VALIDATION OF PROGNOSTIC BIOMARKERS FOR OVERALL SURVIVAL IN MELANOMA PATIENTS UNDERGOING AUTOLOGOUS VACCINATION

A THESIS

SUBMITTED TO THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE OF BILKENT UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

BY

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APRIL, 2014

I certify that I have read this thesis and that in my opinion it is fully adequate,

in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

Validation of Prognostic Biomarkers for Overall Survival in Melanoma Patients Undergoing Autologous Vaccination

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M.Sc.in Molecular Biology and Genetics

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April 2014

Malignant Melanoma is deadliest type of skin cancer, seen with low frequency. Lack of diagnostic and prognostic markers are important obstacles during early detection and selection of appropriate adjuvant therapy for malignant melanoma patients. Upon early diagnosis of melanoma in patients with stage IA and IB, the survival rates for 5 to 10 years is above 85% based on American Cancer Society statistics. However, with increasing stage and low survival rates, selection of appropriate post surgical adjuvant therapy has high impact to increase the survival and life quality of patients. Immunotherapy trials targeting malignant melanoma (MM) patients have been developing in larger extents in the last 2 decades, considering melanoma is the most immugenic cancer type among all cancers. Based on these facts, we decided to search for prognostic biomarkers which will guide clinicians to define appropriate malignant melanoma patients to apply autologous vaccination as a post surgical adjuvant therapy. With the contribution of our collaborator research group, we were able to obtain 28 primary cell cultures of MM patients with known survival outcomes, and their gene expression profiles in Luminex platform. Grounding from the results of Unsupervised Survival Analysis Tool (USAT; a software designed to determine prognosis-associated mRNAs, developed in our laboratory), we decided to validate the expression profiles of prospective prognostic biomarker genes in the primary cell culture samples, with qRT-PCR.

Subsequent to elimination of samples determined as being altered in culture over time, and thus no longer representing their expression profile, based on Sum of Absolute Rank Difference (SARD) analysis, 8 genes validated out of 10 in 21 samples were as follows; CT genes (SSX1, MAGEA1), EMT markers (CDX2, CLDN1), DNA repair genes (ERCC1, LIG3), transforming growth factor alpha (TGFα), A-kinase anchor protein 13 (AKAP13). Previous studies of our laboratory, concerning high immunogenic potential of CT genes in cancers, made SSX1 and MAGEA1 our major interest in autologous vaccinated MM patients. Our findings suggest that autologous vaccination might increase overall survival especially in malignant melanoma patients whose tumors show higher CT gene expression. We show that high expression of TGFα can also be used as a prognostic marker for identifying patients who will benefit from this treatment.

Keywords: Malignant Melanoma, Autologous Vaccination, Cancer Testis Genes, TGFα.

ÖZET

Otolog Aşı yapılmış Malign Melanoma hastalarında prognoz ile ilgili belirteç onaylaması

Zeynep Özge AYYILDIZ

Moleküler Biyoloji ve Genetik Yüksek Lisansı

Tez Yöneticisi: Yrd. Doç.Dr. Ali OSMAY GÜRE

Nisan 2014

Malign Melanoma, nadir görülen en ölümcül deri kanseri türüdür. Malign melanoma hastalarının tanı ve tedavisi için belirteçlerin eksikliği, hastaların erken teşhisi ve hastalar için uygun destekleyici tedavi yöntemlerinin seçilmesinin önünde önemli bir engeldir. Melanomanın erken teşhis edilmesi takdirde IA ve IB evresindeki hastaların 5-10 yıllık hayatta kalma oranları Amerika Kanser Topluğunun istatistiklerine gore %85'in üzerindedir. Bunun yanı sıra, ilerleyen evre ve düşen hayatta kalma oranları ile birlikte, cerrahi müdahele sonrası uygun destekleyici tedavinin seçiminin hastaların hayatta kalma oranlarına ve hayat kalitelerin artırılmasına etkisi yüksektir. Melanomanın kanser türleri arasında en immunojenik kanser türü olması sebebiyle, malign melanoma hastalarını hedef alan immunoterapi denemeleri son 20 yılda daha geniş kapsamlı olarak geliştirilmektedir. Bunlara dayanarak, klinisyenlerin cerrahi müdahele sonrası otolog aşı yapılması için uygun malign melanom hastalarını belirlemelerinde kullanabilecekleri prognoz ile ilişkili belirteç araştırmaya karar verdik. İş birliği içinde olduğumuz araştırma grubunun katkısıyla, 28 malign melanom hastası primer hücre kültürü ve Luminex platformunda gen ekspresyon profilerini temin ettik. Güdümsüz Sağkalım Analiz Programı (USAT: laboratuarımızda geliştirilmiş sağ kalımla ilişkili genleri tespite yarayan bir yazılım) sonuçları temelinde, prognozla ilişkisi muhtemel genlerin gen ifade profilerini primer hücre kültürlerinde, qRT-PCR ile onamaya karar verdik.

Hücre kültürü sırasında değişen ve dolaysıyla gen ifadeleri Luminex verileriyle örtüşmeyecek hale gelmiş hücre kulturlerinin Mutlak Sıra Farkı Toplamı (SARD) analizine dayanarak elenmesinden sonra, 21 örnekte çalıştığımız 10 genin doğrulanabilen 8'i; CT genleri (SSX1, MAGEA1), epitelyal mezenkimal belirteç genleri (CDX2, CLDN1), DNA onarım genleri (ERCC1, LIG3), transforme edici büyüme fakötürü (TGFα), kinaz A taşıyıcı proteini (AKAP13) idi. Laboratuarlarımızdaki CT genlerinin kanserlerdeki yüksek immunojenik potensiyellerine ait geçmiş çalışmalar, SSX1 ve MAGEA1 genlerini otolog aşılanmış malign melanoma hastalarında esas ilgimiz haline getirdi. Sonuçlarımız CT gen ifadesi yüksek olan malign melanom hastalarına otolog aşı yapılmasının genel sağkalımı artırmada faydalı olduğunu düşündürtüyor. Buna ek olarak, yüksek TGFα ifadesinin de otolog aşılamaya tabi tutulacak hastalarda prognozla ilişkili belirteç olduğu anlaşılmaktadır.

Anahtar kelimeler: Malign Melanom, Otolog Aşılama, Kanser testis genleri, TGFα.

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during the day, made me feel safe and loved. It's been my privilege to know and have you in my life as valuable friends.

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To my mother and sister for their endless

support and love

and

my father whom I lost in 2008…

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1.1 Histology and Anatomy of the Skin

Skin with total 16% of body weight and 1.8 m^2 surface areas, is the largest organ of the body. Water and electrolyte balance, protections against UV, microorganism, mechanical insults or toxic agents are maintained by skin. Skin consists of three structural layers; (1) epidermis, (2) dermis, (3) subcutis. Structure of the skin (Figure 1.1) is constant though, thickness of it may vary depending on the location.

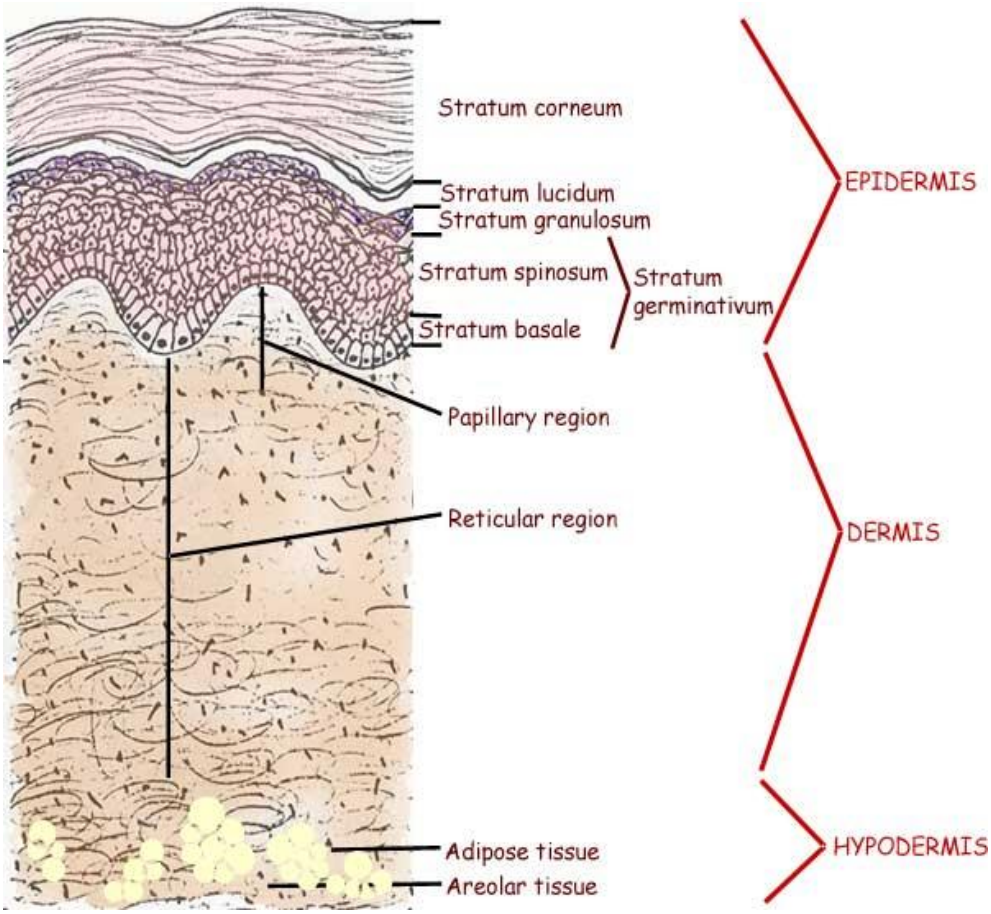


Figure 1.1 General structure of the skin (microscopic cross section)

Besides keratinocytes, which are the main cell type in epidermis, melanocytes function to (1) form a connection between neighbouring keratinocytes in stratum basale, (2) produce

and (3) store melanin in their melanosome. Melanin is transferred to keratinocytes, where they reside in granules, will be beneficial against harmfull effects of UV.[1]

1.2 Melanocyte development of the Skin

Skin melanocytes are originated from the neural crest cells (NCC) resides in the embryonic germlayer ectoderm. Multipotent properties of NCCs are lost gradually in developmental process and they become lineage restricted cells.[2, 3] Lineage restriction counts on the location of NCCs. Traditionally, NCCs located in dorsal trunk region give rise to epidermal melanocytes migrating dorsa-laterally.[4] However, NCCs travelling ventrally can be maintained as multipotent cells which will turn into myelin sheats or melanocytes.[5] Embryonic cutaneous innervation hosts invasion of epidermis by ventrally migrating cells. [6] Evidences suggest that development of congenital nevi may arise from response to epidermal cells secreted growth factors by precursors of Schwann cells reach epidermis during cutenous innervation.[7] Studies demonstrating molecular difference between dermal and epidermal melanocytes support the double origin of skin melanocytes idea.[8]

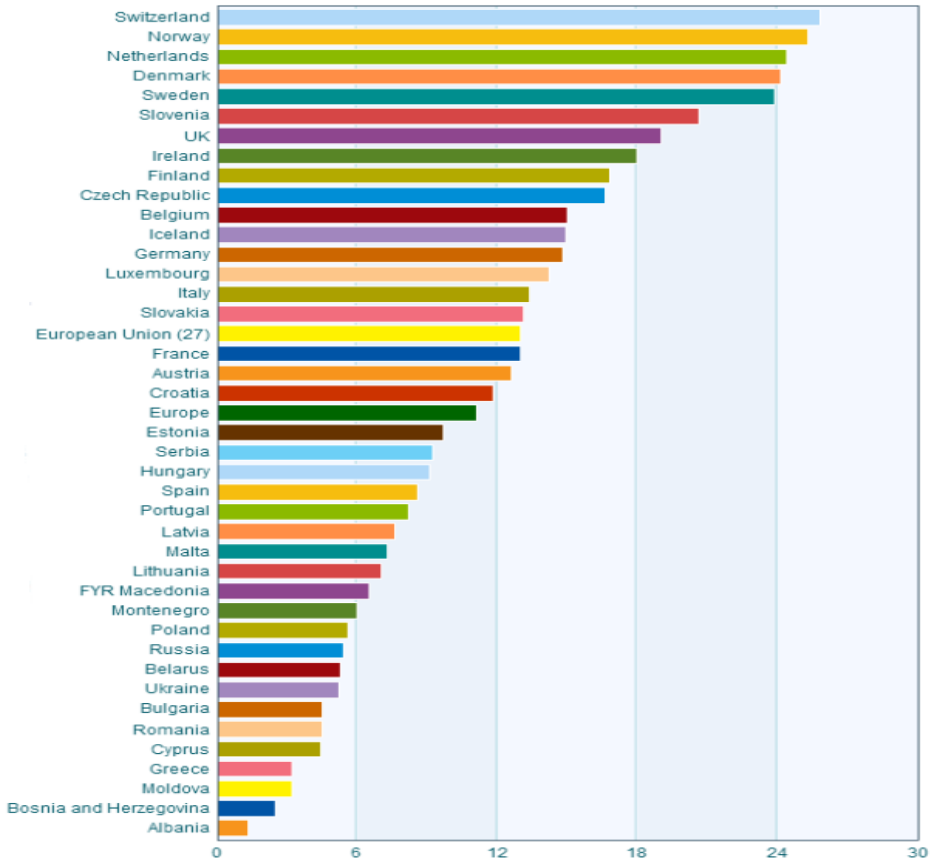
At the molecular level, intact BMP signalling partly contributes to early neural crest induction.[9] Fate of trunk NCCs counts on Notch signalling activation acts on early progenitors cells preventing them taking neuranal fate instead of becoming neural crest cells.[10] Subsequent to induction, neural crest cells undergo epitheliel-mesencymal transition, lose their attachment to neighbouring cells and migrate. This mobility acquired by repression of E-cadherin expresion via Snail/Slug transcription factors.[11] WNT signalling is essential for its role in promoting cell fate towards melanoblast and mature melanocyte through B-catenin expression.[2, 12-14] Further receptor tyrosine kinase c-KIT and its ligand; G protein coupled receptor EDNRB2 and its ligand endothelin-3; transcription factors Sox10, Pax3, MITF; are demonstrated to play crucial roles in melanocyte formation during embryogenesis.[5, 15-18]

1.3 Epidemiolgy of Cutaneous Malignant Melanoma

1.3.1 Incidence of Cutaneous Malignant Melanoma

Melanoma of the skin is the 5th common cancer in USA after prostate, breast, lung, colon and rectum cancers. Although 5% of skin cancer defined as melanoma, 75% of the deaths occur as a result of melanoma. American Cancer Society estimated 4.6% of the new cancer cases would be diagnosed as melanoma in 2013. Out of estimated 76,690 new melanomas, approximately 45,060 cases would be diagnosed in men. Expected number of people died from malignant melanoma is 9,480 which are equal to 1.6 % of cancer death.

Geographical variations, age, gender, anatomic location of the primary tumor may affect the incidence of melanoma. Latitude gradient for melanoma is taken into account as a feature of melanoma.[19, 20] Fair skinned people living at low gratitudes in high incidence countries such as Australia, New Zealand, USA, Isaril, have highest rates of melanoma compared to high latitude counterparts and dark skinned residents of the countries.[21-23] Southern Europe populations (Italy, southern Spain and Balkans) are exceptions in the context of latitude graident. Assumed to be predominated by the overall darker pigmentary chracteristics, southern Europe populations demonstrates low incidence of melanoma. Compatible with these findings World Health Organisation estimated incidence of melanoma to be high in Scandinavian countries in Europe (Fig 1.2).



Age Standardised Rate (European) per 100,000

Figure1.2 Estimated Incidence of Malignant Melanoma of skin in both sexes, 2012

Source: Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JWW, Comber H, Forman D, Bray F. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer. 2013 Apr; 49(6):1374-403.

Starting from 40 years of age, age-dependent incidence of melanoma gradually rises and peaks at the seventh and eight decades. [24-28] People around 50-60 years of age have high incidence of primary tumor on the trunk compared to those around 80s whose primary tumor locate in the head and neck.[27-31] Males are more prone to melanoma in low latitude countries. Thus, melanoma incidence have been higher among men in Australia and USA or have been increased in New Zealand compared to females.[32]

1.3.2 Risk Factors of Cutaneous Malignant Melanoma

Genetic predisposition and exposure to environmental agents promote incidence of melanoma. Elevated numbers of atypical moles inherited in a familial setting defined as dysplastic nevus syndrome and may delevop melanoma with a higher penetrance rate and earlier onset than sporadic melanoma.[33-35]

A Germline mutation in the cyclin dependent kinase inhibitor 2 gene (CDKN2A, chromosome 9p21) is a major indicator of susceptibility to melanoma progression in familial type of melanoma.[36, 37] 20-40% of the familial melanoma is originated from germline alterations in CDKN2A gene.[38, 39] Coinheretance of a low –risk susceptibility gene, melanocortin-1 receptor (MC1R) in red haired vairants with CDKN2A mutations has found to increase the risk of melanoma from 50% to 80%.[40, 41] With less frequency, abberations in CDK4 or CDK6 can be detected in melanoma. Mutations in CDK4 binding site of p16 INK4A (Arg24CyS Arg24 His mutation) resulting in impaired binding of p16INK4A to CDK4 may seen in melanoma prone families. [42-45]

The only environmental factor sunlight may increase the risk of melanoma through its UV components. UV exposure is indicated to be a risk factor for melanoma in several studies such as, (1) increased incidence up to 10 to 20 fold in fair skinned people located in close proximity to equator[46]; (2) XP diagnosed people have 1,000 fold increase incidence to melanoma compared to average population[47]; (3) UV radiation induced other types of skin cancer history elevates the risks up to 3 fold compared to patients without history[48, 49] and (4) tendency to burn and freckles are phenotypic measures raise risk nearly two fold in all populations.[50] Exposure pattern of sunlight is also important in melanoma. Common view supports higher risks of melanoma caused by intermittent sun exposure compared to chronic or cumulative sun exposure.[51-53] Differences in sun exposure patterns on specific locations with abberant genetic backgrounds also have influence on types of melanoma. [54]

1.4 Cutaneous Malignant Melanoma Genetics

A better understanding of molecular changes in melanoma pathogenesis is beneficial for a better classification of patients and thus improving treatment options. So far, molecular researchs in melanoma have provided insights about abberations in cell cycle regulatory and cell signalling processes contributing melanomagenesis. Vast majority of malignant melanoma cases resulting from either presence of germline or somatic mutations in these pathways. Cross-talk between these pathways either is helpfull to partially control the resulting impairment or worsen the pathogenesis depending on the number of effected processes.

1.4.1 Abberant Cell Cycle Regulation of CMM

Phosphorylation status of pRB in the G1/S phase is a crucial step for cell cycle regulation thus, pRB serves as gatekeeper protein. .Entrance to S phase from G1 phase requires phosphorylation of pRB by cyclin-Cdk complexes, resulting in releasing E2F transcription factor from pRB, leading to expression of essential genes for G1/S transition.

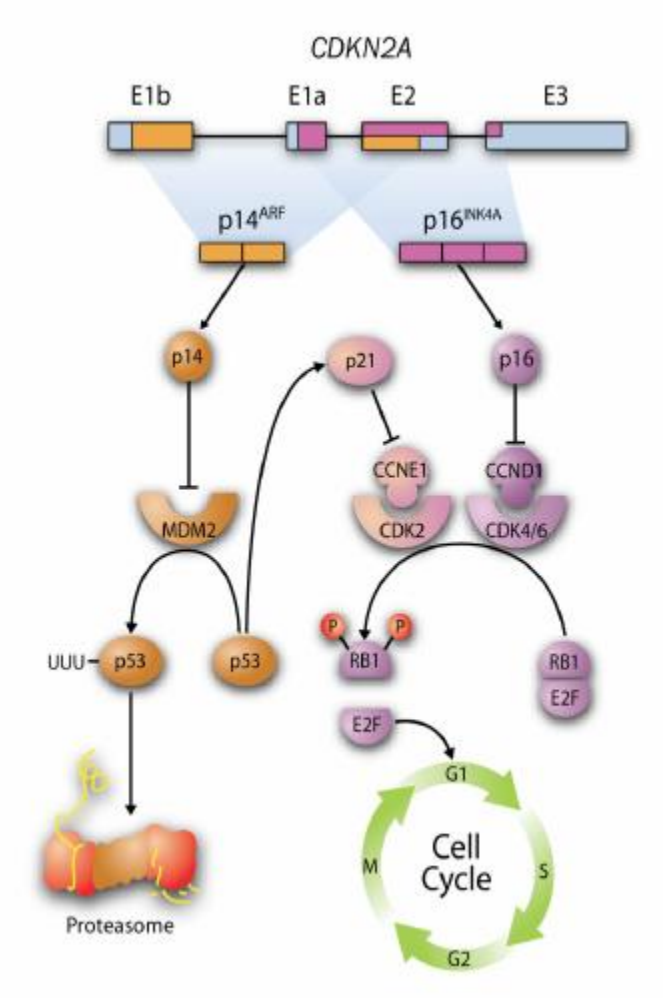


Figure 1.3 The CDKN2A locus and cell cycle control

Source: Aleksandar Sekulic, MD, PhD, Paul Haluska Jr, MD, PhD, Arlo J. Miller, MD, PhD, Josep Genebriera De Lamo, MD, Samuel Ejadi, MD, Jose S. Pulido, MD, MS, MPH, MBA, Diva R. Salomao, MD, Erik C. Thorland, PhD, Richard G. Vile, PhD, David L. Swanson, MD, Barbara A. Pockaj, MD, Susan D. Laman, MD, MPH, Mark R. Pittelkow, MD, and Svetomir N. Markovic, MD, PhD. Malignant Melanoma in the 21st Century: The Emerging Molecular Landscape. Mayo Clin Proc. 2008.

CDKN2A gene encodes 2 structurally particular proteins, p16 INK4a and p14 ARF, functioning in the cell cycle regulation as tumor supressors.[55] Hypophosparylation status of pRB is maintained directly by p16 INK4a or indirectly by p14 ARF. In presence of cdk

inhibitor kinase (CKI); p16 INK4a inhibits cyclinD1-cdk4/6 complex activity, enabling pRB to sequester E2F and subsequently, lead to cell cycle arrest. Alternative splice variant of CDKN2A, p14 ARF protein indirectly controls pRB status in a same manner of p16 INK4a, via stabilizing levels of p53 protein, inhibiting its interaction with MDM2. In normal cellular conditions, p53 is target of MDM2 protein leading its ubiqutination and subsequent degradation in proteosomes.[56] DNA damage triggers p53 accumulation in the cell via sequestering of MDM2 by p14 ARF.[57] Activation of p53 induces another CKI protein p21, which, prevents phosphorylation of pRB via cyclin E/Cdk2 complex.(Fig1.3) In contrast to other malignancies, p53 mutation is very low[58] in melanoma confirming reliance to p14 ARF control.

Melanoma associated CDKN2A mutations either resulted in low expression of p16 INK4A[59, 60] or p14 ARF alone[61, 62] or both [63-67] indicates that each tumor supressor fulfill its own particular role against development of melanoma.

1.4.2 Abberant Cell Signalling in CMM

Receptor tyrosine kinases (RTKs) initiated proliferation via mitogen activated protein kinase (MAPK) pathway and survival via phosphoinosite 3kinase/ AKT (PI3K/AKT) pathway are in the centre of sporadic melanoma pathogenesis.

1.4.2.1 MAPK pathway

Activation upon RTKs ligand to RTKs or integrin adhesion to extracellular matrix; GDP binding to RAS protein (NRAS/KRAS/HRAS) is replaced with GTP, transfering RAS to its active state.[68, 69] RAS activation triggers its downstream effectors; RAF/MEK/ERK serine threonine kinases. Any changes resulting in hyperphosphorylation of ERK through MAPK cascade contributes to cellular proliferation and survival of melanoma patients in the

90% of the cases.[70] 50-70% mutation is detected in RAF gene, and with a 90% frequency in BRAF resulting from substitution of valine with glutamic acid at 600 position. [71-74] RAF mutation increases the kinase activity to 10-480 fold compared to wild type.[75] Transcription factors; MITF [76](microphthalmia-associated transcription factor)and BRN-2 [77](POU domain class 3 transcription factor), the cell cycle regulators cyclin D1 and p16 CDKN2A [78, 79]; and the tumor maintenance enzymes, matrix metalloproteinase-1 (MMP-1) [80] and inducible nitric oxide synthase (iNOS)[81] act as downstream effectors of BRAF mutation in melanoma. Neoangiogenesis is also maintained by BRAFV600E mutation via inducing VEGF secretion.[82] BRAF mutation is found to be present in 70-80 % common benign nevi suggesting that BRAF mutation is a very early event in melanomagenesis and by its own is not sufficient to drive melanoma development. [83-85]

Independent of PI3K-AKT induced mTOR activation, MAPK induced ERK1/2 and ribosomal S6 kinase (RSK1) also act on TSC1/TSC2 complex in a same manner of AKT. MNK1 and MNK2, downstream kinases of ERK, also directly phosporylate eukaryotic translation initiation factor 4E (eIF4E). Upon mTORC 1 activation, eIF4E is released from its binding protein; leading to subsequent formation of eIF4E multisubunit complex, inducing cap-dependent protein translation, and increased translation of mRNAs with regulatory elements in the 5'-untranslated terminal regions (5'-UTR) of its downstream target genes (e.g., c-myc, ornithine decarboxylase and cyclin D1), which are required for G1-to-S phase transition.[86, 87]

cAMP has demonstrated to have an influence on MAPK pathway.[88, 89] The mechanism how cAMP regulates MAPK pathway was brought up by Scott et al. A kinase anchoring protein 13 (AKAP13) plays a crucial role in this regulation by translocating cAMP induced PKA to near vicinity of RAF-MEK-ERK scaffold protein KSR1 and sustaining tha MAPK

stimulation by phosphorylation of KSR1. Moreoever, AKAP13 constitutes a core complex with KSR1 and functions as enhancer of the process as it keeps RAF close to MEK. [90]

1.4.2.2 PI3K-AKT Pathway

Loss of intrinsic GTPase activity of NRAS due to substitution of glutamine to arginine at 61 position (Q61R) is seen 90% of overall 30% mutations among RAS family.[91, 92] BRAF and NRAS mutations rarely coexist, regarding implications in the same cascade.[72, 93] However, NRAS mutations can activate both MAPK and PI3K signalling simultaneously.

Oncogenic NRAS activates PI3K to phosphorylate the second mesenger phosphotadylinositol 4,5 biphosphate(PIP2) to PIP3.[94] Protein kinase B (AKT) activation is maintained by PIP3 via phosphoinositide-dependent kinase-1(PDK1).[95-97] AKT is a serine/threonine kinase which is involved in cell cycle progression, survival, metabolism and migration via phosphorylation of many physiological substrates upon activation. [98-102] Among 3 isoforms of AKT (AKT1, AKT2, AKT3), AKT3 is preferentially activated in sporadic melanomas up to 60% of the cases.[103, 104] This additional activation of AKT3 is separate from loss of functional phosphatase and tensin homolog (PTEN) which acts as a lipid phosphatase that regulates intracellular PIP3 levels.[75] Thus, homozygous mutations or deletions in tumor supressor PTEN contribute abberant activation of AKT.

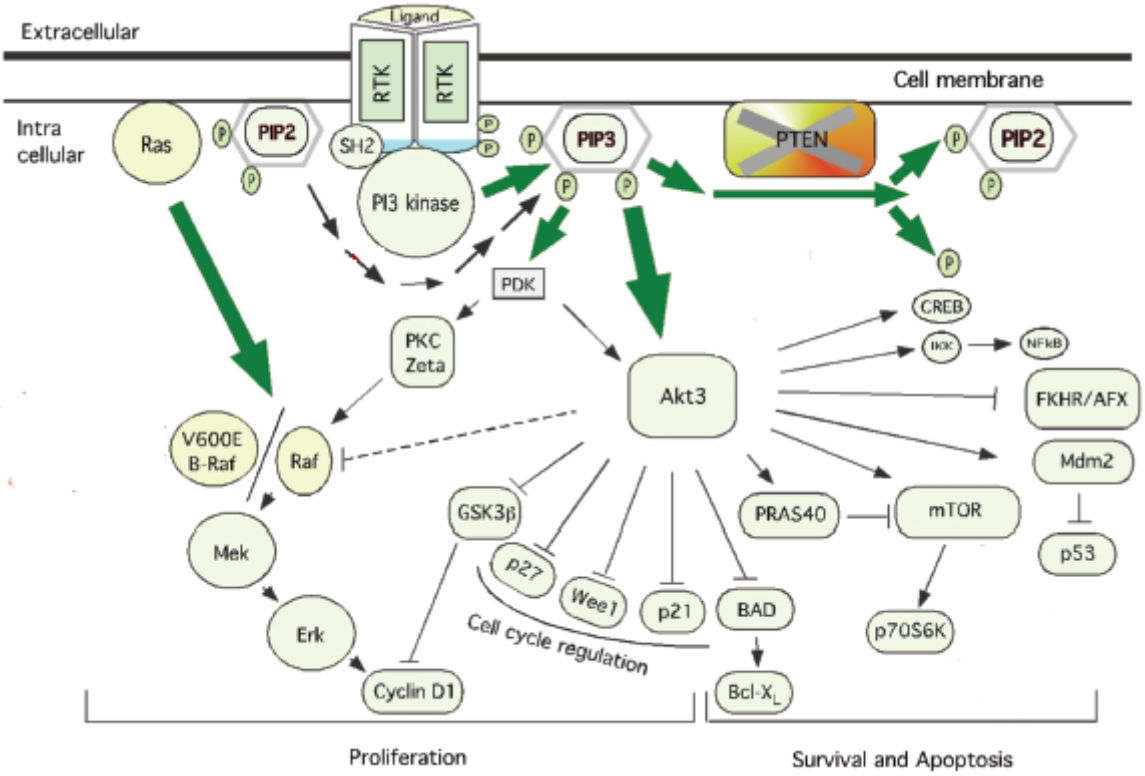


Figure 1.4 schematic representations of key therapeutic targets in the PI3K/Akt and MAPK pathways

Source: Adapted from SubbaRao V. Madhunapantula, Paul J. Mosca8 and Gavin P. Robertson. The Akt signaling pathway. Cancer Biology & Therapy, 2011.

Phosphorylation of proapoptotic proteins; BAD[105, 106], caspase-9[107] ,MDM2 [108-110], forkhead transcription factors[111, 112] leading to inactivation of these proteins whereas NF-kB activation[104, 113, 114] is the result of phosphorylation in AKT driven prevention of apoptosis. Down regulation of Cdk inhibitor proteins p21 cif1/WAF1, p27 kip1 and stabilization of CylinD1 and Cyclin E at G1/S transition and inhibition of Wee1 at G2/M transition is controlled under phosphorylation of AKT in the process of cellular proliferation. [115-117] AKT is also shown to interact with GSK3Beta, which is a part of multiprotein complex regulating B-catenin degradation in the cytosol in the absence of WNT signalling, leading its ubiqutination and nuclear translocation of B catenin.[118] (Fig 1.4)

Major downstream effector of AKT is mammalian target of rapamycin (mTOR) which is a serine/threonine kinase. mTOR has two distinct multiprotein complexes mTORC1 and mTORC2. mTORC1 gathers information from various extra and intra cellular signals like growth factor induced PI3K –AKT pathway, controlling processes such as protein and lipid synthesis and autophagy. AKT can activate mTOR via phosphorylation directly by acting on the negative regulator PRAS40 of mTORC1 complex, or indirectly by inhibiting GAP activity of TSC1/TSC2 heterodimer on Rheb which stimulates mTORC1. mTORC2 comlex of mTOR is a positive coregulator of AKT by phosphorylating it on hydrophobic motif Ser437 for its maximal activation. However, defeciency in ser437 phosphorylation of AKT by mTORC2 is only related with phosphorylation capability of AKT to its some targets such as FOXO but not all.[119]

1.4.2.3 Regulation of MITF

Microphthalmia associated transcription factor (MITF) regulates melanocytic development, differentiation and maintenance.[120, 121] Pigmentation enzyme genes, as TYR, TYRP1, and DCT; melanoma marker genes including SILV/gp100/, HMB45, MLANA/MART1, and AIM1 and prognostically relevant melanoma marker TRPM1 is regulated by MITF dependent fashion.[122-130] MITF expression control needs fine tunning since high and low levels of MITF results in cycle arrest and differentiation via p16 CDKN2A and p21cip1 WAF1 induction or apoptosis, respectively.[131, 132] However, melanoma cells are thought to maintain an intermediate level of MITF expression to promote cell proliferation along with tumorigenesis. MITF activation relies on cross talk between MAPK and melanocortin pathway (MC1R).(Fig 1.5) MC1R activation is needed for MITF expression whereas MAPK is needed for MITF activation.[55] BRAF mutation in MAPK is shown to control MITF protein by increasing MITF expression through BRN2 transcription factor counterbalance to MAPK dependent degradation of MITF.[133, 134] Further downstream effector

of WNT signaling, B-catenin is shown to regulate MITF expression in TCF/LEF/DNA consensus dependent transactivation of MITF promoter.[135]

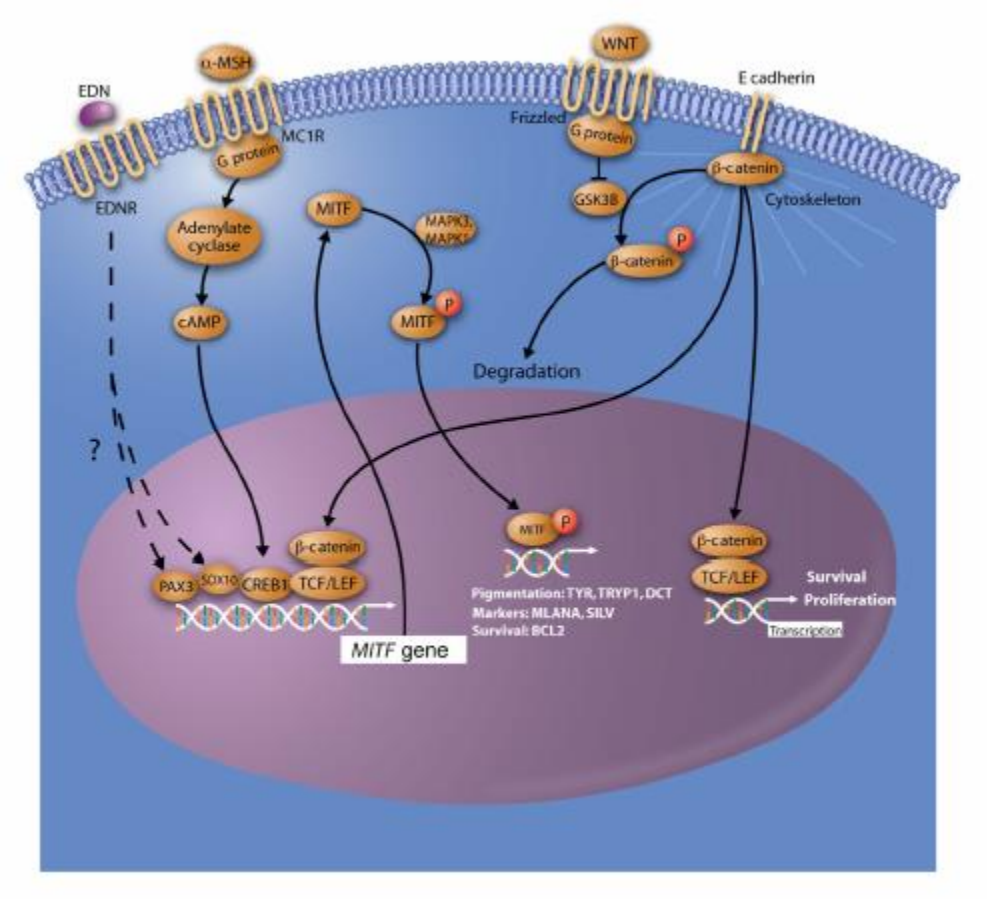


Figure 1.5 MITF signaling

Source: Aleksandar Sekulic, MD,PhD, Paul Haluska Jr, MD, PhD, Arlo J. Miller, MD, PhD, Josep Genebriera De Lamo, MD, Samuel Ejadi, MD, Jose S. Pulido, MD, MS, MPH, MBA6, Diva R. Salomao, MD, Erik C. Thorland, PhD, Richard G. Vile, PhD, David L. Swanson, MD, Barbara A. Pockaj, MD, Susan D. Laman, MD, MPH, Mark R. Pittelkow, MD, and Svetomir N. Markovic, MD, PhD. Malignant Melanoma in the 21st Century: The Emerging Molecular Landscape. Mayo Clin Proc. 2008.

1.4.2.4 Deficiency in KIT and MC1R Receptors

Abberant activation or deactivation of receptors contributes to melanoma progression independent of downstream mutations. So far changes in KIT and MC1R genes have been detected in melanoma. KIT also known as c-KIT encodes a receptor tyrosine kinase, belonging to PDGF family. Although it is essential for melanocyte survival, its expression is lost in nevi. Mutation or gene amplification dependent overexpression of KIT triggers downstream proliferative and pro survival pathways. Mutations occurred in K642E, L576P, D816H, and V559A positions may either provoke KIT dimerization in the absence of stem cell factor or hinder an auto control conformational change to its unstimulated status. [136-138] It is also noted that abberant expression of KIT is found in some cases that do not harbor any gene amplifications or mutation.

Polymorphisms seen in MC1R which is a G-protein coupled receptor increase the UV induced susceptibility to melanoma. Upon UV exposure; keratinocytes, increase transcription and secretion of alfa MSH, activating intracellular melanocortin pathway to overcome UV effects by producing melanin resulting in tanning.[139-141] Germline polymorphisms in MC1R receptor lessen expression of MITF and prevents tanning in light skinned and red headed people, making them vulnerable to melanoma.[142] [143, 144]

1.4.2.5 Notch Signalling

Expression levels of Notch receptors and ligands are found to be elevated in atypical nevi and melanoma compared to common mole. [145, 146] Notch1 interacts with (1)Beta-catenin , which acts as a downstream effector of WNT signaling and interacts with N-cadherin[147]; (2) MAPK and PI3K/AKT with an unknown way and stimulate transition to vertical growth phase[148]; (3) NF-kB and provides prolonged residence in nucleus of T cells.[149] Further, notch1 signaling is essential for RAS mediated transformation where impairments in notch1 signalling cause significant reduction RAS mediated signaling.[150, 151]

1.5 Current Evaluation of Cutaneous Malignant Melanoma

A patient can be diagnosed with melanoma depending on clinical and histopathological assesments. Clinical diagnostic approaches differ from histopathological ones in the devices they use. Especially radiological devices are used in clinical assessment of node involvements where as light microscopy is used in the latter one. Besides, with a slight difference in details, both methods come up with same result considering stages. Presence or absence of node involvement or distant metastasis is sufficient for clinical staging. However a deeper understanding of primary tumor, number of nodes, locations of distant metastasis is given by histopathological staging.

1.5.1 Clinical Evaluation

An experienced dermatologist can diagnose the lesion of interest according to the clinical ABCDs criterias of the melanoma.[152] ABCD refers to assymetry, border irregularity, color distribution, and diameter of the lession that can be detected by naked eye.(Table 1.1) Since asymmetrical changes in size, shape or color, accompanied with itching and bleeding may develop in time, "E" of evoltion is added to ABCD rule. [153] Atypical nevus can be identified more easily beacuse of the differences seen in patterns compared to benign nevus of the same patient examined prior to selected nevi. This is called ugly duckling sign.[154] For questionable lessions non-invasive dermascopy method can be applied to distinguish between atypical nevus and melanoma.[54] Methods give insight about architecture of the tumor.[155] Algorithmic methods such as the ABCD rule of dermoscopy[156], the 7-point checklist of dermoscopy [157], or Menzies' scroring method[158] are valid and reliable during dermascopy. Dermascopy may increase detection of cutaneous melanoma with a sensitivity of up to 92%and a specificity of up to 99% depending on the individual experience of the clinician.[159]

A	Asymmetry	Benign nevi are symmetric, their shape is round or oval. In contrast, cutaneous melanomas grow faster in several areas and are consequently asymmetric.
B	Border irregularity	Common nevi are bounded regularly to normal skin, whereas cutaneous melanomas often exhibit ragged and dull margins.
C	Color variegation	Benign nevi usually show homogeneous brown shade of color, whereas cutaneous melanomas exhibit different colors like black, brown, red or slate blue.
D	Diameter > 6 mm	After a growth period in infancy and adolescence, common nevi remain stable in size, whereas melanomas often grow rapidly. A diameter more than 6 mm is considered to be suspicious.

Table 1.1 Clinical diagnostic rules for melanoma, the ABCD criteria.

Source: Franziska Brehmer, M.D., Martina Ulrich, M.D., Holger A. Haenssle, M.D. Strategies for early recognition of cutaneous melanoma—present and future. Dermatol Pract Concept 2012.

1.5.2 Histopathological Evaluation

Histopathological evaluation can be used for both diagnostic and prognostic purposes. When a suspicious lesion is removed by either excisional or incisional surgery; tumor thickness (Breslow's Depth), ulceration of the tumor, and mitotic rate are the first three parameters to define the primary tumor (T). [54]

Tumor thickness so called Breslow's depth is the major definer of the T category. It's the vertical measurement of the specimen in millimeters from the top of the granular layer of epidermis to the deepest point of invasion.[160] Though thickness negatively corellates with survival, thinner melanomas have shown to metastasize with a rate of 4.8%.[161, 162] Therefore, Clark's level, ulceration, mitotic rate and regression rate should be taken into account for further assessment of thin melanomas.[161]

Second determinant and contributor of T staging is ulceration. Ulceration is defined as the absence of stratum corneum and basal membrane accompanied with host response and

thinning, effacement or reactive hyperplasia of the surrounding epidermis without any known prior surgery.[163] Ulceration can be refered as a sign of metastatis, since the survival rates of any T stage with ulceration is equal to following T stage without ulceration.[164]

Clark's level of invasion for the discremination of T1a and T1b is replaced with mitotic rate in the 7th edition of AJCC melanoma staging. [54]Clark's level of invasion is based on relating anatomic landmarks to tumor depth for melanomas ≤1mm thin.[165] Compared to Breslow depth which is a continous variable, a discrete variable such as Clark's level has shown weaker correlation with patient survival.[163, 166] In addition, discremination differences in Clark's levels originating from variation in pathologists, lead to classification errors. Prognostic significance of mitotic rate and ulceration in concert with Breslow depth ruled out the importance of Clark's level only if these two parameters can be assessed. [165] Mitotic rate corresponds to the number of mitotis in the hotspots in dermis within a range of $1mm^2$.[167] The significant threshold relevant to survival is 1mitosis/mm^2 for classification of T1a and T1b melanomas.[161, 163]

Regional lymph node involvement in the prognosis of melanoma is a major prognostic factor that strongly correlates with 10 year survival rates of the patients.[167] Node involvement can be classified in three groups; micrometastatsis, macrometastasis and intralymphatic metastasis. Lymph nodes metastasis which can not be diagnosed clinically by palpation or imagining techniques but can be assesed histopathologically following sentinel lymph node biopsy (SLNB) or elective lymphadenectomy is defined as micrometastasis.[168] Macrometastasis is the case where node involvelment is identified clinically, then confirmed histopathologically. Intralympatic metastasis can be either in form of in- transit or satellite lessions related with poor prognosis. Both are discontinous lessions apart from the primary tumor with a differential in length of location. Discontinous lesions within the 5cm or more than 5 cm of primary tumor are called satellite and in-transit metastasis, respectively.[169]

Microsatellites are nests of tumor cells with a ≥0.05 diameter disconnected from the primary tumor by only dermis, categorized with in the same group as satellite or in-transit metastasis.

Distant metastasis is categorized by the site of metastasis and lactate dehydrogenase (LDH) levels. The survival rates of patients with nonvisceral, lung or visceral metastasis differ greatly, with a poor prognosis in the direction of visceral metastasis.[167] Serum LDH level is an independent prognostic indicator of survival in stage IV melanoma patients. [167, 170-172]Therefore confirmation of high level LDH without any detection of distant metastasis in any site still defined as distant metastasis.[167]

Any type of metastasis detected without any primary site is considered to be equivalent of metastasis with known primary sites when staging. The circumstances are described as spontenous regression of the primary site before detection. [173] A similar or in some cases better survival of metastatic melanoma patients without known primary sites compared to patients with known primary sites supports the idea of effective immune response in those patients contributing primary site regression and better prognosis.[174, 175]

The criterias mentioned so far are placed in the current tumor classification system of melanoma described in the 7th edition of AJCC (Table 1.2).

T	Thickness (mm)	Ulceration status/mitoses
Tis	NA	NA
T1	≤1.00	a: Without ulceration and mitosis <1/mm^2 b: With ulceration or mitoses ≥1/mm^2
T2	1.01–2.00	a: Without ulceration b: With ulceration
T3	2.01–4.00	a: Without ulceration b: With ulceration
T4	>4.00	a: Without ulceration b: With ulceration
N	**No. of Metastatic Nodes**	**Nodal Metastatic Burden**
N0	0	NA
N1	1	a: Micrometastasis* b: Macrometastasis†
N2	2–3	a: Micrometastasis* b: Macrometastasis† c: In-transit metastases/satellites without metastatic nodes
N3	≥4 metastatic nodes, or matted nodes, or in-transit metastases/satellites with metastatic nodes	
M	**Site**	**Serum LDH**
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous, or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastasis	Elevated

Table 1.2 TNM staging categories for Cutaneous Malignant Melanoma

Source: Zendee Elaba, Michael J. Murphy, Philip Kerr, and Jane M. Grant-Kels. Staging of Melanoma. Diagnostic and Prognostic Biomarkers and Therapeutic Targets in Melanoma, 2012.

1.6 Melanoma Biomarkers

Parameters used to assign patients in to subgroups are either morphological or histopathological. This approach is a crude way of subgrouping patients since the genetic dynamics of patients are variable. Thus, conventional treatments do not accomplish the proposed outcome. For this reason, markers such as tissue-based, serological and molecular are under investigation for a better diagnostic and prognostic classification. Imminohistochemistry, gene expression profiling, gene mutation analysis and comparative genomic hybridization techniques are used in this purpose.

Immunohistochemical staining (IHC) of a panel including melanoma lineage markers such as s100, MART-1, gp100/HMB45 are qualified to be diagnostic discriminators between melanoma and non-melanoma cells. IHC staining of Ki67 with MIB-1 antibody is widely used to distinguish benign from malignant melanoma.[176] AJCC also finds this approach alone acceptable in the cases of evident morphological malignant cells features in the IHC staining of nodes to approve nodal metastasis.[176]

So far there are three serological markers identified as prognostic markers of metastasis; S100-beta, melanoma inhibitory activity (MIA) and LDH. Crediting S100-beta as inferior to MIA, S100-beta and MIA are used as prognostic indicators of relapse and metastasis in the follow up of the patients.[172, 177] Besides in the absence of metastasis or in tumor free situation, both have no significance to give insight into earlier stages.[178] Moreover in the case of detection of micrometastasis, S100-beta also fails to identify the micrometastasis where SLNB still remains as identification approach.[179] Among these 3 serological markers, lactate dehydrogenase (LDH) has shown to be the strongest independent prognostic factor that its elevated amounts in the serum is accepted to be the indicator of distant metastasis regardless of approved presence and accrued into AJCC classification system as stage M1c.[180]

Discovery of BRAF mutations in the MAPK pathway with a incidence rate of 70% in melanoma patients led to personalized treatment with BRAF inhibitors. Especially, testing BRAFV600E mutation which accounts for 90% of the BRAF mutations is an eligibility criterion for patients with metastatic or unresectable melanoma to be treated with BRAF and MEK inhibitors.

1.7 Autologous Vaccination and Melanoma, Impact of Autologous Vaccination on Survival of Cutaneous Malignant Melanoma Patients

1.7.1 Autologous Vaccination

Discovery of tumor antigen presentation is recognized by T cells on solid tumors, yields invesigators to develop immunotherapeutical approaches against cancer. Among them vaccination strategies are evaluated as an adjuvant treatment for melanoma. Aim of vaccination is to sensitize T cells against relevant tumor antigens.[181]Developing immune response through vaccination involves autologous or allogenic whole cell vaccines, tumor specific peptide presentations or delivery of specific antigen presenting dendritic cells.[182]

The rationale behind the whole cell vaccination is to introduce adaptive immune response companents with high amount of tumor antigens on actual tumor cells delivered by injection. Therefore, whole cell vaccination is known to be polyvalent, eliciting immune response against multiple antigens.[183] Depending on source, whole cell vaccination is categorized as (1) autologous, originated from and delivered to same patient or (2) allogenic, originated from various patients and delivered to a different recipient. Autologous whole cell vaccination has its own advantages over the latter one, such as having unique patient specific antigens and appropriate HLA match for ideal antigen presentation. However, obstacles remain in the procedure against effective application. Patients need to have available tumors for resection to obtain tumor cells. Nonetheless amount of cells occupied from any of them, aren't sufficient to produce more than two or three doses. Meanwhile the duration of harvesting the tumor and production of vaccine may give rise to unexpected tumor progression that would rule out the effect of vaccine. On the other hand, allogenic whole cell vaccines have shared antigens relevant to specific tumor from various patients in all stages. Moreoever, availability of abundant source provides administration of vaccines for a

prolonged time. The only drawback of this approach is lack of important antigens to provoke immune response in specific patients.[184]

An established autologous whole cell vaccine for melanoma relies on studies done in murines with metastatic mammary carcinoma.[185] Adaptation and optimization of this approach into clinic trial applications of melanoma is perfomed by Berd et al. Basically, previously harvested melanoma tumor cells are first irradiated and conjugated to DNPs mixed then mixed with BCG prior to administration are delivered via intradermal injection on the day of treatment. [186] Administration of the vaccine is as cruical as preparation for its utmost effect in patients. [187] Procedure starts with DNP and cyclophosphamide injection prior to vaccination. Cyclophosphamide is used for its immunopotentiating properties [188-190] Vaccination continues in intervals up to 8 doses. [191]

1.7.2 Role of Autologous Vaccination on Cutaneous Malignant Melanoma Patient's Survival

The settled preparation and administration of melanoma vaccination called MVAX was subjected to clinical trials to assess its effect on patient's survival. Phase I trial was evaluated on 83 stage IV metastic melanoma patients. In total 11 of the patients responded including; 2 complete, 4 partial and 5 mixed. Subjects of complete responses and 2 of partial response had lung metastasis. Response duration of complete responses were 12, 29 months whereas partial response last for 5, 6, 8 and 47+ months in patients. [192] As a post-surgical adjuvant application, 214 stage III patients treated with MVAX. 5 year overall survival was increased to 44% when compared to 20-25% resulted from surgical alone. [193] Phase III trials were conducted to evaluate effeciency of MVAX over HDI in stage IIIB –IIIC melanoma patients as post surgical adjuvant therapy. At the edge of marketing approval, MVAX was found to be inadequate to pass sterility testing since fresh vaccine shelf life of MVAX was too

short. A great deal of vaccine amount were found to be contaminated with aneorobic *Propionibacterium acnes.*[187]

Following FDA termination of fresh MVAX trial, a frozen version of MVAX was developed, having shelf life of months instead of hours. FDA acknowledged it as a new vaccine which should be subjected to trials from the beginning due to dead tumor cell content of the vaccine. Frozen MVAX was demonstrated to be equally effective and more importantly safe in comparison to fresh MVAX in phase I –II trials.[187]Contraversial results from a previous study[194] and insufficient funding were the end of planned phase III trial for frozen MVAX. [187]

1.8 A Crosstalk between CT Gene Expression and Tregs in Melanoma

Expressions of CT genes are normally restricted to immune privileged germ cells of testis and placenta but abnormal expression patterns are also found in various cancer types. [195]Their importance for immunotherapy resides in the fact that any therapy developed against them will directly affect the tumor cells in the relevant cancer, considering their tumor specific expression. Tumors expressing cancer testis antigens may demonstrate heterogeneous expression, increasing with elevated stages of cancer. Moreover, a CT gene expression in any type of cancer is found to be expressed in concert with multiple CT genes in that cancer. This escalating expression of co existing CT genes correlates with worse prognosis in increasing cancer stages of 1-3.[196]

In a simplistic approach, considering these facts and high immunogenic potential of CT genes, one should expect to see elevated immune response in parallel with higher CT expression and elimination of cancer. However, the microenvoriment established and maintained by tumors seem to have immunosuppressive properties, preventing recognition of presented CT epitopes on MHC class I and II molecules.

T regulatory cells of immune system have been demonstrated to take a part in diminishing antitumor T cell immunity.[197]Their normal role in immune system is to hinder autoimmune diseases by repressing numbers and cytokine products of CD4CD25 T cells, CD8 T cells, already existing Th1 and Th2 cells. Therefore, presence of Tregs in tumor microenvironment suggested being one of the possible ways of immunosuppression.[198]

Contribution of Tregs to melanoma progression has been evaluated. Study conducted by Mourmouras et al., underlines the importance of Tregs presence from atypical nevi to vertical growth phase melanoma, proposing immunotolerance maintained by Tregs in the early melanomagenesis and support melanoma growth.[199] Also, Tregs amounts are shown to be high in tumor infiltrated lymp nodes to peripheral blood in patients and peripheral blood of melanoma patients to healthy controls. [200]Concordant with these results, Cebon et al. detected 40% Treg population to all T cells infiltrating metastatic melanoma tumor tissues.They related presence of Tregs to immune suppression in their studies, concerning effects of NY-ESO-1 ISCOMATRIX cancer vaccine in melanoma NY-ESO-1 positive melanoma patients after surgery with minimal residual disease (MRD) to metastic NY-ESO-1 positive patients. It's been demonstrated that vaccine results in high immune response in MRD patients to metastatic patients; in terms of providing strong DTH, circulating CD4 and CD8 T cells against NY-ESO-1 epitopes and prolonged disease free survival. [197] Higher abundance of Tregs in advanced disease compared to MRDs in concert with previous findings is a possible ground of tumor induced immune suppression in melanoma patients. Moreover, an additional contribution from melanoma cells expressing FoxP3, acting like Tregs and enhancing local immune suppressive environment has been demonstrated.[198]

1.9 An Overview of TGFα, CLDN1, CDX2, ERCC1 and LIG3 in Melanoma

1.9.1 TGFα

Abnormal expression of growth factors, receptors and their related signalling pathways are thought to have a role in transformation of melanocytes to melanomas. Among the mRNA levels of 11 growth factors that were compared between 19 human metastatic melanoma cell lines and 14 normal human foreskin melanocytes, mRNA levels of TGFα was demonstrated to be characteristically higher in melanoma cell lines as well as TGFβ2 and bFGF.[201] TGFα has also been detected in the urine of primary and metastatic melanoma patients.[202, 203] High structural homology in the receptor binding site with EGF, TGFα can stimulate autocrine mediated proliferation via binding and activating EGFR.[204] However, EGFR activation is only detected in BRAF V600E mutant melanomas treated with BRAF inhibitors.[205, 206]

1.9.2 CLDN1 and CDX2

Tight junctions are important for their role in maintaining cellular polarity, and contribution to cellular transport, growth and differentiation.[207] Among the tight junction proteins, claudins are most implicated and studied in the tumor formation and progression. [208] Differential expression patterns of claudins are tissue specific and may end up in both tumor suppression and promotion. [209] In the case of CLDN1 expression in melanoma patients, it has been demonstrated that in the presence of high PKC, CLDN1 expression will increase and atypical localization of CLDN1 to nucleus or cytoplasm will occur. [210] By this way, not only the amount of CLDN1 in cell membrane lessens but also MMP-2 activation due to increased CLDN1 will be obtained. This will lead a decrease in tight junction integrity and an increase for higher capacity of motility and metastasis in melanoma patients. Further, the same group implicated the particular effect of PKA on trans locating CLDN1 to cytoplasm from nucleus which is a critical in the progression of melanoma. [211] The intermediate localization of

CLDN1 to nucleus has been proposed to be related with non-canonical WNT signaling which leads EMT transition in melanoma cells under PKC dependent manner. [212] On the contrary, Cohn et al. demonstrated that loss CLDN1 expression in metastatic melanoma when compared to benign or primary lesions. [213]

Though there are no published literature about CDX2 gene function in melanoma genesis, CDX2 is related to be transcriptional regulator of CLDN1 in colon cancer with CDX1 and GATA4 in concert with WNT signaling. Overexpression of these transcription factors impact on CLDN1 expression was also searched in renal epithelial and breast cancer and no change has been observed suggesting, these transcription factors have control over up regulation of CLDN1 in colon specific manner. [214]

1.9.3 ERCC1 and LIG3

ERRC1 and LIG3 are genes both participating in DNA repair mechanisms. ERCC1/ XPF heterodimer endonuclease is functional in nucleotide exchange repair (NER) and has roles in interstrand crosslinks repair (ICL) and double strand break repair (DSB)[215]. Platinum drugs used as chemotherapeutics in cancer treatment such as cisplatin cause inter and intra strand crosslinkings.ERCC1/XPF are important for repairing cisplatin caused DNA damage, making them critical for resistance. It has been demonstrated that melanoma cell lines may have intrinsic and acquired resistance to cisplatin. The former may result from high expression pattern of ERCC1 and XPF in melanoma cells occurred spontaneously. The latter may arise from cisplatin induced increase of these two genes via MAPK pathway with minor contribution of low expreesion DUSP6, resulting in continuum ERK activation and high ERCC1 expression in particular.[216]

LIG3, which is vertebrate restricted, along with XRRC1 have roles in short patch base excision repair (BER), single strand break repair, NER in dividing cells and DNA ligase IV dependent non homologous end joining pathway as back up. [217] No direct linking of

expression of LG3 to any cancer type has been proposed including melanoma. Though contribution of LIG3 SNPs to increased risk for cancer types such as breast[218], pancreatic[219], young on set lung[220], colorectal[221], non-small cell lung cancer[222] have been searched. Only cancer which one LIG3 SNP is linked significantly with altered risk of cancer was found to be pancreatic cancer

.

2 OBJECTIVES AND RATIONALES

Due to lack of diagnostic markers, curable malignant melanoma in the early stages escapes from detection and thus results in uncontrollable disease. Although surgery is the only treatment of stage I melanoma upon early detection, there are various post surgical treatment options in the following stages. Yet, still choosing the appropriate post surgical adjuvant therapy for a patient is an issue, considering absence of informative prognostic biomarkers.

Melanoma, being highly immunogenic, has been a target for immunologists and oncologist to elaminate cancer. Autologous vaccination of malignant melanoma patients subsequent to surgery has been one of the approaches trialed so far. Still, to determine the patients who will gain favour from this treatment is critical.

Therefore we aimed to identify prognostic marker/s to define a subpopulation of malignant melanoma patients who could be candidates for autologous vaccination post surgically.

In this study, we started with a short list of potential prognostic biomarkers identified using USAT analysis, a unique bioinformatics tool developed in our laboratory. USAT is composed of statistical packages (cox propotional hazards regression model, maximally selected rank statistics, log-rank test) found in R which seeks correlation of gene expression profiles and survival. In addition to EMT markers, DNA repair genes, EGFR ligand and AKAP gene in our list; cancer testis genes, SSX1 and MAGEA1 are the ones that gain our full attention. CT genes are known to be highly immunogenic; however, increased expressions of them are related with poor prognosis in melanoma. [223, 224] So, we hypothesized that autologous vaccination may favour patients with expressing high CT genes by sensitizing their immune systems with these antigens and overcome the immunesuppression. With the other selected prospective biomarker genes, I tried to demonstrate a possible impact of

autologous vaccination in a specific subset of malignant melanoma patients expressing those genes.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 General Laboratory Regeants

A variaty of regaeants and kits branded from SIGMA-ALDRICH (St.Louis, MO, USA), Ambion (Carlsbad, CA, U.S.), Invitrogen (Carlsbad, CA, USA) ,Cayman Chemical (Ann Arbor, MI, USA), Applied Biosystems (Carlsbad, CA, U.S.) used in this thesis to accomplish the project. Chemical Reagents such as Ethanol, Choloroform, Isopropanol were acquired from SIGMA-ALDRICH (St.Louis, MO, USA). RNAse Zap, Nuclease Free Water (not DEPC-Treated) were aquired from Ambion (Carlsbad, CA, U.S.). DMSO (Dimethyl sulfoxide) was acquired from AppliChem (Darmstadt, Germany). Tri-Reagent was aquired from Ambion (Carlsbad, CA, USA).Qubit™ RNA BR Assay Kit and DNA-*free*™ Kit were acquired from Invitrogen (Carlsbad, CA, USA). Agilent RNA 6000 Nano Kit was acquired from Agilent Technologies (Santa Clara, CA, USA)**.** RevertAid First Strand cDNA Synthesis Kit was acquired from Thermo Scientific (Waltham, MA, USA). One*Taq* Quick-Load 2X Master Mix with Standard Buffer was acquired from New England Biolabs (Ipswich, MA, USA). Taqman Gene Expression Assays and TaqMan Universal PCR Master Mix were acquired from Applied Biosystems (Carlsbad, CA, U.S.) Gefitinib was acquired from Cayman Chemnicals (Ann Arbor, MI, USA).

3.1.2 Cell Culture Materials and Reagents

Materials used in Cell Culture procedures; serological pipettes, cell culture scrapers and flasks were purchased from Costar Corning Incorporated (NY, USA), Sarstedt (Nümbrecht, Germany) and Greiner Bio One (Monroe, NC, USA), resprectively. SFCA (Surfactant-Free Cellulose Acetate) membrane Serum filter unit were purchased from Thermo Fisher – Nalgene (Waltham, MA, USA). Millex-FG Syringe Filter Unit was purchased from Merck MilliPore (Billerica, MA, USA). Rosswell Park Memorial Institute Medium (RPMI) was purchased from both Biochrom (Berlin, Germany) and Lonza (Basel, Switzerland). Cell culture solutions; Trypsin-EDTA and Fetal Bovine Serum (FBS) were purchased from SIGMA-ALDRICH (St.Louis, MO, USA) and GIBCO (Carlsbad, CA, USA) as in order. L-Glutamine and Pencilin –Streptomycin were purchased from HyClone (Rockford, USA).

3.1.3 Gel Electrophoresis, Nanodrop, Qubit Fluorimeter, Agilent Bioanalyzer

Gel Electrophoresis tank and power supply were bought from Hoefer Inc (Holliston, MA). NanoDrop (ThermoScientific, Wilmington, USA) was used to asses the quantity and purity of the RNA samples.Further, Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) was used to determine RNA quantity in a more precise manner. Bioanalyzer 2100 instrument (Agilent Technologies, Santa Clara, CA, USA) was used to qualify the RNA integrity of the samples.

3.1.4 Solutions and Media

3.1.5 General Solutions

Ethidium Bromide In order to obtain 100 ml Agarose Gel, 5 µl Ethidium Bromide is used from 10 mg/ ml stock solution with a final concentraion of 1X.

50X Tris-Acetate-EDTA (TAE) 242 gr of Tris base; 37.2 g of Tritiplex III (EDTA); 57.1 ml of glacial acetic acid, with 1 L ddH2O left to dissolve overnight and autoclaved.

3.1.5.1 Cell Culture Solutions and Media

RPMI Complete Medium 10% or %20 FBS, 1% L-Glutamine and 1% Penicilin-Streptomycin of the total media amount were filtered with 0.2µm syringe filter and added to the media. Complete Media stored at 4 ºC.

Freezing Medium Freezing medium was obtained from FBS and DMSO with 9:1 ratio at the final amount.

10X Phosphate Buffered Solution 80 gr of NaCl; 2 gr of KCl; 2.4 gr of KH_2PO_4, and 14.4 gr of Na_2HPO_4 were dissolved in 1 L of ddH_2O. 10X PBS buffer was diluted to 1X PBS buffer by adding 450ml of ddH_2O to 50 ml of 10X PBS. 1X solution was autoclaved and filtered with serum filter unit before use.

0.25% Trypsin-EDTA (1X), Phenol Red Acquired from SIGMA-ALDRICH (St.Louis, MO, USA)

L-Glutamine Acquired from HyClone (Rockford, USA).

Fetal Bovine Serum (FBS) Acquired from GIBCO (Carlsbad, CA, USA)

Penicilin-Streptomycin Acquired from HyClone (Rockford, USA).

3.2 METHODS

3.2.1 Cell Culture

3.2.1.1 Origin of the Cell Lines Used in this Research

36 melanoma cell lines derived from autologous vaccinated cutaneous malignant melanoma patients at Hadassah Hebrew University Hospital, Jerusalem, Israel (M1, M133, M154, M193, M202, M2025, M217, M299, M307, M336, M51, M77, M13, M139, M144, M145, M187, M189, M207, M21, M221, M24, M241, M332, M34, M346, M369, M371, M425, M433, M465, M517, M7, M84, SHM21.5, SHM20A2) and kindly provided by Dr.

Michael Lotem (Jerusalem, Israel). 8 Cell lines (M154, M202, M51, M13, M369, M371, SHM21.5, and M202) were lost due to contamination.

3.2.1.2 Thawing the Cell Lines

The Cryotubes of the cell lines filled with cell culture stocks of the relevant cells were taken from nitrogen tank. Each cell line was thawn by hand and immediately the content of the cryotubes transferred to the 15ml falcon tubes containing 5 ml medium at room temprature. Each tube was centrifuged at 1500 rpm for 3 min. The supernatant in each tube was aspirated and 2 ml fresh media was added to resuspend the cells. The cells were seeded on 25 mm culture flasks containing 4 ml media used during resuspension (alternatively, 75mm culture flasks containing 10 ml media can be used based on the pellet amount). Then, each flask was placed in incubator. The cells with low amounts of pellets compared to others after centrifugation and a history with slow growth rate, were cultured with media supplemented 20% FBS due to thawing. When the attachment and the recovery of the thawn cells were accomplished, media was changed directly to 10% FBS enriched media.

3.2.1.3 Maintenance of the Cell Lines

Each cell line was cultured in 1640 –RPMI media (BioChrom) enriched with either %10 or 20% Fetal Bovine Serum (Gibco), 1% L-Glutamine (HyClone), 1% penicillin and streptomcyn (HyClone). All cell lines were incubated at 37 C°, with 5% CO2. They were supplemented with new media as often as they needed during daily regular controls.

3.2.1.4 Passage of the Cell Lines

Each cell line was seeded in to new flask when they reached the optimum confluency (70-80%). First, the media in each flask was aspirated.5 ml PBS was used to wash the cell lines in 75mm flask (1.5ml for 25 mm flask). Following removal of the PBS, 1.5 ml Trypsin-EDTA was added to each flask and placed into incubator for 1-2min. The detached cell were resuspended with 5ml media and transferred in to 15ml falcon tubes. Each tube was

centrifuged at 1500 rpm for 3 minutes. The supernatant was removed and the pellet in each tube was resuspended with 2 ml media and seeded in to new flask containing 10ml media used in resuspension.

3.2.1.5 Freezing the Cell Lines

The confluence cell lines were subjected to sucking of the media and washing with 5ml PBS, and trypsinization with 2 ml Trypsin-EDTA respectively. After the removal of the supernatant containing media with trypsin-EDTA, the pellet of each cell line was resuspended in freezing media. The amount of freezing media was directly related to the confluency. Each cryotubes was filled with 1ml freezing media- cell suspension and immediately put into -20 °C for 1-2 hours and transferred to -80°C for overnight incubation. Then the cryotubes were placed into nitrogen tank for long period storage.

3.2.2 RNA Isolation

Cell lines with an 80% confluency ratio treated with trypsin-EDTA and placed in incubator for 1-2min. Effect of tyrpsin neutralized with addition of fresh media to the flasks. Resuspension was taken into 15ml falcon tubes and cetrifuged at1500rpm for 3 min. The pellet of each cell line homogenized with 1 ml of Tri-Reagent (Molecular Research Center) and centrifuged at 13000rpm for 15min at 4 C° subsequent to incubation in room temprature(RT) for 5 min. Choloroform was added to each sample, incubated for 10min at RT and centrifuged (13000rpm, 15min, 4C°). RNA containing upper phases transferred into new 1.5 ml eppendorfs. Centrifugations (13000rpm, 15min, 4C°) in between and after the addition of isopronal and alcohol, aided to precipitate and wash RNAs. In each step after centrifugation pellets were spotted and supernatans were carefully removed. Following 5 min air dry after alcohol washing, each sample dissolved in adequate amount of Nuclease Free Water (Ambion Carlsbad, CA, USA) depending on the pellet amount. First assesment of quantity and purity of the samples were performed by a Nanodrop spectrophotometer ND-

1000 (NanoDrop Technologies; Wilmington, DE). Samples were stored at - 80 C° for further experiments.

3.2.3 DNase Treatment

A possible contamination of DNA during RNA isolation was elimanted by using DNA-Free kit (Ambion, Carlsbad, CA, USA). Kit consists of rDNAse I, 10X DNAse Buffer, 10 X DNAse Inactivation reagents. 1µl rDNAse I for routine DNAse treatment (samples containing 200ng/ µl RNA or less) and 5µl DNAse Buffer with a final 1X concentration in 50µl reaction were added to each sample with maximum amount of 10µg RNA. Process ensued with an incubation period for 30min at 37 C°. 5µl DNAse Inactivation reagent was added at a final 1X concentration to each sample and pippetted for 2 min. All samples were centrifuged at 10000 rpm for 2 min. Supernatants consisted of DNA free RNAs were transferred into new eppendorf tubes.

3.2.4 Quality and Quantity assesment of the samples

3.2.4.1 Qubit Quantification

The quantity of RNA samples were measured with Qubit Fluorometer (Invitrogen) which is when compared to NanoDrop, more precise because of the direct binding of fluorescents to the nucleic acid of interest. Quant-it Reagent, buffer and standards were supplied with the Quant-it Kit (Invitrogen, Carlsbad, CA, USA). Working Solution was prepared by addition of 1 µl Quant-it reagent to 199 µl Quant-it buffers for each sample. First for the standards samples, 10 µl of each added to 190 µl working solution, incubated at RT for 2 min and measured in order to calibrate the instrument. Later, 2µl of each sample mixed with 198 µl working solution, incubated at RT for 2 min and measured with Qubit Flurometer.

3.2.4.2 Agilent Bioanalyzer Qualification

The integrity of RNA samples were determined by using Bioanalyzer 2100 instrument (Agilent Technologies, Santa Clara, CA, USA). Components of Agilent RNA 600 Nano Kit

are as follows; Agilent RNA 6000 Nano Chips, 2 Electrode Cleaners, RNA 6000 NanoLadder, RNA 6000 Nano Dye Concentrate, RNA 6000 Nano Marker, RNA 6000 Nano Gel Matrix. RNAse zap and nuclease free water (Ambion, Carlsbad, CA, U.S.) were loaded onto relevant chips and placed in the instrument in order to decontaminate and clean the electrodes of the instrument. First 550 µl of gel matrix was pippetted into a spin filter, centrifuged at 3500 rpm for 10 min at RT and aliquoted. 65 µl of filtered gel mixed with 1 µl gel dye and centrifuged at 10000 rpm for 10 min at RT. 9 µl of gel dye mix pipetted into corresponding wells on the chip. After pipetting the first well, it was diffused to the chip by the help of a pressure provided by a syringe. RNA marker and the ladder pipetted in to chip, first one to sample wells and ladder well and the second one only to the ladder well, resprectively. 1 µl of each sample loaded to each well on the chip and chip was replaced into the instrument. 2100 Expert Software (Agilent Technologies, Santa Clara, CA, USA) determined a RIN (RNA integrity number) with in a range 1 to 10, 10 meaning the intact RNA. All RNA preparations have a RIN (RNA integrity number) in a range between 8.5-10, with two exceptions (M34, RIN: 7.4; M77, RIN: 7.7) where base RIN is proposed to be 5.1 for further qRT-PCR experiments.

3.2.5 cDNA synthesis

1350 ng of total RNA from each sample except M193, M217, M207, M425 with an amount of 900 ng and 240ng for M34 due to low concentrations, were used to synthesize first strand cDNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) in a total amount of 60 µl in separate 2 reactions which are simultaneously prepared. RNAs were incubated with appropriate amount of random hexamer and water at 65 C° for 5 min. Then a mixture containing 5X Reaction Buffer, Ribolock RNase Inhibitor, 10 mMdNTP Mix, M-MuLV Reverse Transcriptase added to each sample (RT+ samples). For the reverse transcriptase negative (RT-) controls; 1 µl of RNA of randomly

selected samples were taken, incubated with random hexamer and water under the same conditions as RT+ samples. Further same mixture without M-MuLV Reverse Transcriptase was added to obtain 20 µl reaction. Conditions for cDNA syntesis were 25°C for 10 min, 42°C for 60 min and 70°C for 5 min.

3.2.6 EndPoint Polymerase Chain Reaction

EndPoint PCR was performed to confirm that cDNAs were free of genomic DNA. 18S RNA primers were used to identify gDNA contamination since it consists of only 1 exon and contamination would be observed in the gel electrophrases of the RT- samples. Primer sequence used for 18S RNA was as follows; Forward 5 ’CGTGCATTTATCAGATCAAAACCAACC-3’ and Reverse 5’-ATGGTAGGCACGGCGACTAC-3’. 1 µl of RT+ and RT- samples and a mixture of OneTaq™ Quick Load® Master Mix (New England BioLabs), reverse and forward primers and nuclease free water were added to accomplish 25 µl reactions each. The process consisted of 30 cycle; 30sec. of denaturation at 94 C°, 30sec of annealing at 60 C° and 30sec of extension at 72 C° with an initial denaturation step for 5 min at 94 C° and final extensin for 5 min at 72 C°.

3.2.7 Agarose Gel Electrophoresis

To eliminate the possibility of gDNA contamination EndPoint PCR products were visiualized. 100 ml 1X TAE was mixed with 1 gr Agarose in order to obtain 1% agarose gel. The mixture was boiled in the microwave for a complete dissolution of agarose in 1XTAE. Ethidium Bromide of 5 µl was added subsequent after the agarose gel cooled down. The gel was poured to appropriate tank and 10 µl of ready to load samples were loaded into gel. Gene Ruler 50bp DNA ladder (#SM0373 Thermo Scientific Waltham, MA, USA), ready to use, was also loaded into gel to visiualize and confirm the size of 18S RNA product. The gel was run for 30 min at 120V prior to visiualization.

3.2.8 Quantitative Real time Polymerase Chain Reaction (qRT-PCR)

Applied Biosystems 7500 Instrument (Applied Biosystems, Carlsbad, CA, and U.S.A) was used to perform qRT-PCR experiments. Each sample was run in triplicates for each target gene.A triplicate no template control were also prepared for each gene. Each reaction set as 20 µl, including undiluted 2 µl cDNA of relevant sample, 10 µl 1X TaqMan Universal PCR Master Mix, 1 µl Taqman Gene Expression Assay (Applied Biosystems) primer-probes and 7 µl nuclease free water. Applied Biosystems pre-made and custom primer probes designed with NCBI Primer –Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) were used in reactions. Reaction conditions were as follows; holding stage to prevent the reamplification of carryover PCR products by activating AmpErase UNG enzyme at 50 C° for 2 min, holding at 95 C° for 10 min for the inactivation of the former, and 45 cycles of 15 sec.at 95 C° and 1 min at 60 C°. GAPDH was used for normalization as an endogeneous reference control. Data analysis was calculated manually using ΔΔ CT Method.[225] Primer probe Assay IDs - product catalog numbers and custom designed primer probe sequances are listed in Table 3.2 and Table 3.3, respectively.

Table 3.1 Custom Design Primer-Probe List

Gene	Exon Spanning	Sequence (5'-3')	Tm °C
TGFA-F	E5-6	AAGAGACGGACTCCTGTTCACCTA	59.3
TGFA-R	E5-6	TGTGTTGGGCAGAATTCTGTTG	59.6
TGFA-P	E5-6	TTGGTGTCTGAGTCCAC	69
ERCC1-F	E9-10	GTGGGCACCTTCAGCTTTCT	58
ERCC1-R	E9-10	CCGCCACTGAATTCAGAGTCT	58
ERCC1-P	E9-10	CAGACTACACAGGCTGC	70

AKAP13-F	E35-36	CAGCAGAAGAAGGGCACATACC	59.7
AKAP13-R	E35-36	CCTCATCAGCTTGGTATGTGGAT	59.0
AKAP13-P	E35-36	ACAGCTTGAGAGGGAA	68

Table3.2 Pre -Design Primer-Probe List

Genes	**Assay ID**	**Catalog No**	**Probe Dye**
AKAP13	Hs00180747_m1	4331182	FAM
TGFA	Hs00608187_m1	4331182	FAM
ERCC1	Hs01012158_m1	4331182	FAM
CDX2	Hs01078080_m1	4331182	FAM
CLDN1	Hs00221623_m1	4331182	FAM
MAGEA1	Hs00607097_m1	4331182	FAM
SSX1	Hs00846692_s1	4331182	FAM
MICA	Hs00792195_m1	4331182	FAM
LIG3	Hs00242692_m1	4331182	FAM
EWSR1	Hs01580532_g1	4331182	FAM
GAPDH	Hs00205046_m1	4331182	FAM

3.2.9 Software Programs Used in This Research

3.2.9.1 NCBI (National Center Biotechnology Information)

mRNA sequence of all variants of all genes validated by qRT-PCR in this research (AKAP13, TGFα, ERCC1, CDX2, CLDN1, MAGEA1, SSX1, MICA, LIG3, EWSR1) were reached from NCBI web site in FASTA format.

Table 3.3 Selected 10 Genes and Corresponding Links to NCBI

Genes	Link
AKAP13	http://www.ncbi.nlm.nih.gov/gene/11214
TGFα	http://www.ncbi.nlm.nih.gov/gene/7039
ERCC1	http://www.ncbi.nlm.nih.gov/gene/2067
CDX2	http://www.ncbi.nlm.nih.gov/gene/1045
CLDN1	http://www.ncbi.nlm.nih.gov/gene/9076
MAGEA1	http://www.ncbi.nlm.nih.gov/gene/4100
SSX1	http://www.ncbi.nlm.nih.gov/gene/6756
MICA	http://www.ncbi.nlm.nih.gov/gene/100507436
LIG3	http://www.ncbi.nlm.nih.gov/gene/3980
EWSR1	http://www.ncbi.nlm.nih.gov/gene/2130

3.2.9.2 USCS (University of California, Santa Cruz) Blat Search Genome

USCS Blat search tool was used to determine all cDNA sequences of each variant of each gene in an exon depended manner.

(http://genome.ucsc.edu/cgi-bin/hgBlat?command=start)

3.2.9.3 Affymetrix NetAffyx Analysis Center

Affymetrix NetAffyx Analysis Center, Batch Query was used to download the sequence of all probes of genes which were validated in this research (AKAP13, TGFA, ERCC1, KIR3DX1, CDX2, CLDN1, MAGEA1, SSX1, SSX4, MICA, LIG3, and EWSR1).

3.2.9.4 Cluster 3.0 and Treeview

Hierarchical clustering and heatmap of CT genes and TGFα in USAT analysis concerning Luminex data and Luminex samples were performed with cluster 3.0 and

treeview. To plot the trees, Euclidean distances were calculated for both genes and samples and average linkage method was used. In each heatmap, samples are represented according to their gene expression in Luminex data for the selected genes with red, black and green corresponding to high, intermediate and low expression, respectively.

3.2.9.5 GraphPad Prism 5.0

GraphPad Prism 5.0 developed by GraphPad Software Inc (La Jolla, CA, USA) was used to perform Pearson r correlation and survival analysis of the datas and subsequently to draw figures. All values considering Pearson r correlation coefficient values, linear regression p values and Log-rank p values were obtained using this software.

3.2.10 Algorithms used in this Research

3.2.10.1 Adjustment of qRT-PCR values into the Luminex Gene Expression Range

qRT-PCR results of samples for each gene were converted into the numbers corresponding to gene expression values of samples for that gene. Following formula was used for conversion;

NewValue = (((OldValue - OldMin) * (NewMax - NewMin)) / (OldMax - OldMin)) + NewMin

Since luminex gene expression values are log 2 transformed, all qRT-PCR results of all genes concerning every sample were first transformed in to log 2. Subsequently, the minimum qRT-PCR value for a particular gene was subtracted from a qRT-PCR value of a sample for that gene and multiplied with new range to old range ratio. The minimum value of the new range was added to the outcome, leading to adjusted value of that particular sample in the new range. This equation was used to calculate the adjusted values of qRT-PCR result of all samples for all genes.

3.2.10.2 Sum of Absolute Rank Difference (SARD) Analysis

Sum of absolute rank difference analysis algorithm was developed in our laboratory (developer, Murat işbilen). It's an algorithm used for estimating how differentiated a primary cell culture is from the day luminex gene expression profile was obtained until the validation experiments were performed. Principles of algorithm are grounded on the rankings of samples in the corresponding luminex gene expression and qRT-PCR log 2 transformed adjusted expression ranges for each gene underinvestigation. Rank difference of each sample is calculated between these rankings for each gene. Subsequently, absolute sum of each rank difference and sum of absolute rank difference were calculated for each sample concerning all genes underinvestigation. Samples were sorted from lowest to highest based on their sum of absolute rank differences. The sample with lowest sum of absolute rank difference was assumed to be least differentiated sample among all samples. Pearson correlation and linear regression p values were calculated beginning with the first 3 samples and kept calculated with adding 1 sample at a time in SARD increasing order of samples. Numbers of significant genes were identified for each sample, assuming them as the last sample included for that particular analysis. A subset of samples was defined to be undifferentiated where the ultimate number of validated genes obtained.

4 RESULTS

4.1 Selection of Genes to be validated as Prognostic Biomarkers

To determine the genes which are going to be our subject in this project, we analyzed the normalized Luminex gene expression data of the autologous vaccinated 36 malignant melanoma samples with USAT (developer Murat İşbilen). USAT is composed of biostatistic R packages: CoxPh, Maxstat and Log-rank, and searches for a correlation between survival data and gene expression values.[226, 227] Out of 121 significant genes we identified by USAT analysis, we selected10 genes based on their functions and possible contribution to melanomagenesis. Names of selected gene, corresponding affymetrix probeset IDs, hazard ratio (HR) values, Cox Proportional Hazard Regression (CoxPH) p values, Expression range of the genes, Maximally Selected Rank Statistics (Maxstat) cut-off values, Maxstat p values, Log-Rank p values and median survival values for high and low expression groups are demonstrated in Table 4.1.

Table 4.1: Genes Significant in USAT Analyses and Corresponding Statistics

Gene Name	Affymetrix Probeset	HR	CoxPH p Value	Expression Range	Maxstat Cut-Point	Maxstat p Value	LogRank p Value	MS for Low Exp. (Months)	MS for High Exp. (Months)
TGFA	205016_at	0.6651	0.0185	2.4403-9.2642	4.0735	0.0111	3.34E-06	15.35	NA
CDX2	206387_at	0.5335	0.0076	5.456-7.2806	6.0212	0.0176	0.0009	25.70	NA
CLDN1	218182_s_at	0.5625	0.0051	1.7968-6.3888	3.3640	0.0176	0.0009	25.70	NA
SSX1	206626_x_at	0.6304	0.0432	2.448-5.9426	3.2411	0.0353	0.0012	27.97	NA
MICA	205904_at	0.4962	0.0153	6.1012-8.7776	7.7953	0.0183	0.0013	24.32	NA
MAGEA1	207325_x_at	0.6877	0.0278	4.3814-9.4401	5.5536	0.0422	0.0014	22.06	NA
ERCC1	203719_at	0.4150	0.0196	6.5139-8.9225	8.0085	0.0071	0.0017	27.97	NA
AKAP13	222024_s_at	1.9880	0.0153	6.3388-8.5806	7.2267	0.0481	0.0036	NA	27.97
LIG3	207348_s_at	0.5350	0.0495	5.0414-7.2648	6.1751	0.0175	0.0062	28.66	NA
AKAP13	221718_s_at	1.9394	0.0186	7.3045-9.8984	7.6655	0.0499	0.0086	NA	28.66
EWSR1	210012_s_at	0.5026	0.0301	5.6571-6.8475	6.4669	0.0458	0.0154	32.61	NA

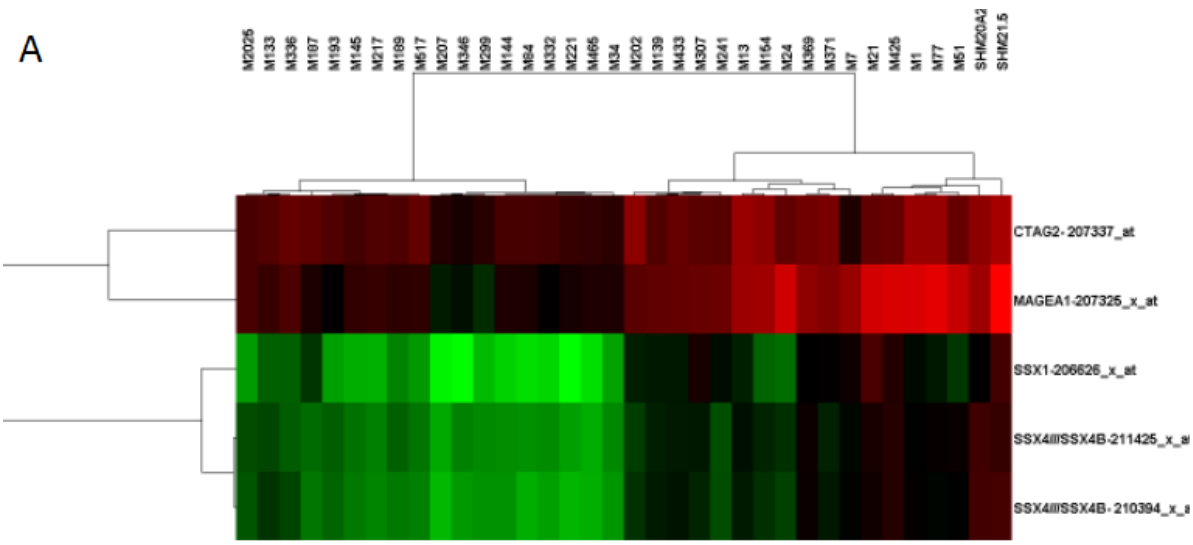
In addition, cancer testis antigens which were present in the out put captured our attention and we decided to study them further. . CT genes that were significantly associated with prognosis by USAT in this analysis are shown in Table 4.2.

Table 4.2: CT Genes Significant in USAT Analyses and Corresponding Statistics

Gene Name	Affymetrix Probeset	HR	CoxPH p Value	Expression Range	Maxstat Cut-Point	Maxstat p Value	LogRank p Value	MS for Low Exp. (Months)	MS for High Exp. (Months)
CTAG2	207337_at	0.5792	0.0151	5.2398-7.4981	5.8949	0.0137	0.0001	16.14	NA
MAGEA1	207325_x_at	0.6877	0.0278	4.3814-9.4401	5.5536	0.0422	0.0014	22.06	NA
SSX1	206626_x_at	0.6304	0.0432	2.4428-5.9426	3.2411	0.0353	0.0012	27.97	NA
SSX4 /// SSX4B	211425_x_at	0.5821	0.0176	3.1633-5.7720	3.8465	0.0079	0.0001	22.06	NA
SSX4 /// SSX4B	210394_x_at	0.5821	0.0176	3.0679-5.8641	3.6162	0.0077	0.0001	22.06	NA

4.1.1 *In silico* based Analysis of CT genes and TGFα as Prognostic Markers

Independent of USAT analysis, the distribution of these significant CT genes among malignant melanoma patients was assessed by performing hierarchical clustering and creating a heatmap image with Cluster 3 and Treeview software (Figure 4.1A). For its ranking in USAT as being the most significant gene, TGFα was chosen for further evaluation in terms of separating the patients in concert with CT genes (Figure 4.1B).



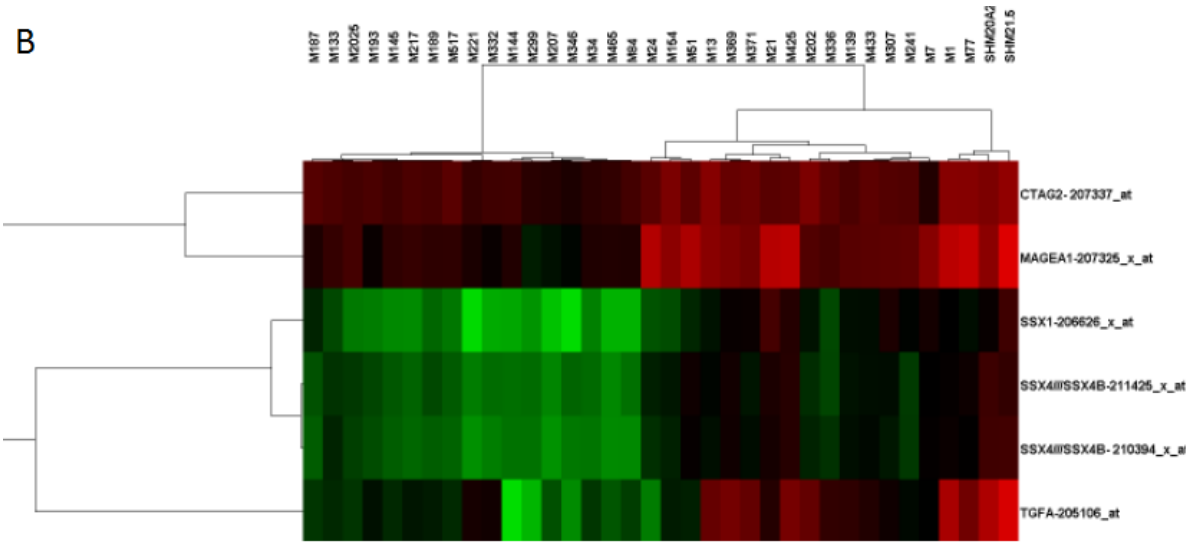


Figure 4.1 Heatmap and hierarchical clustering of significant CT genes and TGFα of Luminex data and Luminex samples. In the heatmap; red, black and green squares show that the corresponding sample has high, moderate and low expression for the corresponding probeset respectively. According to the gene tree, there are two major clusters. (**A**) Heatmap of only CT genes (**B)** Heatmap of CT genes with TGFα.

According to Figure 4.1 A, it can be interpreted that samples are discriminated based on their Luminex data in to 2 main clusters. SSX family genes and MAGEA1 – CTAG2 are a subclassification for genes based on their expression range among samples. In concert with CT genes clusters, in figure 4.1B, it's been demonstrated that TGFα clusters the same groups of samples except 1 sample M336.Expression profile of TGFα resembles both CTAG2-MAGEA1 in high expression and SSX family genes in low expression patterns among samples.

Survival analysis of these CT genes and TGFα based on heatmap distribution (Figure 4.2) and Maxstat values set by USAT analysis (Figure 4.3) was performed to find out a possible relation with the prognosis of samples *in silico*.

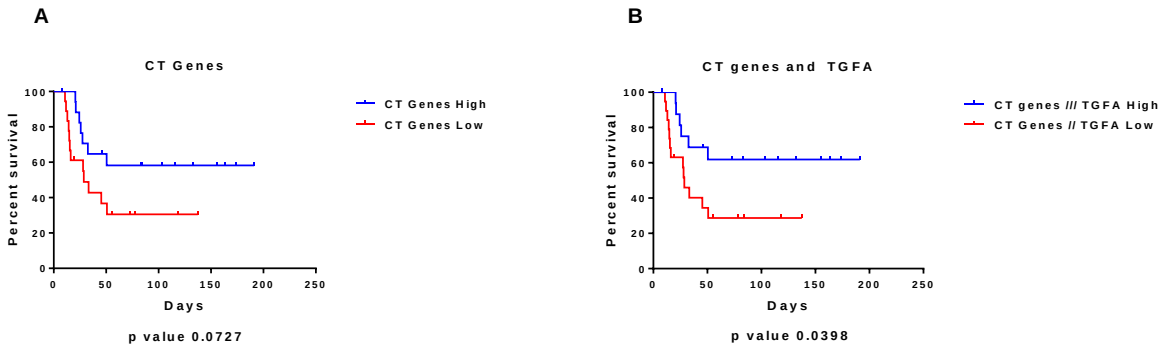


Figure 4.2 Kaplan Meier curves of Heatmap data in Figure 4.1. Log –Rank p value is calculated for each gene, present in the figure. Blue and red lines are corresponding to survival duration of samples having higher and lower expression of interested genes based on heatmap data, respectively. X axis indicates days and Y axis corresponds to survival percentage of samples when analysed based on days. Every vertical line in each colored line is an indicator of cencored sample.(A) Kaplan Meier curve of samples based on figure 4.1 A considering SSX1-MAGEA1 genes (B) Kaplan Meier curve of samples based on figure 4.1B considering SSX1-MAGEA1-TGFα.

Considering heatmap distribution of samples in terms of CT genes alone (p value, 0.0727) and combined with TGFα (p value, 0.0398) suggests a possible impact of CT genes on prognosis only when combined with TGFα. How ever, TGFα and each CT gene were found to contribute the prognosis in USAT, independently.

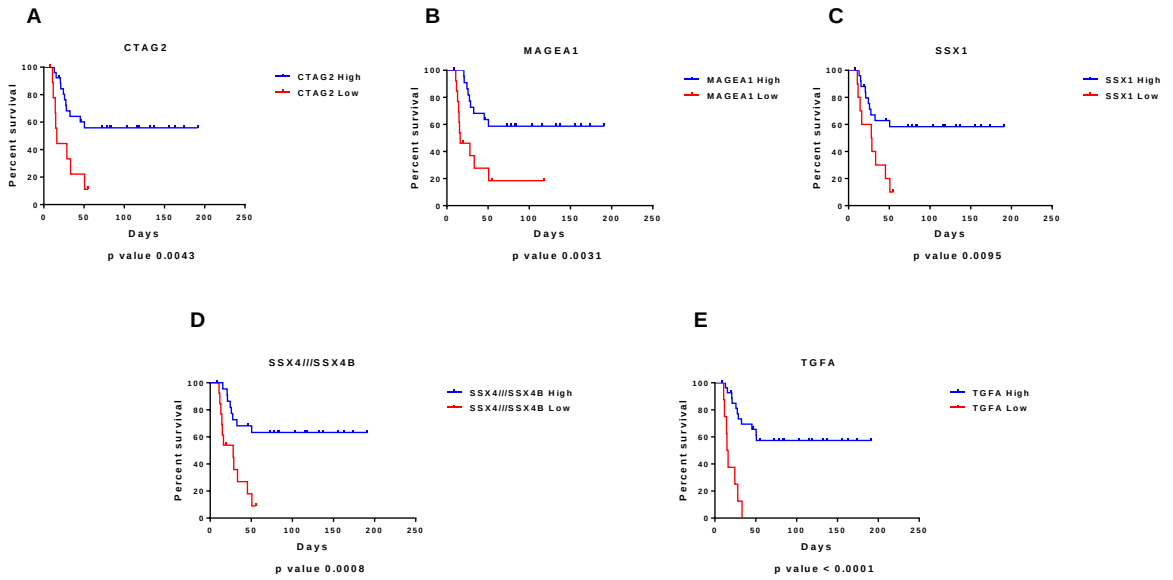


Figure 4.3 Kaplan Meier curves of CT genes and TGFα based on their Luminex Expression profile and Maxstat cutpoint calculated using Luminex dependent-USAT analysis. Log –Rank p value is calculated for each gene, present in the figure. Blue and red lines are corresponding to survival duration of samples having higher and lower expression of interested genes, respectively. X axis indicates days and Y axis corresponds to survival percentage of samples when analysed based on days. Every vertical line in each colored line is an indicator of cencored sample. Genes demonstrated in the figure are; (A) CTAG2 (B) MAGEA1 (C) SSX1 (D) SSX4///SSX4B (E) TGFα.

Based on these *in silico* results concerning not only the selected 10 genes but also the relation of CT genes with TGFα, we continued with in vitro confirmation of these genes on relative primary cell lines.

4.2 Validation of First 10 sample of MM patients with qRT-PCR

First round qRT-PCR experiments with the selected genes were performed with the Taqman Primer Probes (details of Primer Probes were given in Methods part). However, out of 11 probesets corresponding 10 genes, only 3 of them including 2 probesets of AKAP13 (p value 0.0299, 0.0124) and LIG3 (p value, 0.0485) were concordant with the Luminex data.

With a borderline p value of ERCC1 (p value, 0.0886), rest of the genes were discordant (p value > 0.05) with the Luminex data for 10 samples (Figure 4.4, Supplementary Figure 1).

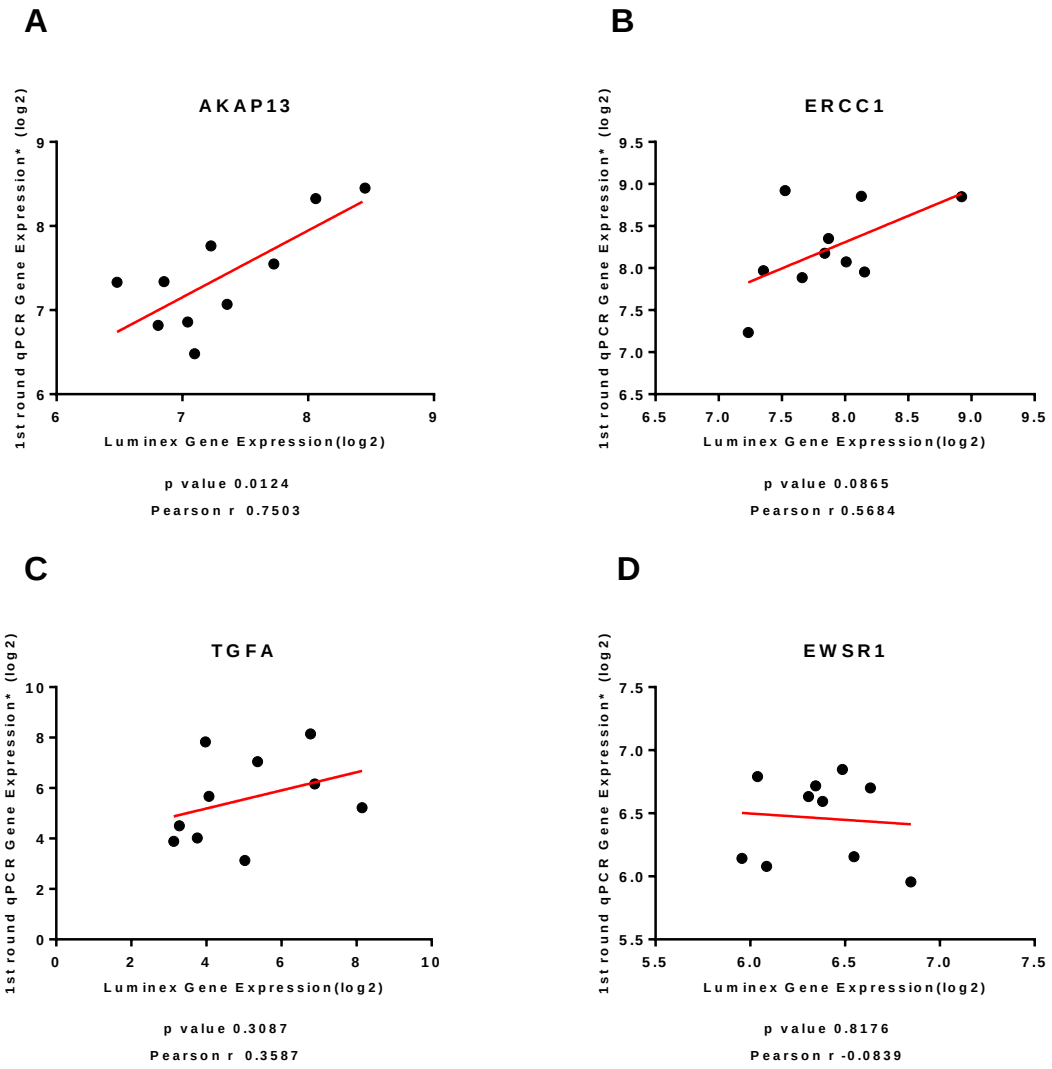


Figure 4.4 Scatter plots of a sample set of selected genes analysed based on their qPCR and Luminex expression values from the 1st round experiments of 1st 10 samples. For each gene, Pearson correlation coefficient and linear regression p value are calculated and present in the figure. Red line corresponds to line of concordance drawn based on Pearson correlation

coefficient. Selected representatives genes are as follows; (A) AKAP13 (B) ERCC1 (C) TGFα (D) EWSR1.

*qPCR expression range is adjusted according to Luminex expression range for each gene

Discorrelation of qRT-PCR experiments results and Luminex data for the 1st 10 samples was further evaluated in 2nd round. None of the genes evaluated in the 2nd round showed concordance with Luminex data either (Figure 4.5, Supplementary Figure 2).

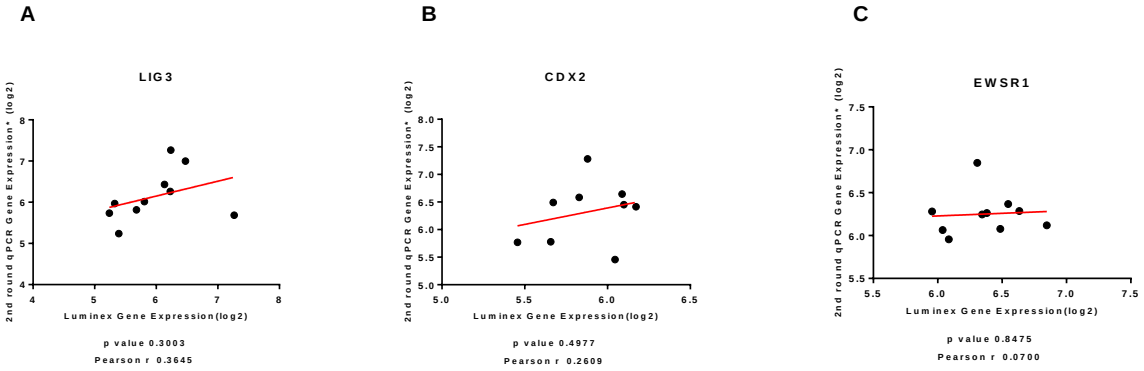


Figure 4.5 Scatter plots of a sample set of selected genes analysed based on their qPCR and Luminex expression values from the 2nd round of 1st 10 samples. For each gene, Pearson correlation coefficient and linear regression p value are calculated and present in the figure. Red line corresponds to line of concordance drawn based on Pearson correlation coefficient. Selected representatives genes are as follows; (A) LIG3 (B) CDX2 (C) EWSR1.

*qPCR expression range is adjusted according to Luminex expression range for each gene

To rule out any possible mistakes than can be originated from experimental procedure in the first 2 rounds of qRT-PCR results of 1st 10 samples Pearson correlation coefficient and linear regression p value were calculated with the results of 2 rounds. Out of 5 common genes detected in both of the rounds, the results of 3 genes demonstrated high correlation with a

Pearson r value greater than 0.95 (Figure 4.6). For the rest 2 genes; LIG3 demonstrated a border line correlation whereas, EWSR1 showed discordant results between rounds (data not shown).

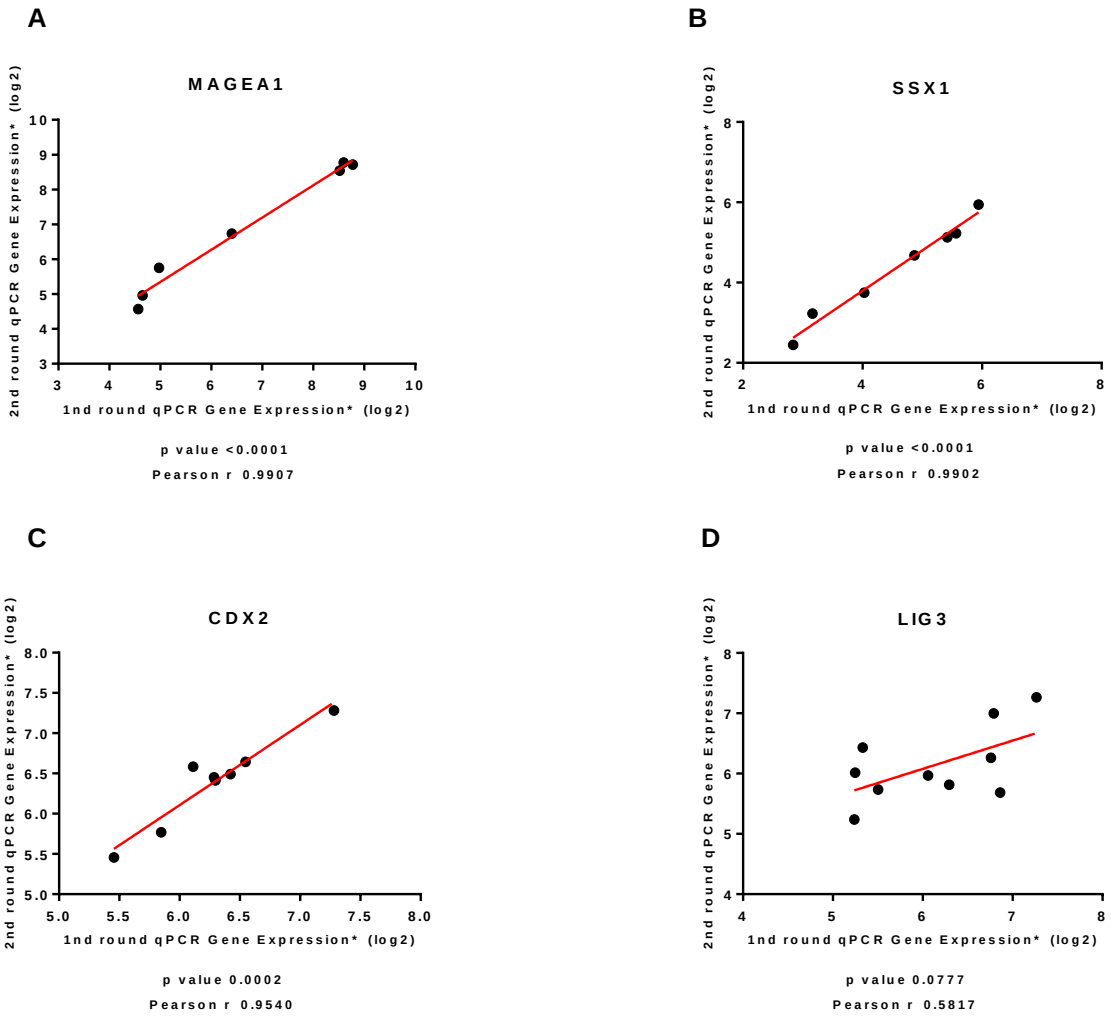


Figure 4.6 Scatter plots of common genes analysed based on their qPCR results from 1st and 2nd round of 1st 10 samples. For each gene Pearson correlation coefficient and linear regression p value are calculated and present in the figure. Red line corresponds to line of concordance drawn based on Pearson correlation coefficient. Genes are: (A) MAGEA1 (B) SSX1 (C) CDX2 (D) LIG3.

*qPCR expression range is adjusted according to Luminex expression range for each gene.

Besides one border line result in LIG3 and an insignificant result in EWSR1 (data not shown), results from both rounds have a significant Pearson r value and p value indicating that discordant results from both rounds for different genes might be independent of experimental error.

4.3 Evaluating effect s of Custom Design and Pre Designed Primer Probes on Validation Experiments

We considered that pre-designed primer probes might not match specific transcripts of the relevant genes which are detected in Luminex platform. To overcome this issue; we selected AKAP13, ERCC1 and TGFα as representatives of significant, border line significant and insignificant genes found in 1st round experiments and specifically designed primers and probes for each gene corresponding to regions interrogated by Affymetrix probesets, which Luminex data is based on. We downloaded all transcript variants of AKAP13, ERCC1 and TGFα from NCBI database and compared them to probeset sequences corresponding to each gene. To detect all transcript variants for each gene, primer probes were designed to common exons in exon spanning fashion, including the sequences amplified by Luminex Platform.

As demonstrated in Figure 4.7, we obtained similar Pearson correlation coefficient r values and p values for AKAP13 and TGFα when using both pre and custom design primer probes. On the other hand, ERRC1 displayed an inversed correlation.

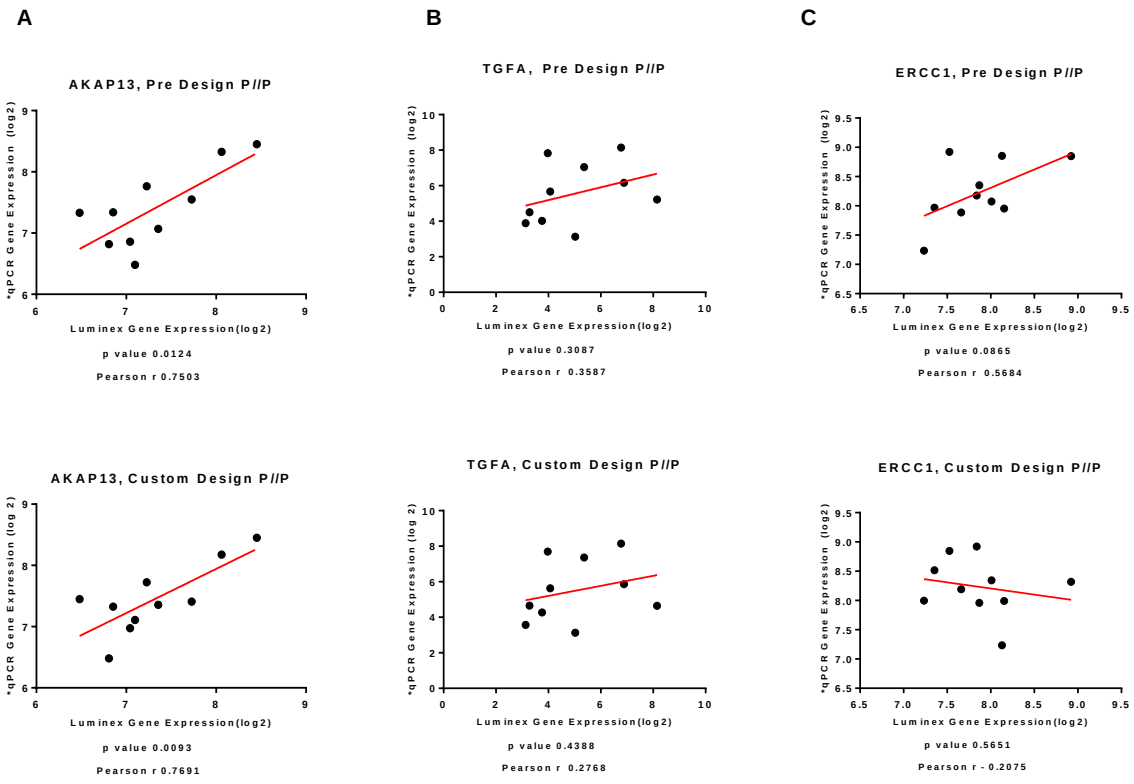


Figure 4.7 Scatter Plots of pre design qPCR results and Luminex vs custom design qPCR results and Luminex expression values of 3 selected genes in the 1st 10 samples. Correlation calculated with pre design qPCR results are in the first line and corresponding correlation of custom design qPCR results with Luminex data are in the second line for (A) AKAP13, (B) TGFα, (C) ERCC1. For each gene, Pearson correlation coefficient and linear regression p value are calculated and present in the figure. Red line corresponds to line of concordance drawn based on Pearson correlation coefficient.

*qPCR expression range is adjusted according to Luminex expression range for each gene.

2 out of 3 selected genes demonstrated a high correlation between results of pre and custom design experiments for the 1st 10 samples (Figure 4.8). Based on these results, we conclude that custom design and pre designed primer probes detect the same transcript with that identified by Luminex analysis. The discordant results from ERCC1 can be based on the low numbers of samples detected in the experimental procedure.

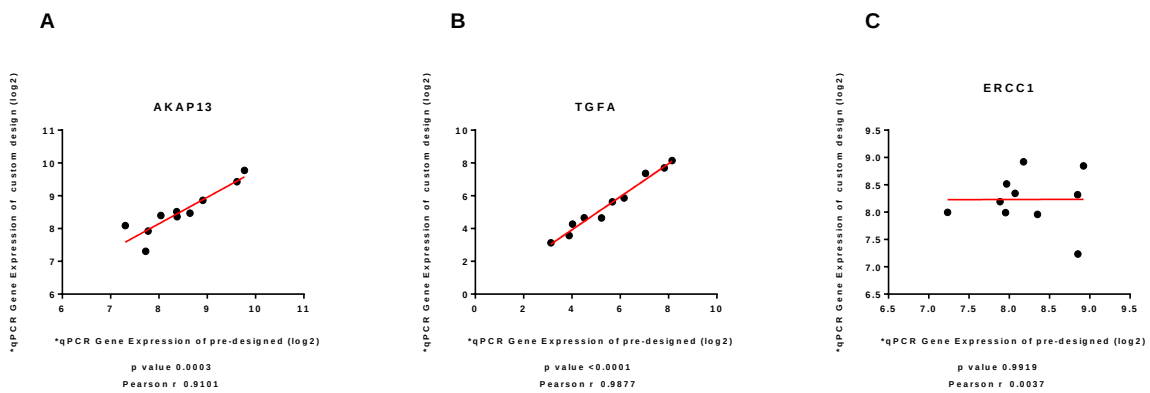


Figure 4.8 Scatter Plots of pre design and custom design qPCR expression values of selected 3 genes in the 1st 10 samples. For each gene, Pearson correlation coefficient and linear regression p value are calculated and present in the figure. Red line corresponds to line of concordance drawn based on Pearson correlation coefficient. Genes are; (A) AKAP13, (B) TGFα, (C) ERCC1.

*qPCR expression range is adjusted according to Luminex expression range for each gene.

4.4 Validation of 28 sample of MM patients with qRT-PCR

We next increased the sample size to 28 samples and repeat tha validation experiments. However, we could only validate 4 out of 10 genes which are LIG3, MAGEA1, TGFα and ERRC1 (Figure 4.9and Supplementary Figure 3).

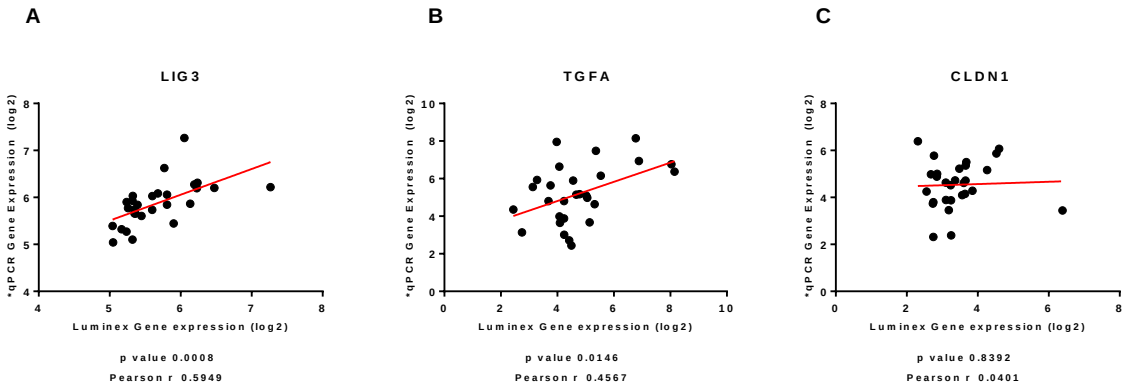


Figure 4.9 Scatter plots of a sample set of selected genes analysed based on their qPCR and Luminex expression values for 28 samples. For each gene, Pearson correlation coefficient and linear regression p value are calculated and present in the figure. Red line corresponds to line of concordance drawn based on Pearson correlation coefficient. Selected representatives genes are as follows; (A) LIG3, (B) TGFA; (C) CLDN1.

*qPCR expression range is adjusted according to Luminex expression range for each gene.

4.5 Summary of Sum of Absolute Rank Differences (SARD) based identification of concordance

So far, we eliminated potential facts, such as experimental faults, inappropriate primer probe usage for detection and small sample size that could lead to discordants results. However, another potential factor could be the primary cell cultures that we use to validate Luminex data. Primary cell cultures are composed of all cells present in a tumour at the time of surgery they are obtained. In time, one subpopulation of cells present in the primary cell cultures may expand more and be favoured during culturing and resulting cell lines with altered gene expression. To asses the state of alteration of our primary cell cultures, we used SARD analysis, developed by Murat Isbilen. Based on SARD analysis, 7 of the samples were shown to be discordant in terms of qPCR and Luminex concordance (Figure 4.10).

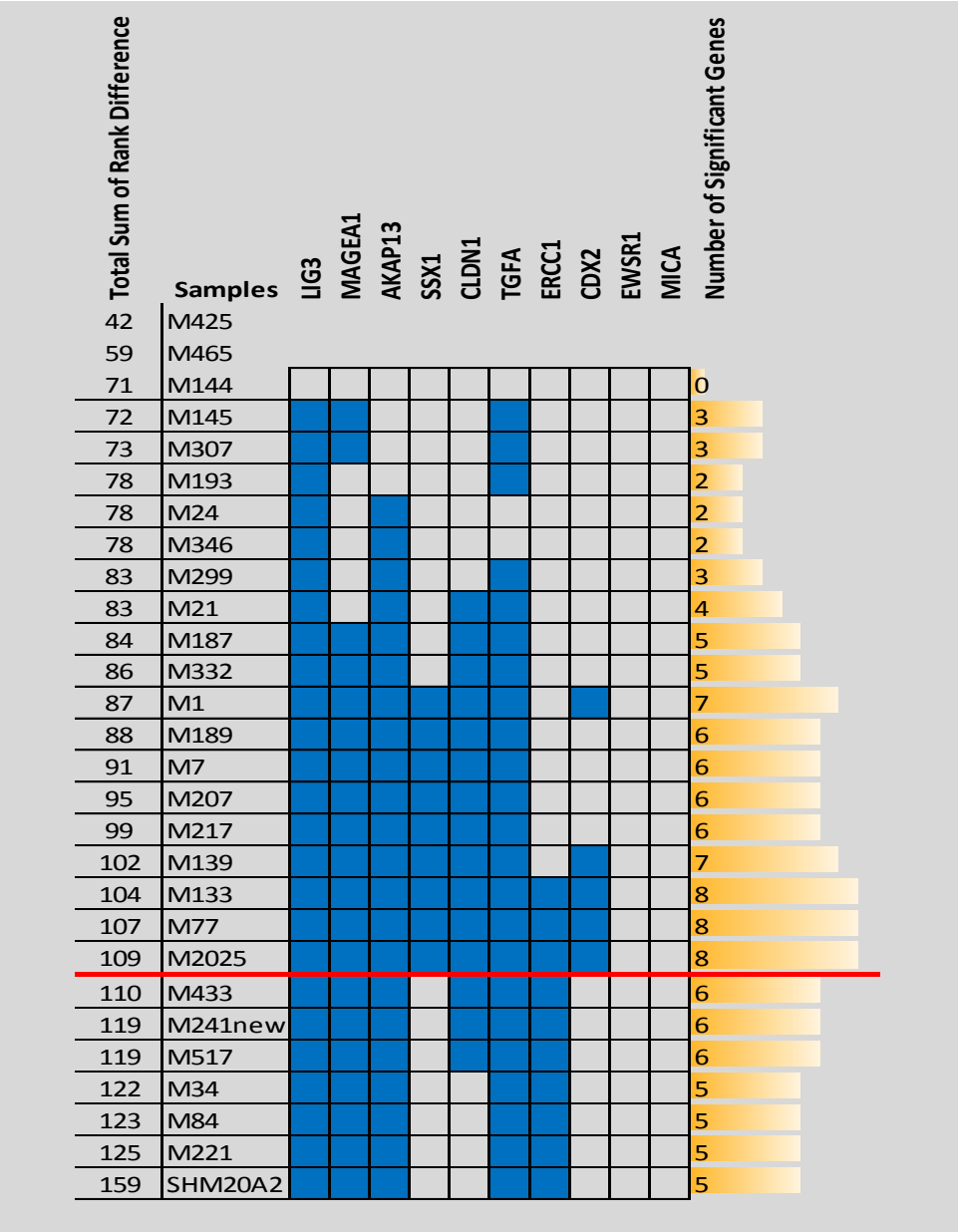


Figure 4.10 Summary of Sum of Absolute Rank Differences (SARD) analysis results. The samples are sorted (from top to bottom) according to total sum of absolute rank differences. Each blue square shows the significant correlation when the samples from top to the corresponding sample are included in correlation analysis. Empty squares are non-significant correlations. The red line shows the sample cut-off that gives the best correlation for all genes. Yellow bars show the number of significant genes for each sample threshold.

When those 7 differentiated samples were excluded from the correlation analysis, both Pearson r correlation coefficient values and p values were improved significantly (Table 4.3).

Table 4.3 Correlation improvement between Luminex microarray data and qPCR data

	p value		**Pearson r**	
Genes	**28 Sample**	**21 Sample**	**28 Sample**	**21 Sample**
LIG3	0,0012	0,0003	0,580	0,713
CLDN1	0,8922	0,0011	0,027	0,662
AKAP13	0,0098	0,0031	0,479	0,614
TGFA	0,0149	0,0049	0,455	0,590
ERCC1	0,0015	0,0064	0,570	0,575
MAGEA1	0,0393	0,0083	0,464	0,673
CDX2	0,6350	0,0203	0,120	0,611
SSX1	0,1398	0,0252	0,362	0,639
MICA	0,8931	0,7310	-0,027	0,080
EWSR1	0,9426	0,9455	0,014	0,016

A better understanding of improved correlation and election of concordants samples according to SARD analysis is demonstrated in CT genes dependent and independent manner in Figure 4.11 and Figure 4.12, respectively. Finally, we were able to demonstrate concordance for 8 selected genes out of 10 with the exceptions of MICA and EWSR1 (Supplementary Figure 4).

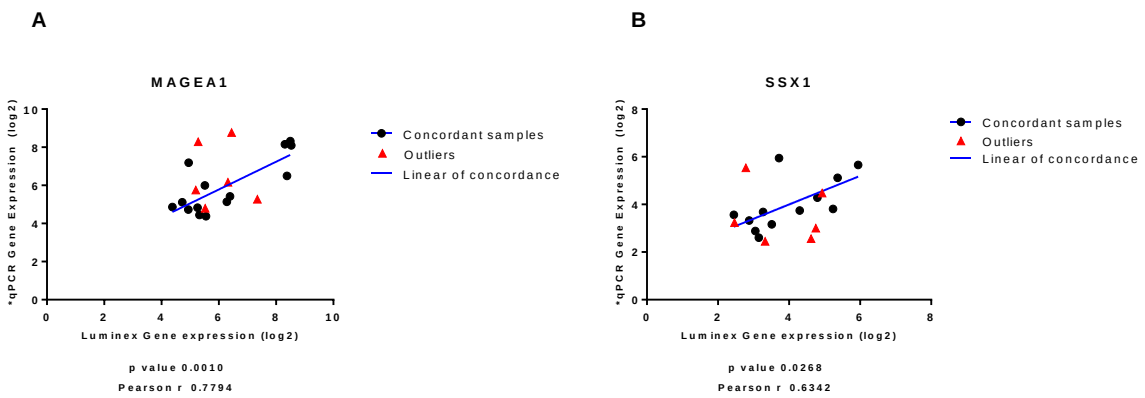


Figure 4.11 Scatter Plots of validated CT genes after SARD analysis. For each gene, Pearson correlation coefficient and linear regression p value are calculated and present in the figure. Red triangles correspond to discordant samples, black dots correspond to concordant samples and blue line corresponds to line of concordance drawn based on Pearson r value calculated with the concordant samples. Genes are; (A) MAGEA1, (B) SSX1.

*qPCR expression range is adjusted according to Luminex expression range for each gene

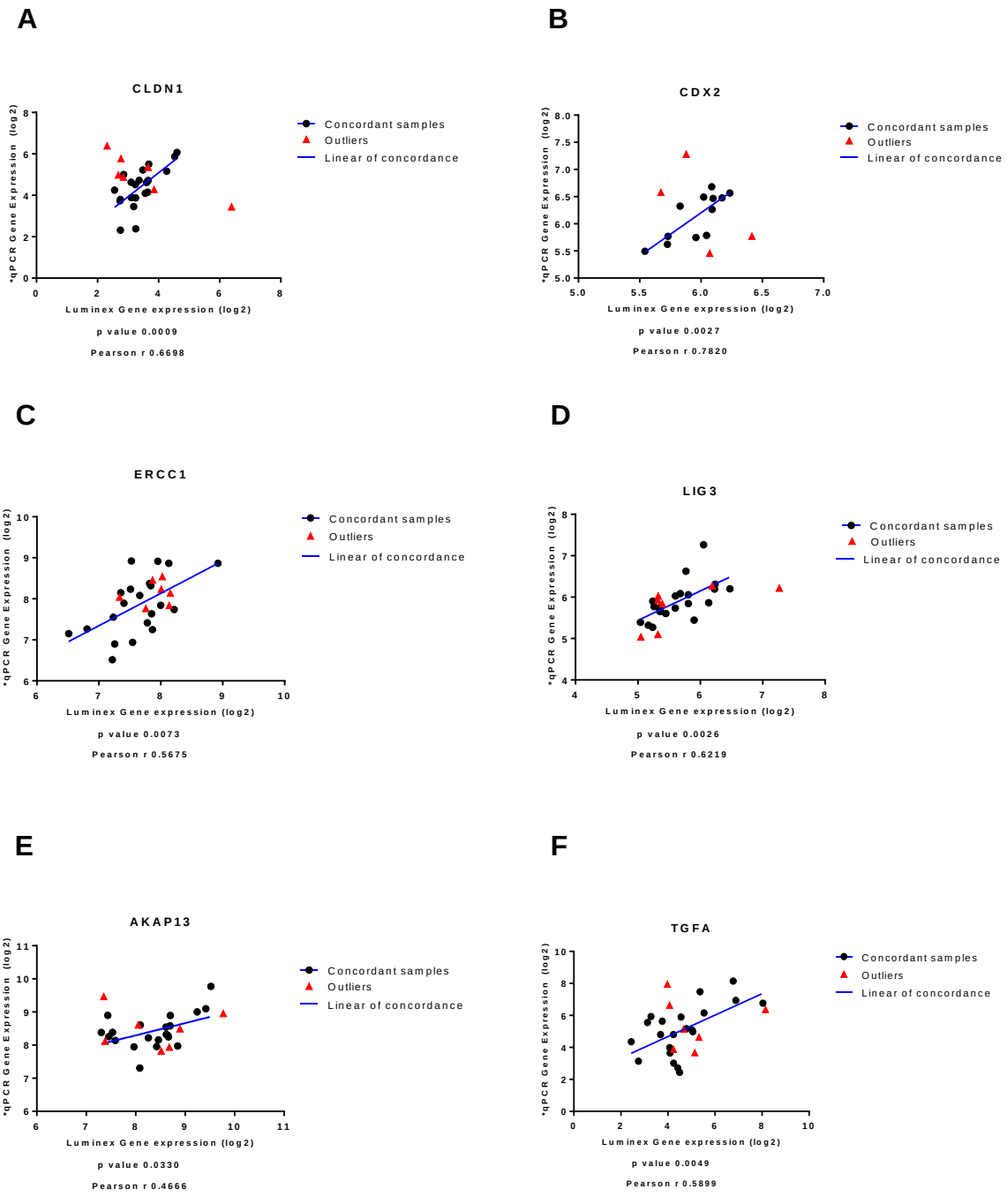

A
CLDN1
*qPCR Gene Expression (log2)
Luminex Gene expression (log2)
p value 0.0009
Pearson r 0.6698
Concordant samples
Outliers
Linear of concordance
B
CDX2
*qPCR Gene Expression (log2)
Luminex Gene expression (log2)
p value 0.0027
Pearson r 0.7820
Concordant samples
Outliers
Linear of concordance
C
ERCC1
*qPCR Gene Expression (log2)
Luminex Gene expression (log2)
p value 0.0073
Pearson r 0.5675
Concordant samples
Outliers
Linear of concordance
D
LIG3
*qPCR Gene Expression (log2)
Luminex Gene expression (log2)
p value 0.0026
Pearson r 0.6219
Concordant samples
Outliers
Linear of concordance
E
AKAP13
*qPCR Gene Expression (log2)
Luminex Gene expression (log2)
p value 0.0330
Pearson r 0.4666
Concordant samples
Outliers
Linear of concordance
F
TGFA
*qPCR Gene Expression (log2)
Luminex Gene expression (log2)
p value 0.0049
Pearson r 0.5899
Concordant samples
Outliers
Linear of concordance

Figure 4.12 Scatter Plots of validated CT independent genes after SARD analysis. For each gene, Pearson correlation coefficient and linear regression p value are calculated and present in the figure. Red triangles correspond to discordant samples, black dots correspond to concordant samples and blue line corresponds to line of concordance drawn based on Pearson r value calculated with the concordant samples. Genes are; (A) CLDN1, (B) CDX2, (C) ERCC1, (D) LIG3, (E) AKAP13, F) TGFα.

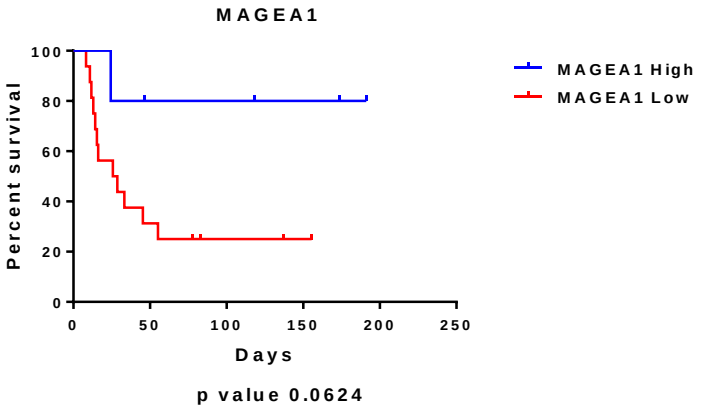
*qPCR expression range is adjusted according to Luminex expression range for each gene.

4.6 Survival Analysis with Genes concordant with Luminex data

To determine the relationship between For qRT-PCR results and survival, we used each gene Maxstat to determine the best threshold.for thecut point is calculated with the qRT-PCR results of 8 validated genes (MAGEA1, SSX1, TGFα, CLDN1, CDX2, ERCC1, LIG3, and AKAP13) and the survival data of the corresponding 21 concordant samples. For the MAGEA1, SSX1; CT genes and TGFα, the result presented in Figure 4.13 shows that we can validate the *in silico* gene-survival association of each gene identified by USAT analysis in the same direction with a significant Log-rank p value when tested by qRT-PCR as well. For the rest of the genes (CLDN1, ERCC1, LIG3), all genes have a significant Log- rank p value with the given cut point by Maxstat (Figure 4.14).

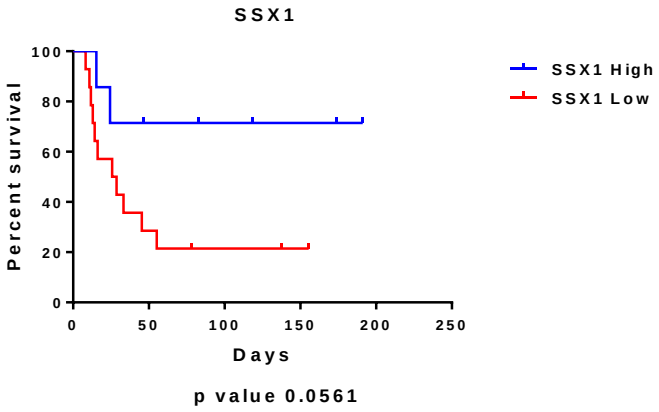
These results confirm that 7 out of 8 genes which we were able to validate, are significantly related with the prognosis of autologous vaccinated malignant melanoma patients as we proposed at the beginning based on USAT analysis.

A



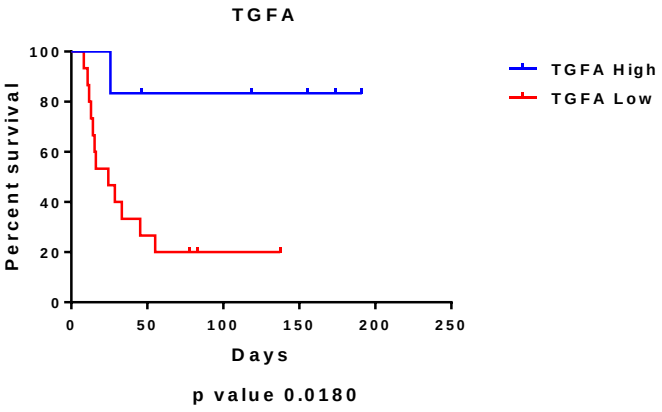
p value 0.0624

B



p value 0.0561

C



p value 0.0180

Figure 4.13 Kaplan Meier curves of each validated CT genes and TGFα based on their qPCR results. For each gene Maxstat cut point is calculated using qPRC results of relevant genes. Log-rank p values of each gene are present in the figure. Blue and red lines are corresponding to survival duration of samples having higher and lower expression of interested genes, respectively. X axis indicates days and Y axis corresponds to survival percentage of samples when analysed based on days. Every vertical line in each colored line is an indicator of cencored sample. Genes are; (A) MAGEA1 with a Log rank p value 0.0624, (B) SSX1 with a Log rank p value 0.0561, (C) TGFα with a Log rank p value 0.0180.

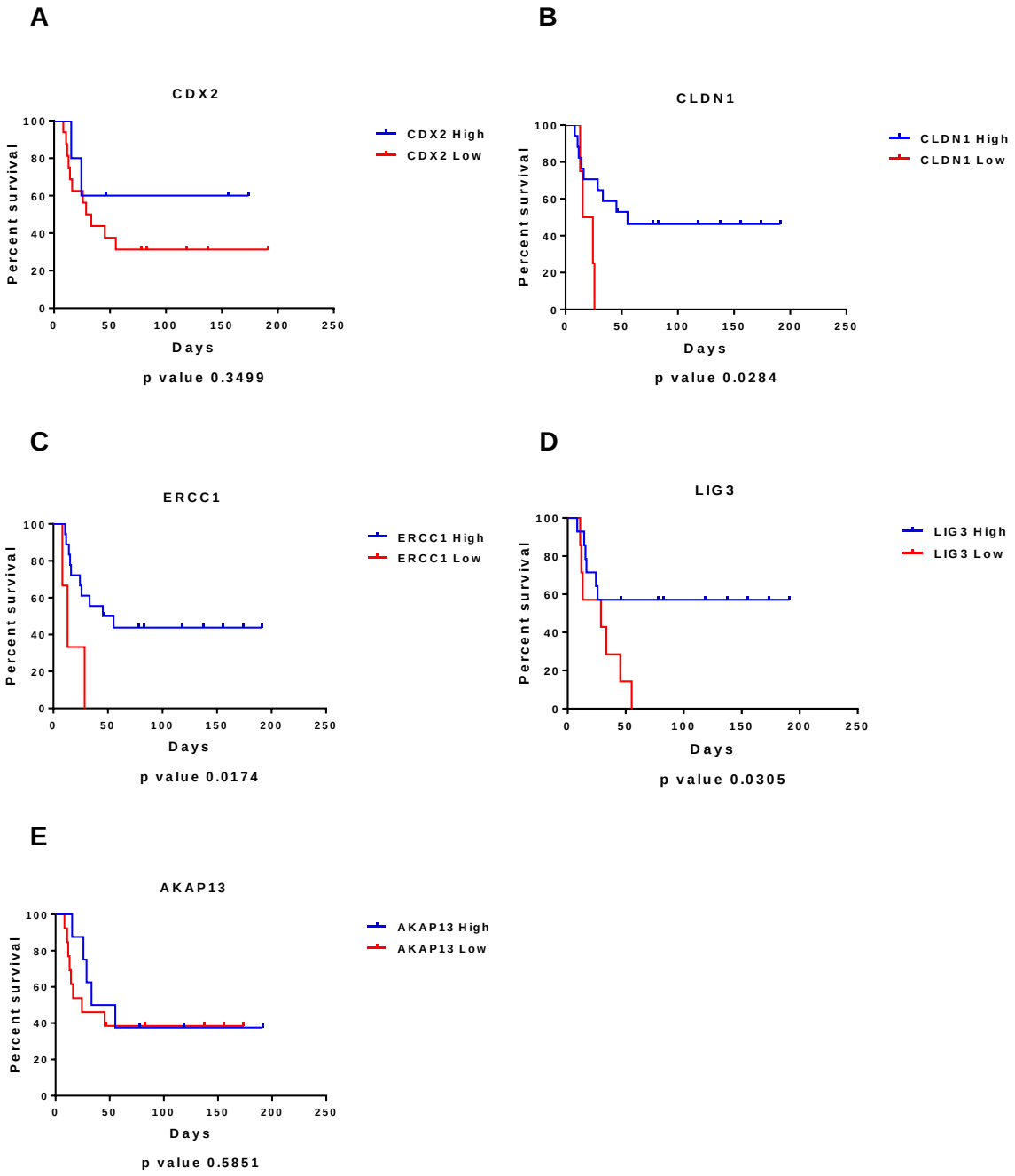


Figure 4.14 Kaplan Meier curves of each validated CT independent genes based on their qPCR results. For each gene Maxstat cut point is calculated using qPRC results of relevant genes. Log-rank p values of each gene are present in the figure. Blue and red lines are corresponding to survival duration of samples having higher and lower expression of interested genes, respectively. X axis indicates days and Y axis corresponds to survival percentage of samples when analysed based on days. Every vertical line in each colored line

is an indicator of cencored sample. Genes are; (A) CDX2 with a Log rank p value 0.3499, (B) CLDN1 with a Log rank p value 0.0284, (C) ERCC1 with a Log rank p value 0.0174, (D) LIG3 with a Log rank p value 0.0305, (E) AKAP13 with a Log rank p value 0.5851.

5 DISCUSSION

This study was conducted to determine mRNA based biomarkers which could identify a sub-population of melanoma patients who might benefit from autologous vaccination. In this thesis, I could validate 5 genes out of 8 genes by qRT-PCR as prognostic biomarkers, which were identified by a unique *in silico* analysis tool (USAT). These genes are; TGFα, CLDN1, ERCC1, LIG3, and SSX1. Besides the first 4 validated genes, SSX1 is particularly interesting since being a CT antigen makes it direct target of an immune response when expressed in tumours.[196]

5.1 CT gene as a prognostic biomarker

Previously, MAGEA1 is found to be highly expressed in 48% of metastatic melanomas compared to 16% of primary melanomas.[223] Further, SSX1 is shown to be expressed 8% in various cancer types in the study conducted by Türeci et al. [228] In the same study, it's shown that 43% of malignant melanoma samples have SSX family gene expression, including SSX1-2-4. In contradiction with these literatures, this study suggests that higher expression of SSX1 and MAGEA1 are correlated with better survival in autologous vaccinated melanoma patients. This situation might be explained with the studies of Cebon et al where he and his group proposed that the suppression of immune system via Tregs overcome the NY-ESO-1 vaccination triggered immune response before the surgery. [197]Supporting the study of Cebon et al., this study once more proposes that presence of CT gene expression such as MAGEA1 and SSX1 in melanoma may not be detected by the immune system due to immune suppressor environment, especially Tregs, established by the tumour. Removing the tumour might destroy the immune-suppressive tumour microenvironment and therefore, increase the impact of autologous vaccination in melanoma patients.The vaccination protocol used in this cohort, increase the efficacy of treatment and introduce CT epitopes (MAGEA1 and SSX1) to

immune system of patients when they are in minimal residual disease (MRD) state , thereby triggering immune response and increase the survival of patients.

5.2 CT independent prognostic biomarkers

TGFα can be secreted autonomously by melanoma cells; can be UVR induced or might be produced exogenously by keratinocytes; it can not trigger cell growth in proliferating melanocytes.[229] This might be due to two facts; (1) EGFR mediated pathway is less accessible or (2) EGFR is not the preferred as a transducer of mitogenic signals primarily.[229] However high expression of TGFα is characteristic of metastatic melanoma cell lines.[201] Further , TGFα is proposed to activate EGFR or EGFR/ErbB2 signalling leading migration in one of the metastatic melanoma cell lines studied by Thomson et al.[230] Activation of EGFR pathway is demonstrated after development of resistance to BRAF inhibitors in melanoma.[206] Since the samples we used are no metastatic cell cultures and they were not treated with any BRAF inhibitor agents leading EGFR pathway activation, increased expression of TGFα may trigger immune response against tumour and increase survival upon autologous vaccination.

High expression of CLDN1, one of the EMT markers studied in this thesis, proposed to be related with both metastasic[210] and primary melanomas.[213] The issue here is the localization of CLDN1 when it's upregulated. If there are not any PKC involvement in the up regulation of CLDN1 and no relocalization to nucleus or cytoplasm, better outcome in the case of CLDN1 up regulation may be expected in primary tumours. This work supports the study of Cohn et al. which demonstrates that upregulation of CLDN1 expression in benign and primary lesions compared to metastatic melanoma, favours patients survival.[213] High expression of CLDN1 validated in stage 1 and 2 patients of our cohort suggests that CLDN1 presence in the membrane of early melanoma patients can be used as a trigger upon autologous vaccination.

ERCC1 and LIG3 are involved in nucleotide excision repair and base excision repair, respectively. Up-regulated ERCC1 is known to induce chemoresistance against cisplatin in melanoma. [216] Higher expression of ERCC1 suggests cisplatin resistance upon administration. However, LIG3 hasn't been found to be related with prognosis or chemosensitivity in melanoma. Up-regulation of both enzymes is observed in patients with better overall survival.

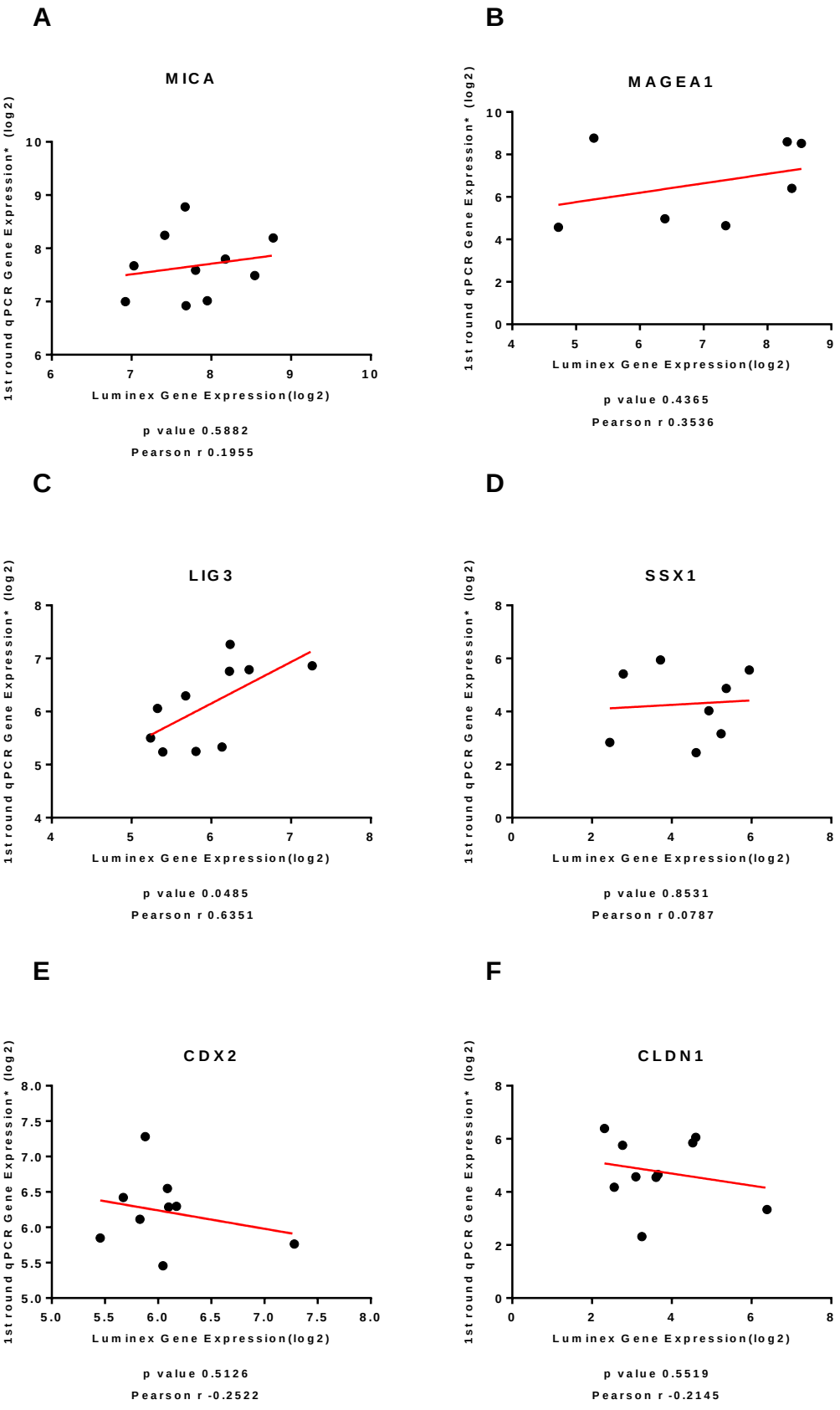
In this study, I was able to validate prospective biomarkers of malignant melanoma corresponding to better survival of patients undergoing autologous vaccination treatment. Autologous vaccination is a method which needs further evaluation to assess its benefits, particularly for escalating recognition of tumour antigens by T cell lymphocytes following surgery. Its impact on impeding immune surveillance and letting antigen recognition, in particular CT antigen recognition, is a major contribution for directly detecting and eliminating tumour cell thus decreasing tumour progression and increasing tumour relapse duration in malignant melanoma patients.Additional high TGFA expression patterns seen in melanoma patients are in concert with CT genes profiles and may be considered as an alternative prognostic biomarker to distinguish the autologous vaccination beneficial group for melanoma patients.

6 FUTURE PERSPECTIVES

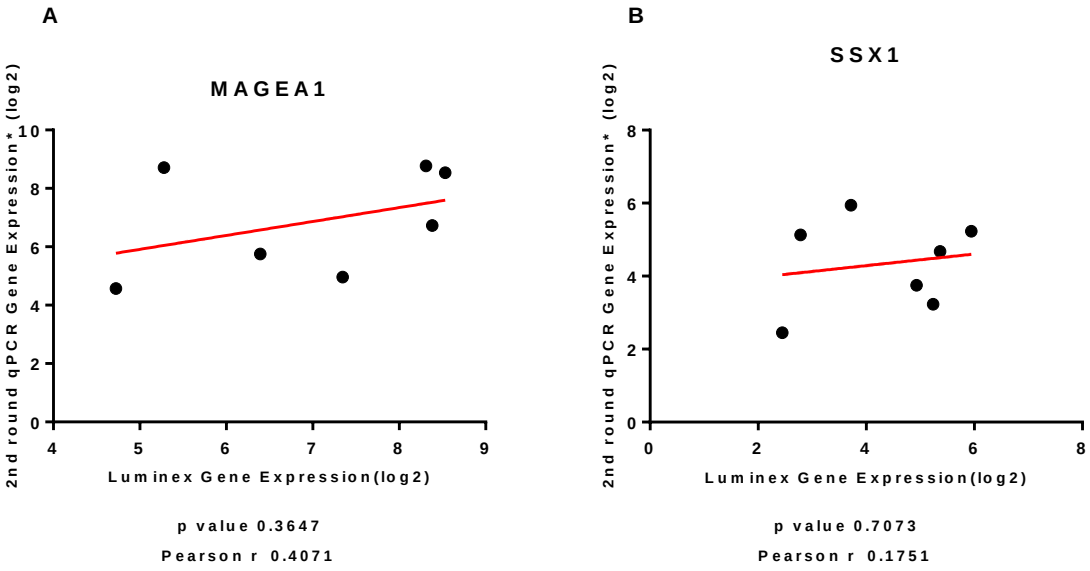
In this thesis, five genes including one cancer testis gene (SSX1) were shown to be related with better survival of malignant melanoma patients undergoing autologous vaccination. To reproduce and improve the significance of this relation, the patient's size can be increased in scientifically similar projects. In addition, malignant melanoma patients can be treated with demethylating agents such as 5-aza-2-deoxycytidine, known to increase the cancer testis gene expression, prior to surgery. This might help to increase cancer testis antigen specific immune responses following surgery upon autologous vaccination and thus increase the overall survival rate.

Validated prospective biomarker genes in this thesis might give insights to other scientist and further clinicians to define appropriate treatments such as autologous vaccination centered immunotherapy and combined chemotherapy options for malignant melanoma patients. Moreover, roles of CT independent genes in the melanomagenesis and reasons to be immunoresponse related can be leaned and their functions can be searched in wide range.

7 SUPPLEMENTARY FIGURES

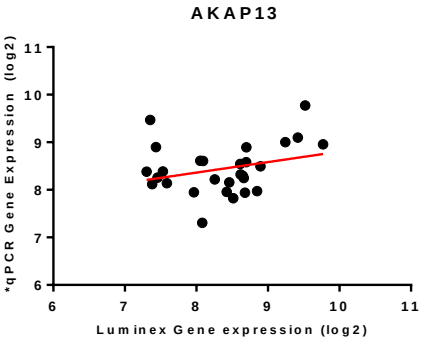


Supplementary Figure 1 Scatter plots of the selected genes analysed based on their qPCR and Luminex expression values from the 1st round experiments of 1st 10 samples. For each gene, Pearson product moment correlation coefficient and linear regression p value are calculated and present in the figure. Red line corresponds to line of concordance drawn based on Pearson product moment correlation coefficient. Genes are as follows; (A) MICA, (B) MAGEA1, (C) LIG3, (D) SSX1, (E) CDX2, (F) CLDN1.



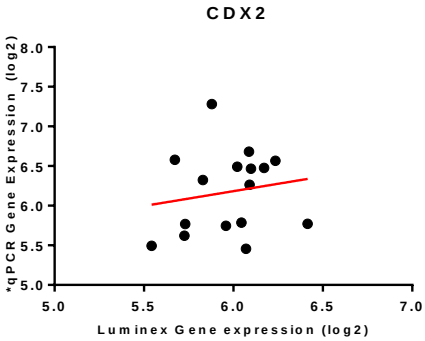
Supplementary Figure 2 Scatter plots of the selected genes analysed based on their qPCR and Luminex expression values from the 2nd round of 1st 10 samples. For each gene, Pearson correlation coefficient and linear regression p value are calculated and present in the figure. Red line corresponds to line of concordance drawn based on Pearson correlation coefficient. Genes are as follows; (A) MAGEA1, (B) SSX1.

A



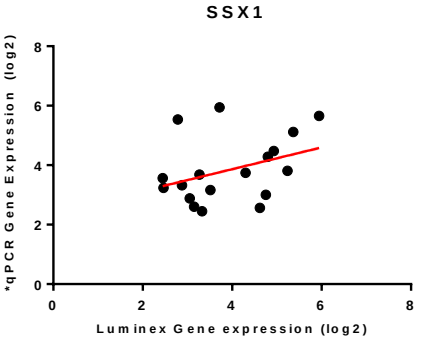
p value 0.1328
Pearson r 0.2912

B



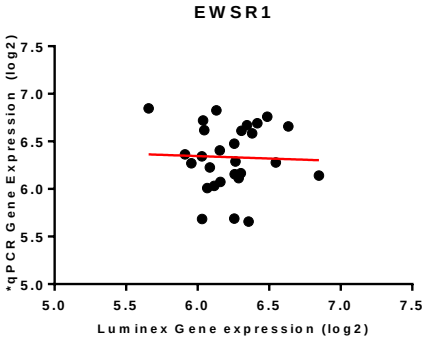
p value 0.5422
Pearson r 0.1647

C

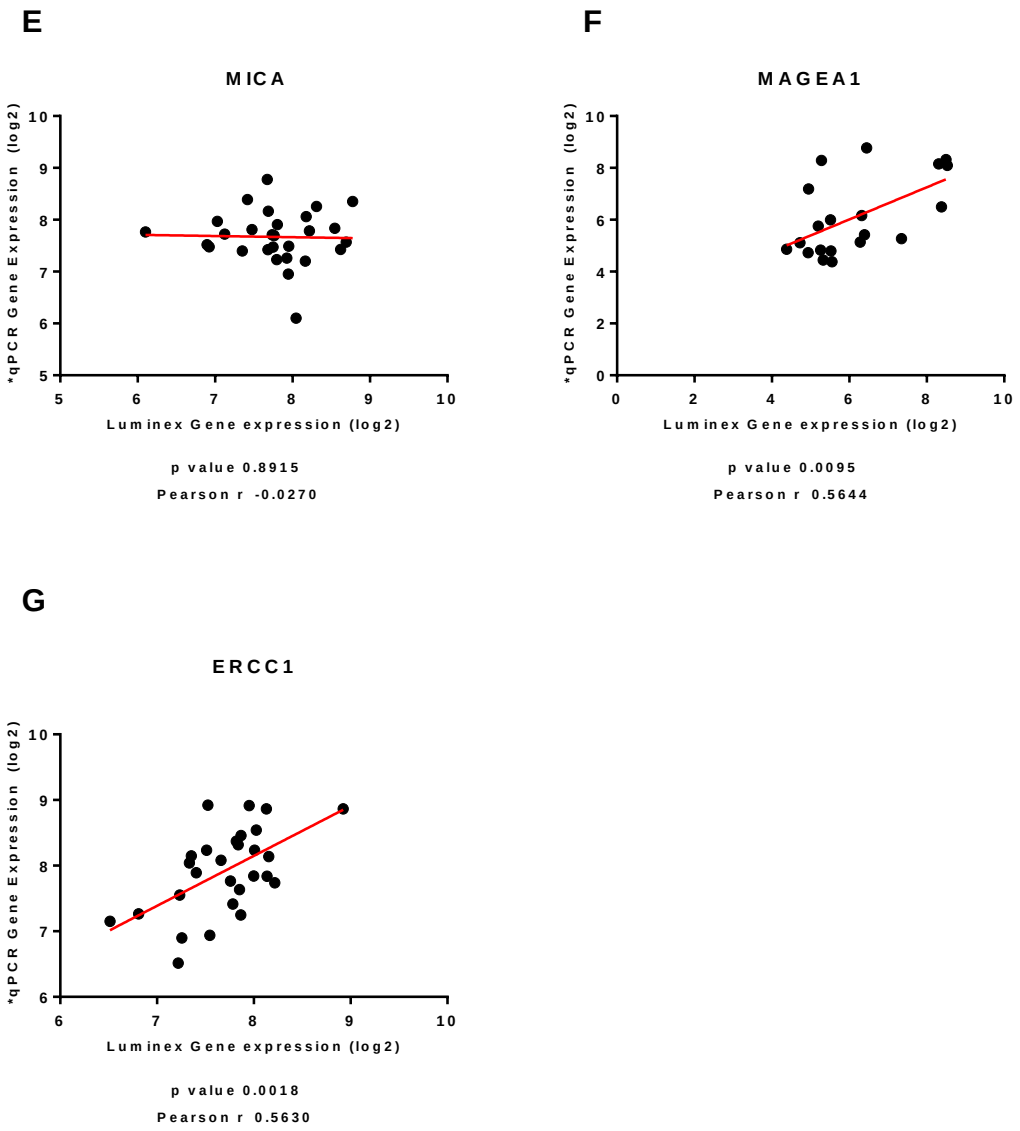


p value 0.1423
Pearson r 0.3600

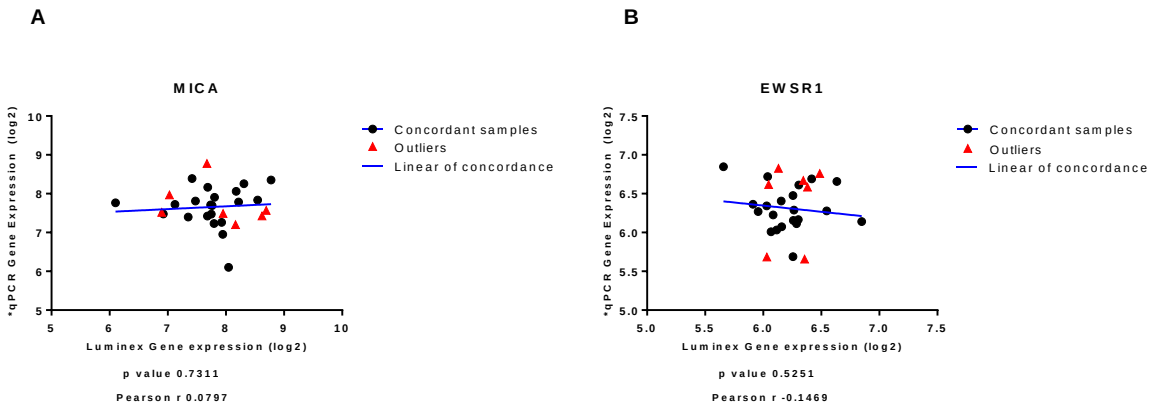
D



p value 0.8560
Pearson r -0.0359



Supplementary Figure 3 Scatter plots of selected genes analysed based on their qPCR and Luminex expression values for 28 samples. For each gene, Pearson correlation coefficient and linear regression p value are calculated and present in the figure. Red line corresponds to line of concordance drawn based on Pearson correlation coefficient. Genes are as follows; (A) AKAP13, (B) CDX2, (C) SSX1, (D) EWSR1, (E) MICA, (F) MAGEA1, (G) ERCC1.



Supplementary Figure 4 Scatter Plots of discordant genes after SARD analysis. For each gene, Pearson correlation coefficient and linear regression p value are calculated and present in the figure. Red triangles correspond to discordant samples, black dots correspond to concordant samples and blue line corresponds to line of concordance drawn based on Pearson r value calculated with the concordant samples. Genes are; (A) MICA, (B) EWSR1

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