After which cells were replated in low attachment flasks. This was carried out for 6 passages.

Quantitative RT-PCR

Total RNA was isolated using Trizol (Ambion) and was treated with DNase (Ambion) according to manufacturer's protocols. RNA was reverse transcribed by Revert-Aid first strand cDNA synthesis kit (Thermo Fisher Scientific) according to the supplier's protocol using random hexamer primers. Quantitative profiling of selected genes by

qRT-PCR was performed in triplicates using Light Cycler 480 (Roche) with iTaq Universal SYBR Green Supermix (Bio-Rad). Primers were designed using Primer3 online tool (<http://primer3.ut.ee/>) and then validated with NCBI Primer-Blast Tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primer sequences are shown in **Supplementary Table 2**. GAPDH was used as endogenous reference control. All expression data were calculated using the $\Delta\Delta CT$ Method²⁴.

Cell viability analyses

Cell viability was analyzed in quadruplicates using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega) and CyQUANT Cell Proliferation Assay Kit (Thermo Fisher Scientific) according to manufacturer's instructions. For drug cytotoxicity in 2D culture, 3000 cells were plated in each well of 96 well plates and after 24 hours were treated with drugs (Lapatinib, Midostaurin) at 10 different doses (Lapatinib: 50 μ M-0.001 μ M, Midostaurin: 5 μ M-0.0001 μ M). Percent cell viability was calculated 72 hours after drug treatment. These values were then used to draw dose-inhibition curves using 6 models as (3 parameter, 3 parameter Top 100, 3 parameter Bottom 0, 4 parameter, 4 parameter Top 100 and 4 parameter Bottom 0) using R software. The model with the lowest standard error was used to calculate IC50 and Activity area values. Same procedures were used to calculate cytotoxicity for mammosphere cultures except mammosphere generated cells were cultured in 96 well ultra-low attachment plates in mammosphere media.

siRNA knockdown and cytotoxicity change

Knockdown of ZEB1 and SNAI2 genes was performed using commercially available siRNAs, targeting the respective transcripts (D-006564-04 (SiZEB1) and D-017386-02 (SNAI2), Dharmacon Lafayette, CO, USA) along with control siRNA from Qiagen (CAT# SI03650318, Qiagen, Hilden, Germany) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in serum free Opti-MEM at a concentration of 20 nM according to the manufacturer's protocol. In brief, cells were seeded at a density of 2×10^5 cells/well in complete medium. When the cells reached 50-60 % confluency, medium was replaced with medium without antibiotics. The siRNA-Lipofectamine 2000™ mixture was added to the medium and cells were incubated at 37°C and 5% CO₂ for 48 hours. Cells were collected 72 h after transfection to analyze changes in transcript levels of related genes using qRT-PCR. For cytotoxicity experiments with Lapatinib and Midostaurin MDA-MB-157 cells (3000 cells/well) were seeded in 96-well plates and

twenty-four hours after seeding, cells were transfected with the siZEB1 and siSNAI2 at a final concentration of 20 nM, using Lipofectamine 2000™ (Invitrogen, CA) transfection reagent according to the manufacturer's protocol. After 24 hours of transfection, cells were treated with at 4 concentrations for Lapatinib (1, 5, 10 and 50 μ M) and Midostaurin (0.1, 0.5, 1 and 5 μ M). Cell viability was assessed 72 hours after treatment with CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) as per the manufacturer's instructions. All treatment groups were set up in quadruplicate.

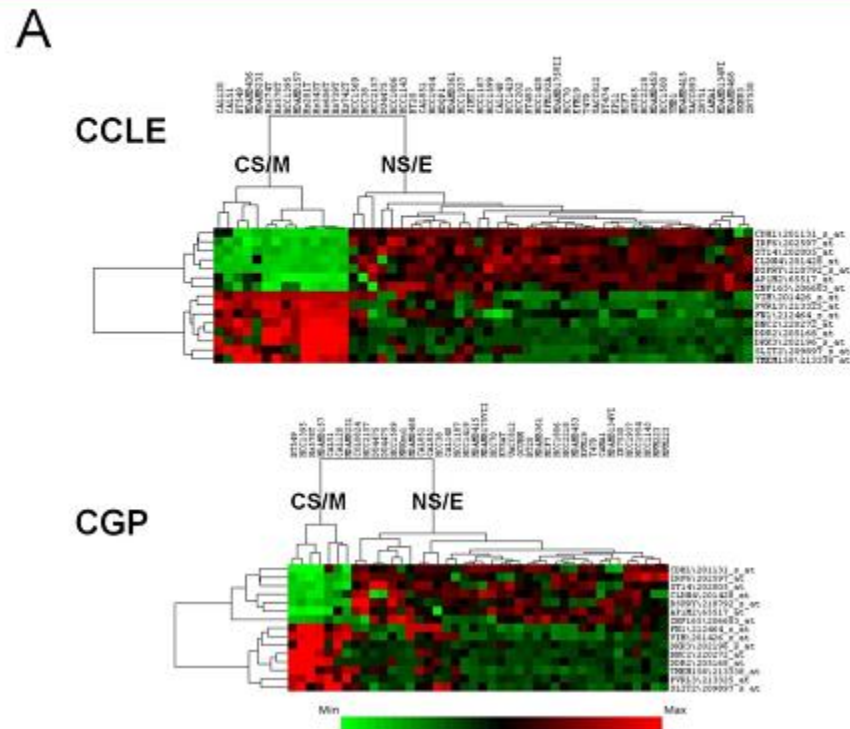
Statistical analysis

Different treatment groups were compared using "t-test" and graphs were generated using GraphPad Prism v 6.01 (GraphPad Software Inc.). For survival analysis, Cox proportional hazard regression and Log-Rank tests were performed using the "Survival" package in R^{25,26}. Kaplan Meier analysis was performed using IBM SPSS Statistics v.19.

Results

Identification of the CSC/non-CSC gene list (CNCL)

We previously showed²⁷ that breast cancer cell line sub-groups obtained by a clustering approach using highly variable gene expression differences²⁸ overlapped with that generated by a cytotoxicity based approach¹⁶. Thus, we could classify all breast cancer cell lines either as CSC-like or non-CSC-like. To generate a robust gene list that could be used to separate these two groups *in silico* and *in vitro*, we identified the most differentially expressed ($p < 0.05$, by t-test) 200 genes among the CSC-like or non-CSC-like cells using both the GSE36139 (CCLE)²¹ and E-MTAB-783 (CGP)²² datasets, which contain 56 and 39 breast cancer cell lines, respectively. One hundred and thirty three probesets, corresponding to 97 genes were common to both datasets, and 129 had highly correlated expression patterns (**Supplementary Figures 1A and 1B**). Of these, we selected 15 genes/probesets (8 up and 7 down-regulated in CSC-like cell lines), which had a minimum fold-change of 3 and a p value below 0.0002 and showed strong intergenic correlation (**Supplementary Tables 3A and 3B**); thus forming the CSC/non-CSC gene list (CNCL). A hierarchical clustering analysis using CNCL shows two clearly distinct cell clusters in CCLE and CGP datasets (**Figure 1A**). These two clusters were designated cancer-stem-cell-like and mesenchymal (CS/M) and non-cancer-stem-cell-like and epithelial (NS/E) as explained below.



B

Gene Symbol	CCLE		CGP	
	P (t-test)	Fold change	P (t-test)	Fold change
CD44	<0.0001	2.62	0.005	1.89
CD24	<0.0001	-4.82	0.003	-3.90
ALDH1	0.792	0.19	0.526	0.68

Figure 1: Clustering of cell lines in CCLE and CGP datasets by CNCL. CNCL generates two distinct clusters for CCLE and CGP cell lines (A). CS/M and NS/E classification correlates with stem cell markers CD44 and CD24 but not with ALDH1 in both datasets (see also **Supplementary Table 6**). CD44 and CD24 expression is significantly different between CSC-like and non-CSC-like clusters by t-test but ALDH1 did not show the same pattern in CCLE and CGP datasets (B).

We then used these cell clusters to perform gene-set-enrichment analyses (GSEA)²⁹. Genesets enriched in both CSC-like and non-CSC-like groups were ranked by significance, and rank numbers obtained in both analyses were combined generating a ranksum value to identify commonly enriched genesets. As shown in **Supplementary Tables 4A and 4B**, gene sets indicative of a mesenchymal phenotype such as "Extracellular Matrix", "Basement Membrane" and "Cell Migration" were enriched in CSC-like cells, while gene sets related to an epithelial phenotype like "Tight Junction" were enriched in

non-CSC-like cell lines, suggesting that the CSC-like phenotype is similar to a "mesenchymal", and non-CSC-like to an "epithelial" phenotype. In support of this, an *in silico* analysis of CDH1 protein expression from two independent sources^{30,31} showed that CDH1 was significantly up-regulated among non-CSC-like cell lines compared to those with a CSC-like phenotype (**Supplementary Figure 2**). We thus conclude that CNCL classifies cell lines into relatively epithelial and mesenchymal phenotypes and therefore name these two types of cells CS/M and NS/E.

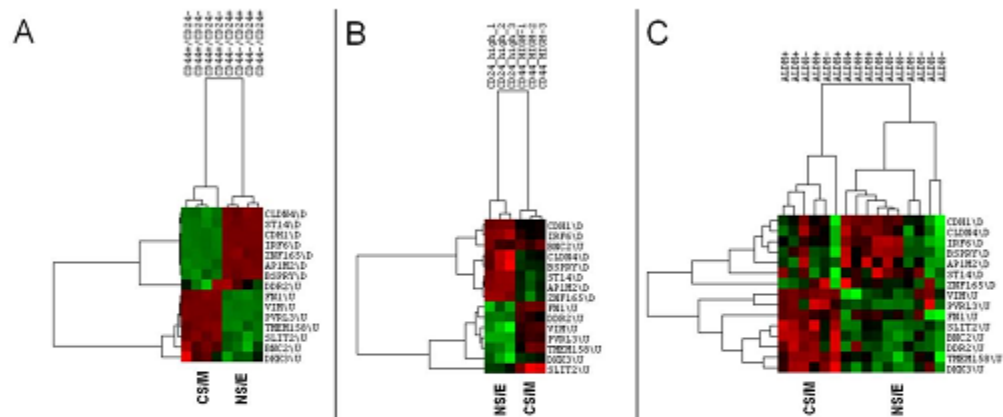


Figure 2: CNCL identifies stemness in silico. CNCL generates two clusters of CD44+/CD24- and CD44-/CD24+ MCF10A breast cancer cells (GSE15192) (A), as well as in Ras-transformed human mammary epithelial cells sorted based on their CD44 or CD24 expression (GSE36643) (B). In primary breast cancer cells sorted for ALDH+ expression (GSE52327), CNCL based differentiation is not clear (C).

To test the robustness of the CNCL gene list we asked if cell lines defined as CS/M or NS/E in CGP and CCLE would be classified consistently by CNCL in other datasets as well. Cell line gene expression data from 3 datasets: GSE24717³² and GSE50811³³ (microarray) and GSE73526³¹ (RNA sequencing) was used for this purpose. Of 123 cell line assignments, only 1 (HCC1419 in GSE50811) was classified inconsistently across datasets. We therefore, conclude that CNCL behaves consistently across different platforms and is unaffected from inter-laboratory variation (Supplementary Table 5).

We then asked if the CNCL gene list could provide biologically significant distinctions among cell and tissue subtypes *in silico* and *in vitro*.

CNCL reflects stemness in silico

NS/E and CS/M cells identified by CNCL showed significantly different levels of expression, *in silico* for CD24 and CD44 in both CCLE and CGP datasets, while another stem-cell marker, ALDH did not show differential expression among cell groups (Figure 1B, and Supplementary Table 6). CNCL could clearly identify CD44+/CD24- stem cells and CD44-/CD24+ non stem cells derived from MCF10A breast cancer cells (GSE15192)³⁴ (Figure 2A). Similarly, CNCL could distinguish stem and non-stem HMLER cells (Ras-transformed human mammary epithelial cells) obtained by CD44/CD24 expression sorting (GSE36643)³⁵ (Figure 2B). The distinction between CS/M and NS/E type cells in primary breast cancer cells (GSE52327)³⁶ was less clear (Figure 2C) possibly reflecting the heterogeneity of these cells. ALDH expression was not related to CNCL defined phenotypes in this analysis as well.

Expression of all 15 genes in CNCL correlated with CD24 and CD44 but not with ALDH in GSE15192 (Supplementary Table 7A) and in GSE36643 (Supplementary Table 7B), similar to CCLE or CGP. Cumulatively, these data suggest NS/E cells are highly similar non-stem cancer cells, while the opposite is true for CS/M cells.

CNCL can identify EMT in silico

We next analyzed the relation of CNCL determined clusters with those determined in the context of EMT. When the GSE9691 dataset that contains expression data from HMLER cells in which E-Cad gene was downregulated either by shRNA treatment or by the transfection of a dominant negative version of E-Cad, or cells in which both E-Cad and β -Cat were downregulated³⁷ were analyzed, CNCL correctly classified all E-Cad down-regulated cells as CS/M, and all control cells, and those where effects of E-Cad downregulation are reversed by simultaneous β -Cat downregulation, as NS/E cells (Figure 3A). In a separate analysis, HMLER cells that were retrovirally transduced with TGF β , Twist, Gsc or Snail inducing EMT³⁸, were identified by CNCL as CS/M cells (GSE24202) (Figure 3B). Unexpectedly when E-cad expression was downregulated by shRNA in these cells, they were still identified as NS/E. However, shRNA dependent downregulation of E-cad in these cells was significantly less efficient compared to the other experiments (TGF β , Twist, Snail and Gsc), suggesting that a conversion to a more mesenchymal state possibly requires E-cad downregulation below a certain threshold (Figure 3B, bottom). To test if CNCL could classify primary tumor cells we analyzed the

GSE7515 dataset³⁹. Of 11 primary tumor cells, 10 were classified either as NS/E or intermediary (I) phenotypes by CNCL, while 1 was of a CS/M phenotype. In contrast, among 15 mammospheres, generated from primary tumor cells, in which EMT would be expected to occur, 12 were characterized as CS/M, and 3 as either NS/E or I cells (Figure 3C). Therefore, 22 of 26 primary cell and mammosphere cultures were clustered by the CNCL list in an expected fashion. Exceptions could be due to the fact that primary cells can have a mesenchymal phenotype to start with, and because *in vitro* mammosphere cultures might not always generate mesenchymal cells. Cumulatively these results indicate that CNCL is able to distinguish cells with relatively epithelial features from those with mesenchymal features for both cell lines and primary tumor cells.

CNCL can predict drug sensitivity *in silico*

As both cancer stem cells and mesenchymal cancer cells have been reported to resist the majority of cancer chemotherapy and/or targeted therapy regimens, we asked if CNCL could identify drug sensitive and resistant cells *in silico*. As shown in Figure 4A, CNCL could clearly distinguish Doxorubicin sensitive MCF7 cells from those that were made resistant to this drug via prolonged exposure (GSE24460)¹⁷. Development of drug resistance *in vivo* is a major cause of therapy failure. To investigate if the CNCL list could identify a phenotype switch in *in vivo* drug resistance, we utilized the GSE10281 dataset which contains pre- and post- Letrozol treated tumor tissue from 18 patients³⁹. CNCL based analysis of this dataset revealed that after a 3 month treatment period, out of 14 tumors with a pre-treatment NS/E phenotype, 7 had converted to CS/M tumors post-therapy. And among 4 tumors with a CS/M phenotype, pre-therapy, one converted to a NS/E tumor post-therapy (Figure 4B). We interpret these results to mean that most tumors with a NS/E phenotype pre-therapy, are prone to generate CS/M tumors post-therapy, and thus possibly become drug resistant.

Analyses based on a quantitative stemness score (SS)

We generated two matrices, one for CS/M related-, and one for NS/E related genes (Supplementary Table 8) based on the median expression levels for each gene in the CNCL for CS/M and also for NS/E cells in the CCLE database after mean standardization. We then correlated each tumor sample's standardized CNCL gene expression values to both the CS/M and NS/E matrices generating two correlation values (Pearson's *r*) for each matrix,

CS/M(*r*) and NS/E(*r*), respectively. Analysis of cells based on their correlation scores clearly distinguished Paclitaxel sensitive and resistant MDA-MB-231 cells in GSE12791, as well as cells re-sensitized to Paclitaxel through differentiation inducing Bexarotene treatment⁴⁰ (Figure 5A). In another case (GSE23399), we found that the same analysis was able to reveal the phenotypic change observed in cancer-associated fibroblasts (CAF) in response to paclitaxel therapy⁴¹. As shown in Figures 5B, the longer CAFs were exposed to Paclitaxel, the more CS/M-like, and less NS/E-like they became. A similar switch to a more CS/M-like phenotype was also observed in BT20 breast cancer cells made resistant to a targeted therapy agent, Lapatinib (GSE16179) (Figure 5C)⁴². Next we generated the SS (Delta (*r*)) by calculating the difference of two Pearson *r* values for each sample (i.e.: CS/M (*r*) - NS/E (*r*)), where negative values indicate a more NS/E phenotype. Using SSs we found that tumor tissues from 29 breast cancer patients obtained before Anthracycline and Taxane based neo-adjuvant chemotherapy were more NS/E-like, compared to post-therapy tumor tissues from 32 patients (GSE28844)⁴³, suggesting that chemotherapy exposure had resulted in a general phenotype switch in most of these tumors (Figure 5D). Therefore, just like the hierarchical clustering based experiments summarized in Figure 4, the SS based approach could identify cells and tumor tissues which responded to chemotherapy treatment. Based on these results, we conclude that CNCL can trace tumor phenotype plasticity.

Utilization and *in vitro* validation of CNCL as a drug sensitivity predictor

To identify drugs which could preferentially target CS/M and NS/E cells, we analyzed the CCLE database and found 4 drugs that affect these subtypes differentially: Midostaurin and Elesclomol were significantly more effective on CS/M cells, whereas Lapatinib and Panabinoestat were cytotoxic for NS/E cells (Figure 5E). To validate these *in silico* findings *in vitro*, we first tested whether the CNCL based classifications could be reproduced using qPCR generated data. Indeed, qPCR data obtained for genes in the list were highly concordant with CCLE (GSE36139) microarray expression data (Supplementary Table 9A), and inter-gene correlations were as expected (Supplementary Table 9B). We then generated a SS matrix based on qPCR data, similar to that generated for *in silico* analyses: BC cell lines were hierarchically clustered using standardized qPCR expression data thus generating CS/M and NS/E cell groups. Each group was used

independently to calculate average expression values for all 15 genes which were then used to generate the CS/M and NS/E matrices and ultimately SSs (delta (r) values).

In vitro, Midostaurin was preferentially cytotoxic for CS/M cells in line with our *in silico* findings. We

observed that Lapatinib was more effective on NS/E cells (Figure 6A and Supplementary Figure 3). We were unable to show a differential effect for Elexclomol and Panobinostat on CS/M vs. NS/E cells *in vitro* (data not shown).

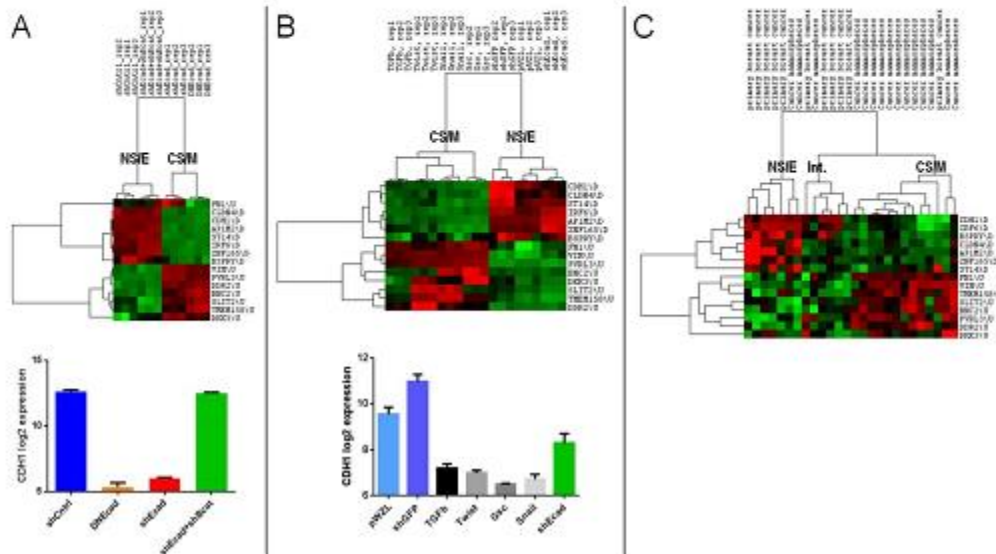


Figure 3: CNCL identifies EMT *in silico*. CNCL identifies cells in which CDH1 was downregulated using either shRNA (shEcad) or via dominant-negative CDH1 expression (DNEcad), as CS/M cells, when compared to controls (shGFP) or cells where both CDH1 and CTNNB1 were downregulated (shEcad+shBcat) which are of the NS/E phenotype (GSE9691). CDH1 expression was significantly lower in CS/M as compared to NS/E cells (CDH1 vs. shGFP: $p < 0.0001$, CDH1 vs. shEcad+shBcat (CTNNB1) $p < 0.0001$). (A) In GSE24202, cells over-expressing TGF- β , Twist, Gsc or Snail are of the CS/M phenotype compared to controls (shGFP and pWZL). Although cells treated with shCDH1 downregulated CDH1, its expression remained higher than TGF- β ($p = 0.02$), Twist ($p < 0.01$), Gsc ($p < 0.01$), and Snail ($p < 0.01$). (B) For primary breast cancer cells and mammospheres generated from those samples CNCL identified most of the mammospheres (12/15) as CS/M and all but one primary cancer samples as NS/E and intermediary, in GSE7515 (C).

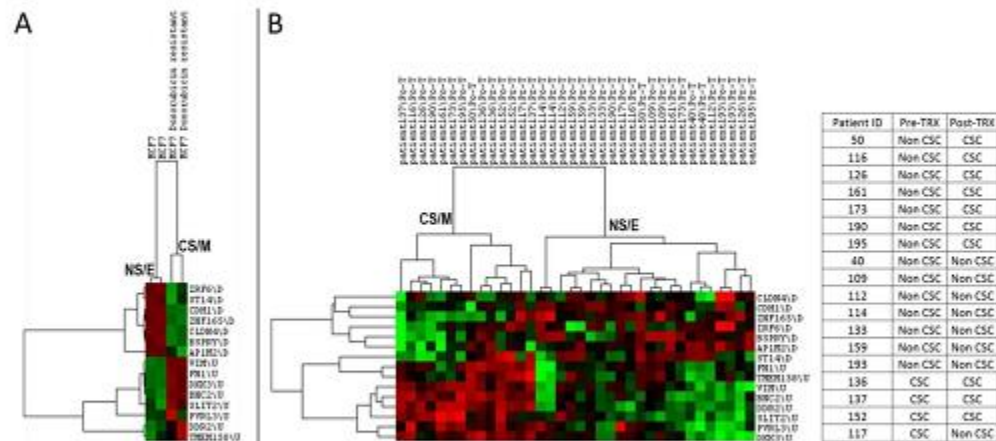


Figure 4: CNCL can predict drug sensitivity *in silico*. CNCL identifies doxorubicin resistant MCF7 cells as CS/M, compared to control cells (GSE24460) which are NS/E (A). (B) CNCL identifies Letrozol resistance in pre- and post- Letrozol treated tumor tissue (GSE10281) from 14 NS/E tumors, 7 converted to a CS/M phenotype, post treatment.

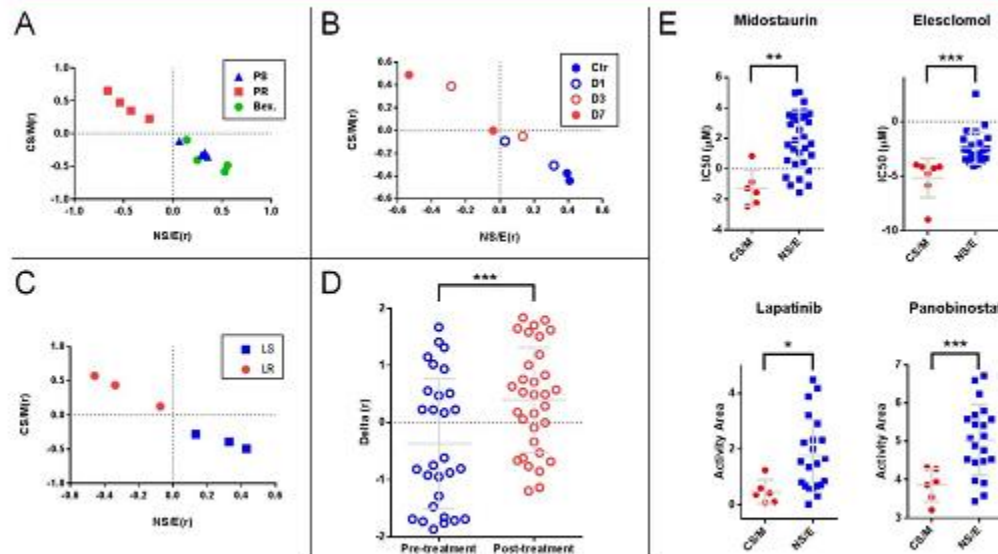


Figure 5: Stemness score (SS) based analyses show that the CNCL can reflect tumor plasticity. **A:** (GSE12791) Paditaxel resistant MDA-MB-231 cells (PR) show high CS/M (r) and low NS/E (r) as compared to Paditaxel sensitive cells (PS) or those PR cells treated with Bexarotene (Bex). **B:** (GSE23399) Paditaxel treated carcinoma associated fibroblasts (CAFs) treated for 3 and 7 days (D3 and D7) show higher CS/M (r) and low NS/E (r) when compared to cells treated for 1 day or control cells (D1 and Ctr). **C:** (GSE16179) Lapatinib-resistant BT474 cell line (LR) show high CS/M (r) and low NS/E (r) when compared to sensitive cells (LS). **D:** (GSE28844) Patient tumors post-anthracycline and taxane chemotherapy, show high Delta (r) when compared to pre-treatment where Delta (r) = [CS/M(r)] - [NS/E(r)]. **E:** Midostaurin and Etesclomol are cytotoxic preferentially for CS/M while Lapatinib and Panobinostat target NS/E. * $p < 0.05$ (t-test), ** $p < 0.01$, *** $p < 0.001$.

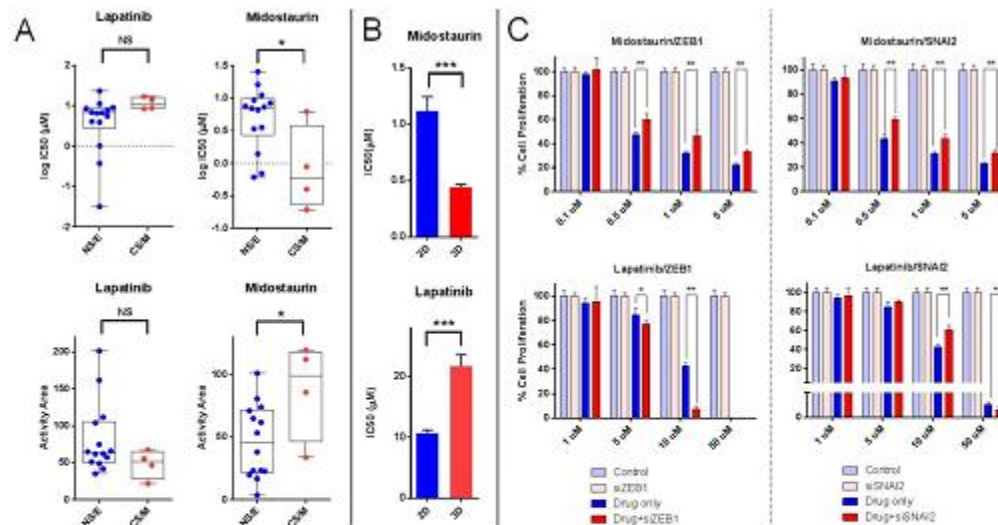


Figure 6: In vitro validation of CNCL as a drug sensitivity predictor. **A:** Midostaurin is preferentially cytotoxic for CS/M cells compared to NS/E cells, while the reverse is observed for Lapatinib (CyQUANT assay). **B:** MDA-MB-157 spheroids showed increased sensitivity to Midostaurin and resistance to Lapatinib. **C:** ZEB1 and SNAI2 knock down results in decreased sensitivity to Midostaurin and increased sensitivity to Lapatinib in MDA-MB-157 cells. * $p < 0.05$ (t-test), ** $p < 0.01$, *** $p < 0.001$, NS: not significant.

To test if the CNCL signature reflected tumor phenotype plasticity *in vitro* and whether this would relate to a change in drug sensitivity, we generated spheroids from MDA-MD-157 cells and measured gene expression by qPCR, and the simultaneous change in drug sensitivity. The qPCR based initial SS for MDA-MD-157 cells (-0.145) increased to +0.145 upon spheroid formation, reflecting a shift towards a CS/M phenotype. This happened concurrently with a significant increase in sensitivity to Midostaurin and a decrease in sensitivity to Lapatinib (Figure 6B). Therefore, spheroid generation from MDA-MD-157 cells caused a change in phenotype that could be quantitated by the CNCL, concurrent with a change in drug sensitivity *in vitro*.

As ZEB1 and SNAI2 are considered critical genes in maintaining a mesenchymal/CSC-like phenotype, we knocked down both genes individually in MDA-MD-157 (Supplementary Figure 4) and determined changes in CNCL gene expression and drug sensitivity. SS Delta (r) values changed from +5.1 in control siRNA transfected cells, to -0.12 and -0.01 in ZEB1 and SNAI2 siRNA transfected cells, respectively, indicating a switch from a CS/M to a NS/E phenotype. In parallel to this change in phenotype, we observed significantly increased sensitivity to Lapatinib and decreased sensitivity to Midostaurin (Figure 6C). The fact that the change in sensitivity to Lapatinib was more obvious for ZEB1 knock-down cells is likely due to the fact that these underwent a stronger shift towards a NS/E phenotype, compared to SNAI2 knock-down cells.

Cumulatively, these results indicate that tumor cells that are innately of a CS/M phenotype, or those that are induced to become so, are less sensitive to Lapatinib and more sensitive to Midostaurin, compared to cells that are innately NS/E or are induced to become so, which show the opposite pattern of sensitivity.

Evaluation of CNCL as a prognostic tool for breast cancer

To determine if a CNCL based classification of tumor samples could be used to predict prognosis, we generated SS values for all samples within several breast cancer datasets: Metabric cohorts (British (n=994) and Canadian (n=997))⁴¹, GSE1456 (n=159)⁴⁵, GSE2034 (n=286)⁴⁶, GSE2603 (n=82)⁴⁷, GSE3494 (n=251)⁴⁸, GSE4922(n=249)⁴⁹, GSE6532 (n=380)⁵⁰, GSE7390 (n=198)⁵¹, GSE11121(n=200)⁵², GSE12276 (n=204)⁵³, GSE19615 (n=115)⁵⁴, GSE20685(n=327)⁵⁵, GSE21653 (n=266)⁵⁶, GSE25066(n=508)⁵⁷ and GSE58812 (n=107)⁵⁸. We included all cohorts with survival data and did not use filtering criteria as our

aim was to discover any prognostic association the CNCL had, independent of confounding factors. As shown in Supplementary Table 10, SS delta (r) based evaluation of prognosis of 15 patient cohorts in 29 tests with various end-point measures revealed statistically significant differences between CS/M and NS/E groups in only 7 tests. And in only two of these (GSE25066 and GSE2603) patients with primarily CS/M tumors had worse prognosis compared to NS/E tumor-harboring patients. Interestingly the GSE25066 cohort, where this trend was most obvious, consisted of patients who had received Taxane (incl. Paclitaxel) based neo-adjuvant therapy. Patients in this cohort with NS/E tumors had longer distant relapse free survival compared to those with CS/M tumors (Figure 7A, B). Although SS distributions were significantly different for PAM50 subtypes in this cohort (Figure 7C), the CNCL based cut-off was a statistically significant prognostic factor in a multivariate analysis that included N stage, T stage and PAM50 subtypes (Figure 7D). In line with our results summarized in Figure 5, these show a clear relation between Paclitaxel sensitivity and CNCL identified groups, results obtained for this cohort suggest that the CNCL list might be especially beneficial in determining outcome in patients receiving Taxane based treatment as neo-adjuvant therapy. In 22 tests, we did not observe a significant association between SSs and survival end-measures, and in 5 tests we observed the opposite of what we expected, a direct relation between better prognosis and the CS/M phenotype. Given the very consistent results we obtained for the experiments summarized in the previous sections, we think that the lack of a clear relation between CS/M and worse prognosis in patient cohorts is due to the heterogeneity of tumors, and also because information related to chemotherapy or other treatment was not available for most samples.

Discussion

There exists a large array of markers that have been reported to identify different subtypes of breast cancer cells⁵⁹. We find that CNCL correlates with CD24 and CD44 expression but not with ALDH. EMT markers for breast cancer seem to be shared to a greater extent among cancers and can be used to classify tumors across tissue-types⁶⁰. The CNCL gene list defined in this study is related to a previously defined EMT gene list⁶⁰ as indicated by the high concordance between the two (data not shown). Similar to the EMT gene list, CNCL can classify both primary tumor tissues as well as cell lines.

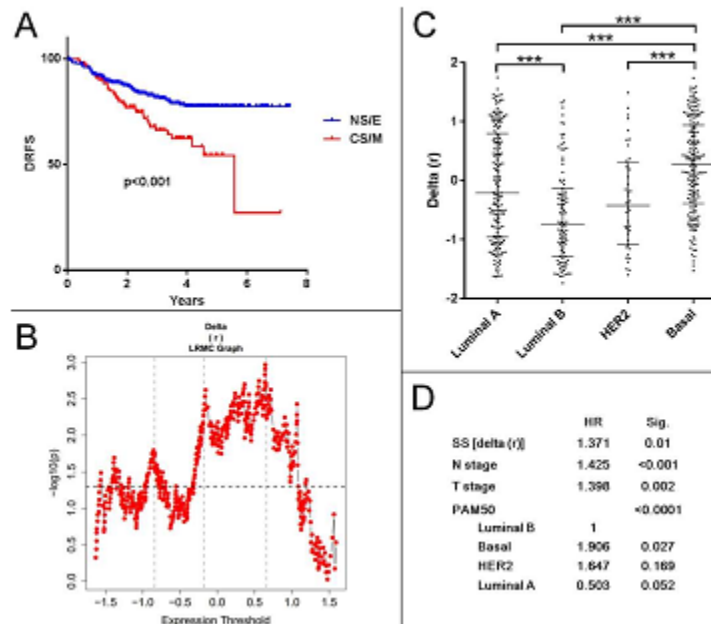


Figure 7: CNCL can predict prognosis for patients treated with Taxane based neo-adjuvant therapy. **A:** (GSE25066) Patients with CS/M tumors show worse prognosis compared to NS/E group, when a SS cut-off of 0.6459 is used. **B:** Log rank test with multiple cut-offs (LRMC). **C:** Delta (r) value distribution among breast cancer subtypes. **D:** Multivariate analysis with multiple clinical parameters showing SS is an independent prognostic factor for patients with paclitaxel neo-adjuvant treatment. ***p<0.001.

The differentiation state of a tumor is accepted as the key determinant of resistance to therapy⁶¹. However, tumors have the capability of altering their phenotype, which is referred to as phenotype plasticity. This term is used to define both de-differentiation as well as trans-differentiation in cancer cells⁶². Our findings show that phenotype plasticity occurs almost without exception in tumor cells, *in vivo* and *in vitro*, in response to chemotherapy and CNCL is able to dynamically follow the phenotype switch between these two states. As both Zeb1 and Snail1 induction have been associated with EMT and drug resistance⁶³, and as Zeb1 is also a critical component of tumor cell plasticity⁶⁴, it is not surprising that the down-regulation of these would correlate with increased sensitivity to anti-cancer agents. Our results clearly show that the CNCL can identify tumor states and cell lines undergone through EMT or stemness as a result of phenotype plasticity. CNCL is therefore, a novel gene list that can account for both stemness and EMT in breast cancer, indicating that the two are related when analyzed in the context of this signature.

Metastasis is considered to be the major reason for tumor related mortality. Although EMT clearly

has a role in tumor progression and metastasis, recent data suggests that the metastatic potential of tumor cells and their ability to undergo EMT are not necessarily related⁶⁵. These studies suggest that instead of classifying tumors either as epithelial or mesenchymal, to determine the rate at which plasticity occurs might be a better indicator of prognosis and drug responses⁶². It is therefore, important to be able to quantify the exact phenotypic status of a tumor within the EMT scale and to be able to trace its change. In this line, we believe the CNCL defined here is worth validating in larger studies.

Gene signatures in BC have been shown to be able to predict sensitivity to various drugs *in vitro*⁶⁶ and EMT has been reported to result in resistance to the EGFR inhibitor Gefitinib⁶⁷. Our results suggest the best strategy for BC treatment is possibly the utilization of two drugs simultaneously, each which would target either NS/E or CS/M cells. In line with our earlier findings²⁷, we show here for the first time *in vitro*, that Lapatinib and Midostaurin are candidates for such combination therapy, as they target selectively either NS/E or CS/M cells, respectively. Even though EMT might be a signature shared among tumor types, combination treatment

suggestions obtained for breast cancer in this study are different from those we found for other tumor types, such as colon cancer and melanoma^{68,69}. One explanation for this can be that NS/E and CS/M cell types defined for tumors originating from different tissues actually define different phenotypes in the EMT or plasticity scale. Indeed most CS/M cells of colon tumors are similar to NS/E cells of breast cancer, while melanoma cells are among the most CS/M cells when compared to all other tumors⁶⁰.

Our results clearly show that most tumors with a pre-therapy NS/E phenotype will switch to a CS/M phenotype post-therapy. This might explain why most tumors are resistant to secondary treatment. Changes in gene expression following NACT have shown that highly proliferative tumors are more sensitive to chemotherapy^{70,71}. Here we show that the CNCL gene list can also robustly distinguish patients who will have a better prognosis post taxane-based NACT. However, in light of our findings summarized here and those of others⁷², it should be noted that NACT might induce a CS/M phenotype in tumors, reducing drug responsiveness and thus, overall or disease-free survival. Therefore, it is critical to conclusively determine if NACT is actually decreasing survival of patients whose tumors switch to a CS/M state, following therapy, rather than improving survival. The CNCL could be utilized to follow tumor plasticity in response to such therapies to evaluate responses to NACT and to help answer such questions.

Conclusions

We show in this study for the first time that the CNCL gene list, which includes stem-cell and EMT features, reflects tumor plasticity and thus a shift in cytotoxicity profiles, especially in response to Midostaurin and Lapatinib in breast cancer; and that it is applicable both *in silico* and *in vitro*. Although the gene list is not uniformly predictive of prognosis, it can be utilized to identify patients who might benefit from Taxane-based neo-adjuvant therapy.

Abbreviations

CSC: Cancer stem cells; BC: Breast cancer; PR: Progesterone receptor; ER: Estrogen receptor; EMT: Epithelial to mesenchymal transition; MET: Mesenchymal to epithelial transition; CS/M: Cancer-stem-cell-like and mesenchymal; NS/E: Non cancer stem cell like and epithelial; GSEA: Gene set enrichment analysis; CNCL: CSC/non-CSC gene list.

Supplementary Material

Supplementary figures and tables.

<http://www.jcancer.org/v11p0949s1.pdf>

Acknowledgements

This work was partially funded by a grant from the Turkish Scientific and Technological Research Council/TUBITAK (117S058) to AOG and one from The Higher Education Commission of Pakistan to MWA.

Contributions

MWA: conceptualization, methodology, validation, data analysis, writing original manuscript. **MI:** conceptualization, data analysis. **NB:** acquisition of data. **SD:** formal analysis. **BK:** acquisition of data. **CT:** acquisition of data. **OS:** conceptualization and design, development of methodology, writing review. **AOG:** conceptualization, methodology, data analysis, writing original manuscript, acquisition of funds, study supervision.

Competing Interests

The authors have declared that no competing interest exists.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin* 2016;66:7-30.
2. van 't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002;415:530-536.
3. Parker JS, Mullins M, Cheung MC, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. *J Clin Oncol* 2009;27:1160-1167.
4. Al-Hajj M, Wicha MS, Benito-Hernandez A, et al. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* 2003;100:3983-3988.
5. Balic M, Lin H, Young L, et al. Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. *Clin Cancer Res* 2006;12:5615-5621.
6. Clarke MF, Dick JE, Dirks PB, et al. Cancer stem cells—perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res* 2006;66:9339-9344.
7. Wang Y, Zhou BP. Epithelial-mesenchymal transition in breast cancer progression and metastasis. *Chin J Cancer* 2011;30:603-611.
8. Floor S, van Staveren WC, Larsson D, et al. Cancer cells in epithelial-to-mesenchymal transition and tumor-propagating-cancer stem cells: distinct, overlapping or same populations. *Oncogene* 2011;30:4609-4621.
9. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* 2009;9:265-273.
10. Yilmaz-Ozcan S, Sade A, Kucukkaraduman B, et al. Epigenetic mechanisms underlying the dynamic expression of cancer-testis genes, PAGE2, -2B and SPANX-B, during mesenchymal-to-epithelial transition. *PLoS One* 2014;9:e107905.
11. Gilbertson RJ, Graham TA. Cancer: Resolving the stem-cell debate. *Nature* 2012;488:462-463.
12. Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res* 2008;10:R25.
13. Charafat-Jauffret E, Ginestier C, Iovino F, et al. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. *Cancer Res* 2009;69:1302-1313.
14. Grimshaw MJ, Cooper L, Papazisis K, et al. Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. *Breast Cancer Res* 2008;10:R52.
15. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008;133:704-715.
16. Gupta PB, Onder TT, Jiang G, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* 2009;138:645-659.

17. Calcagno AM, Salcido CD, Gillet JP, et al. Prolonged drug selection of breast cancer cells and enrichment of cancer stem cell characteristics. *J Natl Cancer Inst* 2010;102:1637-1652.
18. Simon R, Lam A, Li MC, et al. Analysis of gene expression data using BRB-ArrayTools. *Cancer Inform* 2007;3:11-17.
19. de Hoon MJ, Imoto S, Nolan J, et al. Open source clustering software. *Bioinformatics* 2004;20:1453-1454.
20. Saldanha AJ. Java Treeview—extensible visualization of microarray data. *Bioinformatics* 2004;20:3246-3248.
21. Barretina J, Caponigro G, Stransky N, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* 2012;483:603-607.
22. Garnett MJ, Edelman EJ, Heidorn SJ, et al. Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature* 2012;483:570-575.
23. Balic M, Schwarzenbacher D, Stanzler S, et al. Genetic and epigenetic analysis of putative breast cancer stem cell models. *BMC Cancer* 2013;13:358.
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402-408.
25. Team RC. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria ISBN 2007.
26. Therneau TM, Grambsch PM. Modeling survival data: extending the Cox model. Springer Science & Business Media, 2013.
27. Ishilen M, Senses KM, Gure AO. Predicting Chemotherapy Sensitivity Profiles for Breast Cancer Cell Lines with and Without Stem Cell-Like Features. *Current Signal Transduction Therapy* 2013;8:268-273.
28. Kao J, Salari K, Bocanegra M, et al. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PLoS One* 2009;4:e6146.
29. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005;102:15545-15550.
30. Prat A, Adamo B, Cheang MC, et al. Molecular characterization of basal-like and non-basal-like triple-negative breast cancer. *Oncologist* 2013;18:123-133.
31. Marcotte R, Sayad A, Brown KR, et al. Functional Genomic Landscape of Human Breast Cancer Drivers, Vulnerabilities, and Resistance. *Cell* 2016;164:293-309.
32. Shais I, Gatzia ML, Chang JT, et al. Using a stem cell-based signature to guide therapeutic selection in cancer. *Cancer Res* 2011;71:1772-1780.
33. Denzo Z, Östreich J, Weaver A, et al. Gene expression profiling reveals epithelial-mesenchymal transition (EMT) genes can selectively differentiate eribulin sensitive breast cancer cells. *PLoS One* 2014;9:e106131.
34. Bhat-Nakshatri P, Apputiah H, Ballea C, et al. SLUG/SNAI2 and tumor necrosis factor generate breast cells with CD44+/CD24- phenotype. *BMC Cancer* 2010;10:411.
35. Battula VL, Shi Y, Evans KW, et al. Ganglioside GD2 identifies breast cancer stem cells and promotes tumorigenesis. *J Clin Invest* 2012;122:2066-2078.
36. Liu S, Cong Y, Wang D, et al. Breast cancer stem cells transition between epithelial and mesenchymal states reflective of their normal counterparts. *Stem Cell Reports* 2014;2:78-91.
37. Onder TT, Gupta PB, Mani SA, et al. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* 2008;68:3645-3654.
38. Taube JH, Herschkowitz JL, Komurov K, et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. *Proc Natl Acad Sci U S A* 2010;107:15449-15454.
39. Creighton CJ, Li X, Landis M, et al. Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proc Natl Acad Sci U S A* 2009;106:13820-13825.
40. Luo W, Schork NJ, Manschke KB, et al. Identification of polymorphisms associated with hypertriglyceridemia and prolonged survival induced by docetaxel in treating non-small cell lung cancer. *Anticancer Res* 2011;31:2303-2311.
41. Chan TS, Hsu CC. Metronomic chemotherapy prevents therapy-induced stromal activation and induction of tumor-initiating cells. *J Exp Med* 2016;213:2967-2988.
42. Liu L, Greger J, Shi H, et al. Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. *Cancer Res* 2009;69:6871-6878.
43. Vera-Ramirez L, Sanchez-Rovira P, Ramirez-Tortosa CL, et al. Transcriptional shift identifies a set of genes driving breast cancer chemoresistance. *PLoS One* 2013;8:e53983.
44. Curtis C, Shah SP, Chin SF, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 2012;486:346-352.
45. Pawitan Y, Bjohle J, Amler L, et al. Gene expression profiling spares early breast cancer patients from adjuvant therapy: derived and validated in two population-based cohorts. *Breast Cancer Res* 2005;7:R953-964.
46. Wang Y, Klijn JG, Zhang Y, et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* 2005;365:671-679.
47. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature* 2005;436:518-524.
48. Müller LD, Smeds J, George J, et al. An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. *Proc Natl Acad Sci U S A* 2005;102:13550-13555.
49. Ivshina AV, George J, Senko O, et al. Genetic reclassification of histologic grade delineates new clinical subtypes of breast cancer. *Cancer Res* 2006;66:10292-10301.
50. Loi S, Haibe-Kains B, Desmedt C, et al. Definition of clinically distinct molecular subtypes in estrogen receptor-positive breast carcinomas through genomic grade. *J Clin Oncol* 2007;25:1239-1246.
51. Desmedt C, Piette F, Loi S, et al. Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series. *Clin Cancer Res* 2007;13:3207-3214.
52. Schmidt M, Böhm D, von Törne C, et al. The humoral immune system has a key prognostic impact in node-negative breast cancer. *Cancer Res* 2008;68:5405-5413.
53. Bos PD, Zhang XH, Nadal C, et al. Genes that mediate breast cancer metastasis to the brain. *Nature* 2009;459:1005-1009.
54. Li Y, Zou L, Li Q, et al. Amplification of LAPTM4B and YWHAZ contributes to chemotherapy resistance and recurrence of breast cancer. *Nat Med* 2010;16:214-218.
55. Kao J, Chang KM, Hsu HC, et al. Correlation of microarray-based breast cancer molecular subtypes and clinical outcomes: implications for treatment optimization. *BMC Cancer* 2011;11:143.
56. Sabatier R, Finetti P, Adelaide J, et al. Down-regulation of ECRG4, a candidate tumor suppressor gene, in human breast cancer. *PLoS One* 2011;6:e27636.
57. Hatzis C, Paszai L, Valero V, et al. A genomic predictor of response and survival following taxane-anthracycline chemotherapy for invasive breast cancer. *Jama* 2011;305:1873-1881.
58. Jerezuel P, Loussouarn D, Guerin-Charbonnel C, et al. Gene-expression molecular subtyping of triple-negative breast cancer tumours: importance of immune response. *Breast Cancer Res* 2015;17:43.
59. Bai X, Ni J, Beretov J, et al. Cancer stem cell in breast cancer therapeutic resistance. *Cancer Treat Rev* 2018;69:152-163.
60. Tan TZ, Miao QH, Miki Y, et al. Epithelial-mesenchymal transition spectrum quantification and its efficacy in deciphering survival and drug responses of cancer patients. *EMBO Mol Med* 2014;6:1279-1293.
61. Housman G, Byler S, Heerboth S, et al. Drug resistance in cancer: an overview. *Cancers (Basel)* 2014;6:1769-1792.
62. Gupta PB, Pastushenko I, Skibinski A, et al. Phenotypic Plasticity: Driver of Cancer Initiation, Progression, and Therapy Resistance. *Cell Stem Cell* 2019;24:65-78.
63. Shang Y, Cai X, Fan D. Roles of epithelial-mesenchymal transition in cancer drug resistance. *Curr Cancer Drug Targets* 2013;13:915-929.
64. Krebs AM, Mitschke J, Lasierra Losada M, et al. The EMT-activator Zeb1 is a key factor for cell plasticity and promotes metastasis in pancreatic cancer. *Nat Cell Biol* 2017;19:518-529.
65. Pastushenko I, Brisebarre A, Sifrim A, et al. Identification of the tumour transition states occurring during EMT. *Nature* 2018;556:463-468.
66. Hsu YC, Chen HY, Yuan S, et al. Genome-wide analysis of three-way interplay among gene expression, cancer cell invasion and anti-cancer compound sensitivity. *BMC Med* 2013;11:106.
67. Weng CH, Chen LY, Lin YC, et al. Epithelial-mesenchymal transition (EMT) beyond EGFR mutations per se is a common mechanism for acquired resistance to EGFR TKI. *Oncotarget* 2019;38:455-468.
68. Demirkol S, Gomceli I, Ishilen M, et al. A Combined UHRF2 and SEMASA Expression Signature as a Prognostic and Predictive Biomarker for Colon Cancer. *J Cancer* 2017;8:1113-1122.
69. Senses KM, Ghasemi M, Akbar MW, et al. Phenotype-based variation as a biomarker of sensitivity to molecularly targeted therapy in melanoma. *MolChromom* 2017;8:88-95.
70. Klintman M, Buus R, Cheang MC, et al. Changes in Expression of Genes Representing Key Biologic Processes after Neoadjuvant Chemotherapy in Breast Cancer, and Prognostic Implications in Residual Disease. *J Exp Med* 2016;224:2405-2416.

71. Klintman M, Buus R, Cheang MC, et al. Changes in Expression of Genes Representing Key Biologic Processes after Neoadjuvant Chemotherapy in Breast Cancer, and Prognostic Implications in Residual Disease. *Clin Cancer Res* 2016;22:2405-2416.
72. Liu L, Yang L, Yan W, et al. Chemotherapy Induces Breast Cancer Stemness in Association with Dysregulated Monocytosis. *Clin Cancer Res* 2018;24:2370-2382.

RESEARCH ARTICLE

A novel 20-gene prognostic score in pancreatic adenocarcinoma

Seçil Demirkol Canlı^{1*}, Ege Dedeoğlu², Muhammad Waqas Akbar², Banş Küçükkaraduman², Murat İşbilen^{2c}, Özge Şükrüoğlu Erdoğan³, Seda Kılıç Erciyas³, Hülya Yazıcı³, Burçak Vural⁴, Ali Osmay Güre²

1 Molecular Pathology Application and Research Center, Hacettepe University, Ankara, Turkey, **2** Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey, **3** Cancer Genetics Division, Department of Basic Oncology, Institute of Oncology, Istanbul University, Istanbul, Turkey, **4** Department of Genetics, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey

* Current address: DNAFect Genetics Consulting R&D and Biotechnology Inc., Kocaeli, Turkey
* sevil.demirkol@hacettepe.edu.tr



OPEN ACCESS

Citation: Demirkol Canlı S, Dedeoğlu E, Akbar MW, Küçükkaraduman B, İşbilen M, Erdoğan ÖŞ, et al. (2020) A novel 20-gene prognostic score in pancreatic adenocarcinoma. PLoS ONE 15(4): e0231835. <https://doi.org/10.1371/journal.pone.0231835>

Editor: Nicola Silvestris, Cancer Institute, ITALY

Received: February 21, 2020

Accepted: April 1, 2020

Published: April 20, 2020

Copyright: © 2020 Demirkol Canlı et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All data used in this study is from public databases as described in the methods.

Funding: This study was supported by the Research Fund of Istanbul University (Project no: ONAP 46784) to BV. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal cancers. Known risk factors for this disease are currently insufficient in predicting mortality. In order to better prognosticate patients with PDAC, we identified 20 genes by utilizing publically available high-throughput transcriptomic data from GEO, TCGA and ICGC which are associated with overall survival and event-free survival. A score generated based on the expression matrix of these genes was validated in two independent cohorts. We find that this "Pancreatic cancer prognostic score 20—PPS20" is independent of the confounding factors in multivariate analyses, is dramatically elevated in metastatic tissue compared to primary tumor, and is higher in primary tumors compared to normal pancreatic tissue. Transcriptomic analyses show that tumors with low PPS20 have overall more immune cell infiltration and a higher CD8 T cell/Treg ratio when compared to those with high PPS20. Analyses of proteomic data from TCGA PAAD indicated higher levels of Cyclin B1, RAD51, EGFR and a lower E-cadherin/Fibronectin ratio in tumors with high PPS20. The PPS20 score defines not only prognostic and biological sub-groups but can predict response to targeted therapy as well. Overall, PPS20 is a stronger and more robust transcriptomic signature when compared to similar, previously published gene lists.

Introduction

PDAC is among the most lethal cancer types world-wide with records of 5 year survival of less than 5% [1]. A projection of cancer incidence and death rates in US showed that pancreas cancer will become the second cause of cancer-related death by 2030 [2].

Currently the only curative option for PDAC is surgical resection (pancreaticoduodenectomy), however less than 20% of patients have resectable tumor due to the aggressiveness of the disease [3]. Standard therapy is gemcitabine and gemcitabine combinations with other drugs

Abbreviations: PDAC, Pancreatic ductal adenocarcinoma; PPS20, Pancreatic cancer prognostic score 20; ICGC, International Cancer Genome Consortium; PACA-CA, Pancreatic Cancer Canadian; PACA-AU, Pancreatic Cancer Australian; GEO, Gene Expression Omnibus; TCGA, The cancer genome atlas; PAAD, Pancreatic Adenocarcinoma; HR, Hazard ratio; RPKM, Reads per kilo base per million mapped reads; CTRP, Cancer Therapeutic Response Portal; AUC, Area under curve; CCLE, Cancer Cell Line Encyclopedia; GSEA, Gene set enrichment analysis.

[4]. In recent years FOLFIRINOX and targeted EGFR inhibition by erlotinib combined with gemcitabine showed only modest improvements in response rates and overall survival [5]. A deeper understanding of the biological factors contributing to the treatment response may have the potential to improve these outcomes.

A comprehensive study of death rate trends in pancreatic cancer since 1970 revealed complexity of the disease, is largely unexplainable by known risk factors [6]. Currently the only FDA approved biomarker is CA19-9 for the management of pancreatic ductal adenocarcinoma, despite its limitations [2]. AJCC TNM staging and performance status are critical indicators of prognosis in clinical practice [8, 9]. Although multiple gene expression based prognostic prediction methods have been developed [10–16], no such test is currently used in routine practice and very few of these tests can be used as predictors of response to therapy [17], or *in vitro* sensitivity to chemotherapeutic agents in cell lines [18]. Therefore there is still much to contribute to the discovery of new risk predictors and new methodologies for prognostic assessments that can guide clinical approaches.

In this study we aimed to define a novel panel of prognostic genes for PDAC based on *in silico* analysis of microarray and RNA sequencing datasets. We thus generated a 20-gene based risk scoring method, PPS20, which can stratify patients when either overall survival or event-free survival are used as end-point measures. We show that tumors from these two patient groups are biologically distinct: tumors with high PPS20 show a higher proliferation, whereas tumors with low PPS20 have a higher rate of lymphocyte infiltration. By analyzing public cytotoxicity databases, we find that these two tumor types show differential sensitivity to specific agents.

Methods

Processing public datasets

RNA-sequencing raw counts and clinical data of patients with pancreatic tumors from International Cancer Genome Consortium Pancreatic Cancer Canadian and Australian (ICGC, PACA-CA and PACA-AU) cohorts were downloaded from ICGC data portal (<https://dcc.icgc.org/>). Primary tissue was selected as Pancreas and the Specimen Type was selected as "Primary Tumor". Samples with sequencing based expression data "EXP-S" were downloaded. 156 and 81 patients in PACA-CA and PACA-AU cohorts, respectively, with available survival data were included in the study and raw read counts were downloaded. CPM values for each gene were calculated by the formula: (read count of the gene/total read count of sample) * 1000000. TCGA PAAD RNAseqv2 Level3 RSEM normalized data and clinical data were downloaded from Broad Institute GDAC data portal (<http://gdac.broadinstitute.org/>). Samples with available overall survival data (n = 178) and event-free survival data (n = 133) were used. Genes which had 0 counts in more than 85% of samples were eliminated from the prognostic analyses in all RNAseq datasets. Mutational data of 150 pancreatic cancer patients within TCGA cohort was obtained from <https://www.cbioportal.org/> [19]. Sample "TCGA-IB-7651-01" was excluded from chi square analyses since over 80% of the screened genes are altered. RNA-seq based gene counts of 51 pancreatic adenocarcinoma tissues comprising the GSE79668 dataset were downloaded from GEO (<https://www.ncbi.nlm.nih.gov/geo/>). CPM values were calculated using the same formula used for ICGC cohorts. Log expression was calculated as log (CPM+0.001) values for ICGC CA, ICGC AU and GSE79668 and Log2 (RSEM+0.001) for TCGA PAAD. Raw CEL files of GSE28735 dataset including 45 paired pancreatic tumor and normal tissues were downloaded from GEO and RMA normalized using BRB-array tools developed by Dr. Richard Simon and the BRB-ArrayTools Development Team. Clinical data were extracted from the series matrix file for GSE28735. 42 patients with survival data were

included in survival analyses. Series matrix file of GSE21501 (n = 102) and GSE71729 (n = 123) datasets and the annotation files for GPL4133 and GPL20769, respectively, were downloaded from GEO and annotated accordingly. Clinical data was derived from the series matrix file for GSE21501. Patients with survival value "0" were eliminated from survival analyses in all datasets.

Survival analyses and prognostic gene ranking

Log expression of all the genes and the corresponding survival data of PACA-CA, PACA-AU, TCGA and GSE21501 datasets were analyzed by an in-house R script utilizing "survival" library [20] and "coxph" functions. For each gene, Cox proportional hazards regression p value and hazard ratio were obtained separately in each dataset. Genes were ranked based on (1) Cox p value- the smaller the p value the smaller the rank, (2) Hazard ratio (HR), the greater the HR the smaller the rank (HRs smaller than 1 were included as 1/HR to make them comparable to the HRs above 1), in PACA-CA, PACA-AU, TCGA. Summation of ranks generated "ranksum" which was considered as the criteria for priority sorting genes from the smallest ranksum to the biggest. Stromal score for TCGA PAAD samples were downloaded from (<https://bioinformatics.mdanderson.org/estimate/disease.html>) under "RNA-Seq-V2" platform type. Residual tumor information for TCGA PAAD patients were obtained from supplementary data of the TCGA PDAC study [21]. Overall survival was used as "days to last follow-up" for censored patients and "days to death" for others, and event-free survival was calculated as "days to new tumor events" for patients with an event, "last follow time" or "days to death" for censored patients in TCGA PAAD. All survival times in days were converted to months via division by 30 for consistency in Kaplan Meier curves. Original survival values (days/months) were used for all datasets in cox regression analyses.

Calculation of the PPS20

Log expression of the 20 genes were used in the following formula for the calculation of the prognostic score. $PPS20 = ARNTL2 + KANK1 + MAP4K4 + LDHA + SLC20A1 + TRIO + ZNF557 + EPS8 + CBX7 + RAB7A + POLR3H + STX16 + PITPNB + TFG + CADPS2 + ERF1 + GSK3B + NDUF2 - C2orf42 - MIA3$. A PPS20 score was derived by the summation of the log expression of 11 genes related to worse outcome and subtraction of expression values of 9 genes related to favorable outcome. For genes with multiple probesets, their mean expression value was used in PPS20. For GSE28735 the score was calculated without STX16 gene as the gene was absent from the array platform. The median value of PPS20 was used as the cut-off for categorical comparisons generating "high PPS20" and "low PPS20" groups.

Gene set enrichment analysis (GSEA)

All GSEA analyses were run using Broad Institute's GSEA software (<http://www.broad.mit.edu/gsea/>) [22]. For TCGA, PACA-CA, PACA-AU and GSE71729, a pre-ranked GSEA, based on the log fold change of the expression of each gene between high PPS20 and low PPS20 groups was performed. C5.all.v6.2.symbols.gmt [Gene Ontology] was used as "Gene sets database" for all analyses. Gene sets with less than 50 genes were not included in the analyses.

Drug sensitivity prediction analysis

To find drugs which can target low PPS20 and high PPS20 groups differentially, we used RNA sequencing data-RPKM values- produced by Cancer Cell Line Encyclopedia [23] and drug cytotoxicity data published by Cancer Therapeutic Response Portal (CTRP) [24]. CTRP

dataset contains cytotoxicity data corresponding to 543 drugs screened against 38 pancreatic cancer cell lines, among other cancer cell lines. The parameter used for reporting drug cytotoxicity in this dataset is area under curve (AUC). We restricted our analyses to the compounds which are used in treatments in 16 different concentrations. Additionally not all cell lines were screened against all drugs resulting in missing values for some combinations. So we chose drugs which were screened against at least 10 cell lines (475 drugs).

We calculated PPS20 for all 37 cell lines using CCLE (Cancer Cell Line Encyclopedia) expression data and correlated it with AUC values from CTRP data using Pearson correlation (QGP1 cell line was removed as its gene expression score resulted in highly skewed data and was truly an outlier). The drugs which showed positive correlation with the score are differentially effective on high PPS20 group and drugs with negative correlation are effective on low PPS20 group.

Re-evaluation of previously published signatures

Three previously published prognostic gene signatures for PDAC were compared to PPS20. Chen's signature [11] is a 15 gene signature which we applied to our validation datasets (GSE62452, GSE79668). For GSE79668, staging information, which was available in T, N, and M format, was converted to TNM stage based on AJCC Staging system as a means to better compare all the validation datasets. The platform (Affymetrix Human Gene 1.0 ST Array) that was used in GSE62452, did not contain a probe for CAPN8, a gene that is part of Chen's Signature [11]. Therefore, when calculating risk scores for Chen's signature CAPN8 was not considered. The coefficients supplied by the authors were used and median dichotomization was used to determine the high and low risk groups. Yan's signature [16] includes 4 genes, and the risk groups were calculated as described in the original manuscript. The third signature (Shi et al.) [17] was adapted with an approximation method. Utilizing the TCGA PAAD (Illumina HiSeqV2) based outputs of Shi et al., a coefficient was generated for each gene via dividing the cut-off value of the gene that Shi et al. determined in their own article by median expression value of that gene. This coefficient was then used in other datasets to generate each dataset's specific cut-off values by multiplication of the coefficient by the median of the specific genes. Then each gene was considered absent or present depending on that cut-off value. Absent genes were given the value 0 and present genes 1. Based on this 0–1 matrix and the cut-offs in the TCGA outputs of the paper, Shi et al. risk score was calculated as described [17]. A threshold of 1.709 was used to determine high and low risk groups.

Statistical analysis

Kaplan-Meier curves were generated and log-rank tests and Cox regression analyses were performed using SPSS Statistics v.19 (IBM, 2010, Chicago, IL, USA). Statistical analyses were done using GraphPad Prism 5.0 (Graphpad Prism 5 Software, San Diego, CA, USA). Student's t-tests were performed to determine differences between two groups (unpaired and not assuming equal variance—except for GSE28735, for which paired t-test was performed). Chi-squared test with Yates' continuity correction was performed using "stats" package in R Bioconductor for mutational frequency comparisons [25]. P values below 0.05 were considered statistically significant.

Results

PPS20 can predict clinical outcome in pancreatic cancer

To identify individual genes related to prognosis in pancreatic adenocarcinoma we performed cox regression analyses with overall survival as an end-point measure in three discovery

datasets: TCGA, PACA-CA and PACA-AU. Based on resulting HR and p values, we ranked the genes in all three datasets (see [Methods](#)). Among the top 500 genes of each dataset, 85 genes that were common in at least 2 datasets were retested utilizing the GSE21501 dataset in order to restrict the number of genes as well as to further eliminate cohort specific effects. Thus, we identified 11 and 9 genes that were significantly associated with shorter and longer overall survival, respectively ([S1 Table](#)). A prognostic scoring system was generated based on log expression of these 20 genes in pancreatic cancer primary tumors (see [Methods](#) for details), that we designate "Pancreatic cancer prognostic score 20 –PPS20". As can be seen in [Fig 1](#), PPS20 can stratify the patients into prognostically distinct subgroups with HR (95% CI) and p values of, 2.016 (1.362–3.088) and 0.007, 2.040 (1.498–3.089) and <0.0001, 2.416 (1.696–4.635) and <0.0001, 2.492 (1.551–5.044) and 0.0009, in TCGA, PACA-CA, GSE21501 and PACA-AU, respectively. PPS20 was then validated in GSE62452 and GSE79668 datasets, with HR (95% CI) and p values of 2.099 (1.335–4.241) and 0.0051, 2.438 (1.486–5.106) and 0.0017, respectively ([Fig 2](#)). A MVA that included patients without residual disease after operation and patients who did not receive targeted molecular therapy using the TCGA cohort, showed that PPS20 can predict overall survival independent of confounding factors ([S2 Table](#)). We then asked if PPS20 could also stratify patients when event-free survival (EFS) is used as an end-point. Indeed in the TCGA cohort, PPS20 is associated with event-free survival with an HR (95% CI) of 2.312 (1.393–3.982), p value of 0.0015 ([Fig 3](#)). Overall, we conclude that, PPS20 is a robust independent prognostic signature for pancreatic cancer.

PPS20 compared to other prognostic signatures

We aimed to assess how our scoring performs when compared to previously published prognostic classifiers, Chen et. al. [11], Yan. et. al [16], and Shi et. al. [17]. A multivariate cox regression analysis which included parameters significant by univariate analyses (PPS20, Shi signature and tumor subtype) identified PPS20 as the only independent prognostic factor in GSE71729 ([Tables 1 and 2](#)). Similarly, PPS20 in GSE62452, PPS20 and Shi signature in GSE79668 were the only independent prognostic molecular signatures among the parameters significantly associated with survival in univariate analyses ([S3 and S4 Tables](#)). Therefore, PPS20 remains significant in all three analyses performed in separate cohorts; indicating it is a superior and independent prognostic classifier of PDAC.

Molecular characteristics of PPS20-identified PDAC sub-groups

In order to understand the biological mechanisms underlying the differences in outcome in PPS20 identified PDAC sub-groups, we performed GSEA (Gene set enrichment analysis) between the high PPS20 and low PPS20 tumors in TCGA, PACA-CA, PACA-AU and GSE71729 datasets. The gene sets with nominal p values below 0.01 and FDR q-values below 0.25 were considered enriched. Among the enriched gene sets in high PPS20 and low PPS20 groups, those common to all four datasets are listed in ([S5 and S6 Tables](#)), representative enrichment plots are shown in ([S1 Fig](#)).

Enrichment of digestion and potassium channel related gene sets in low PPS20 group supports a 'normal like' phenotype, as pancreas is a ductal organ where digestive enzymes are secreted. Secretion of insulin from pancreatic beta cells is regulated by ATP-sensitive K(+) (K(ATP)) channel dependent pathways [26]. In addition HCO₃⁻ secreted by pancreatic ductal epithelial cells to duodenum neutralizing chyme acidity is transported by multiple ion exchangers including Na⁺–K⁺–Cl⁻ co-transporter (NKCC1) and Na⁺–K⁺-pump on the basolateral membrane [27]. Therefore the GSEA results suggest that tumors with a more favorable outcome are more differentiated, compared to those with worse outcome. In this line, we

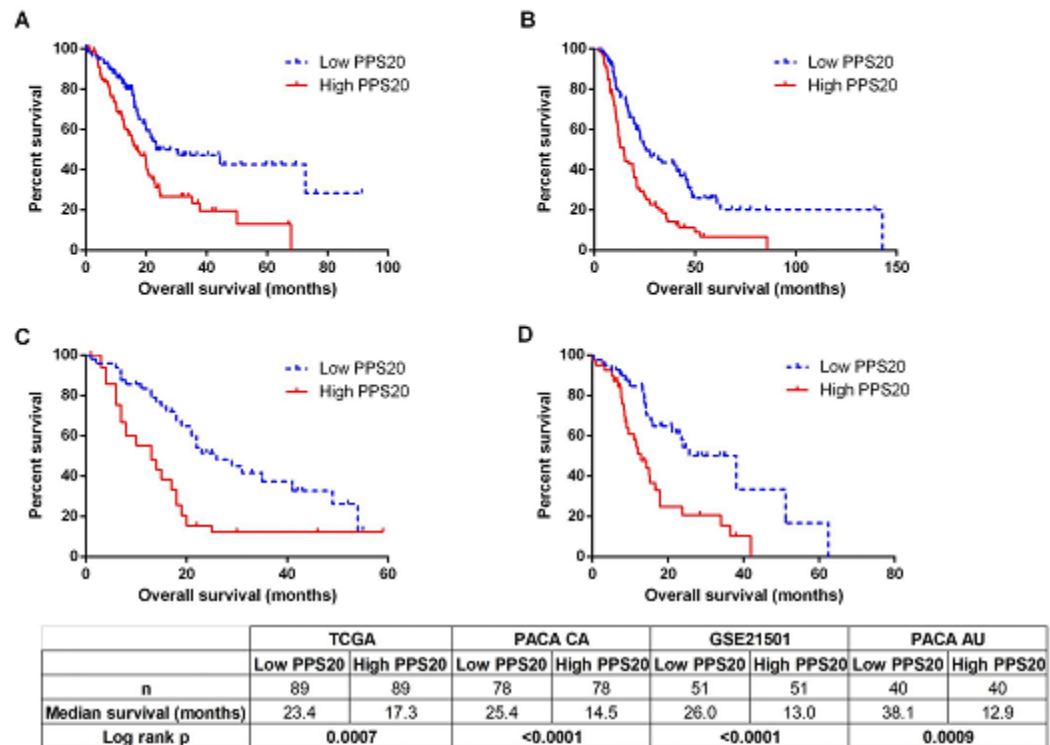


Fig 1. Prognostic stratification by PPS20 in discovery cohorts. Kaplan Meier graphs based on PPS20 for TCGA (A), PACA-CA (B), GSE21501 (C), PACA-AU (D). Cut-off for the score is the median in all cohorts. Statistics are given below the figure.

<https://doi.org/10.1371/journal.pone.0231835.g001>

observe that the PPS20 score is lowest in normal pancreas when compared to primary tumors, and most elevated in metastatic tissues (Fig 4). The enrichment of protein activation cascade gene set, which mainly includes complement system proteins, might suggest a relatively higher immune activity in these tumors.

Keratinocyte differentiation, skin development and epidermis development gene sets enriched in the high PPS20 group include many genes belonging to the keratin family, among which KRT16 has been used as a basal cell marker [28, 29]; KRT17 has been shown to induce cancer stem cell-like properties in cervical cancer [30] and tumor growth, motility and invasion in gastric cancer [31], which is also associated with poor prognosis in breast cancer [32]. Formation of primary germ layer and endoderm gene sets were also enriched in tumors with high PPS20. They include many genes related to extracellular matrix, collagens, laminins, integrins, fibronectin which are known mesenchymal markers [33] and matrix metalloproteinases which are involved in tumor growth, invasion and metastasis [34], as well as HMGA2 which is known to maintain oncogenic RAS-induced epithelial-mesenchymal transition (EMT) in pancreatic cancer [35]. These results suggest that high PPS20 tumors have relatively

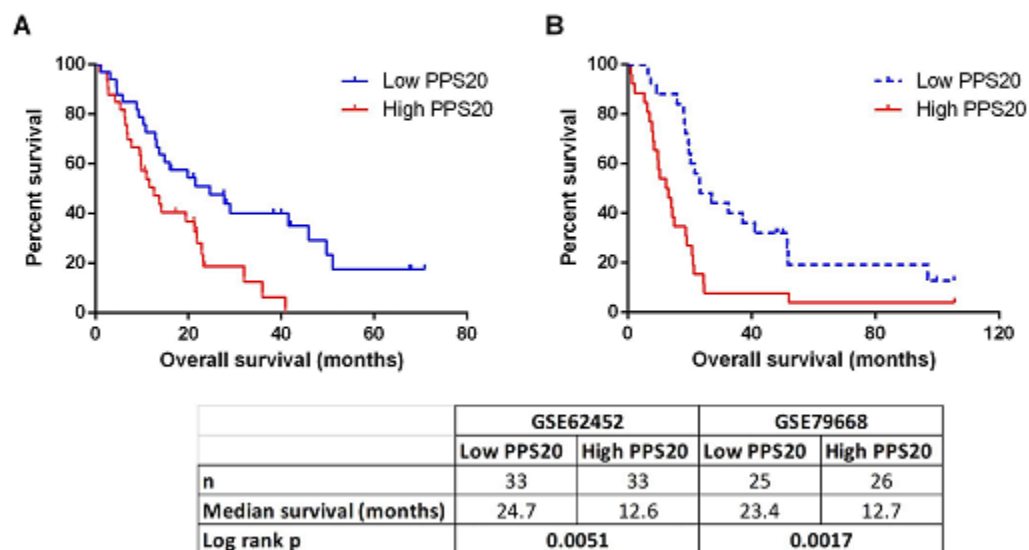


Fig 2. Prognostic stratification by PPS20 in validation cohorts. Kaplan Meier graphs based on PPS20 for GSE62452 (A), GSE79668 (B). Cut-off for the score is median in all cohorts. Statistics are given below the figure.

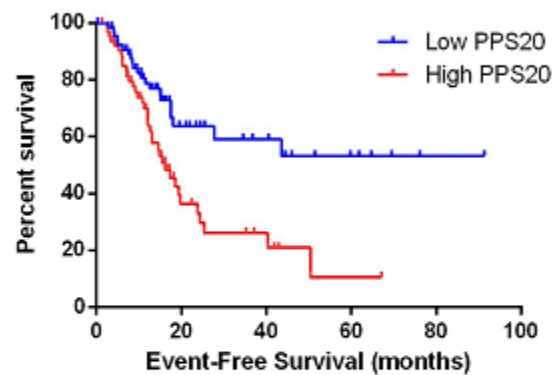
<https://doi.org/10.1371/journal.pone.0231835.g002>

more invasive and mesenchymal properties which is consistent with shorter event-free survival times (Fig 3). The same group has a lower E-cadherin/Fibronectin ratio, in line with the fact that downregulation of E-cadherin and upregulation of Fibronectin are two indicators of EMT [36] (S2 Fig).

In order to understand the immune involvement in the prognostic sub-groups, we analyzed TCGA PAAD tumor RNAseq data using CIBERSORT Absolute mode which enables us to assess involvement of 22 immune cell types in absolute fraction scores [37]. There is a dramatically higher "CD8 T cell-T regulatory cell" score in low PPS20 tumor together with lower scores of "Plasma cell-B cell naïve" (S3 Fig). These observations, together with those showing a slight increase of M2 macrophages in low PPS20 tumors are in line with an inhibition of anti-tumor immune responses. High-PPS20 tumors, however, have a larger proportion of M1 macrophages (S3 Fig).

The tumor-absolute score-, indicating the overall immune cell content is elevated in low PPS20 tumors (S3 Fig). PD-1 gene expression is significantly higher in the low PPS20 group (S4 Fig), when there is no significant difference in PD-L1 and CTLA-4 among PPS20 low and high groups in the TCGA dataset (S4 Fig).

To evaluate the mutational profile of the PPS20 groups, we utilized TCGA mutational data. Among the genes that have been altered in at least 10 patients, TP53 was the only gene which showed significantly different mutation frequency between PPS20 groups; with 79.3% mutated in high PPS20 and 56.4% mutated in low PPS20 tumors (S7 Fig). A multivariate analyses of PPS20 and TP53 mutation status resulted cox regression outputs of $p = 0.02$ HR = 1.74 (95% CI: 1.07–2.83) for PPS20 whereas no significance obtained for TP53. Overall these results



	Low PPS20	High PPS20
n	67	65
Median survival (months)	Undefined	16.2
Log rank p	0.0015	

Fig 3. PPS20 can predict event-free survival. Kaplan Meier graph based on PPS20 in TCGA PAAD tumors. Statistics are given below the figure.

<https://doi.org/10.1371/journal.pone.0231835.g003>

Table 1. Univariate analyses.

GSE71729 (n = 123)	Nr.	HR*	P*	95% CI
PPS20				
High	64	1.781	0.011	1.142–2.779
Low	59			
Chen Signature				
High	64	1.173	0.471	0.760–1.809
Low	59			
Shi Signature				
High	45	1.670	0.024	1.070–2.608
Low	78			
Yan Signature				
High	57	0.841	0.436	0.544–1.300
Low	66			
Tumor Subtype				
Basal (ref)	35	1.831	0.012	1.142–2.936
Classical	88			
Stroma Subtype**				
Low	17	1.216	0.251	0.871–1.700
Normal	29			
Activated	77			

*Cox proportional hazards regression performed with overall survival

**Stroma Subtype's were treated as continuous variables 1: Low, 2: Normal, 3: Activated

<https://doi.org/10.1371/journal.pone.0231835.t001>

Table 2. Multivariate analyses (Backward wald).

GSE71729 (n = 123)		HR ^a	P ^a	95% CI
Step 1	PPS20	1.416	0.195	0.837–2.397
	Shi Signature	1.264	0.371	0.756–2.114
	Tumor Subtype	1.409	0.209	0.825–2.406
Step 2	PPS20	1.24	0.087	0.940–2.512
	Tumor Subtype	1.221	0.133	0.886–2.509
Step 3	PPS20	1.335	0.003	1.142–2.779

^a Cox proportional hazards regression performed with overall survival

<https://doi.org/10.1371/journal.pone.0231835.t002>

indicate that there is no relationship between PPS20 and mutational status except for TP53, and PPS20 can predict prognosis independent of TP53 mutation status.

PPS20 as a predictor of response to targeted therapy

When we stratified TCGA PDAC patient data by PPS20, we observed that high PPS20 patients who received molecular targeted therapy had significantly better prognosis compared to patients who did not receive the same therapy (S5 Fig) while no significant difference was observed in the low PPS20 group, or in case of radiation therapy response (S5 Fig). Unfortunately, the specific drug information used in molecular targeted therapy of these patients is not given in TCGA. We also noted that EGFR, RAD51, Cyclin B1 protein level expressions are significantly higher in high PPS20 patients (S2 Fig), indicating a proliferative activity in this group. Overall, these results show that the PPS20 score can be an identifier of response to molecular targeted therapy in PDAC, especially for high PPS20 patients.

Identification of compounds selectively targeting individual risk groups

In order to identify compounds targeting low PPS20 and high PPS20 groups, the score was applied to CCLE pancreatic cancer cell lines. Pearson correlation analyses between the score and AUC of drugs in CTRP database resulted in the discovery of 40 drugs (S7 Table, most significant 5 drugs are shown for each group). The most effective drug for the high PPS20 group was BIRB-796, which is a p38 MAPK inhibitor (S6A Fig). Among the drugs that are effective on low PPS20 group, Ouabain was the most significant which is the inhibitor of the Na⁺/K⁺-ATPase (S6B Fig). This observation is also in line with our GSEA results showing enrichment for potassium ion transport and potassium channel activity.

Discussion

In contrast to other cancer types like colon and breast for which multiple molecular tests are available for risk prediction and/or molecular subtyping, such as PAM50 [38], MammaPrint [39], Oncotype Dx Breast [40], Oncotype Dx Colon [41], there are limited number of molecular signatures defined for prognostication of pancreatic cancer in the literature and none are available to guide clinical therapeutic decisions in practice. A major reason for this is the lack of validation of the present signatures in multiple patient cohorts. As the number of publically available high-throughput transcriptomic data has increased over time, it became possible to include higher number of patient samples/cohort into biomarker discovery methodologies that enabled identification of more robust biomarkers which are not cohort specific. Using

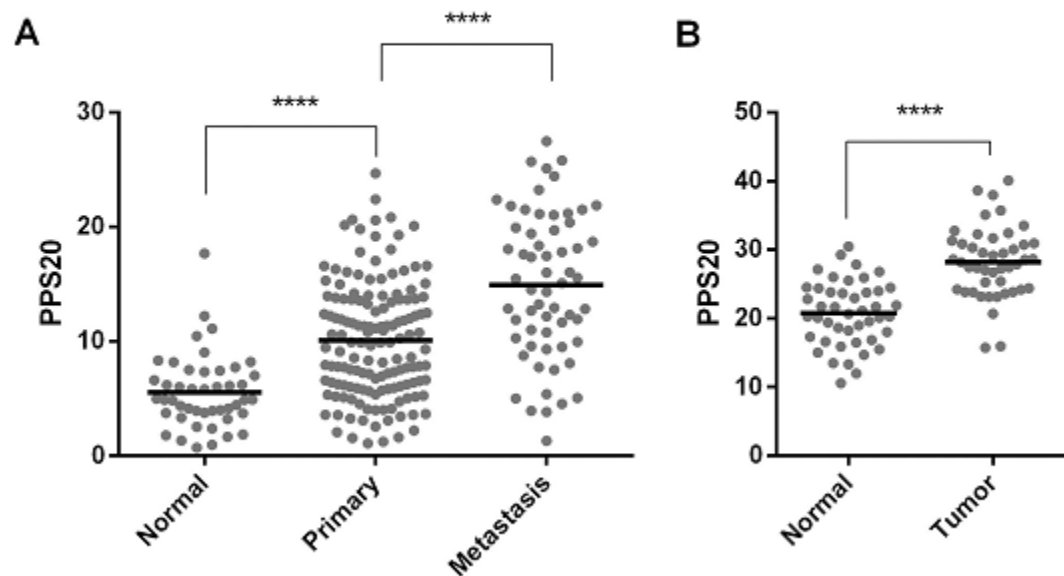


Fig 4. PPS20 is significantly different between normal, tumor and metastatic tissues. PPS20 in normal ($n = 46$), primary ($n = 145$) and metastatic tissues ($n = 61$) of GSE71729 (A). PPS20 in 45 paired tumor and normal tissues in GSE28735 (B). Horizontal lines indicate mean. Statistical comparisons were performed with unpaired and paired test in A and B, respectively. **** $p < 0.0001$.

<https://doi.org/10.1371/journal.pone.0231835.g004>

this strategy, we previously identified two mRNA based biomarkers, ULBP2 and SEMA5A, for the prognostication of colon cancer [42]; and identified an independent gene panel for prediction of prognosis in both diffuse and intestinal type gastric cancer (unpublished data); which shows that the growing transcriptomic data enables discovery of such biomarkers which could have been missed when less patients studied.

Therefore, in this study, we aimed to enlarge the number of *in silico* cohorts and used 4 discovery and 2 validation PDAC gene expression datasets, including data from RNA sequencing and multiple microarray platforms. Indeed, eighteen of 20 genes identified in this study were not included in previously published prognostic gene signatures; two, ARNTL2 and SLC20A1, were used in Shi et al. [17], and Haider et al. [10], respectively. Our results show the robustness of PPS20 even when different assay platforms are used. Although tumor stage (I–IV), differentiation status (poor–moderate–well) and clinical characteristics as such as age and gender varied highly among the datasets analyzed, PPS20 could stratify prognostic subgroups independent of clinical confounding parameters in all cohorts. Indeed these results clearly will be clinically more relevant when validated *ex vivo* in a large patient cohort.

We compared three different prognostic signatures generated for PDAC to PPS20. We applied these predictive signatures as they were described in their respective publications with modifications as described in the methods section. Two of these (Chen et al (Moffit) [11] & Yan et al [16]) were predictors of overall survival. The third signature that was compared to PPS20 was Shi et al's signature [17] which predicts recurrence free survival. Although the coefficients that were determined from the original study were utilized in determining the cutoff

values for different datasets, the expression values of each gene might not be identical to those used in the original publication and this in turn might have caused some genes that would normally be assigned a value of 1 to have a value of 0. Therefore our application of the Shi signature should be considered only an approximation. In summary, although the aforementioned signatures are, in our analyses, not superior to PPS20, they would need to be further validated utilizing identical tumor samples for a conclusive analysis.

The risk groups identified in this study have distinct molecular features. In high PPS20 group, we found increased protein level expression of Cyclin B1, which is a marker of cell proliferation and as well as DNA repair [43] which is consistent with increased RAD51 which is involved in double stranded break repair, tumor progression and resistance to anti-cancer treatments [44]. TRIO, SLC20A1, MAP4K4 and ERFF1 genes which are upregulated in high PPS20 patients, were also shown to be involved in cellular proliferation and/or tumor growth [45–48]. EGFR, which is one of the major drivers of cell proliferation [49], was also higher in the high PPS20 group at the protein level. EGFR expression has been detected in 25–90% of the pancreatic adenocarcinomas in different studies and is associated with stage, metastasis, poor differentiation and survival [50].

We find a differential response to molecular targeted therapy, in high PPS20 group. When the patients are first stratified by PPS20, we have showed that high risk patients who were treated with targeted therapy have significantly longer overall survival (Log rank HR: 5.104, p value < 0.0001) compared to untreated group, with median survivals of 23 and 6.1 months, respectively. Therefore PPS20 stratification can enable a striking contribution to prediction of targeted therapy success, from an overall survival benefit of 0.33 months to 16.9 months. Overall, a better prediction of responders to molecular targeted therapy can be achieved when *ex vivo* validations are performed for PPS20 and when specified agents are known.

E-cadherin/Fibronectin ratio is slightly lower in high risk group, indicating mesenchymal properties, in line with the association of high PPS20 with shorter event-free survival. Consistently, EPS8, one of the genes related to worse prognosis in PPS20 which functions as part of the EGFR pathway also regulates actin cytoskeleton and promotes EMT [51, 52]. Among the genes associated with shorter survival in PPS20, TRIO, LDHA, MAP4K4 and ARNTL2 contribute to cellular motility/invasiveness/tumor aggressiveness and thus can also be contributing to shorter event-free survival [45, 48, 53, 54]. Similarly, LDHA, EPS8, SLC20A1 and ARNTL2 expression are also related to metastasis or shorter survival in pancreatic cancer [10, 17, 55, 56].

We interpret the dramatically higher “CD8 T cell–T regulatory cell” score in low PPS20 tumors to show the presence of larger amounts of CD8 CTLs in these tumors. The higher scores of “Plasma cell–B cell naïve” in high PPS20 tumors, suggesting a stall in B cell activation, might support an ineffective CTL response in this tumor group. The dominance of M2 macrophages which are known to inhibit anti-tumor T cell responses [57] in low PPS20 tumors, further supports this hypothesis. On the other hand a larger M1 proportion in high-PPS20 tumors correlates with their mesenchymal phenotype, as pro-inflammatory cytokines produced by M1 macrophages are known to promote EMT [58]. As PD-1 gene expression is significantly higher in the low PPS20 group, despite the absence of a significant difference in PD-L1 and CTLA-4 among PPS20 low and high groups, these findings cumulatively suggest that PPS20 low tumors might benefit from anti-PD-1 therapy.

GSEA results indicate an enrichment of complement system proteins which can promote T cell activation and maturation [59]. Our GSEA analysis also indicated a “normal like”, more differentiated phenotype of low PPS20 tumors with an enrichment of digestion and ion channel transport related gene sets. Among genes upregulated in this group, CBX7, MIA3 and KANK1 have been shown to have tumor suppressive activities including roles in inhibition of

cellular motility/migration and/or proliferation, and induction of cell cycle arrest in various malignancies [60–62]. PTPN14 which we found as a good prognostic indicator, is suggested as favorable prognostic marker in pancreatic, endometrial and renal cancers in the Human Protein Atlas (www.proteinatlas.org) [68], and its overexpression was associated with longer survival in PDAC [69]. Human protein atlas also suggests POLR2A and C2ORF42 are favorable prognostic markers in pancreatic cancer.

Via analyzing cell line drug cytotoxicity data we identified a list of compounds which are selectively effective on either low PPS20 or high PPS20 tumors. The most significant associations were with BIRB-796, a p38 MAPK inhibitor for high PPS20 group and quabain which is a Na⁺/K⁺ATPase inhibitor for low PPS20 group. The role of p38 MAPK in regulating tumor cells' proliferation, apoptosis and metastasis has been previously extensively reviewed [70]. Specifically in pancreatic tumors, p38 MAPK expression has been shown to be increased compared to normal tissues, and targeting p38 α has shown to inhibit pancreatic cancer cell proliferation; in addition a higher expression level in tumors was associated with worse overall survival [71]. Another study showed that activation of p38 results in proliferation, invasion, and metastasis of pancreatic cancer cells leading to worse prognosis and its suppression prevents the progression of pancreatic cancer [72, 73]. Additionally, under hyperglycemic conditions, increased p38 MAPK signaling is also responsible for epithelial to mesenchymal transition of pancreatic cells and upon p38 MAPK inhibition, these cells reverted back to a relative epithelial phenotype and tumor volume was decreased [74]. Increased expression of p38-MAPK has been also related to chemotherapy resistance in human gastric cancer cells [75]. The compound identified by our analysis has previously been shown to inhibit all 4 types of p38 MAPK isoforms both *in vivo* and *in vitro* [76] and has been shown to increase the efficiency of other chemotherapeutic agents in drug resistant models [77]. Here in our analysis, we found that BIRB-796 (p38 α inhibitor [78]) differentially target high PPS20 group.

In esophageal squamous cell carcinoma, overexpression of Na⁺/K⁺ATPase is associated with severity of the disease and is also reported in medulloblastoma, glioblastoma, melanoma, hepatomas, and non-small-cell lung cancer [79], and in breast cancer, it is reported to increase invasion of endocrine resistant cancer cells [80]. Although Quabain causes Na⁺/K⁺ATPase to interact with Src and EGFR, and can activate ERK1/2, it also results in growth arrest in human breast cancer cells, possibly by increasing the expression of p53 and p21 [81]. Its antiproliferative effects have been also shown in prostate cancer cells, and in pig kidney epithelial cells [82–84]. Since our GSEA results showed enrichment of potassium ion transport and potassium channel activity in low PPS20 group, an inhibitor of a Na⁺/K⁺ pump can be a potential drug for this group of patients subsequent to *in vitro* and *in vivo* validation.

Conclusions

We identified a gene signature composed of 20 prognostic genes (PPS20), and a score generated based on expression of these genes. PPS20 identifies both prognostically and biologically distinct sub-groups among PDAC tumors, and has potential as a predictive marker of response to molecular targeted therapy for PDAC.

Supporting information

S1 Fig. Examples to genesets enriched in low PPS20 and high PPS20 groups in TCGA and PACA CA datasets.

(TIF)

S2 Fig. Proteome analysis of pancreatic tumors in TCGA. Normalized protein expression values shown for low PPS20 (n = 53) and high PPS20 (n = 70) TCGA PAAD primary tumors. Horizontal lines indicate mean. Unpaired ttest results are given. **p<0.01, *p<0.05. (TIF)

S3 Fig. CIBERSORT based immune cell content of PPS20 based groups in TCGA. Immune cell fraction scores were obtained for each tumor sample using <https://cibersort.stanford.edu>. The samples with a deconvolution p value below 0.05 were included in the analysis. The differences between two immune cell fractions are given for "T cells CD8 and Tregs", "M1 and M2 macrophages", and "Plasma cells and naive B cells". Unpaired ttest results are given. ****p<0.0001, ***p<0.001 (TIF)

S4 Fig. Patients with low PPS20 have elevated PD-1 expression. Log transformed RSEM values plotted for low PPS20 (n = 89) and high PPS20 (n = 89) TCGA PAAD primary tumors. Horizontal lines indicate mean expression. Unpaired ttest was performed. *p<0.05. (TIF)

S5 Fig. High PPS20 group shows a favorable outcome when treated with targeted molecular therapy. Kaplan Meier graphs stratified by PPS20 in TCGA PAAD comparing patients who received and did not receive radiation therapy and molecular targeted therapy. Statistics are shown below the figure. (TIF)

S6 Fig. BIRB-796 sensitivity is negatively correlated with PPS20 and can preferentially target cells with high PPS20 (A). Ouabain shows the opposite pattern (B). (TIF)

S7 Fig. Comparison of mutation frequencies between PPS20 groups in TCGA. The genes which have been altered in at least 10 patients are shown. Chi-squared test with Yates' continuity correction was performed for each gene (synonymous variants excluded). Blue: No alterations, Yellow: Synonymous variants, Red: Nonsynonymous variants. * indicates a p value smaller than 0.05. (TIF)

S1 Table. Genes involved in PPS20.
(DOCX)

S2 Table. Cox regression analyses of PPS20 and clinicopathological parameters—OS.
(DOCX)

S3 Table. A: Univariate Analyses. B: Multivariate Analyses (Backward Wald).
(DOCX)

S4 Table. A: Univariate Analyses. B: Multivariate Analyses (Backward Wald).
(DOCX)

S5 Table. Gene sets enriched in high PPS20 tumors.
(DOCX)

S6 Table. Gene sets enriched in low PPS20 tumors.
(DOCX)

S7 Table. A: 5 drugs targeting cells with 'high PPS20'. Negative correlation between PPS20 and drug cytotoxicity data (AUC) shows drugs targeting 'high PPS20' group. B: 5 drugs

targeting cells with 'low PPS20'. Positive correlation between PPS20 and drug cytotoxicity data (AUC) shows drugs targeting 'low PPS20' group.
(DOCX)

Author Contributions

Conceptualization: Seçil Demirkol Canlı, Ali Osmay Güre.

Data curation: Seçil Demirkol Canlı, Ege Dedeoğlu, Muhammad Waqas Akbar, Barış Küçükkaraduman.

Formal analysis: Seçil Demirkol Canlı, Ege Dedeoğlu, Muhammad Waqas Akbar, Barış Küçükkaraduman.

Funding acquisition: Burçak Vural.

Investigation: Seçil Demirkol Canlı.

Methodology: Seçil Demirkol Canlı, Ali Osmay Güre.

Software: Murat İşbilen.

Supervision: Ali Osmay Güre.

Visualization: Seçil Demirkol Canlı.

Writing – original draft: Seçil Demirkol Canlı, Barış Küçükkaraduman, Özge Şükrüoğlu Erdoğan, Seda Kılıç Erciyas, Hülya Yazıcı, Burçak Vural, Ali Osmay Güre.

Writing – review & editing: Seçil Demirkol Canlı, Muhammad Waqas Akbar, Barış Küçükkaraduman, Özge Şükrüoğlu Erdoğan, Seda Kılıç Erciyas, Hülya Yazıcı, Burçak Vural, Ali Osmay Güre.

References

- Hidalgo M. Pancreatic cancer. *N Engl J Med*. 2010; 362(17):1605–17. Epub 2010/04/30. <https://doi.org/10.1056/NEJRa0801557> PMID: 20427809
- Rahib L, Smith BD, Alizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res*. 2014; 74(11):2913–21. Epub 2014/05/21. <https://doi.org/10.1158/0008-5472.CAN-14-0155> PMID: 24840647
- Spanknebel K, Conlon KC. Advances in the surgical management of pancreatic cancer. *Cancer J*. 2001; 7(4):312–23. Epub 2001/09/20. PMID: 11561607
- Burris HA 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol*. 1997; 15(6):2403–13. Epub 1997/06/01. <https://doi.org/10.1200/JCO.1997.15.6.2403> PMID: 9196156
- Saung MT, Zheng L. Current Standards of Chemotherapy for Pancreatic Cancer. *Clin Ther*. 2017; 39(11):2125–34. Epub 2017/09/25. <https://doi.org/10.1016/j.clinthera.2017.06.015> PMID: 28939405; PubMed Central PMCID: PMC5705388
- Ma J, Siegel R, Jemal A. Pancreatic cancer death rates by race among US men and women, 1970–2009. *J Natl Cancer Inst*. 2013; 105(22):1694–700. Epub 2013/11/10. <https://doi.org/10.1093/jnci/djt292> PMID: 24203988
- Winter JM, Yeo CJ, Brody JR. Diagnostic, prognostic, and predictive biomarkers in pancreatic cancer. *J Surg Oncol*. 2013; 107(1):15–22. Epub 2012/06/26. <https://doi.org/10.1002/jso.23192> PMID: 22728569
- Allen PJ, Kuk D, Castillo CF, Basturk O, Wolfgang CL, Cameron JL, et al. Multi-institutional Validation Study of the American Joint Commission on Cancer (8th Edition) Changes for T and N Staging in Patients With Pancreatic Adenocarcinoma. *Ann Surg*. 2017; 265(1):185–91. Epub 2016/05/11. <https://doi.org/10.1097/SLA.0000000000001763> PMID: 27163957; PubMed Central PMCID: PMC5611666

9. Le N, Sund M, Vinci A, Pancreas Gogo. Prognostic and predictive markers in pancreatic adenocarcinoma. *Dig Liver Dis*. 2016; 49(3):223–30. Epub 2016/01/16. <https://doi.org/10.1016/j.dld.2015.11.001> PMID: 26769569.
10. Halder S, Wang J, Nagano A, Desai A, Arumugam P, Dumartin L, et al. A multi-gene signature predicts outcome in patients with pancreatic ductal adenocarcinoma. *Genome Med*. 2014; 6(12):105. Epub 2015/01/15. <https://doi.org/10.1186/s13073-014-0105-3> PMID: 25587357; PubMed Central PMCID: PMC4293116.
11. Chen DT, Davis-Yadley AH, Huang PY, Husain K, Centeno BA, Permut-Wey J, et al. Prognostic Fifteen-Gene Signature for Early Stage Pancreatic Ductal Adenocarcinoma. *PLoS One*. 2015; 10(8): e0133562. Epub 2015/08/08. <https://doi.org/10.1371/journal.pone.0133562> PMID: 26247463; PubMed Central PMCID: PMC4527782.
12. Newhook TE, Blais EM, Lindberg JM, Adair SJ, Xin W, Lee JK, et al. A thirteen-gene expression signature predicts survival of patients with pancreatic cancer and identifies new genes of interest. *PLoS One*. 2014; 9(9):e105631. Epub 2014/09/03. <https://doi.org/10.1371/journal.pone.0105631> PMID: 25180633; PubMed Central PMCID: PMC4152146.
13. Raman P, Maddipati R, Lim KH, Tozeren A. Pancreatic cancer survival analysis defines a signature that predicts outcome. *PLoS One*. 2018; 13(8):e0201751. Epub 2018/08/10. <https://doi.org/10.1371/journal.pone.0201751> PMID: 30092011; PubMed Central PMCID: PMC6084949 currently employed by Stoke Therapeutics, his contributions occurred entirely during his time at Drexel University. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.
14. Stratford JK, Bentrem DJ, Anderson JM, Fan C, Volmar KA, Marron JS, et al. A six-gene signature predicts survival of patients with localized pancreatic ductal adenocarcinoma. *PLoS Med*. 2010; 7(7): e1000307. Epub 2010/07/21. <https://doi.org/10.1371/journal.pmed.1000307> PMID: 20644708; PubMed Central PMCID: PMC2903589.
15. Birnbaum DJ, Finetti P, Lopresti A, Gilbert M, Poizat F, Raoul JL, et al. A 25-gene classifier predicts overall survival in resectable pancreatic cancer. *BMC Med*. 2017; 15(1):170. Epub 2017/09/21. <https://doi.org/10.1186/s12916-017-0936-z> PMID: 28927421; PubMed Central PMCID: PMC5606023.
16. Yan X, Wan H, Hao X, Lan T, Li W, Xu L, et al. Importance of gene expression signatures in pancreatic cancer prognosis and the establishment of a prediction model. *Cancer Manag Res*. 2019; 11:273–83. Epub 2019/01/16. <https://doi.org/10.2147/CMAR.S185205> PMID: 30643453; PubMed Central PMCID: PMC6312063.
17. Shi G, Zhang J, Lu Z, Liu D, Wu Y, Wu P, et al. A novel messenger RNA signature as a prognostic biomarker for predicting relapse in pancreatic ductal adenocarcinoma. *Oncotarget*. 2017; 8(67):110849–60. Epub 2018/01/18. <https://doi.org/10.18632/oncotarget.22861> PMID: 29340021; PubMed Central PMCID: PMC5762289.
18. Kirby MK, Ramaker RC, Gertz J, Davis NS, Johnston BE, Oliver PG, et al. RNA sequencing of pancreatic adenocarcinoma tumors yields novel expression patterns associated with long-term survival and reveals a role for ANGPTL4. *Mol Oncol*. 2016; 10(8):1169–82. Epub 2016/06/11. <https://doi.org/10.1016/j.molonc.2016.05.004> PMID: 27282075; PubMed Central PMCID: PMC5423196.
19. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. 2012; 2(5):401–4. Epub 2012/05/17. <https://doi.org/10.1158/2158-8290.CD-12-0095> PMID: 22588877; PubMed Central PMCID: PMC3956037.
20. Therneau T.M. GPM. Modeling Survival Data: Extending the Cox Model. New York: Springer; 2000.
21. Network CGAR. Integrated Genomic Characterization of Pancreatic Ductal Adenocarcinoma. *Cancer Cell*. 2017; 32(2):185–203 e13. Epub 2017/08/16. <https://doi.org/10.1016/j.ccr.2017.07.007> PMID: 28810144; PubMed Central PMCID: PMC5964983.
22. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005; 102(43):15545–50. Epub 2005/10/04. <https://doi.org/10.1073/pnas.0506580102> PMID: 16199517; PubMed Central PMCID: PMC1239896.
23. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 2012; 483(7391):603–7. Epub 2012/03/31. <https://doi.org/10.1038/nature11003> PMID: 22460905; PubMed Central PMCID: PMC3320027.
24. Seashore-Ludlow B, Rees MG, Cheah JH, Cokol M, Price EV, Coletti ME, et al. Harnessing Connectivity in a Large-Scale Small-Molecule Sensitivity Dataset. *Cancer Discov*. 2015; 5(11):1210–23. Epub 2015/10/21. <https://doi.org/10.1158/2158-8290.CD-15-0235> PMID: 26482930; PubMed Central PMCID: PMC4631646.

25. Team RC. R: A language and environment for statistical computing. Vienna, Austria.: R Foundation for Statistical Computing; 2019.
26. MacDonald PE, Wheeler MB. Voltage-dependent K(+) channels in pancreatic beta cells: role, regulation and potential as therapeutic targets. *Diabetologia*. 2003; 46(8):1046–62. Epub 2003/06/28. <https://doi.org/10.1007/s00125-003-1159-8> PMID: 12830383
27. Hayashi M, Novak I. Molecular basis of potassium channels in pancreatic duct epithelial cells. *Channels (Austin)*. 2013; 7(6):432–41. Epub 2013/08/22. <https://doi.org/10.4161/chan.26100> PMID: 23862792; PubMed Central PMCID: PMC4042478.
28. Yu KD, Zhu R, Zhan M, Rodriguez AA, Yang W, Wong S, et al. Identification of prognosis-relevant subgroups in patients with chemoresistant triple-negative breast cancer. *Clin Cancer Res*. 2013; 19(10):2723–33. Epub 2013/04/04. <https://doi.org/10.1158/1078-0432.CCR-12-2986> PMID: 23549873; PubMed Central PMCID: PMC3655097.
29. Pirsko V, Cakstina I, Priedite M, Dortane R, Feldmane L, Nakazawa-Mikasevica M, et al. An Effect of Culture Media on Epithelial Differentiation Markers in Breast Cancer Cell Lines MCF7, MDA-MB-436 and SkBr3. *Medicina (Kaunas)*. 2018; 54(2). Epub 2018/10/23. <https://doi.org/10.3390/medicina54020011> PMID: 30344242; PubMed Central PMCID: PMC6037242.
30. Wu L, Han L, Zhou C, Wei W, Chen X, Yi H, et al. TGF-beta1-induced CK17 enhances cancer stem cell-like properties rather than EMT in promoting cervical cancer metastasis via the ERK1/2-MZF1 signaling pathway. *FEBS J*. 2017; 284(18):3000–17. Epub 2017/07/14. <https://doi.org/10.1111/febs.14162> PMID: 28703907.
31. Chivu-Economescu M, Dragu DL, Necula LG, Matei L, Enciu AM, Bleotu C, et al. Knockdown of KRT17 by siRNA induces antitumoral effects on gastric cancer cells. *Gastric Cancer*. 2017; 20(6):949–59. Epub 2017/03/17. <https://doi.org/10.1007/s10120-017-0712-y> PMID: 28299464.
32. Merkin RD, Vanner EA, Romeiser JL, Shroyer ALW, Escobar-Hoyos LF, Li J, et al. Keratin 17 is overexpressed and predicts poor survival in estrogen receptor-negative/human epidermal growth factor receptor-2-negative breast cancer. *Hum Pathol*. 2017; 62:23–32. Epub 2016/11/07. <https://doi.org/10.1016/j.humpath.2016.10.006> PMID: 27816721.
33. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. 2008; 133(4):704–15. Epub 2008/05/20. <https://doi.org/10.1016/j.cell.2008.03.027> PMID: 18485877; PubMed Central PMCID: PMC2728032.
34. Nabeshima K, Inoue T, Shimao Y, Samashima T. Matrix metalloproteinases in tumor invasion: role for cell migration. *Pathol Int*. 2002; 52(4):255–64. Epub 2002/05/29. <https://doi.org/10.1046/j.1440-1827.2002.01343.x> PMID: 12031080.
35. Watanabe S, Ueda Y, Akaboshi S, Hino Y, Sekita Y, Nakao M. HMG2 maintains oncogenic RAS-induced epithelial-mesenchymal transition in human pancreatic cancer cells. *Am J Pathol*. 2009; 174(3):654–68. Epub 2009/01/31. <https://doi.org/10.2353/ajpath.2009.080523> PMID: 19179606; PubMed Central PMCID: PMC2665746.
36. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol*. 2014; 15(3):178–96. Epub 2014/02/22. <https://doi.org/10.1038/nrm3758> PMID: 24556840; PubMed Central PMCID: PMC4240281.
37. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods*. 2015; 12(5):453–7. Epub 2015/03/31. <https://doi.org/10.1038/nmeth.3337> PMID: 25822800; PubMed Central PMCID: PMC4739640.
38. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. *J Clin Oncol*. 2009; 27(8):1160–7. Epub 2009/02/11. <https://doi.org/10.1200/JCO.2008.18.1370> PMID: 19204204; PubMed Central PMCID: PMC2667820.
39. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med*. 2002; 347(25):1999–2009. Epub 2002/12/20. <https://doi.org/10.1056/NEJMoa021967> PMID: 12490681.
40. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med*. 2004; 351(27):2817–26. Epub 2004/12/14. <https://doi.org/10.1056/NEJMoa041588> PMID: 15591335.
41. O'Connell MJ, Lavery I, Yothers G, Paik S, Clark-Langone KM, Lopatin M, et al. Relationship between tumor gene expression and recurrence in four independent studies of patients with stage II/III colon cancer treated with surgery alone or surgery plus adjuvant fluorouracil plus leucovorin. *J Clin Oncol*. 2010; 28(25):3937–44. Epub 2010/08/04. <https://doi.org/10.1200/JCO.2010.28.9538> PMID: 20679606; PubMed Central PMCID: PMC2940392.
42. Demirkol S, Gomceli I, Isbilen M, Dayanc BE, Tez M, Bostanci EB, et al. A Combined ULBP2 and SEMA5A Expression Signature as a Prognostic and Predictive Biomarker for Colon Cancer. *J Cancer*.

- 2017; 8(7):1113–22. Epub 2017/06/14. <https://doi.org/10.7150/ica.17872> PMID: [28607584](#); PubMed Central PMCID: [PMC5463424](#).
43. Schnittger A, De Veylder L. The Dual Face of Cyclin B1. *Trends Plant Sci.* 2018; 23(6):475–8. Epub 2018/04/24. <https://doi.org/10.1016/j.tplants.2018.03.015> PMID: [29680634](#).
 44. Chen Q, Cai D, Li M, Wu X. The homologous recombination protein RAD51 is a promising therapeutic target for cervical carcinoma. *Oncol Rep.* 2017; 38(2):767–74. Epub 2017/06/20. <https://doi.org/10.3892/or.2017.5724> PMID: [28627709](#); PubMed Central PMCID: [PMC5561999](#).
 45. Debant A, Serra-Pages C, Selpel K, O'Brien S, Tang M, Park SH, et al. The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac-specific and rho-specific guanine nucleotide exchange factor domains. *Proc Natl Acad Sci U S A.* 1996; 93(11):5466–71. Epub 1996/05/28. <https://doi.org/10.1073/pnas.93.11.5466> PMID: [8643598](#); PubMed Central PMCID: [PMC39269](#).
 46. Beck L, Leroy C, Salaun C, Margall-Ducos G, Desdouets C, Friedlander G. Identification of a novel function of PTT1 critical for cell proliferation and independent of its phosphate transport activity. *J Biol Chem.* 2009; 284(45):31363–74. Epub 2009/09/04. <https://doi.org/10.1074/jbc.M109.053132> PMID: [19726692](#); PubMed Central PMCID: [PMC2781533](#).
 47. Cairns J, Fridley BL, Jenkins GD, Zhuang Y, Yu J, Wang L. Differential roles of ERK1 in EGFR and AKT pathway regulation affect cancer proliferation. *EMBO Rep.* 2018; 19(3). Epub 2018/01/18. <https://doi.org/10.15252/embr.201744767> PMID: [29335246](#); PubMed Central PMCID: [PMC5835844](#).
 48. Zhao G, Wang B, Liu Y, Zhang JG, Deng SC, Qin Q, et al. miRNA-141, downregulated in pancreatic cancer, inhibits cell proliferation and invasion by directly targeting MAP4K4. *Mol Cancer Ther.* 2013; 12(11):2569–80. Epub 2013/09/10. <https://doi.org/10.1158/1535-7163.MCT-13-0296> PMID: [24013097](#).
 49. Wee P, Wang Z. Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. *Cancers (Basel).* 2017; 9(5). Epub 2017/05/18. <https://doi.org/10.3390/cancers9050052> PMID: [28513565](#); PubMed Central PMCID: [PMC5447982](#).
 50. Guo M, Luo G, Liu C, Cheng H, Lu Y, Jin K, et al. The Prognostic and Predictive Role of Epidermal Growth Factor Receptor in Surgical Resected Pancreatic Cancer. *Int J Mol Sci.* 2016; 17(7). Epub 2016/07/12. <https://doi.org/10.3390/ijms17071090> PMID: [27399694](#); PubMed Central PMCID: [PMC4964466](#).
 51. Innocenti M, Frittoli E, Ponzanelli I, Falck JR, Brachmann SM, Di Fiore PP, et al. Phosphoinositide 3-kinase activates Rac by entering in a complex with Eps8, Abi1, and Sos-1. *J Cell Biol.* 2003; 160(1):17–23. Epub 2003/01/08. <https://doi.org/10.1083/jcb.200206079> PMID: [12515821](#); PubMed Central PMCID: [PMC2172734](#).
 52. Fang D, Chen H, Zhu JY, Wang W, Tang Y, Ding HF, et al. Epithelial-mesenchymal transition of ovarian cancer cells is sustained by Rac1 through simultaneous activation of MEK1/2 and Src signaling pathways. *Oncogene.* 2017; 36(11):1546–58. Epub 2016/09/13. <https://doi.org/10.1038/onc.2016.323> PMID: [27617676](#); PubMed Central PMCID: [PMC5346482](#).
 53. Im DK, Cheong H, Lee JS, Oh MK, Yang KM. Protein kinase CK2-dependent aerobic glycolysis-induced lactate dehydrogenase A enhances the migration and invasion of cancer cells. *Sci Rep.* 2019; 9(1):5337. Epub 2019/03/31. <https://doi.org/10.1038/s41598-019-41852-4> PMID: [30926903](#); PubMed Central PMCID: [PMC6441004](#).
 54. Mazzocco G, Pazienza V, Panza A, Valvano MR, Benegiamo G, Vinciguerra M, et al. ARNTL2 and SERPINE1: potential biomarkers for tumor aggressiveness in colorectal cancer. *J Cancer Res Clin Oncol.* 2012; 138(3):501–11. Epub 2011/12/27. <https://doi.org/10.1007/s00432-011-1126-6> PMID: [22198637](#).
 55. Ohshima K, Hatakeyama K, Kanto K, Ide T, Watanabe Y, Moromizato S, et al. Comparative proteomic analysis identifies exosomal Eps8 protein as a potential metastatic biomarker for pancreatic cancer. *Oncol Rep.* 2019; 41(2):1019–34. Epub 2018/11/16. <https://doi.org/10.3892/or.2018.6866> PMID: [30431134](#).
 56. Fukuhisa H, Seki N, Idichi T, Kurahara H, Yamada Y, Toda H, et al. Gene regulation by antitumor miR-130b-5p in pancreatic ductal adenocarcinoma: the clinical significance of oncogenic EPS8. *J Hum Genet.* 2019; 64(6):521–34. Epub 2019/03/13. <https://doi.org/10.1038/s10038-019-0584-6> PMID: [30858505](#).
 57. Lee KY. M1 and M2 polarization of macrophages: a mini-review. *Med Biol Sci Eng.* 2019; 2(1):1–5. Epub 2019/01/31. <https://doi.org/10.30579/mbse.2019.2.1.1>
 58. Bednarczyk RB, Tuli NY, Hanly EK, Rahoma GB, Maniyan R, Mittelman A, et al. Macrophage inflammatory factors promote epithelial-mesenchymal transition in breast cancer. *Oncotarget.* 2018; 9(36):24272–82. Epub 2018/06/01. <https://doi.org/10.18632/oncotarget.24917> PMID: [29849939](#); PubMed Central PMCID: [PMC5966261](#).

59. Kwan WH, van der Touw W, Heeger PS. Complement regulation of T cell immunity. *Immunol Res*. 2012; 54(1–3):247–53. Epub 2012/04/06. <https://doi.org/10.1007/s12026-012-8327-1> PMID: [22477527](#); PubMed Central PMCID: [PMC4081859](#).
60. Pallante P, Sepe R, Federico A, Forzati F, Bianco M, Fusco A. CBX7 modulates the expression of genes critical for cancer progression. *PLoS One*. 2014; 9(5):e98295. Epub 2014/05/29. <https://doi.org/10.1371/journal.pone.0098295> PMID: [24865347](#); PubMed Central PMCID: [PMC4035280](#).
61. Nawaz Z, Patil V, Arora A, Hegde AS, Arivazhagan A, Santosh V, et al. Cbx7 is epigenetically silenced in glioblastoma and inhibits cell migration by targeting YAP/TAZ-dependent transcription. *Sci Rep*. 2016; 6:27753. Epub 2016/06/14. <https://doi.org/10.1038/srep27753> PMID: [27291091](#); PubMed Central PMCID: [PMC4904208](#).
62. Peng X, Guan L, Gao B. miRNA-19 promotes non-small-cell lung cancer cell proliferation via inhibiting CBX7 expression. *Oncotargets Ther*. 2018; 11:8865–74. Epub 2018/12/26. <https://doi.org/10.2147/OTT.S181433> PMID: [30584339](#); PubMed Central PMCID: [PMC6290863](#).
63. Bao Z, Xu X, Liu Y, Chao H, Lin C, Li Z, et al. CBX7 negatively regulates migration and invasion in glioma via Wnt/beta-catenin pathway inactivation. *Oncotarget*. 2017; 8(24):39048–63. Epub 2017/04/08. <https://doi.org/10.18632/oncotarget.16587> PMID: [28388562](#); PubMed Central PMCID: [PMC5503594](#).
64. Yu T, Wu Y, Hu Q, Zhang J, Nie E, Wu W, et al. CBX7 is a glioma prognostic marker and induces G1/S arrest via the silencing of CCNE1. *Oncotarget*. 2017; 8(16):26637–47. Epub 2017/05/04. <https://doi.org/10.18632/oncotarget.15789> PMID: [28460453](#); PubMed Central PMCID: [PMC5432285](#).
65. Gao H, Cong X, Zhou J, Guan M. MicroRNA-222 influences migration and invasion through MIA3 in colorectal cancer. *Cancer Cell Int*. 2017; 17:78. Epub 2017/09/01. <https://doi.org/10.1186/s12935-017-0447-1> PMID: [28555850](#); PubMed Central PMCID: [PMC5576312](#).
66. Amdt S, Bosserhoff AK. Reduced expression of TANGO in colon and hepatocellular carcinomas. *Oncol Rep*. 2007; 18(4):885–91. Epub 2007/09/06. PMID: [17786351](#).
67. Kakinuma N, Roy BC, Zhu Y, Wang Y, Kiyama R. Kank regulates RhoA-dependent formation of actin stress fibers and cell migration via 14-3-3 in PI3K-Akt signaling. *J Cell Biol*. 2008; 181(3):537–49. Epub 2008/05/07. <https://doi.org/10.1083/jcb.200707022> PMID: [18458160](#); PubMed Central PMCID: [PMC2364688](#).
68. Uhlen M, Zhang C, Lee S, Sjostedt E, Fagerberg L, Bidkhori G, et al. A pathology atlas of the human cancer transcriptome. *Science*. 2017; 357(6352). Epub 2017/08/19. <https://doi.org/10.1126/science.aan2507> PMID: [28818916](#).
69. Birnbaum DJ, Bertucci F, Finetti P, Adelaide J, Giovannini M, Turrini O, et al. Expression of Genes with Copy Number Alterations and Survival of Patients with Pancreatic Adenocarcinoma. *Cancer Genomics Proteomics*. 2016; 13(3):191–200. Epub 2016/04/24. PMID: [27107061](#).
70. Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer*. 2009; 9(8):537–49. Epub 2009/07/25. <https://doi.org/10.1038/nrc2694> PMID: [19629068](#).
71. Yang L, Sun X, Ye Y, Lu Y, Zuo J, Liu W, et al. p38alpha Mitogen-Activated Protein Kinase Is a Drug-Targetable Target in Pancreatic Adenocarcinoma. *Front Oncol*. 2019; 9:1294. Epub 2019/12/13. <https://doi.org/10.3389/fonc.2019.01294> PMID: [31828036](#); PubMed Central PMCID: [PMC6890821](#).
72. Chen J, Zhao T, Jia S, Zhou S, Zhou L, Wang S, et al. High Expression of P38α and Preoperative Carbohydrate Antigen 19–9 Indicate Poor Prognosis in Patients with Pancreatic Ductal Adenocarcinoma. *Journal of Cancer*. 2018; 9(4):650. <https://doi.org/10.7150/jca.21683> PMID: [29556322](#).
73. Handra-Luca A, Lesty C, Hammel P, Sauvanet A, Rebours V, Martin A, et al. Biological and prognostic relevance of mitogen-activated protein kinases in pancreatic adenocarcinoma. *Pancreas*. 2012; 41(3):416–21. <https://doi.org/10.1097/MPA.0b013e318238379d> PMID: [22158075](#).
74. Wang L, Bai YY, Yang Y, Hu F, Wang Y, Yu Z, et al. Diabetes mellitus stimulates pancreatic cancer growth and epithelial-mesenchymal transition-mediated metastasis via a p38 MAPK pathway. *Oncotarget*. 2016; 7(25):38539–50. Epub 2016/10/23. <https://doi.org/10.18632/oncotarget.9533> PMID: [27413117](#); PubMed Central PMCID: [PMC5122409](#).
75. Guo X, Ma N, Wang J, Song J, Bu X, Cheng Y, et al. Increased p38-MAPK is responsible for chemotherapy resistance in human gastric cancer cells. *BMC cancer*. 2008; 8(1):375.
76. Kuma Y, Sabio G, Bain J, Shpiro N, Márquez R, Cuenda A. BIRB796 inhibits all p38 MAPK isoforms in vitro and in vivo. *Journal of Biological Chemistry*. 2005; 280(20):19472–9. <https://doi.org/10.1074/jbc.M414221200> PMID: [15755732](#).
77. He D, Zhao X-q, Chen X-g, Fang Y, Singh S, Talele TT, et al. BIRB796, the inhibitor of p38 mitogen-activated protein kinase, enhances the efficacy of chemotherapeutic agents in ABCB1 overexpression cells. *PLoS one*. 2013; 8(1):e54181. <https://doi.org/10.1371/journal.pone.0054181> PMID: [23349819](#).

78. Pargellis C, Tong L, Churchill L, Cirillo PF, Gilmore T, Graham AG, et al. Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. *Nat Struct Biol*. 2002; 9(4):268–72. Epub 2002/03/16. <https://doi.org/10.1038/nsb779> PMID: 11895401.
79. Wu I-C, Chen Y-K, Wu C-C, Cheng Y-J, Chen W-C, Ko H-J, et al. Overexpression of ATPase Na⁺/K⁺-transporting alpha 1 polypeptide, ATP1A1, correlates with clinical diagnosis and progression of esophageal squamous cell carcinoma. *Oncotarget*. 2016; 7(51):85244. <https://doi.org/10.18632/oncotarget.13267> PMID: 27845894
80. Khajah MA, Mathew PM, Luqmani YA. Na⁺/K⁺ ATPase activity promotes invasion of endocrine resistant breast cancer cells. *PLoS one*. 2018; 13(3):e0193779. <https://doi.org/10.1371/journal.pone.0193779> PMID: 29590154
81. Kometiani P, Liu L, Askari A. Digitalis-induced signaling by Na⁺/K⁺-ATPase in human breast cancer cells. *Mol Pharmacol*. 2005; 67(3):929–36. Epub 2004/12/17. <https://doi.org/10.1124/mol.104.007302> PMID: 15602003
82. Huang YT, Chueh SC, Teng CM, Guh JH. Investigation of ouabain-induced anticancer effect in human androgen-independent prostate cancer PC-3 cells. *Biochem Pharmacol*. 2004; 67(4):727–33. Epub 2004/02/06. <https://doi.org/10.1016/j.bcp.2003.10.013> PMID: 14757172
83. Yeh JY, Huang WJ, Kan SF, Wang PS. Inhibitory effects of digitalis on the proliferation of androgen dependent and independent prostate cancer cells. *J Urol*. 2001; 166(5):1937–42. Epub 2001/10/05. PMID: 11586264
84. Tian J, Li X, Liang M, Liu L, Xie JX, Ye Q, et al. Changes in sodium pump expression dictate the effects of ouabain on cell growth. *J Biol Chem*. 2009; 284(22):14921–9. Epub 2009/03/31. <https://doi.org/10.1074/jbc.M808355200> PMID: 19329430; PubMed Central PMCID: PMC2685674.

RESEARCH ARTICLE

Renin angiotensin system genes are biomarkers for personalized treatment of acute myeloid leukemia with Doxorubicin as well as etoposide

Seyhan Turk^{1*}, Can Turk², Muhammad Waqas Akbar³, Baris Kucukkaraduman³, Murat Isbilen³, Secil Demirkol Canli⁴, Umit Yavuz Malkan⁵, Mufide Okay⁶, Gulberk Ucar¹, Nilgun Sayinalp⁶, Ibrahim Celalettin Haznedaroglu⁶, Ali Osmay Gure³

1 Department of Biochemistry, Hacettepe University, Ankara, Turkey, **2** Department of Medical Microbiology, Lokman Hekim University, Ankara, Turkey, **3** Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey, **4** Molecular Pathology Application and Research Center, Hacettepe University, Ankara, Turkey, **5** Department of Hematology, University of Health Sciences, Ankara, Turkey, **6** Department of Hematology, Hacettepe University, Ankara, Turkey

* seyhan.turk@hacettepe.edu.tr



OPEN ACCESS

Citation: Turk S, Turk C, Akbar MW, Kucukkaraduman B, Isbilen M, Demirkol Canli S, et al. (2020) Renin angiotensin system genes are biomarkers for personalized treatment of acute myeloid leukemia with Doxorubicin as well as etoposide. PLoS ONE 15(11): e0242497. <https://doi.org/10.1371/journal.pone.0242497>

Editor: Francesco Bertolini, European Institute of Oncology, ITALY

Received: April 3, 2020

Accepted: November 4, 2020

Published: November 25, 2020

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.pone.0242497>

Copyright: © 2020 Turk et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its [Supporting Information](#) files.

Abstract

Despite the availability of various treatment protocols, response to therapy in patients with Acute Myeloid Leukemia (AML) remains largely unpredictable. Transcriptomic profiling studies have thus far revealed the presence of molecular subtypes of AML that are not accounted for by standard clinical parameters or by routinely used biomarkers. Such molecular subtypes of AML are predicted to vary in response to chemotherapy or targeted therapy. The Renin-Angiotensin System (RAS) is an important group of proteins that play a critical role in regulating blood pressure, vascular resistance and fluid/electrolyte balance. RAS pathway genes are also known to be present locally in tissues such as the bone marrow, where they play an important role in leukemic hematopoiesis. In this study, we asked if the RAS genes could be utilized to predict drug responses in patients with AML. We show that the combined *in silico* analysis of up to five RAS genes can reliably predict sensitivity to Doxorubicin as well as Etoposide in AML. The same genes could also predict sensitivity to Doxorubicin when tested *in vitro*. Additionally, gene set enrichment analysis revealed enrichment of TNF-alpha and type-I IFN response genes among sensitive, and TGF-beta and fibronectin related genes in resistant cancer cells. However, this does not seem to reflect an epithelial to mesenchymal transition per se. We also identified that RAS genes can stratify patients with AML into subtypes with distinct prognosis. Together, our results demonstrate that genes present in RAS are biomarkers for drug sensitivity and the prognostication of AML.

Funding: This study was supported by The Scientific and Technological Research Council of Turkey (116S350).

Competing interests: The authors have declared that no competing interests exist.

Introduction

Leukemia, lymphoma and multiple myeloma are the three main types of highly heterogeneous hematological malignancies that are derived from myeloid and lymphoid cell lineages [1]. Acute myeloid leukemia (AML) is characterized by abnormal expansion of immature myeloid cells and their accumulation in the bone marrow and blood, interfering with normal cellular growth [2]. AML is a highly aggressive cancer with poor prognosis. It is also the most common type of acute leukemia in adults. Treatment strategies and success rates vary depending on many factors, including the subtype of AML, prognostic factors, age and general health status of the patient [3]. Standard treatment regimens based on patient stratification include the combination of chemotherapeutics such as Cytarabine, Daunorubicin and Etoposide with or without radiotherapy. However, high heterogeneity of clinical outcomes in AML patients suggests that current classifications fail to distinguish patient subgroups sufficiently [4].

A not so well studied protein network in the context of AML is the Renin-Angiotensin System (RAS). RAS is composed of several gene products which play a critical role in regulating blood pressure, renal vascular resistance and the fluid/electrolyte balance [5, 6]. The idea of a local RAS operating independent of the circulating RAS was brought into light by demonstrating localized RAS elements in organs other than liver (angiotensinogen), kidney (renin) and lung (ACE). Localized RAS elements were found in many organs such as the brain, blood vessels and heart [7, 8]. It is predicted that locally produced angiotensins have important homeostatic functions and may contribute to local tissue dysfunction and diseases [8]. The presence of local RAS specific to the hematopoietic bone marrow microenvironment was reported for the first time in 1996 [9]. Major RAS molecules have been identified in the bone marrow microenvironment, such as renin, angiotensinogen, angiotensin receptors and angiotensin converting enzymes (ACEs) [10]. Locally active bone marrow RAS affects important stages of physiological and pathological blood cell production through autocrine, paracrine and intracrine pathways [11, 12]. Local bone marrow RAS peptides control the development of hematopoietic niche, myelopoiesis, erythropoiesis, thrombopoiesis and other cellular lineages [13–19]. Local RAS is also active in the primitive embryonic hematopoiesis phase [20–23]. The presence of renin, ACE, angiotensin II (Ang-II) and angiotensinogen in leukemic blast cells has been demonstrated, and local bone marrow RAS has been shown to play a role in the development of neoplastic malignant blood cells [24–26].

Establishing a role for genes involved in the development and biology of cancers, as prognostic and chemotherapeutic markers, is one of the most effective and successful approach used in the classification of malignancies. Thus, here we aimed to define AML subgroups based on expression of RAS genes. We also aimed to test if the resulting tumor subtypes differ in their responses to drugs and to demonstrate distinct prognostic profiles.

Materials and methods

In silico

Datasets. Cancer Genome Project (CGP) gene expression data (E-MTAB-783) [27, 28] was downloaded from ArrayExpress website (<https://www.ebi.ac.uk/arrayexpress/>), and drug screening data [29] was downloaded from the CGP database. Microarray dataset GSE12417 [30], corresponding to AML patients, was downloaded from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database. The training cohort of 163 patients in GSE12417 [30] was used for our analyses since this was the same microarray platform as the one used for CGP.

Data normalization and variance analysis. CGP gene expression data was normalized by the RMA method using the BRB-Array Tools software [31].

In order to choose the RAS genes that would be used in real-time PCR for validation studies, we first aimed to choose the most variable genes that would likely give detectable fold differences *in vitro* by PCR. Analysis of variance was performed using all 39 probesets corresponding to the 25 genes in the RAS and genes with at least 0.8 of variance. Above these thresholds, the mean expression was at least 5.5 and the log fold difference between min to max was above 3 for all probesets. Thus, nine probesets corresponding to eight genes (*CTSG*, *CPA3*, *AGT*, *ANPEP*, *IGF2R* (two probesets), *RNPEP*, *ATP6AP2* and *CTSA*) were selected to be used in further analyses (S1 Table).

IC50 calculation methods. In order to calculate drug response parameters such as IC50, EC50, activity area and Amax, the growth rate of the cells were depicted as a function of drug concentration by being modeled with non-linear logistic regression as explained in De Lean *et al.* [22], which is also reported in NIH/NCIC assay guidelines [33]. While the non-linear logistic regression function used to model data is used widely for cytotoxicity calculations, here for the first time we used six different versions of this function and selected the one with the lowest standard error rate among all for the calculation of cytotoxicity values. We name this approach the 6-model (6M).

Thus, six different models were derived from the following non-linear logistic regression function:

$$Y = (a - d / (1 + (X/c)^b) + d)$$

where Y is the percent growth of the cells, X is the arithmetic drug concentration, a is the percent growth of the cells when the cells are not treated with the drug, d is the percent growth of the cells for infinite dose, i.e. a dose for which there is no additional effect when increased, c is the dose corresponding to percent growth exactly between a and d , and b is the Hill slope factor that is used to define the steepness of the curve fitted.

The following are the conditions required for the generation of 6 models:

1. **3-Parameter model:** Curves were fitted without using Hill slope factor b .
2. **3-Parameter Top 100 model:** Curves were fitted without using Hill slope factor b and with $a = 100$.
3. **3-Parameter Bottom 0 model:** Curves were fitted without using Hill slope factor b and with $d = 0$.
4. **4-Parameter model:** Formula is used as it is.
5. **4-Parameter Top 100 model:** Curves were fitted with $a = 100$.
6. **4-Parameter Bottom 0 model:** Curves were fitted with $d = 0$.

Six different drug response parameters are calculated out of the fitted curves as follows:

- **IC50:** Value of X when $\hat{Y} = 50\%$
- **IC90:** Value of X when $\hat{Y} = 90\%$
- **IC95:** Value of X when $\hat{Y} = 95\%$
- **EC50:** Value of X when $\hat{Y} = a + d$
- **Amax:** $a - d$

- **Activity Area:** $\Sigma \hat{Y}X$, (sum of $\hat{Y}$ s for each 0.01 increment of X fitted), where $\hat{Y}$ is the predicted value of Y by the curve fitted.

With the 6M approach we recalculated IC50 values that were also included in the raw CGP data for the 17 AML cell lines treated with four drugs (ATRA, Cytarabine, Etoposide and Doxorubicin) using an in-house R script "SixModelIC50 V3.r" (https://github.com/muratibilen/6-Model_IC50_CalculationV3.git).

These drugs were selected as we obtained AML chemotherapy treatment protocols from the Department of Hematology, Hacettepe University and compiled a list for all drugs in these protocols. Among these only ATRA, Cytarabine, Etoposide and Doxorubicin were present in the CGP database.

We referred to the recalculated IC50 data as 6M IC50 and performed a Pearson r correlation analysis between CGP IC50s and recalculated 6M IC50s to test the compatibility.

In addition, IC50 values were calculated using the 6M approach on the data obtained from in vitro analysis in which nine AML cell lines were treated with Doxorubicin and Etoposide.

Linear regression analyses. We performed correlation analysis between expression values of the eight genes and drug data (both CGP IC50 data and 6M IC50 data) individually. To identify if multiple genes can be used to better identify the relationship between gene expression and drug sensitivity data, linear regression analyses were performed using the Minitab 17 software (<https://www.minitab.com>). Seventeen AML cell lines from the CGP database were either randomly divided into two groups, the discovery group (12 cell lines) and the test group (five cell lines), or chosen manually so that the sensitivity range of cells in both groups spanned as large variance as possible. To generate a linear regression model for each drug (ATRA, Cytarabine, Etoposide, Doxorubicin), IC50s of the discovery cell line group obtained either from CGP or recalculated as 6M IC50, and expression of the eight RAS genes which were selected from variance analysis, were used as predictors. As a measure of the response variable variation explained by each linear regression model, we used the adjusted (adj.) R^2 values. To test consistency of the linear regression models generated with the eight genes, we replicated the random division of groups ten times and reported the average of the adjusted R^2 .

Furthermore, to identify a minimal gene list for the prediction of chemosensitivity, the discovery group was used to fit a model explaining the drug response using "best subsets" function of the software, which runs all possible regression models with one variable, two variables and so on, based on a list of predictors, enabling the user to choose a smaller set of predictors that can explain the response. The subset with the highest R^2 (adj.) was selected as the best model. Regression formula of the best models ($y = \pm a + [n1 \times x1] \pm [n2 \times x2] \pm [n3 \times x3] \pm [n4 \times x4] \dots$) were applied for the test group of each drug. In the regression formula y (predicted IC50 values) were calculated where a and n are the constant values, x : gene expression values of the 12 cell lines in the discovery group. Also, the goodness of fit measure $Sy.x$ were computed by Graphpad. $Sy.x$ is a standard deviation of the residuals that here has been used to describe the difference in standard deviations of CGP IC50 and 6M IC50 versus predicted IC50s. It is a goodness-of-fit measure used to show how well our predicted IC50s fit with CGP and 6M IC50 values. All the correlations were calculated with Graphpad software as Pearson's r and p values.

Hierarchical clustering analysis. Cluster 3.0 (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>) [34] and Java Treeview (<http://jtreeview.sourceforge.net/>) [35] software were used for hierarchical clustering analysis with mean standardized gene expression values for each dataset. Hierarchical clustering was performed by clustering both genes and arrays using Euclidian distance as similarity metric and complete linkage as clustering method.

Gene sets enrichment analysis—GSEA. Gene set enrichment analysis was performed using the GSEA guideline (<https://www.gsea-msigdb.org/gsea/index.jsp>) [36].

Briefly, dataset E-MTAB-783 [27, 28] has 22277 probesets IDs and these were collapsed into 13321 genes. For genes with more than one probeset, one with the highest expression was selected. “C5_all Gene ontology v6.1 database” was used for the analysis which has gene sets that contain genes annotated by the same GO term. We used default filtering criteria in GSEA for gene set sizes, which includes genesets with sizes between 15–500. After applying this filter, analysis was performed for 4081 gene sets.

Mutation analyses. Mutation data of AML cell lines was downloaded from Genomics of Drug sensitivity in Cancer database (https://www.cancerrxgene.org/downloads/bulk_download) [37]. 14 out of the 17 AML cell lines used in our analyses were available. Seven genes which were mutated in at least three AML cell lines were analyzed further.

In vitro

Cell lines and cytotoxicity experiments. HEL92.1.7 (2111706), and QIMR-WIL (86030601) cell lines were purchased from Sigma Aldrich (St. Louis, Mo., USA), KASUMI-3 (CRL-2725), GDM-1 (CRL-2627) and CESS (TIB-190) cell lines were purchased from ATCC (Virginia, USA) and P31/FUJ (JCRB0091), NOMO-1 (IFO50474), KASUMI-1 (JCRB1003) and SKM-1 (JCRB0118) cell lines were purchased from JCRB Cell Bank (Osaka, Japan). Cell lines were authenticated by manufacturers, all cell lines were morphologically checked by microscope and routine mycoplasma testing was performed by PCR. HEL92.1.7, GDM-1, CESS, P31/FUJ and NOMO-1 were cultured and maintained in RPMI-1640 medium (Sigma-Aldrich, R0883 (St. Louis, Mo., USA)) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, F6178 (St. Louis, Mo., USA)), 1% penicillin-streptomycin (Sigma-Aldrich, 11074440001 (St. Louis, Mo., USA)), and 1% 200 mM L-glutamine (Sigma-Aldrich, G7513 (St. Louis, Mo., USA)). KASUMI-1, SKM-1 and KASUMI-3 were cultured in RPMI-1640 medium but with 20% FBS. QIMR-WIL was cultured in DMEM medium (Sigma-Aldrich, D6546 (St. Louis, Mo., USA)) but with 10% FBS, 1% penicillin-streptomycin, and 1% 200 mM L-glutamine. All cell lines were cultured at 5% CO₂ and 37 °C in a humidified incubator.

Doxorubicin (D1515) and Etoposide (E1383) were purchased from Sigma-Aldrich (St. Louis, Mo., USA) and were dissolved in DMSO. Cell viability was measured using CellTiter-Glo reagent (G7572, Promega, Fitchburg, Wisconsin, USA). 7000 cells/well in 90 µl medium were plated in each well of a 96-well plate. Cells were treated with six different concentrations of Doxorubicin or Etoposide separately (20, 10, 2, 1, 0.2, 0.1 µM). After 72 hours of drug treatment, cells were treated with CellTiter-Glo reagent and the luminescence signal was then recorded with a microplate luminometer (Turner Designs, CA, USA). All drug treatment experiments were repeated three times. Growth percentages were calculated for each drug and cell line, and cytotoxicity values were calculated using the 6M approach.

qRT-PCR. *AGT, ANPEP, ATP6AP2, CPA3, CTSA and IGF2R* genes' expression was quantified using SYBR™ Green master mix (Bio-Rad, #1725150, (USA)). PCR reactions were run under cycling conditions according to manufacturer's instructions. GAPDH was used as a reference gene in all reactions. qRT-PCR relative gene expression data was calculated using ddCT method [38].

Using qRT-PCR relative gene expression data, predicted IC₅₀ values were calculated with the formulas generated by linear regression analyses of *in silico* data using qRT-PCR based expression values as predictors. Primers used in this study are shown in Table 1. GAPDH was used as endogenous control.

Table 1. Primer sequences are for selected genes.

Gene Name	Forward Primer	Reverse Primer
AGT	GGCCAGCAGCAGATAACAACC	AACTGGGAGGTGCATTTGTGC
ANPEP	CGTTCTCTCTGCTGTGAGC	AGGCCGTTCATTGTCCATCG
ATP6AP2	GATCCTTGTTGACGCTCTGC	CTTGCTGGGTCTTCGCTTG
CPA3	TGCCCTCTGTTTGAATAAGCC	CGTGGGTCCAACTTCACCTGG
CTSA	CTCTACCGAAGCATGAATCCC	TACTTCACTAACCAGGGCCG
IGF2R_probe1	CTCCACCCAGTGAAGAACG	TGTCATGGAGGACACCCAG
IGF2R_probe2	GGTGTTCTTATCTGGGGGC	CAACCAAGCCAGCCAAACCG
GAPDH	GGAGCGAGATCCCTCCAAAT	GGCTGTTGTCATCTTCTCATGG

<https://doi.org/10.1371/journal.pone.0242497.t001>

Clinical data validation

Log Rank with Multiple Cutoffs (LRMC) and survival analysis. In regression analysis, four formulas were generated for Doxorubicin and Etoposide using both CGP and 6M IC50 data. *IGF2R*, *CTSA*, *ATP6AP2* are common in three of the four formulas except for 6M IC50 data for Etoposide. Therefore, these genes were chosen to test relationships with clinical outcome.

Clinical data were obtained from the training cohort of the GSE12417 [30] dataset (AML Cooperating Group 1999). In the AMLCG 1999 cohort, patients were treated with TAD: Thioguanine, Cytarabine and Daunorubicin, or HAM: Cytarabine, Mitoxantrone protocols followed by the TAD protocol. We used an in-house R script (<https://github.com/muratisbilen/LRMC.git>) (Log Rank Multiple Cutoff, LRMC) by which log-rank test-based p-values associated with hazard ratio (HR) could be obtained using all possible cutoff values representing each sample in a given dataset and best cutoff is selected as in [39, 40]. Using this approach, we selected best cutoffs for *IGF2R*, *ATP6AP2* and *CTSA* genes to be used for clinical correlation studies and Kaplan-Meier plots.

Patients with gene expression lower than cutoff, for each gene individually, were labeled as 'Low' (low expression) and higher than cutoff were labelled as 'High' (high expression). Univariate cox regression analyses were performed and Kaplan-Meier graphs were drawn using SPSS Statistics 19 (IBM, Chicago, IL, USA).

Additionally, the expression of all these three genes (*IGF2R*, *CTSA* and *ATP6AP2*) was evaluated together as good and bad prognostic groups. Patients were grouped as "Good" if they have high expression levels of *IGF2R* and *CTSA* and low expression levels of *ATP6AP2* defined by expression value cutoffs in previous analysis. Rest of the patients were grouped as "Bad". Then Kaplan Meier survival analysis was performed for these groups.

Results

Discovery of RAS drug sensitivity biomarker genes

The RAS consists of the 25 genes, corresponding to 39 probesets in Affymetrix HG-U133A, a microarray platforms used in the Cancer Genome Project (CGP) [27, 28]. For the 17 AML cell lines, both drug cytotoxicity and gene expression data are available in the CGP database [27, 28]. We focused only on genes which showed high variation in expression for further validation and therefore, selected nine probesets (eight genes) as described in the methods section (S1 Table). We recalculated IC50 values using the 6M approach applied to raw CGP cytotoxicity data (see Materials and methods). Using Pearson correlation we observed strong correlations between CGP IC50 and 6M IC50 for drugs widely used in AML (Cytarabine, Etoposide, Doxorubicin) but not for ATRA (S2 Table). To identify biomarkers of chemosensitivity, we

calculated Pearson correlation between gene expression and IC50 values obtained from CGP and generated by 6M approach. We thus identified six gene/drug cytotoxicity correlations which were significant with CGP IC50 values, and seven significant correlations with 6M IC50 values. Four gene/drug associations were common to both analyses (S3 Table). Linear regression analysis was then performed to test whether the combined expression analyses of genes could correlate better with drug sensitivity data or not. Thus, we generated discovery and test groups. Each group include a wide range of cell line IC50 values as possible. Linear regression models for drug sensitivity prediction were generated for the discovery group (12 cell lines) using expression data of highly variant eight genes and IC50 values obtained from CGP and 6M IC50 of four drugs in Minitab 17. Then, obtained results tested with the validation group (five cell lines). The models generated with combined expression analyses of the eight genes resulted in high R^2 (adj) values for Etoposide, Doxorubicin and Cytarabine but no model could be generated for ATRA (S4 Table). As independent datasets with drug sensitivity data for these compounds do not exist, we utilized a cross-validation method to test the robustness of the proposed models by generating the discovery and test groups 10 times, with 12 and five cell lines, respectively. The average of 10 R^2 values generated from discovery groups was calculated for both CGP and 6M IC50s. Our results showed that the 10 random models of sensitivity to Doxorubicin had an average R^2 above 85% for both CGP and 6M IC50s, but R^2 decreased slightly for models of sensitivity to Etoposide while R^2 values highly decreased for models of sensitivity to Cytarabine (S4 Table) when compared to those generated for cell lines that were manually selected. We therefore, focused on Doxorubicin and Etoposide for further analyses.

We then aimed to identify the minimal number of genes that needed to be included in combinations into the models that would give the highest correlation using the 'best subsets function' of Minitab. The software selected three genes/probesets for Doxorubicin when either CGP and 6M IC50 values were used and, four and five genes/probeset combinations for Etoposide using CGP and 6M IC50 values, respectively; all together corresponding to a total of six genes (AGT, ANPEP, ATP6AP2, CPA3, CTSA and IGF2R (two probesets)) (S5 Table), when the analysis was performed with the discovery group. Applying the resulting models to the test group showed the reliability of all models. As shown in Fig 1, the goodness of fit measures (R^2 sq. and S_y^2) were 0.9 and 0.21 for Doxorubicin as modeled using 6M IC50 data and 0.89 and 0.34 when we used CGP IC50 values. Similarly, for Etoposide, these two measures were 0.78 and 0.34 for 6M IC50 and 0.77 and 0.57 for CGP IC50 values.

In vitro validation of biomarker genes

We next asked if the linear regression models generated *in silico* could predict *in vitro* cytotoxicity. For this purpose, we determined gene expression values by qRT-PCR for the six RAS genes (AGT, ANPEP, ATP6AP2, CPA3, CTSA and IGF2R (two probesets)) and used these to predict *in vitro* IC50 values obtained for Etoposide and Doxorubicin calculated with 6M approach for nine AML cell lines (see Materials and methods section). Correlation analysis showed that *in silico* and *in vitro* gene expression data were highly concordant except for CTSA (r : >0.7 and p -value <0.05) (S6 Table). We applied normalized gene expression values obtained *in vitro* to the *in silico* generated linear regression models (using four regression formulas) (S7 Table). Thus, utilized linear regression formulas with qRT-PCR gene expression data showed a good correlation with *in silico* data for Doxorubicin but not Etoposide (Table 2).

Biological features of drug sensitive and resistant cells

Cell lines sensitive to Etoposide and Doxorubicin were almost identical (S1 Fig). To determine molecular mechanisms underlying differential response to Etoposide and Doxorubicin, we

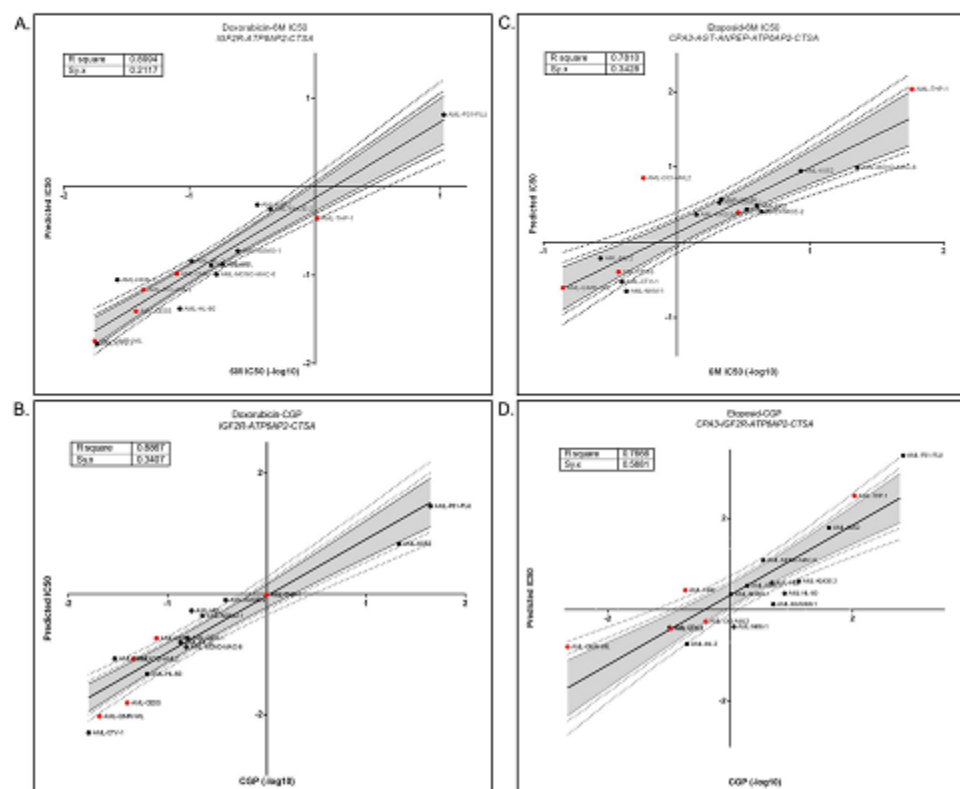


Fig 1. Reliability of Doxorubicin and Etoposide sensitivity predictions in linear regression models generated using the 12 AML cells. Linear regression models were generated using the discovery group and applied to the test group to predict sensitivity values. Reliability of sensitivity predictions was measured with goodness of fit test for Doxorubicin 6M IC50 (A) resulting 0.9 R sq. and 0.21 Sy.x Doxorubicin CGP IC50 resulting (B) 0.89 R sq. and 0.34 Sy.x Etoposide 6M IC50 resulting (C) 0.78 R sq. and 0.34 Sy.x Etoposide CGP IC50 resulting (D) 0.77 R sq. and 0.57 Sy.x with 90, 95, and 99% confidence intervals. Black dots represent cell lines used for discovery group, and red dots for the test/validation group.

<https://doi.org/10.1371/journal.pone.0242497.g001>

Table 2. Pearson's correlation analysis between *in vitro* 6M IC50 values and predicted IC50 values from CGP / 6M IC50 linear regression formulas.

Applied formulas	Pearson's r	p -value
Etoposide (CGP)	0.1271	0.7446
Etoposide (6M IC50)	-0.0579	0.8825
Doxorubicin (CGP)	0.7107	0.0319
Doxorubicin (6M IC50)	0.6925	0.0387

Predicted IC50 values obtained from linear regression formulas generated with 6M IC50 and CGP IC50 values showed high correlation with *in vitro* IC50s obtained from cytotoxicity experiments for Doxorubicin but not for Etoposide. For prediction of IC50s, normalized qRT-PCR gene expression values were used in the linear regression formulas.

<https://doi.org/10.1371/journal.pone.0242497.t002>

performed gene set enrichment analyses (GSEA) with sensitive and resistant subgroups for Gene Ontology (GO) gene sets. Several gene sets were significantly enriched among sensitive and resistant cell lines (FDR q -value < 0.25). Gene sets enriched in sensitive cells with a FDR q -value of lower than 0.25 included TNF-receptor interacting, and response to type I IFN stimulus; while gene sets such as regulation of TGF-beta production and FN-binding were enriched in resistant cells suggesting a mesenchymal phenotype (S2 Fig and S8 Table).

To determine if the differentially expressed genes could be reflecting Epithelial-Mesenchymal Transition (EMT), we compared E-Cadherin (epithelial marker) and Vimentin (mesenchymal marker) expression using t-test, between sensitive and resistant cell groups. EMT is the process that epithelial cells lose the apical-basal polarity and cell adhesion, and transform to invasive mesenchymal cells [41]. It is known to play an important role in biological and pathological processes such as cancer progression, metastasis and drug resistance [42–44]. In our analysis, E-cadherin and Vimentin expression were not significantly different between sensitive and resistant groups defined in S1 Fig ($p > 0.1$) (S3 Fig).

Then, in order to understand whether the mutational profile is involved in sensitivity to Doxorubicin, we analyzed mutational data of sensitive, intermediate and resistant groups of AML cell lines (see Materials and methods). Although we have a small sample size, especially in the resistant group ($n = 2$), we observed that both of the resistant cell lines are NRAS and P53 mutant, whereas all of the sensitive cell lines ($n = 5$) were wild type for these genes. However, these results need to be validated in larger sample sizes to be conclusive (S9 Table).

RAS genes are prognostic biomarkers for AML

We then asked if the RAS gene expression could help prognosticate AML patients. For this purpose, we utilized the training set within the GSE12417 [30] dataset, including 163 samples of bone marrow or peripheral blood mononuclear cells from adult patients with untreated acute myeloid leukemia. Patients in this cohort were also-treated with TAD protocol which contains Daunorubicin, which is also used as the starting material for semi-synthetic manufacturing of Doxorubicin. We found that high expression of genes *IGF2R* and *CTSA* were both associated with better overall survival, while the opposite was true for *ATP6AP2* when patients were classified in either "High" or "Low" groups based upon LRMC cutoffs for each gene separately (see Materials and methods section) (Fig 2). We then stratified patients into "Good" and "Bad" prognosis groups using the best cutoff values obtained for these three genes as explained in the method section. As shown in Fig 3, it was revealed that there was a striking difference in overall survival in the groups that were predicted as "Good" and "Bad". The "Good" group showed better survival than the "Bad" group. Since the patients were all treated with Daunorubicin, these data suggest that the expression pattern of these genes was able to identify patients which are responders of this therapy.

Discussion

RAS' local presence in the marrow affects the most important stages of physiological and pathological blood cell proliferation, and also has important roles in the development of blood cancers. It has been shown that RAS plays important roles in drug resistance to chemotherapeutic agent in addition to angiogenesis, invasion and proliferation [9, 24, 45–47]. Inevitably, most of these processes are interdependent. Most of the increased metastasis and invasion occurs due to an active RAS results in angiogenesis [45, 48, 49]. AT1R upregulation in ovarian cancer and increased expression of AT1R and ACE in prostate cancer, and AGTR1 in breast cancer; localized RAS presence in gastric cancer and its correlation with tumor spread and progression; demonstrate strong associations of RAS with various cancers. Irregularity of RAS components

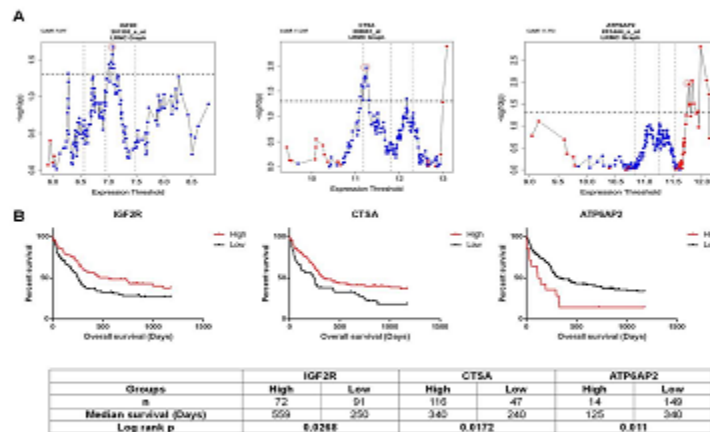


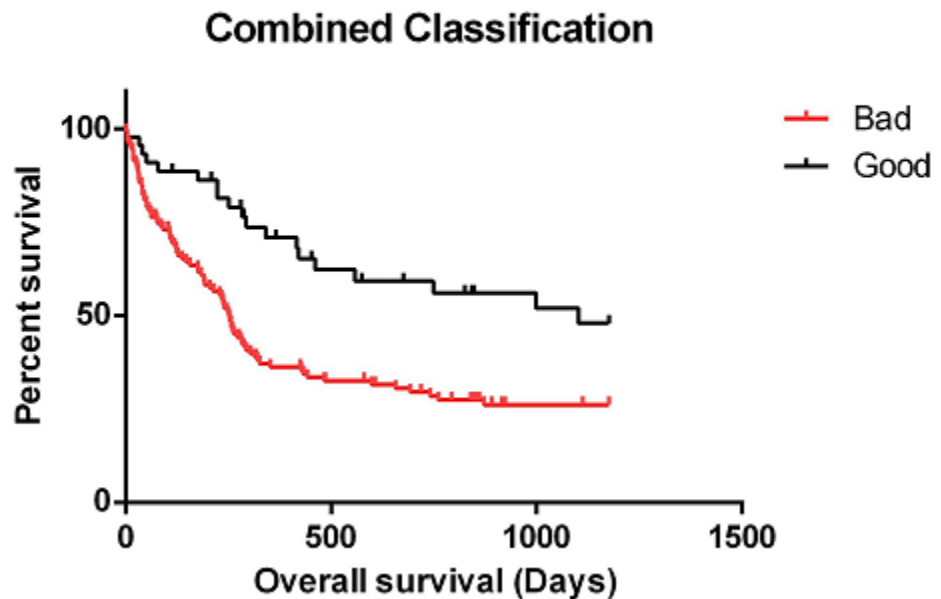
Fig 2. Log Rank Multiple Cutoff (LRMC) plots and Kaplan Meier curves for dataset GSE12417. (A) LRMCs of IGF2R (Probeset: 201392), CTSA (Probeset: 200661) and ATP6AP2 (Probeset: 201444). Graphic shows log rank based p values in the y axis for the "high" and "low" expression groups generated by all possible expression based cutoffs shown on the x axis (for details see Materials and methods). HRs above one and below one are shown with red and blue colors for specific cutoffs, respectively. Vertical dotted lines show 25th, 50th and 75th percentiles and horizontal dotted line shows significance cutoff 0.05 ($-\log_{10}(p) = 1.301$). From LRMC graphs, we selected cutoffs 7.077, 11.247 and 11.773 for IGF2R, CTSA and ATP6AP2 respectively which are highlighted in figure with red circle. Patients were divided into high and low groups based on these cutoffs. (B) Kaplan Meier plots for patients classified in high and low expression based on LRMC cutoffs. Patients classified in high expression group of IGF2R and CTSA showed better overall survival when compared with low expression group and high expression group of ATP6AP2 showed worse survival when compared with low expression group. For all survival plots, overall survival time is shown in days for 163 AML patients. Table at the bottom shows number of patients in each group, median survival for each group and Log rank p value for Kaplan Meier analysis.

<https://doi.org/10.1371/journal.pone.0242497.g002>

in cancer is strongly associated with increased angiogenesis and metastasis, and these parameters are associated with poor prognosis [50–54].

Gene expression profiling has revealed various AML subtypes related to diagnosis, therapy response and prognosis [55, 56]. Although gene expression profiling has not yet been integrated into clinical practice, this is expected to happen in near future.

In our study, we focused on RAS genes and identified their association with Doxorubicin and Etoposide sensitivity. We also show that RAS genes can be used to stratify AML patients into groups with distinct prognoses. Similar to our findings, low expression of IGF2R in non-small cell liver cancer has been associated with poor prognosis and high expression in bladder cancer has been associated with good prognosis [57, 58]. Although high CTSA expression was associated with poorer outcome in breast ductal carcinoma *in situ*, it was also found to suppress invasion and metastasis of colorectal cancer, suggesting tissue-specific differential roles [59, 60]. Recent studies linked ATP6AP2 up-regulation to the progression of glioma and colorectal cancer, due to its roles in aberrant activation of the Wnt/beta-catenin signalling pathway [61, 62]. ATP6AP2 was also shown to be a key component of the pro-angiogenic/proliferative arm of the RAS, which plays a role in the growth and spread of endometrial cancer [63]. Compared to the presence in the lysosome, it is found more in the cell membrane. Thus, it is clear that in this way it induces TGF-beta pathway activation. IGF2R is located in the membrane of organelles and is responsible for the transport to lysosome, and its intracellular functions have



Combined Classification		
Groups	Bad	Good
n	119	44
Median survival (Days)	252	1102
Log rank p	0.001	

Fig 3. Combined classification using IGF2R, CTSA and ATP6AP2 expression. Patients were grouped as "Good" if they have high expression levels of IGF2R and CTSA and low expression levels of ATP6AP2 defined by expression value cutoffs in Fig 2. Rest of the patients were grouped as "Bad". Kaplan Meier plot shows "Good" group showed better survival when compared with "Bad" group as expected. Table at the bottom shows number of patients in each group, median survival for each group and Log rank p value for Kaplan Meier analysis.

<https://doi.org/10.1371/journal.pone.0242497.g003>

not yet been clearly identified. CTSA is a protease found in the lysosome. The fact that these three genes function together in the lysosome suggests that lysosomal functions can contribute to cell sensitivity. ATP6AP2 gene was found to cause disruption of V-ATPase formation and defects in the lysosomal glycosylation and autophagy [64]. Supportively, Doxorubicin has been reported to cause autophagy induced cell death in AML cells [65, 66].

GSEA revealed that sensitive cells were correlated with TNF-receptor interacting and response to type I IFN gene sets and resistant cells were correlated with regulation of TGF-beta production and FN-binding gene sets in AML, suggesting a mesenchymal phenotype.

A good and reliable subgrouping which can predict Doxorubicin sensitivity in AML was performed with the *ATP6AP2*, *IGF2R*, and *CTSA* gene combination. For those analyses, we utilized a Daunorubicin treated cohort, which is used as the starting material for semi-synthetic manufacturing of Doxorubicin. Therefore, the combination of these genes which can predict the sensitivity of Doxorubicin in AML patients may, therefore, be confirmed *ex vivo*.

The mutational analyses performed in this study had a small sample size with only two resistant cells. Therefore more conclusive results would be reached when this type of analysis is performed with larger sample sizes, or when mutational profiling is performed in patients treated with Doxorubicin, which may shed light on future studies.

Conclusions

As a result, we identified *IGF2R*, *CTSA* and *ATP6AP2* gene biomarkers, which can subgroup AML patients into distinct good and bad prognostic groups. *ATP6AP2* was associated to resistance and *IGF2R* and *CTSA* were associated to sensitivity for Doxorubicin *in silico* and *in vitro*. In future studies, it is important to investigate whether these genes can be used for personalized treatment and improve the effectiveness of treatments.

Supporting information

S1 Fig. Hierarchical clustering of AML cell lines by sensitivity profiles for Doxorubicin and Etoposide. The analysis reveals sensitive (six cell lines-green), intermediate (eight cell lines-orange) and resistant (three cell lines-red) subgroups for the 17 AML cell lines. Sensitivity to Doxorubicin and Etoposide is highly concordant in three subgroups. Green indicate low expression, orange indicate intermediate expression and red indicates high expression. (TIF)

S2 Fig. Comparative analysis of differentially enriched gene sets among drug sensitive and resistant cell lines. (A) Plots showing gene sets enriched in sensitive cells, including genes interacting with TNF-receptor and genes affected in response to type I IFN stimulus. (B) Plots showing gene sets enriched in resistant cell lines, including genes having role in regulation of TGF- β production and genes interacting selectively and non-covalently with Fibronectin. (TIF)

S3 Fig. Expression levels of E-cadherin and Vimentin genes in AML cell lines. RMA normalized gene expression values of CGP microarray data (y-axis) were used to determine EMT status of sensitive and resistant AML cell lines (x-axis) defined in [S1 Fig](#). VIM: Vimentin (black bars), CDH1: E-cadherin (white bars). n.s. (not significant). (TIF)

S1 Table. Expression variance of RAS genes in AML cell lines. RMA normalized gene expression values of 25 RAS genes were used to analyze variance, standard deviation (SD), mean and min-max difference among cell lines. Gene names is shown along with probe set ID. (XLSX)

S2 Table. Pearson correlation analysis between CGP IC50s and 6M IC50s. IC50 values recalculated according to 6M approach using CGP raw cytotoxicity measurements were used

to calculate Pearson correlation analysis with CGP IC50 values. Strong correlations are observed for all drugs except for ATRA.

(PDF)

S3 Table. Pearson correlation analysis between CGP gene expression data and CGP / 6M IC50s for 17 AML cell lines. Eight genes expression data (nine probesets) was used to calculate correlation (as R^2 coefficient of determination) with IC50 values of four drugs (ATRA, Cytarabine, Etoposide, Doxorubicin) from CGP database along with recalculated data with 6M approach. Highlighted values with green and red indicate significant correlation in negative and positive manner respectively.

(PDF)

S4 Table. Linear regression analysis between expression values of nine probesets and CGP / 6M IC50s for the discovery group (12 cell lines) and also for the ten times randomly divided different discovery groups (12 cell lines). Adjusted R^2 values were calculated in Minitab 17 with eight genes (nine probesets) for four drugs using CGP gene expression data and CGP / 6M IC50 values of the 12 AML cell lines. High correlations are observed with Etoposide and Doxorubicin (bold, for Etoposide $R^2 > 90\%$, for Doxorubicin $R^2 > 80\%$). Averages of adjusted R^2 values of ten randomly divided groups were calculated in Minitab 17 with eight genes (nine probesets) for four drugs using CGP gene expression data and CGP / 6M IC50 values of the 12 AML cell lines. High correlation is observed with Doxorubicin and fine with Etoposide (bold, for Doxorubicin $R^2 > 85\%$, for Etoposide $R^2 > 60\%$). Asterisk represents the analysis in which Minitab could not perform linear regression analysis.

(PDF)

S5 Table. Generation of linear regression models using CGP gene expression data and CGP / 6M IC50 data of the discovery group (12 AML cell lines) for drug sensitivity predictions. (A) Individual genes and gene combinations were used to generate linear regression models using IC50 values of Doxorubicin and Etoposide from CGP and 6M IC50. Highest correlation is observed in *IGF2R/ATP6AP2/CTSA* combination with Doxorubicin CGP and 6M IC50 values. And, highest correlation is observed in *IGF2R/ATP6AP2/CTSA/CPA3* combination with Etoposide CGP and in *ANPEP/ATP6AP2/CTSA/CPA3/AGT* combination with Etoposide 6M IC50 values. (B) Regression formulas for gene panels with highest correlations.

(PDF)

S6 Table. Relative expression values of *ATP6AP2*, *IGF2R* (two probesets), *CTSA*, *CPA3*, *AGT* and *ANPEP* genes in nine AML cell lines and Pearson's correlation analysis for six genes between CGP gene expression data and *in vitro* qRT-PCR gene expression data of nine AML cell lines. (A) Expressions of all genes was normalized to GAPDH expression. (B) *ATP6AP2*, *IGF2R* (two probesets), *CPA3*, *AGT*, and *ANPEP* gene expression data obtained from CGP *in silico* and *in vitro* qRT-PCR expression data from nine cell lines show significant correlations with *in vitro* qRT-PCR expression data with the exception of *CTSA*.

(PDF)

S7 Table. *In vitro* and predicted IC50s (from CGP and 6M IC50 linear regression formulas) of Doxorubicin and Etoposide for nine AML cell lines. *In vitro* IC50 values were obtained from cell viability measurements of the cell lines that are treated with six different concentrations of Doxorubicin and Etoposide separately (20, 10, 2, 1, 0.2, 0.1 μ M). Predicted IC50s were calculated using the four formulas generated (with CGP / 6M IC50s) in the linear regression analysis with the normalized gene expression data obtained from qRT-PCR.

(PDF)

S8 Table. Gene sets enriched in (A) sensitive cell lines, and (B) resistant cell lines.
(PDF)

S9 Table. Mutational data of sensitive, intermediate and resistant groups of AML cell lines. Seven genes which are mutated in at least three AML cell lines were included to examine the relationship between mutational status and drug sensitivity. Red: mutations that cause change in aminoacid sequence, grey: unknown status of aminoacid change, change at the DNA level; blue: wild type.
(PDF)

S1 File.
(XLSX)

Author Contributions

Data curation: Seyhan Turk, Muhammad Waqas Akbar, Murat Isbilen, Secil Demirkol Canli.

Formal analysis: Seyhan Turk, Can Turk, Ali Osmay Gure.

Investigation: Seyhan Turk, Can Turk.

Methodology: Seyhan Turk, Can Turk, Muhammad Waqas Akbar, Murat Isbilen, Secil Demirkol Canli, Umit Yavuz Malkan, Mufide Okay, Gulberk Ucar, Nilgun Sayinalp, Ibrahim Celalettin Haznedaroglu, Ali Osmay Gure.

Project administration: Seyhan Turk, Ali Osmay Gure.

Resources: Seyhan Turk, Umit Yavuz Malkan, Mufide Okay.

Software: Seyhan Turk, Can Turk, Muhammad Waqas Akbar, Murat Isbilen, Secil Demirkol Canli, Ali Osmay Gure.

Supervision: Seyhan Turk, Gulberk Ucar, Nilgun Sayinalp, Ibrahim Celalettin Haznedaroglu.

Validation: Seyhan Turk, Gulberk Ucar, Nilgun Sayinalp, Ibrahim Celalettin Haznedaroglu, Ali Osmay Gure.

Visualization: Seyhan Turk, Gulberk Ucar, Ali Osmay Gure.

Writing – original draft: Seyhan Turk, Baris Kucukkaraduman, Ali Osmay Gure.

Writing – review & editing: Seyhan Turk, Can Turk, Baris Kucukkaraduman, Ali Osmay Gure.

References

1. Stein GS, Luebbers KP. Cancer: Prevention, Early Detection, Treatment and Recovery. John Wiley & Sons; 2019.
2. Chen SJ, Shen Y, Chen Z. A panoramic view of acute myeloid leukemia. Nat Genet. 2013; 45(6):586–7. <https://doi.org/10.1038/ng-2651> PMID: 23715324
3. De Kouchkovsky I, Abdul-Hay M. 'Acute myeloid leukemia: a comprehensive review and 2016 update'. Blood Cancer J. 2016; 6(7):e441. <https://doi.org/10.1038/bcj.2016.50> PMID: 27367478
4. Chu E. Physicians' cancer chemotherapy drug manual 2018. [S.L.]: Jones & Bartlett Learning; 2017.
5. Chiong JR, Aronow WS, Khan IA, Nair CK, Vijayaraghavan K, Dart RA, et al. Secondary hypertension: current diagnosis and treatment. Int J Cardiol. 2008; 124(1):6–21. <https://doi.org/10.1016/j.ijcard.2007.01.119> PMID: 17462751
6. Bader M. Tissue renin-angiotensin-aldosterone systems: Targets for pharmacological therapy. Annu Rev Pharmacol Toxicol. 2010; 50:439–65. <https://doi.org/10.1146/annurev.pharmtox.010909.105610> PMID: 20055710

7. Kuba K, Imai Y, Penninger JM. Angiotensin-converting enzyme 2 in lung diseases. *Curr Opin Pharmacol*. 2006; 6(3):271–6. <https://doi.org/10.1016/j.coph.2006.03.001> PMID: 16581295
8. Campbell DJ. Clinical relevance of local Renin Angiotensin systems. *Front Endocrinol (Lausanne)*. 2014; 5:113. <https://doi.org/10.3389/fendo.2014.00113> PMID: 25071727
9. Haznedaroglu IC, Tuncer S, Gursay M. A local renin-angiotensin system in the bone marrow. *Med Hypotheses*. 1996; 46(6):507–10. [https://doi.org/10.1016/s0306-9877\(96\)0122-x](https://doi.org/10.1016/s0306-9877(96)0122-x) PMID: 8803932
10. Strawn WB, Richmond RS, Ann Tallant E, Gallagher PE, Ferrario CM. Renin-angiotensin system expression in rat bone marrow haematopoietic and stromal cells. *Br J Haematol*. 2004; 126(1):120–6. <https://doi.org/10.1111/j.1365-2141.2004.04998.x> PMID: 15198742
11. Haznedaroglu IC, Ozturk MA. Towards the understanding of the local hematopoietic bone marrow renin-angiotensin system. *Int J Biochem Cell Biol*. 2003; 35(6):867–80. [https://doi.org/10.1016/s1357-2725\(02\)00278-9](https://doi.org/10.1016/s1357-2725(02)00278-9) PMID: 12676173
12. Haznedaroglu IC, Beyazit Y. Local bone marrow renin-angiotensin system in primitive, definitive and neoplastic haematopoiesis. *Clin Sci (Lond)*. 2013; 124(5):307–23. <https://doi.org/10.1042/CS20120300> PMID: 23157407
13. Hara M O K, Wada H, Sasayama S, Matsumori A. Preformed angiotensin II is present in human mast cells. *Cardiovasc Drugs Ther* 2004; 18. <https://doi.org/10.1007/s10557-004-6218-y> PMID: 15770428
14. Hubert C, Savary K, Gasc JM, Corvol P. The hematopoietic system: a new niche for the renin-angiotensin system. *Nat Clin Pract Cardiovasc Med*. 2006; 3(2):80–5. <https://doi.org/10.1038/nccp.0449> PMID: 16446776
15. Kato H, Ishida J, Imagawa S, Saito T, Suzuki N, Matsuoka T, et al. Enhanced erythropoiesis mediated by activation of the renin-angiotensin system via angiotensin II type 1a receptor. *FASEB J*. 2005; 19(14):2023–5. <https://doi.org/10.1096/fj.05-3820je> PMID: 16223784
16. Kwiatkowski BA R R. Angiotensin II Receptor-Associated Protein (AGTRAP) Synergizes with Mpl Signaling to Promote Survival and to Increase Proliferation Rate of Hematopoietic Cells. *ASH Annual Meeting Abstracts* 2009; 114:3606.
17. Lin C, Datta V, Okwan-Duodu D, Chen X, Fuchs S, Alsabeh R, et al. Angiotensin-converting enzyme is required for normal myelopoiesis. *FASEB J*. 2011; 25(4):1145–55. <https://doi.org/10.1096/fj.10-169433> PMID: 21148418
18. Park TS, Zambidis ET. A role for the renin-angiotensin system in hematopoiesis. *Haematologica*. 2009; 94(6):745–7. <https://doi.org/10.3324/haematol.2009.006965> PMID: 19483149
19. Shen XZ, Bernstein KE. The peptide network regulated by angiotensin converting enzyme (ACE) in hematopoiesis. *Cell Cycle*. 2011; 10(9):1363–9. <https://doi.org/10.4161/cc.10.9.15444> PMID: 21441775
20. Jokubaitis VJ, Sinka L, Driessen R, Whitty G, Haylock DN, Bertoncello I, et al. Angiotensin-converting enzyme (CD143) marks hematopoietic stem cells in human embryonic, fetal, and adult hematopoietic tissues. *Blood*. 2008; 111(8):4055–63. <https://doi.org/10.1182/blood-2007-05-091710> PMID: 17993616
21. Sinka L, Blasch K, Khazaal I, Peault B, Tavian M. Angiotensin-converting enzyme (CD143) specifies emerging lympho-hematopoietic progenitors in the human embryo. *Blood*. 2012; 119(16):3712–23. <https://doi.org/10.1182/blood-2010-11-314781> PMID: 22282502
22. Tavian M, Blasch K, Sinka L, Vallet J, Peault B. Embryonic origin of human hematopoiesis. *Int J Dev Biol*. 2010; 54(6–7):1061–5. <https://doi.org/10.1387/jdb.103097mt> PMID: 20711983
23. Zambidis ET, Park TS, Yu W, Tam A, Levine M, Yuan X, et al. Expression of angiotensin-converting enzyme (CD143) identifies and regulates primitive hemangioblasts derived from human pluripotent stem cells. *Blood*. 2008; 112(9):3601–14. <https://doi.org/10.1182/blood-2008-03-144766> PMID: 18728246
24. Aksu S, Beyazit Y, Haznedaroglu IC, Canpinar H, Kekilli M, Uner A, et al. Over-expression of angiotensin-converting enzyme (CD 143) on leukemic blasts as a clue for the activated local bone marrow RAS in AML. *Leuk Lymphoma*. 2006; 47(5):891–6. <https://doi.org/10.1080/10428190500399250> PMID: 16753875
25. Beyazit Y, Aksu S, Haznedaroglu IC, Kekilli M, Misiriloglu M, Tuncer S, et al. Overexpression of the local bone marrow renin-angiotensin system in acute myeloid leukemia. *J Natl Med Assoc*. 2007; 99(1):57–63. PMID: 17304969
26. Wulf GG, Jahns-Streubel G, Nobling R, Strutz F, Hemmerlein B, Hiddemann W, et al. Renin in acute myeloid leukaemia blasts. *Br J Haematol*. 1998; 100(2):335–7. <https://doi.org/10.1046/j.1365-2141.1998.00565.x> PMID: 9488623
27. Garnett MJ, Edelman EJ, Heidom SJ, Greenman CD, Dastur A, Lau KW, et al. Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature*. 2012; 483(7391):570–5. <https://doi.org/10.1038/nature11005> PMID: 22460902

28. Vankova L, Aliper A, Suntsova M, Kholodenko R, Shepelin D, Borisov N, et al. Combinatorial high-throughput experimental and bioinformatic approach identifies molecular pathways linked with the sensitivity to anticancer target drugs. *Oncotarget*. 2015; 6(29):27227–38. <https://doi.org/10.18632/oncotarget.4507> PMID: 26317900
29. Iorio F, Knijnenburg TA, Vis DJ, Bignell GR, Menden MP, Schubert M, et al. A Landscape of Pharmacogenomic Interactions in Cancer. *Cell*. 2016; 166(3):740–54. <https://doi.org/10.1016/j.cell.2016.06.017> PMID: 27397505
30. Metzeler KH, Hummel M, Bloomfield CD, Spiekermann K, Braess J, Sauerland M-C, et al. An 86-probe gene-expression signature predicts survival in cytogenetically normal acute myeloid leukemia. *Blood*. 2008; 112(10):4193–201. <https://doi.org/10.1182/blood-2008-02-134411> PMID: 18716133
31. Simon RM. Design and analysis of DNA microarray investigations. New York: London: Springer; 2011.
32. DeLean A, Munson PJ, Rodbard D. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *The American journal of physiology*. 1978; 235(2):E97–102. <https://doi.org/10.1152/ajpendo.1978.235.2.E97> PMID: 686171
33. Beck B, Chen YF, Dere W, Devanarayan V, Eastwood BJ, Farnen MW, et al. Assay Operations for SAR Support. In: Sittampalam GS, Grossman A, Brimacombe K, Arkin M, Auld D, Austin CP, et al., editors. *Assay Guidance Manual*. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004.
34. de Hoon MJ, Imoto S, Nolan J, Miyano S. Open source clustering software. *Bioinformatics* (Oxford, England). 2004; 20(9):1453–4. <https://doi.org/10.1093/bioinformatics/bth078> PMID: 14871861
35. Saldanha AJ. Java Treeview—extensible visualization of microarray data. *Bioinformatics* (Oxford, England). 2004; 20(17):3246–8. <https://doi.org/10.1093/bioinformatics/bth349> PMID: 15180930
36. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. 2005; 102(43):15545–50.
37. Yang W, Soares J, Greninger P, Edelman EJ, Lightfoot H, Forbes S, et al. Genomics of Drug Sensitivity in Cancer (GDSC): a resource for therapeutic biomarker discovery in cancer cells. *Nucleic acids research*. 2013; 41(Database issue):D955–61. <https://doi.org/10.1093/nar/gks1111> PMID: 23180760
38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25(4):402–8. <https://doi.org/10.1006/meth.2001.1262> PMID: 11846609
39. Akbar MW, Isbilen M, Beldeir N, Carli SD, Kucukkaraduman B, Turk C, et al. A Stemness and EMT Based Gene Expression Signature Identifies Phenotypic Plasticity and is a Predictive but Not Prognostic Biomarker for Breast Cancer. *Journal of Cancer*. 2020; 11(4):949–61. <https://doi.org/10.7150/jca.34648> PMID: 31949488
40. Demirkol S, Gormcal I, Isbilen M, Dayanc BE, Tez M, Bostanci EB, et al. A Combined ULBP2 and SEMA5A Expression Signature as a Prognostic and Predictive Biomarker for Colon Cancer. *Journal of Cancer*. 2017; 8(7):1113–22. <https://doi.org/10.7150/jca.17872> PMID: 28607584
41. Du B, Shim JS. Targeting Epithelial-Mesenchymal Transition (EMT) to Overcome Drug Resistance in Cancer. *Molecules* (Basel, Switzerland). 2016; 21(7). <https://doi.org/10.3390/molecules21070965> PMID: 27455225
42. Hay ED. An overview of epithelial-mesenchymal transformation. *Acta anatomica*. 1995; 154(1):8–20. <https://doi.org/10.1159/000147748> PMID: 8714286
43. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation*. 2009; 119(6):1420–8. <https://doi.org/10.1172/JCI39104> PMID: 19487818
44. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Current opinion in cell biology*. 2003; 15(6):740–6. [https://doi.org/10.1016/s0950-2688\(03\)00066-0](https://doi.org/10.1016/s0950-2688(03)00066-0) PMID: 14644200
45. Ager EI, Neo J, Christophi C. The renin-angiotensin system and malignancy. *Carcinogenesis*. 2008; 29(9):1675–84. <https://doi.org/10.1093/carcin/bgn171> PMID: 18632755
46. Teresa Gomez Casares M, de la Iglesia S, Perera M, Lemes A, Campo C, Gonzalez San Miguel JD, et al. Renin expression in hematological malignancies and its role in the regulation of hematopoiesis. *Leuk Lymphoma*. 2002; 43(12):2377–81. <https://doi.org/10.1080/1042819021000040080> PMID: 12613527
47. Tawinwung S, Ninsontia C, Chanvorachote P. Angiotensin II Increases Cancer Stem Cell-Like Phenotype in Lung Cancer Cells. *Anticancer Res*. 2015; 35(9):4789–97. PMID: 26254369
48. Egami K, Murohara T, Shimada T, Sasaki K, Shintani S, Sugaya T, et al. Role of host angiotensin II type 1 receptor in tumor angiogenesis and growth. *The Journal of clinical investigation*. 2003; 112(1):67–75. <https://doi.org/10.1172/JCI16645> PMID: 12840060

49. Fujita M, Hayashi I, Yamashina S, Fukamizu A, Itoman M, Majima M. Angiotensin type 1a receptor signaling-dependent induction of vascular endothelial growth factor in stroma is relevant to tumor-associated angiogenesis and tumor growth. *Carcinogenesis*. 2005; 26(2):271–9. <https://doi.org/10.1093/carcin/bgh324> PMID: 15637093
50. Ito K, Shibata K, Kajiyama H, Yamamoto E, Nagasaka T, Nawa A, et al. Angiotensin II type 1 receptor expression in ovarian cancer and its correlation with tumour angiogenesis and patient survival. *Br J Cancer*. 2006; 94(4):552–60. <https://doi.org/10.1038/sj.bjc.6602961> PMID: 16434950
51. Kinoshita J, Fushida S, Harada S, Yagi Y, Fujita H, Kinami S, et al. Local angiotensin II-generation in human gastric cancer: correlation with tumor progression through the activation of ERK1/2, NF-kappaB and survivin. *Int J Oncol*. 2009; 34(6):1573–82. <https://doi.org/10.3892/ijo.00000287> PMID: 19424575
52. Rhodes DR, Ateeq B, Cao Q, Tomlins SA, Mehra R, Laxman B, et al. AGTR1 overexpression defines a subset of breast cancer and confers sensitivity to losartan, an AGTR1 antagonist. *Proc Natl Acad Sci U S A*. 2009; 106(25):10294–9. <https://doi.org/10.1073/pnas.0900351106> PMID: 19487683
53. Rocken C, Lendeckel U, Dierkes J, Westphal S, Carl-McGrath S, Peters B, et al. The number of lymph node metastases in gastric cancer correlates with the angiotensin I-converting enzyme gene insertion/deletion polymorphism. *Clin Cancer Res*. 2005; 11(7):2526–30. <https://doi.org/10.1158/1078-0432.CCR-04-1922> PMID: 15814629
54. Uemura H, Hasumi H, Ishiguro H, Teranishi J, Miyoshi Y, Kubota Y. Renin-angiotensin system is an important factor in hormone refractory prostate cancer. *Prostate*. 2006; 66(8):822–30. <https://doi.org/10.1002/pros.20407> PMID: 16482568
55. Marcucci G, Haferlach T, Döhner H. Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. *J Clin Oncol*. 2011; 29(5):475–86. <https://doi.org/10.1200/JCO.2010.30.2554> PMID: 21220609
56. Haferlach T, Schmidts I. The power and potential of integrated diagnostics in acute myeloid leukaemia. *Br J Haematol*. 2020; 188(1):36–48. <https://doi.org/10.1111/bjh.16360> PMID: 31808952
57. Tian Z, Yao G, Song H, Zhou Y, Geng J. IGF2R expression is associated with the chemotherapy response and prognosis of patients with advanced NSCLC. *Cell Physiol Biochem*. 2014; 34(5):1578–88. <https://doi.org/10.1159/000366361> PMID: 25402559
58. Wu H, Li Y, Cui H, Wu W, Yang H, Yang X, et al. IGF2/IGF2R expression in urothelial bladder cancer and its implications for tumor recurrence and prognosis. *International Journal of Clinical and Experimental Medicine*. 2017; 10:881–8.
59. Ni S, Weng W, Xu M, Wang Q, Tan C, Sun H, et al. miR-106b-5p inhibits the invasion and metastasis of colorectal cancer by targeting CTSA. *Onco Targets Ther*. 2018; 11:3835–45. <https://doi.org/10.2147/OTT.S172887> PMID: 30013364
60. Toss MS, Miligy IM, Haj-Ahmad R, Gorringer KL, AlKawaz A, Mittal K, et al. The prognostic significance of lysosomal protective protein (cathepsin A) in breast ductal carcinoma in situ. *Histopathology*. 2019; 74(7):1025–35. <https://doi.org/10.1111/his.13835> PMID: 30725481
61. Kouchi M, Shibayama Y, Ogawa D, Miyake K, Nishiyama A, Tamiya T. (Pro)renin receptor is crucial for glioma development via the Wnt/beta-catenin signaling pathway. *J Neurosurg*. 2017; 127(4):819–28. <https://doi.org/10.3171/2016.9.JNS16431> PMID: 28059652
62. Wang J, Shibayama Y, Zhang A, Ohsaki H, Asano E, Suzuki Y, et al. (Pro)renin receptor promotes colorectal cancer through the Wnt/beta-catenin signalling pathway despite constitutive pathway component mutations. *Br J Cancer*. 2019; 120(2):229–37. <https://doi.org/10.1038/s41416-018-0350-0> PMID: 30555158
63. Defforce SJ, Lumbers ER, Corbisier de Meaultsart C, Wang Y, Proietto A, Otton G, et al. Expression of renin-angiotensin system (RAS) components in endometrial cancer. *Endocr Connect*. 2017; 6(1):9–19. <https://doi.org/10.1530/EC-16-0082> PMID: 27956412
64. Rujano MA, Cannata Serio M, Panasyuk G, Peanne R, Reunert J, Rymen D, et al. Mutations in the X-linked ATP6AP2 cause a glycosylation disorder with autophagic defects. *J Exp Med*. 2017; 214(12):3707–29. <https://doi.org/10.1084/jem.20170453> PMID: 29127204
65. Goussetis DJ, Altman JK, Glaser H, McNeer JL, Tallman MS, Platanius LC. Autophagy is a critical mechanism for the induction of the antileukemic effects of arsenic trioxide. *J Biol Chem*. 2010; 285(39):29989–97. <https://doi.org/10.1074/jbc.M109.090530> PMID: 20656687
66. Ristic B, Bosnjak M, Arslan K, Mircio A, Suzin-Zivkovic V, Bogdanovic A, et al. Idarubicin induces mTOR-dependent cytotoxic autophagy in leukemic cells. *Exp Cell Res*. 2014; 326(1):90–102. <https://doi.org/10.1016/j.yexcr.2014.05.021> PMID: 24907655