REPUBLIC OF TURKEY
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF NANOTECHNOLOGY AND ENGINEERING SCIENCES

ESTABLISHMENT OF DRUG-RESISTANT CELL LINES IN NON-SMALL CELL LUNG CANCER

AYLİN GÜNDÜZ

MASTER OF SCIENCE

ADANA 2019

REPUBLIC OF TURKEY
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF NANOTECHNOLOGY AND ENGINEERING SCIENCES

ESTABLISHMENT OF DRUG-RESISTANT CELL LINES IN NON-SMALL CELL LUNG CANCER

AYLİN GÜNDÜZ
MASTER OF SCIENCE

SUPERVISOR
ASSIST. PROF. DR. ÖZGÜR CEM ERKİN

ADANA 2019

ESTABLISHMENT OF DRUG RESISTANT CELL LINES IN NON SMALL CELL LUNG CANCER

submitted by **Aylin GÜNDÜZ** in partial fulfillment of the requirements for the degree of **Master of Science in Department of Nanotechnology and Engineering Sciences, Adana Alparslan Türkeş Science and Technology University** by,

Assoc. Prof. Dr.
Gözde BAYDEMİR PEŞİNT
Graduate School Director

Assist. Prof. Dr.
H. İMGE OKTAY BAŞEĞMEZ
Department Chair

Assist. Prof. Dr.
Özgür Cem ERKİN
Supervisor

Thesis Committee

Assist. Prof. Dr. Özgür Cem ERKİN
Adana Alparslan Türkeş Science and Technology University

Assist. Prof. Dr.
Merve ÇAPKIN YURTSEVER
Adana Alparslan Türkeş Science and Technology University

Assist. Prof. Dr.
Aslı GİRAY
Alanya Alaaddin Keykubat University

Thesis Defense Date

17/06/2019

I hereby declare that all information in this thesis has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all information that is not original to this work.

[Signature]

Aylin GÜNDÜZ

ABSTRACT

ESTABLISHMENT OF DRUG-RESISTANT CELL LINES

IN NON-SMALL CELL LUNG CANCER

Aylin GÜNDÜZ

Department of Nanotechnology and Engineering Sciences

Supervisor: Asist. Prof. Dr. Özgür Cem ERKİN

June 2019, 46 pages

Non-small cell lung cancer is one of the leading causes of cancer-related deaths worldwide. One of the major reasons for this is the development of resistance against theurapatic drugs during treatment or due to genetic make up of tumors in patients. Although several molecular agents have been developed to overcome drug resistance, risk of recurrence is still being a challenge for efficient therapy at the present time. Thus, understanding chemoresistance mechanisms is crucial for successful treatment of this disease. In this study, it was aimed to establish drug-resistant sub-cell lines by mimicking treatment conditions of non-small cell lung cancer patients *in vitro* to study drug resistance mechanisms and to generate cell lines for future purposes to discover novel anti-chemoresistance agents. In accordance with this purpose, human non-small cell lung cancer cell line A549 was exposed to increasing concentrations of Epithelial Growth Factor Receptor targeting drug Erlotinib to create an Erlotinib resistant subline. To compare possible morphological and molecular changes during resistance development, a parental A549 subline was maintained in a parallel culture. The cell viability changes in both sublines were demonstrated by cell viability assays. Both groups of sublines were subjected to wound healing assay to monitor the differences in metastatic progression; on the other hand, tumorigenic capacities were measured by soft agar colony formation assays. Furthermore, the effects of Erlotinib on the gene expression changes of epithelial to mesenchymal transition markers, a key feature of chemoresistance, were detected by quantitative reverse transcription polymerase chain reaction analysis. It was found

that besides increased cell viability under drug conditions, Erlotinib resistant subline was more tumorigenic with a greater migration capacity than the parental subline. Finally mesenchymal biomarker N-Cadherin gene expression was significantly increased in Erlotinib resistant subline.

Keywords: Non-small cell lung cancer, drug resistance, Erlotinib, in vitro

ÖZET

KÜÇÜK HÜCRELİ OLMAYAN AKCİĞER KANSERİ'NDE İLACA DİRENÇLİ HÜCRE HATLARININ OLUŞTURULMASI

Aylin GÜNDÜZ
Nanoteknoloji ve Mühendislik Bilimleri Anabilim Dalı
Danışman: Dr. Öğr. Üyesi Özgür Cem ERKİN
Haziran 2019, 46 sayfa

Küçük hücreli dışı akciğer kanseri dünyada kansere bağlı ölümlerin önemli sebeplerinden birisidir. Bunun temel nedenlerinden birisi tedavi sırasında ya da hasta tümörlerinin genetik yapısına bağlı olarak terapi ilaçlarına karşı gelişen kimyasal dirençtir. Her ne kadar ilaç direncini aşmak için çeşitli moleküler ajanlar geliştirilmiş olsa da hastalığın dirence bağlı olarak tekrarlaması, günümüzde halen etkili tedavi için önemli bir engel oluşturmaktadır. Bu nedenle, kemorezistans mekanizmalarını anlamak, hastalığın başarılı bir şekilde tedavisi için gereklidir. Bu çalışmada ileride ilaç direnç mekanizmalarının aydınlatılması ve yeni direnç karşıtı ajanların tespiti için kullanılacak hücre hatlarının oluşturulması için *in vitro* da hasta kemoterapi koşulları taklit edilerek ilaç dirençli hücre hatları oluşturmak amaçlanmıştır. Bu amaç doğrultusunda Epitelyal Büyüme Faktörü Reseptörü'ne hedeflemeli bir ilaç olan Erlotinib'e dirençli hücre alt hatları, A549 küçük hücreli dışı akciğer kanseri hattının Erlotinib'in artan konsantrasyonlarına maruz bırakılmasıyla oluşturulmuştur. Direnç gelişimi sırasında oluşabilecek morfolojik ve moleküler değişimlerin karşılaştırılması amacıyla anaç A549 alt hattının parelel kültürü sürdürülmüştür. Her iki hücre alt hattında meydana gelen canlılık değişimleri hücre canlılık testi ile gösterilmiştir. Her iki hücre grubu, metastatik gelişmelerdeki farklılıkların gözlenmesi için yara kapama testine maruz bırakılmış, öte yandan tümörijenik kapasiteleri yumuşak agar koloni oluşum testi ile ölçülmüştür. Buna ek olarak, Erlotinib'in kimyasal direncin önemli bir özelliği olan epitelyal hücre tipinden ve mezenkimal tipe geçiş işaretleyicilerinin gen ifadeleri üzerindeki etkisi, kantitatif ters transkriptaz polimeraz zincir reaksiyon analizi ile

belirlenmiştir. İlaç koşullarında artan hücre canlılığının yanı sıra, dirençli alt hattın anaç hatta göre daha tümörijenik ve daha yüksek göç kapasitesine sahip olduğu gözlenmiştir. Son olarak Erlotinib dirençli alt hatta mezenkimal hücre tipi işaretleyicisi N-Cadherin ifadesinin önemli derecede arttığı görülmüştür.

Anahtar Kelimeler: Küçük hücreli dışı akciğer kanseri, ilaç direnci, Erlotinib, in vitro

This study is dedicated to my beloved family who has always stood beside me and supported me unconditionally.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Assist. Prof. Dr. Özgür Cem Erkin for his patience, guidance, supports, useful suggestions and his precious time during my study.

I would also like to thank to my jury members Assist. Prof. Dr. Merve Çapkın Yurtsever and Assist. Prof. Dr. Aslı Giray for accepting to be members of the jury for my thesis.

Words cannot express how grateful I am to my family for all of the sacrifices that they have made on my behalf. Their prayers for me were what sustained me thus far.

This thesis was financially supported by Adana Alparslan Türkeş Science and Technology University, Unit of Scientific Research Projects Coordination with project number of 17303005.

.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

ACTB	: Actin Beta
Akt	: Protein Kinase B
ALK	: Anaplastic Lymphoma Kinase
ASP8273	: Naquotinib
BRAF	: Proto-oncogene B-Raf
CDH1	: E-cadherin
CDH2	: N-cadherin
cDNA	: complementary Deoxyribonucleic acid
EDTA	: Etilendiamin Tetraasetic Acid
EGFR	: Epidermal Growth Factor Receptor
EGFR-TKI	: Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor
EGF816	: Nazartinib
EML4	: Echinoderm Microtubule-associated Protein-like 4
EMT	: Epithelial to Mesenchymal Transition
ER	: Erlotinib Resistant Subline
FBN	: Fibronectin
FBS	: Fetal Bovine Serum
FDA	: Food and Drug Administration
FGFR	: Fibroblast Growth Factor Receptor
HGF	: Hepatocyte Growth Factor Receptor
IL-6R	: Interleukin 6 Receptor
JAK1	: Janus Kinase 1
KRAS	: Kirsten Rat Sarcoma Viral Proto-oncogene
MAPK	: Mitogen-Activated Protein Kinase
mTOR	: mammalian Target of Rapamycin
MTT	: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NRAS	: Neuroblastoma RAS Viral Oncogene Homolog
NSCLC	: Non-Small Cell Lung Cancer
OCC	: Occludin

PBS	: Phosphate Buffered Saline
PDGFR	: Platelet-Derived Growth Factor Receptors
PF-06747775	: Mavelertinib
PI3KCA	: Phosphatidylinositol 3-Kinase, Catalytic α Polypeptide
PI3K	: Phosphatidylinositol 3-Kinase
PS	: Penicilin Streptomycin Solution
PT	: Parental Subline
qRT-PCR	: Quantitative Reverse Transcription Polimerase Chain Reaction
qPCR	: Quantitative Polimerase Chain Reaction
RPMI-1640	: Roswell Park Memorial Institute-1640
SCLC	: Small Cell Lung Cancer
STAT3	: Signal Transducer and Activator of Transcription 3
SYBR Green	: Synergy Brands Green
TGFβ	: Transforming Growth Factor β
TJP1	: Tight Junction Protein 1
VEGFR	: Vascular Endothelial Growth Factor Receptor
VIM	: Vimentin
ZO-1	: Zonula Occludens-1

1. INTRODUCTION

Recent research advances in tumor biology and pathogenesis, have resulted in significant progress in lung cancer treatment. This progress has provided accurate histological classification and biomarker information for the treatment of lung cancers. Lung cancer has classified into two main histological types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (Zheng, 2016).

With 85% of total lung cancer occurrence, NSCLC in early stages can be treated with surgery, chemotherapy or radiotherapy. For the treatment of advanced stage NSCLCs, however, a combination of platin based drugs and other conventional drugs is prefered. Molecular biomarkers such as epidermal growth factor receptor (EGFR) mutations can be targeted with relatively newer drugs such as Erlotinib (Zappa, et al. 2016). But possibility of disease recurrence in early stages and low survival rates and development of drug resistance in late stages avoids successful treatment of this disease (Iglesias et al., 2018). Low survival rates in patients with NSCLC has been associated with intrinsic or acquired drug resistance, which reduces chemotherapeutic drugs' efficiency (Wongvaranon et al., 2013). Epithelial mesenchymal transition (EMT) is an acquired resistance mechanism associated with downregulation of epithelial markers such as E-cadherin and upregulation of mesenchymal markers such as N-cadherin (Xu, et al. 2017).

General purpose of this study is to establish drug-resistant cell lines for investigating intrinsic or acquired resistance mechanisms, which cause failure in the treatment of metastatic NSCLC patients. For this purpose, a drug resistant subline A549-Erlotinib Resistant (A549-ER) was generated by using human NSCLC cell line A549 with wild-type EGFR. During this process, A549 cells were treated with EGFR tyrosine kinase inhibitor (EGFR-TKI) Erlotinib in increasing concentrations. To compare possibly differentiated morphological and molecular characters, A549-ER was cultured in parallel with a parental subline A549-Parental (A549-PT) which was treated with Erlotinib solubilizing agent DMSO only. These comparisons were performed with cell culture and molecular biology methods including cell viability, cell wound healing, and soft agar colony formation assays. Moreover, analysis of expression markers for EMT process, which seems to be a challenge for the treatment of advanced

NSCLC patients, was performed by quantitative reverse transcriptase polymerase chain reaction (qRTPCR). Further research will be carried out in future to observe the effects of 2nd and 3rd generation drugs on A549-ER developed in this thesis process.

2. LITERATURE REVIEW

2.1. Lung Cancer

2.1.1. Lung Cancer Epidemiology

Lung cancer originates from the uncontrolled proliferation of aberrant cells in lung tissues (Zhang et al, 2013). Despite improved diagnostic and therapeutic advances, lung cancer remains as the most prevalent fatal disease (Pathak et al., 2004). The number of people who die due to lung cancer exceeds the number of people dying because of breast, colorectal and prostate cancers combined (Cruz et al., 2011). Due to lung cancer, 1.38 million people die each year, i.e. much more than 3,000 deaths a day worldwide, or two deaths in a minute. Lung cancer is a kind of cancer, which causes fatal results in both men and women. Considering the sex of the people, the number of women who diagnosed as lung cancer is increasing compared to men. More specifically, the annual increase for men is 3 % while for women it is 400 % (Bleehen, 1985)

2.1.2. Lung Cancer Classification

Lung cancers are commonly split up into two major types, NSCLC and SCLC depending on their morphology under the microscope (Inamura, 2017). Approximately 95 % of all lung cancers are classified as either SCLC or NSCLC (Bleehen, 1985). The remaining 5 % consists of other histological types of tumors including adenoid cystic carcinomas, lymphomas, sarcomas as well as hamartomas in lung tissue (Bhatia et al., 2006). SCLC is found in 1 out of every 8 lung cancer patients and it exhibits a tendency to expand more swiftly than NSCLC. Thus, it is frequently diagnosed when it has interpenetrated outside of the lung (Nicholson et al. 2002). SCLCs have smaller diameter size when compared to NSCLCs and their clinical treatment regimen is different (National Cancer Institute, 1995). On the other hand, NSCLC is the most prevalent lung cancer type constituting approximately 85% of the diagnosed lung cancers in a global scale (Pathak et al., 2004). NSCLC is further divided into three main histological types; adenocarcinomas, squamous cell carcinomas and large cell carcinomas (Cagle et al., 2013). Adenocarcinomas arise in the periphery of the lungs while squamous cell carcinomas mostly spring up centrally in larger bronch. Large cell carcinomas on the other hand, may take place in any part of the lungs. They have a tendency to grow and scatter more quickly than the other two-carcinoma types (Bleehen 1985).

2.1.2.1. Non-small cell lung cancer and treatment methods

Approximately 20-30 % of patients harboring the predominant NSCLC type of lung cancers are on stage I, II, and IIIA of the disease at the time of diagnosis. Patients at these stages are treated generally with surgery, chemotherapy, radiotherapy and, in some cases, adjuvant cisplatin. However, the patient's response to treatment is unpredictable and there is always a possibility of disease recurrence (Smythe, 2001; Paraschiv et al., 2016). Almost half of the NSCLC patients are diagnosed on stage IIIB and IV that are advanced and metastatic stages of the disease, respectively. The most appropriate treatment regimen for patients with stage III disease is a combination of platinum-based chemotherapy with radiotherapy; while in stage IV it is chemotherapy alone (Hopper-Borge et al., 2014; Cagle et al., 2013). Platinum-based doublet chemotherapy is an important choice of treatment for advanced NSCLC and is aimed to reduce symptoms as well as prolong disease free survival (Tsvetkova et al., 2012). For metastatic NSCLC patients a combination of cisplatin or carboplatin with other conventional drugs such as paclitaxel, docetaxel, gemcitabine and vinorelbine has been favoured (Yu et al., 2015). However, due to intrinsic or acquired resistance developed against chemotherapeutic drugs, survival rates in NSCLC patients still remains low (Wongvaranon et al., 2013).

2.1.2.2. Non-Small Cell Lung Cancer and Novel Treatment Methods

Recent advances towards personalized medicine and omics studies help to develop more effective cancer treatment methodologies (Verma, 2012). For instance, novel molecular targeted therapies increase the rate of progression-free survival and overall survival of NSCLC patients during treatment. Frequently apprehended molecular biomarkers such as mutations or amplification of EGFR, echinoderm microtubule-associated protein-like- 4-anaplastic lymphoma kinase (EML4-ALK) translocation, Notch signal activation, mutations in proto-oncogene B-Raf (BRAF), kirsten rat sarcoma viral proto-oncogene (KRAS) and phosphatidylinositol 3-kinase, catalytic α polypeptide (PI3KCA) can be targeted in NSCLC therapies (Hopper-Borge et al., 2014; Sun et al., 2015).

In studies aiming to resolve the molecular biological basis of NSCLC, frequent abnormal expression and activation of EGFR have been shown to be predominantly critical for the development of a more aggressive form of NSCLC (Ghosh et al., 2012). EGFR

overexpression has occurred in tumors from more than 60 % of patients with metastatic NSCLC and it is associated with poor prognosis. The dysregulation of EGFR leads to increase in malignant cell survival, proliferation, angiogenesis, invasion, and metastasis (Gazdar, 2009). For this reason, monoclonal antibodies targeting various domains of EGFR are developed. The approved monoclonal antibodies, cetuximab that is a chimeric human/murine monoclonal immunoglobulin G1 antibody and panitumumab which is a fully human IgG2 monoclonal antibody, target the extracellular domain of EGFR (Sgambato et al., 2014). In addition, specific small molecule tyrosine kinase inhibitors targeting EGFR such as erlotinib, gefitinib, and EGFR and HER2 suppressing dual kinase inhibitor lapatinib, pan-ErbB inhibitor canertinib suppressing EGFR as well as other ErbB receptors including EGFR and vascular endothelial growth factor receptor (VEGFR) and less specific inhibitors that suppress RET including vandetanib have been in development for clinical use (Kılıç, 2012). Crizotinib initially utilized as a potent, ATP competitive, orally biologically accessible c-MET inhibitor until EML4-ALK fusion had been detected in NSCLC patients. Nowadays crizotinib is used in therapies for EML4-ALK fusion positive and ROS1 overexpressing lung cancer patients (Timm et al., 2013). Among these small molecule inhibitors, the reversible EGFR TKIs gefitinib, erlotinib and irreversible EGFR TKIs, including afatinib, dacomitinib and neratinib, are approved for metastatic NSCLC treatment (Mahipal et al., 2014; Wang et al., 2016). In lung cancer cells with EGFR mutations, small molecule reversible inhibitors selectively bind to the tyrosine kinase region of the EGFR intracellular domain. This binding results in attenuation in autophosphorylation of EGFR and prevention of signal transduction of the phosphatidylinositol-3-kinase/Protein Kinase B/ mammalian target of rapamycin (PI3KCA/Akt/mTOR) and ras/raf/mitogen-activated protein kinase (RAS/RAF/MAPK) pathways. As a result, it leads to a reduction in cell proliferation, survival and angiogenesis in cancer cells. In addition, this binding of small molecule TKIs promote apoptosis by increasing cancer cell sensitivity to the toxic effects of chemotherapy and radiotherapy (Wang et al., 2016; Lopes et al., 2015).

2.2. Drug Resistance and Resistance Mechanisms

The prognosis of lung cancer is very poor, such that nearly 80% of patients die within 1 year of diagnosis. Despite major advances in chemotherapy and radiotherapy has been made over the past decades, long-term survival could be achieved only in 5-10% of the patients (Zappa et al., 2016). The major problem in lung cancer chemotherapy is the manifestation of

inherent and acquired drug resistance of the cancer cells. Resistance to anticancer agents involved in SCLC as well as in NSCLC (Huber, 2004). Most patients with SCLC have an initial response to chemotherapy, but the majority relapses and their tumors are largely refractory to further treatment. Drug resistance or chemoresistance is seen as a challenge during the treatment of lung cancer patients and thus, the prognosis in both SCLC and NSCLC therapy remains low (Škarda et al., 2007). There are two postulated mechanisms related to drug resistance include intrinsic resistance present before diagnosis or acquired resistance due to continuous exposure to drug during treatment.

2.2.1. Intrinsic Resistance Mechanisms

One of the intrinsic resistance mechanisms originates from destabilisation of the equilibrium between the active and inactive states of EGFR kinase activity due to a non-sensitive EGFR mutation (Morgillo et al, 2016). For instance, NCSLC has intrinsic resistance to conventional cisplatin therapy. Resistance mechanisms against platin based chemotherapy include (a) increased repair of platinum-induced DNA damage i.e. increased nucleotide excision repair or loss of DNA mismatch repair, (b) glutathione or metallothion in drug deactivation, (c) reduced cellular uptake of the platinum, and (d) altered apoptosis (Cosaert et al., 2002). Other conventional therapeutics such as tubulin inhibitors vinorelbine and taxanes (paclitaxel and docetaxel) have almost identical resistance mechanisms containing (a) efflux of drug by a membrane pump, (b) altered metabolism or distribution of agent, (c) altered interaction of agent with microtubules and response to cell cycle arrest, and (d) inadequate induction of apoptotic signal. In addition, gemcitabine resistance mechanisms arose from the inadequate intracellular concentration of gemcitabine triphosphate resulting from the failure of an activation/degradation pathway. Also, the sensitivity of tumor cells to the cytotoxic effects of gemcitabine influenced by alterations of apoptosis-regulating genes such as p53 and altered interactions with intracellular targets (Sève et al., 2005).

EGFR-TKIs, included in relatively new treatment strategies, suffer from chemoresistance occur even before treatment as well in advanced NSCLC. Intrinsic resistance in EGFR mutant NSCLC, defined as an instantaneous ineffectiveness of EGFR-TKI, usually occurs as a result of non-sensitive mutations (Wang et al., 2016). Exon 20 insertions or duplications, and EGFR exon 20 mutation, T790M are associated with primary resistance to EGFR-TKIs. T790M mutation returns the L858R mutant receptors affinity for ATP to wild-

type levels to diminish the effects of TKIs (Stewart et al., 2015). The study performed with using cell lines harboring the V843I and L858R mutations has shown resistance to EGFR-TKIs (Prim et al., 2014). On the contrary, although some of the patients have EGFR-activating mutations, they can not initially provide the desired clinical outcomes due to intrinsic resistance against EGFR TKI treatment (Okamoto et al., 2011). Patients with BIM polymorphism have intrinsic resistance to EGFR TKIs treatment. Thus, selection of EGFR mutant NSCLC patients can be used as a predictor for who can benefit from TKIs therapy (Zou et al., 2015).

2.2.2. Acquired Resistance Mechanisms

Despite the advanced progressions in clinical outcomes provided by EGFR TKIs, most of NSCLC patients harboring EGFR-activating mutations, acquire resistance after about 10 months of treatment initiation (Kosaka et al., 2011). Moreover, after prolonged treatment of cultured cells with drugs *in vitro*, secondary or acquired resistance is formed and the resistant phenotype can appear through a few molecular mechanisms (Morgillo et al., 2016). In recent studies targeting acquired resistance mechanisms of EGFR-TKIs, secondary mutations including T790M, D761Y, L747S, and T854A, parallel activation of downstream signaling pathways consisting of c-MET and hepatocyte growth factor receptor (HGF) overexpression as well as a phenotypic transformation into SCLC and EMT were found to be involved (Figure 2.1). T790M mutation, or convertion of methionine for threonine at amino acid position 790, is a secondary point mutation found in approximately 50 % of the lung cancer patients with acquired resistance (Suda et al., 2009). Acquired T790M is seen as a major mechanism of acquired resistance to afatinib. Moreover, it was observed in around a half of the cases with acquired resistance to gefitinib or erlotinib (Wu et al., 2016). T854A detected in erlotinib-resistant laboratory models as well as in clinical specimens may increase the selective pouch size of EGFR protein and prevent erlotinib binding. In addition, a transmembrane tyrosine kinase receptor MET appears a significant cause for acquired resistance to gefitinib or erlotinib in NSCLC patients.

Recent laboratory studies demonstrated that the activation of signaling pathways, such as Wnt and mTOR could form the c-MET resistance. To overcome this resistance, combinatorial treatment with inhibitors can be applied against c-Met, Wnt, and mTOR (Stone et al., 2014). It has been shown that HGF stimulates resistance by activating c-MET which

regulates the phosphorylation of downstream MAPK/ERK1/ERK2 and PI3K/AKT pathways. Resistance caused by HGF can be removed by blocking EGFR downstream pathways (Huang et al., 2015). SCLC transformation as an alternative resistance mechanism is rarely present in lung cancers harboring EGFR gene mutations and it has still been under investigation (Suda et al., 2015; Huang et al., 2015).

EMT which normally takes part in tissue transformations has been found to be one of the acquired resistance mechanisms in NSCLC (Xu et al., 2017). EMT process involves the downregulation of epithelial markers in adherence and tight junctions as well as cytoketarin filament network including E-cadherin, occludins, claudins, desmoplakin, type IV collagen, laminin 1, cytokeratins, catenins, zonula occludens-1 (ZO-1) and mucin1. On the other hand, it includes the upregulation of mesenchymal markers, such as N-cadherin, vimentin, α5β1 and αVβ6 integrins, tenascin C, laminin β1 and 5, type I and VIα collagen, fibronectin, fibroblast specific protein 1 and α-smooth muscle actin (Joyce, 2014). Epithelial-like cancers are more sensitive to targeted therapies, whereas, mesenchymal-like cancers are more susceptible to DNA damaging agents (Xavier et al., 2016). Increased expression of E-cadherin related to the clinical activity of EGFR TKIs in NSCLC patients is proposed as an acquired resistance mechanism defined *in vitro* and *in vivo* whereas N-cadherin expression is said to promote EMT phenotype (Remon et al., 2014). But, the specific mechanism of EMT related to chemoresistance has not been elucidated (Huang et al., 2015). According to Thomson and colleagues, resistance caused by EMT is seen when alteration occurs in kinase pathway activation. They found that phosphorylation of EGFR, ERBB2 and ERBB3 is reduced in mesenchymal-like cells. On the contrary, expression of platelet-derived growth factor receptors (PDGFR), fibroblast growth factor receptor (FGFR) and transforming growth factor β (TGFβ) receptors increased in EMT-induced cells through overexpression of TGFβ (Thomson et al., 2008).

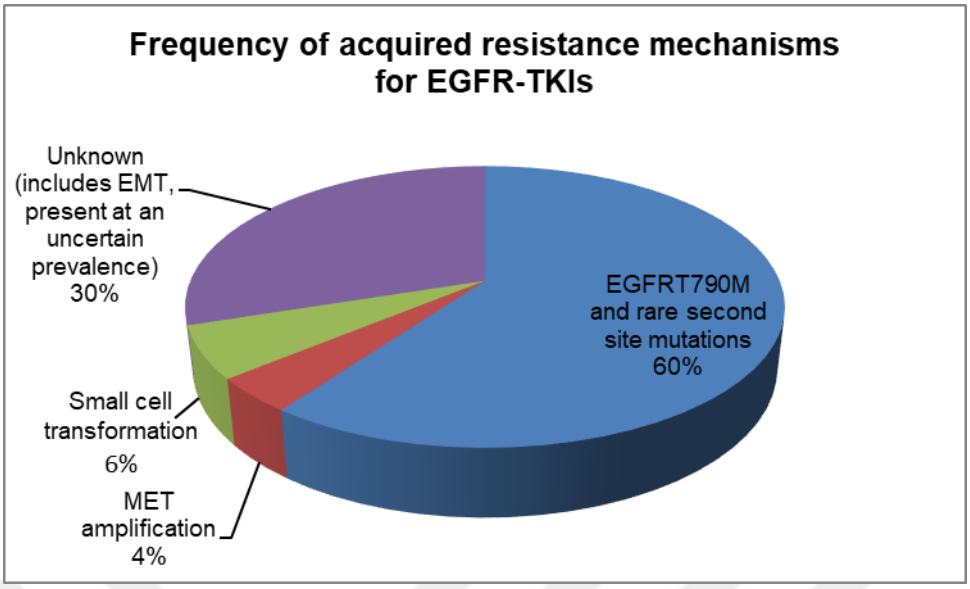


Figure 2.1. Frequency of acquired resistance mechanisms for EGFR-TKIs (http://clincancerres.aacrjournals.org/content/17/17/5530.full-text.pdf)

2.3. Novel Drugs and Their Resistance Mechanisms

Because of the inefficacy of conventional and first-line chemotherapeutic drugs to treat metastatic NSCLC patients, second and third-generation drugs have been developed (Table 2.1). Ceritinib and alectinib are second generation ALK inhibitors approved by American Food and Drug Administration (FDA) to overcome crizotinib resistance, including ALK secondary mutations such as G1202R and F1174C (Gainor et al., 2016; Gou et al., 2016). Brigatinib is a second generation ALK inhibitor used to reveal mutations resistant to crizotinib. Morever, third generation ALK/ROS1 inhibitor lorlatinib could reduce ALK inhibitor resistance (Nursal, 2017). Although ALK inhibitors achieved a good response in therapy, most of the patients beared acquired resistance at last. For instance, MET amplification and MET activation based on autocrine signaling revealed in an alectinib-resistant cell line (Isozaki et al., 2015a).

The second-generation EGFR/HER-TKIs including afatinib, dacomitinib and neratinib were developed to covalently bind to the ATP-binding site of the pan-HER receptors. As a result of this irreversible binding, they inhibit EGFR T790M, enzymatic activation of EGFR and other HER family members including HER2 and HER4 (Hirsh, 2015). Despite an increase in overall survival of NSCLC patients, second-generation EGFR/HER-TKIs could

not overcome the acquired EGFR T790M resistance (Landi et al., 2013). On the contrary, third-generation EGFR/HER-TKIs have been found to be effective in overcoming the acquired EGFR T790M resistance. However, they could not stop the progression of the disease due to involvement of some possible adverse mechanisms. Resistance mechanisms proved in second generation chemotherapeutic drug afatinib involve gatekeeper mutations such as T790M and MET amplification (Stewart et al., 2015). Moreover, FGFR1 amplification, interleukin 6 receptor/ janus kinase1/signal transducer and activator of transcription 3 (IL-6R/JAK1/STAT3) pathway, autophagy and Src upregulation have been associated with resistance to this EGFR TKI. Dacomitinib was found to be effective in both gefitinib-sensitive and gefitinib-resistant NSCLC preclinical models (Wang et al., 2016).

The third-generation EGFR TKIs include osimertinib, rociletinib, olmutinib, naquotinib (ASP8273), nazartinib (EGF816), avitinib, mavelertinib (PF-06747775), and HS-10296. These inhibitors bind to EGFR with T790M and activating mutations to avoid a possible resistance against first- and second-generation EGFR TKIs in NSCLC. Osimertinib is the only third-generation drug approved by FDA to treat metastatic EGFR T790M NSCLC patients (Wang et al., 2016). ASP8273 indicates activity in mutant EGFR cell lines showing resistance to osimertinib and rociletinib. EGFR exon 20 mutation C797S is proposed as a resistance mechanism for osimertinib, rociletinib and olmutinib (Liao et al., 2016). The acquired L798I mutation was reported in one patient treated with rociletinib or osimertinib. As an EGFR-independent mechanism of resistance, neuroblastoma RAS viral oncogene Homolog (NRAS) mutations exhibits acquired resistance to osimertinib, gefitinib and afatinib. Moreover, amplifications in HER2 and MET genes were observed in osimertinib and rociletinib resistant EGFR T790M positive NSCLC patients (Sullivan et al, 2017).

Table 2.1. Features of TKI type drugs used in metastatic NSCLC treatment

Generation	Agent	Molecular Target	Reference
First Generation	Gefitinib	EGFRL858R, EGFRDell9	(Riely et al. 2006)
	Erlotinib	EGFR	(Westover et al. 2018)
	Crizotinib	ALK	(Wu et al., 2016)
Second Generation	Afatinib	wtEGFR (Cys-797), EGFRL858R, EGFRL858R/T790M, EGFRL858R/T854A,wtHER2 (Cys805), HER2 amplification, HER4(Cys803)	(Yu et al., 2018)
	Dacomitinib	EGFRL858R, EGFRDell9, EGFRT790M wtHER2, mutant HER2, HER2 amplification, HER4	(Yu et al., 2013)
	Neratinib	EGFRL858R, EGFRT790M, HER2, HER4	(Vyse, 2019)
	Ceritinib	ALK, ROS1, IFG1R	(Isozaki et al., 2015)
	Alectinib	ALK, RET	(Arai et al. 2017)
	Brigatinib	ALK, EGFR	(Wu et al., 2016)
Third Generation	Osimertinib	EGFRL858R, EGFRDell9, EGFRT790M	(Cortellini et al. 2018)
	Rociletinib	EGFRL858R, EGFRDell9, EGFRT790M	(Tan et al. 2018)
	HM6I7I3	EGFRL858R, EGFRDell9, EGFRT790M	(Wang et al. 2016)
	Olmutinib	EGFRT790M	(Kim, 2016)
	ASP8273	EGFR L858R/T790M	(Azuma et al. 2018)
	EGF816	EGFRT790M, EGFRL858R, EGFRDell9	(Wang et al., 2016)
	PF-06747775	EGFR T790M	(Barnes et al., 2018)
	Avitinib	EGFR T790M	(Gou et al. 2016)
	HS-10296	EGFR T790M	(Sullivan et al., 2017)
	Lorlatinib	ALK, ROS1	(Facchinetti et al., 2018)

2.4. Modeling Drug Resistance *in vitro*

In vitro studies are critical for identifying the mechanisms of drug resistance, and developing new approaches to overcome chemoresistance. There are many methodologies followed to study drug resistance *in vivo* and *in vitro* (Jo et al., 2018). These methodologies can be grouped into clinically relevant and high-level laboratory models in the studies aiming to develop drug-resistant cell lines. Clinically relevant models aim to mimic the treatment regimen of the cancer patients during chemotherapy in the clinic. These models have some disadvantages such as inconsistent and subordinate resistance as well as small molecular changes to observe and analyze (McDermott et al., 2014). Unlike the clinically relevant models, high-level laboratory models are quite simple for creating stable resistant cell lines and detecting the molecular alterations occur during the development of resistance. Generally, the cell lines are treated with increasing concentrations of the drug by a prolonged time during resistance subline development. However, these models can deviate from the clinically relevant range because of final over-increased resistance levels (Lee et al., 2016). In the clinically relevant models, the drug is conducted with minimal increase in dose, while there is no limitation in the high-level laboratory models. Accordingly, the solubility of the selective agent is accepted as a limiting factor used to detect the amount of drug given to cancer cells in culture. A suitable range of chemotherapeutic drug can be selected through a cytotoxicity assay (McDermott et al., 2014).

For developing clinically relevant or high-level laboratory models, a strategy must include the selection of parental cell line, exposing it to a chemotherapeutic agent, collecting surviving cells and comparing them with the original parental cell line. For laboratory modeling of drug-resistant cell lines, the convenient cancer cell line should be selected based on the relatively low IC50 value of the drug (Nikounezhad et al., 2016).

While the pulsed treatment includes the application of the drug to cells for a short period of time, in the prolonged treatment the cells are continuously exposed to the drug. In general, prolonged exposure of cancer cell lines to certain anticancer drug concentration is preferred as a strategy by researchers to create resistant cell lines in the laboratory. For selecting the initial

concentration of the drug, some different approaches are available. Among them, a continuous increase of drug concentration over time, a second and final increase in the drug concentration or maintaining the same drug concentration can be selected. Incubation times may be arranged depending on prolonged or pulsed treatment strategies. After prolonged or pulsed treatment of cells, the less sensitive ones will withstand drugs and divide until confluency. During the development of the resistant cell line, in parallel, the parental cell line should be cultivated for observing alterations in resistant one and to compare. The comparison of the parental cell line and its isogenic resistant pair can be performed through cell viability/proliferation assays (Xavier et al., 2016).

The prolonged and pulsed treatment methods are simple, fast and cost-efficient cell-based assays that use traditional two-dimensional (2D) monolayer cell culture systems. However, 2D cell cultures cannot create *in vivo* natural three-dimensional (3D) micro environment of cells. Therefore, they may induce deceptive and unpredictable clinic responses to result in high-cost. To lower the cost and to remove toxicity especially before a stage of animal tests, 3D cell culture systems mimicking *in vivo* cell behaviors have been developed (Imamura et al., 2015). Two dimensional and 3D culture systems show differences in cellular characteristics such as morphology, proliferation, exposure to medium or drugs, stage of the cell cycle, gene or protein expression as well as drug sensitivity. Two dimensional cultured cells grow quickly, but 3D cultured cells proliferation rate depends on cell types and/or 3D model systems (Edmondson et al., 2014). The monolayer structure of 2D cell culture systems provides cells to exposed to nutrients, growth factors or drugs equally. Ultimately, there is a possibility that many cells in the same stage of the cell cycle. On the other hand, 3D cell cultures have a natural shape in spheroid/aggregate structures. Due to this structure, nutrients, growth factors or drugs cannot pass through spheroid and reach the center of the core. As a result, spheroids are composed of cells found in proliferating, quiescent, apoptotic, hypoxic, and necrotic stages similar to the heterogeneous nature of tumors. Gene or protein expression profiles of 3D cultured cells are more similar to *in vivo* than 2D cultured cells (Antoni et al., 2015). Through having various cell types, 3D culture systems can generate effective *in vitro* models for building multicellular systems. To study stromal cells in the tumor for treating cancer, the multicellular systems are crucial. Due to the connection between stromal cells and metastatic cancer cells, stromal cells are being attractive targets for treating cancer (Edmondson et al., 2014). However, in contrast to all these advantages, there are also

disadvantages of 3D systems. These systems are too expensive for both large-scale studies and high productivity tests compared to 2D cell cultures. The inconsistent results due to variability in biological matrices and the absence of vascularization important for tumor growth/ survival and drug delivery constitutes other disadvantages of 3D cell culture systems (Kapałczyńska et al., 2018).

NSCLC patients start to develop resistance to Erlotinib in approximately 10–14 months after the primary treatment, which results in reoccurring of the disease. In order to better treat patients with NSCLC, further studies should explore the molecular mechanisms behind Erlotinib resistance development (Rho et al, 2009). To that end, it was decided to use EGFR-TKI Erlotinib as a molecular target. KRAS mutant and EGFR wild-type A549 cell line is originated from an adenocarcinoma of the basal squamous epithelial cells. This cell line is widely used as an epithelial cell model for drug metabolism due to easy maintainance and identification in culture. The establishment of chemoresistant subline models *in vitro* is an essential resource for representing the mechanisms involved in drug resistance and for the development of novel drugs against them. For these reasons, A549-ER subline was generated by treating A549 cells with increasing concentrations of Erlotinib and then characterized at the molecular level.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Reagents

Roswell Park Memorial Institute-1640 medium (RPMI-1640, Biowest, Nuaille, France), fetal bovine serum (FBS, Biowest, Nuaille, France), phosphate buffered saline (PBS, Biowest, Nuaille, France), 0.25 % Trypsin- Ethylenediaminetetraasetic acid (Trypsin-EDTA, Gibco, MA, USA), penicilin streptomycin (PS, Gibco, MA, USA), Erlotinib hydrochloride (Santa Cruz Biotechnology, Inc., Hiedelberg, Germany), Dimethylsulphoxide (DMSO, Merck, NJ, USA), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution, 1 % aqueous crystal violet solution, powder agar, and agarose (Sigma Aldrich, MO, USA), Trizol Reagent (Invitrogen, MA, USA), High Capacity Complementary Deoxyribonucleic acid (cDNA) Reverse Transcription Kit (Applied Biosystems, MA, USA), FastStart Universal Synergy Brands Green (SYBR Green) Master (Rox) Mix (Roche, Basel, Switzerland) were purchased from commercial resources.

3.2. Methods

3.2.1. Cell lines and culture conditions

Human non-small cell lung cancer cell line was obtained from Dr. Ece Simsek of Akdeniz University. A549 cells were routinely cultured in RPMI-1640 supplemented with 10 % FBS and 1% PS. The cells were maintained in 5% CO_2 at 37°C and subcultured after reaching about 80 % confluency. The subculture of A549 cells was performed in 3 steps including aspiration of the media, washing cells with PBS and then treating cells with trypsin to harvest cells from the culture plate's surface. The cells at $5x10^5$ cells/mL were resuspended and then transferred to new cell culture plates containing fresh culture media with above ingradients and maintenance conditions. Cells grown in culture were treated by Erlotinib according to treatment strategy created from literature. For this purpose, a stock solution of Erlotinib was prepared by dissolving Erlotinib in DMSO. A working solution was prepared by

diluting stock solution with fresh media and adjusting Erlotinib concentration according to treatment conditions.

3.2.2. Establishment of Erlotinib A549 resistant subline

The concentration of drug at which 50% of the activity of its target is inhibited is called IC50 value. On the other hand, peak plasma concentration for a drug is its highest concentration circulating in the blood. Erlotinib concentrations were adjusted depending on IC50 values and peak plasma concentrations from previous studies. Then, based on the review in literature, treatment strategy of Erlotinib to A549 cells was carried out as shown in Table 3.1. With these findings, it was aimed to establish resistant cell lines by exposing the A549 cells to the increasing concentrations of Erlotinib every two weeks for two months. Finally, the cells were maintained in culture for another month at the final concentration of the Erlotinib to stabilize a resistant phenotype (A549-ER). To develop A549-ER, cells were seeded at the appropriate number in the cell culture medium. After reaching about 80 % confluency, A549 cell line was treated with the initial concentration of Erlotinib and subcultured. Every fortnight, the cells were reseeded for applying the next and higher concentration of Erlotinib. A549-ER was obtained following the end of Erlotinib treatment process as shown in Figure 3.1. Solubilizing agent DMSO treated A549-PT and A549-ER sublines were maintained in parallel in order to monitor the probable effects of Erlotinib on A549 cells.

Table 3.1 : Erlotinib Treatment Strategy for A549 cell line

Drug Name	**Mw (g/mol)**	**Plasma Concentration (ng/mL)**	**IC50(μM)**	**IC50 (μM) (calculated)**	**Concentration (μM)**	**Treatment time**
Erlotinib	393.443	2308	8	6	0, 3, 6, 9, 12	fortnightly

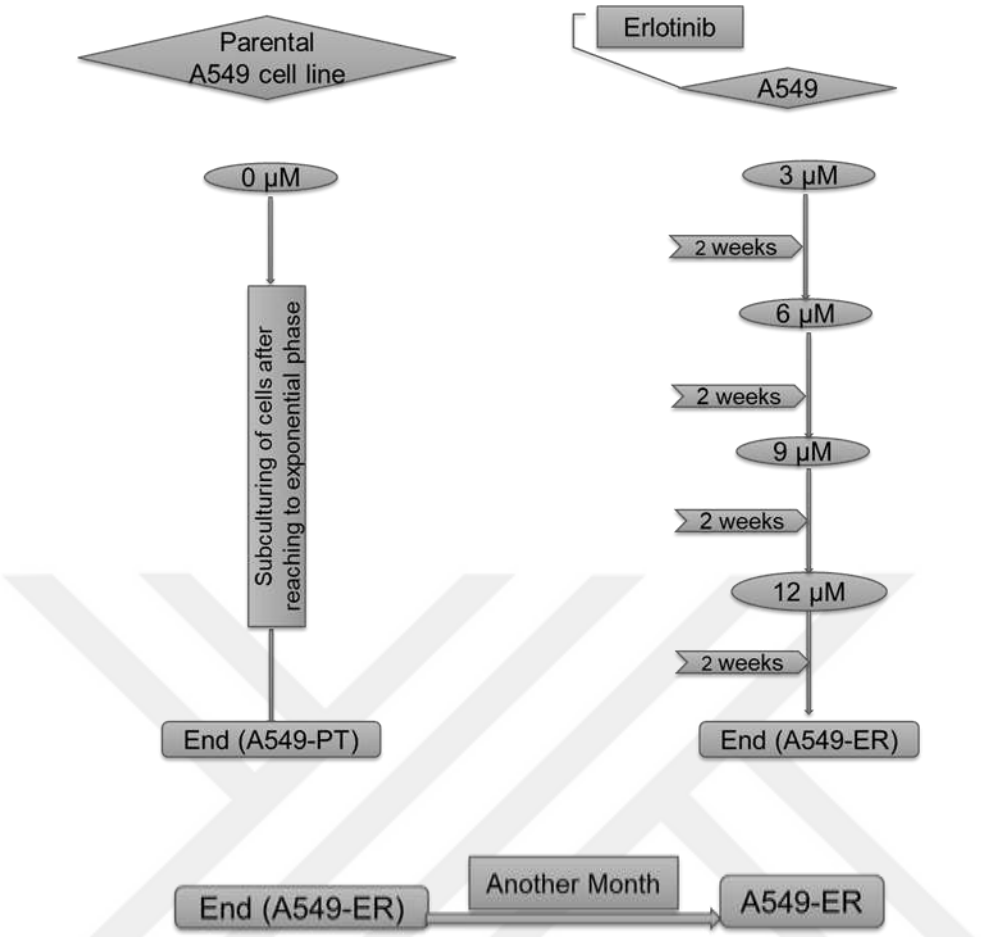


Figure 3.1: Establishment of A549-ER subline and treatment process. A549-ER subline was generated by applying increasing Erlotinib concentration by 3 µM upto 12 µM, every two weeks for two months. Resistant phenotype was stabilized by maintaining cells for another month at 12 µM Erlotinib. For comparison and observing the effects of Erlotinib on A549 cell line, A549-PT was maintained in culture parallel to A549-ER.

3.2.3. Cell Viability Assay

Cell survival was assessed by MTT assay. A549 cells were collected with trypsinization and cell suspensions including $5x10^4$ cells/mL were prepared in RPMI-1640 complete culture medium supplemented with 10 % FBS and 1 % PS. One and a half mL of the cell suspension was added to each well of 6-well plate. Cells were then incubated for 24 h at 37^oC and 5 % CO_2 conditions. After treating cells with Erlotinib at 3, 6, 9 and 12 µM, respectively, they were incubated for further 24 h. Ten percent of MTT working solution was prepared by diluting with complete culture medium. Then, 1 mL MTT solution was added to each well of the 6-well plates. Following 4 h incubation with MTT, 750 µL DMSO was added to each well. The cells were kept in DMSO for 15 minutes to dissolve formazan crystals. Afterward,

the absorbance of the samples at 570 nm were detected by using Shimadzu UV-VIS Spectrophotometer. The results were graphed as the percentage of cell viability & Erlotinib concentration in μM curve.

Cell survival was expressed as a percentage of viability by formula below:

% Cell viability = (absorbance of experiment samples/absorbance of control) x 100

3.2.4. Cell wound healing assay

The cells were trypsinized when they had reached the exponential growth phase. The cell suspensions including $1,5x10^5$ cells/mL were prepared in complete RPMI-1640 growth medium. One and a half mL of cell suspension was added to each well of the 6-well plate. After overnight incubation and cell attachment, a straight wound was formed on cell monolayer through using a 200 μL micropipette tip. Then, the medium was aspirated carefully and fresh media with Erlotinib was added to each well without harming the cells. The plates were placed into the incubator and wound closure images were taken at 0, 24, 48 and 72 hours with a Leica DM IL LED inverted microscope (Leica Biosystems, Wetzlar, Germany). Results were obtained by calculating wound area closure rates at these time intervals, respectively. Wound healing area was calculated by using Image J software. The results were graphed as the percentage of wound closure versus time.

The following formula was used to calculate wound closure:

Wound Closure (%) = [(At=0h-At=Δh)/ (At=0h)]x100%

Where A stands for total area of the wound at a particular time of measurement.

3.2.5. Soft Agar Colony Formation Assay

For base layer, 0.5 % agar solution was melted in microwave about 3 minutes and placed into water bath at 56 ^oC. Agar solution was kept 15 minutes at 56 ^oC and mixed with warmed complete RPMI-1640 media. Each well of a 6-well plate was covered with 1 mL of this mixture. For top layer, 0.3 % agarose was melted in microwave about 3 minutes and then taken to water bath at 40 ^oC. Agarose was kept at 40 ^oC for 20 minutes. Meanwhile, for preparing top agarose solution, $2x10^4$ cells/mL in PBS were mixed with complete RPMI-1640 and 0.3 % agarose. Then, 1 mL top agarose including cells was transferred onto each well without damaging agar base. After 24 hours, cells were fed with 0,5 mL of RPMI-1640 with or without Erlotinib per well for twice a week. After 3 weeks, cells were stained with 0,005 % crystal violet dye. Cells were held with dye an hour and then photographed by Leica EZ50

dissecting microscope (Leica Biosystems, Wetzlar, Germany). Finally, colonies over 0,2 mm were counted by using Image J software and colony number versus to Erlotinib concentration was graphed.

3.2.6. Quantitative Reverse Transcriptase Polymerase Chain Reaction Analysis

3.2.6.1. RNA isolation

RNA isolation was achieved by using the Trizol reagent, following the manufacturer's instructions. Cells were lysed by adding 0,4 mL trizol and were incubated for 5 minutes to dissociate nucleoproteins. Then lysates were incubated for 3 minutes with 0,008 mL of chloroform. Following the centrifugation step with chloroform, aqueous phase containing RNA and organic phase formed in the tube. After transferring aqueous phase to a new tube, RNA was precipitated with 0.2 mL isopropanol and washed with 75 % ethanol. After pelleting in centrifugation step, RNA was dried for 5-10 minutes. Samples were then solubilized with RNase-free water and then heated to 55 ^{o}C on a heat block. RNA quality and quantity were determined by using 2 µL RNA in a Nanodrop instrument (Thermofisher Scientific, MA, USA).

3.2.6.2. cDNA synthesis

Single-stranded cDNA from total RNA was synthesized by using High Capacity cDNA Reverse Transcription Kit. cDNA synthesis requires preperation of the 2× Reverse Transcription Master Mix, addition of total RNA and formation of reverse transcribed cDNA in a thermal cycle. After all these steps, cDNA can be used directly for quantitative-PCR. To prepare the 2× reverse transcription master mix per reaction at 20 µL volume , 4.2 µL of nuclease-free H_2O, 2 µL of 10X RT buffer, 0.8 µL of 25X dNTP mix (100 mM), 2 µL 10X RT random primers, 9 µL of RNA isolate and 1 µL of reverse transcriptase were added to a tube on ice. To perform cDNA reverse transcription reactions, 10 µL of 2X RT master mix and 10 µL RNA sample were placed into each tube. The tubes were centrifuged and placed on ice until loading the reaction mixture into thermal cycler. The reaction volume was set to 20 µL, and then reactions were loaded to thermal cycler (Agilent, CA, USA). Thermal cycler conditions optimized for use with High Capacity cDNA Reverse Transcription Kits include 4 steps. First, RNA sample was incubated at 37 ^{o}C for 120 min followed by incubation at room temperature for 10 minutes. In the next steps, the samples were heated up to 85 ^{o}C for 5

minutes and then incubated at 4 ^{o}C to inactivate Reverse Transcriptase and prevent binding enzyme to cDNA.

3.2.6.3. Quantitative Polimerase Chain Reaction (qPCR)

qPCR was performed by using FastStart Universal Synergy Brands Green Master Mix following the manufacturer's instructions. qPCR was carried out with 41 cycles including denaturation, annealing and elongation steps. Activation of DNA polymerase was achieved at the 1st cycle at 95 ^{o}C for 10 minutes. Afterward, 40 cycles were run for denaturation, and amplification steps. For denaturation step, samples were incubated 95 ^{o}C for 15 seconds followed by incubation at 59 ^{o}C for 60 seconds for amplification. Results were normalized against actin beta (ACTB) reference gene expression to obtain ΔCt values. The list of primers used in this analysis to observe alterations in gene expression of EMT markers is shown in Table 3.2.

Table 3.2: Sequences of oligonucleotide primers used in qRTPCR

Target	Forward Primer	Reverse Primer
CDH1	5'-ACA CTG CCA ACT GGC TGG AGA TTA -3'	5'-TGA TTA GGG CTG TGT ACG TGC TGT-3'
CDH2	5'-ACA GTG GCC ACC TAC AAA GG -3'	5'-CCG AGA TGG GGT TGA TAA TG-3'
VIM	5'-GAG AAC TTT GCC GTT GAA GC-3'	5'-GCT TCC TGT AGG TGG CAA TC-3'
OCC	5'-GCA AAG TGA ATG ACA AGC GG-3'	5'-CAC AGG CGA AGT TAA TGG AAG-3'
TJP1	5'-AGT AAG TCG TCC TGA TCC TGAA-3'	5'-TCG GCC AAA TCT TCT CAC TCC-3'
FBN	5'-CAG TGG GAG ACC TCG AGA AG-3'	5'-GTC CCT CGG AAC ATC AGA AAC-3'

4. RESULTS

4.1. Morphological Analysis in A549-PT and A549-ER Sublines

Erlotinib resistant A549-ER subline was established from A549 cell line by gradually increasing Erlotinib concentrations (0, 3, 6, 9, 12 μM respectively) every two weeks for two months as indicated in Figure 3.1. Cells were maintained another month at final concentration of Erlotinib (12 μM) to stabilize their resistant phenotype. To compare the influence of Erlotinib on A549 cell line's morphology, A549-PT parental subline was developed parallel to A549-ER. It was observed that after 3 months in culture, under different concentrations of Erlotinib, A549-ER subline formed more cell clusters than A549-PT (Figure 4.1). In other words, A549-ER subline developed more mesenchymal appearance by losing epithelial differentiation and communication. A549-ER was observed as spindle-shaped cells through microscopic examination.

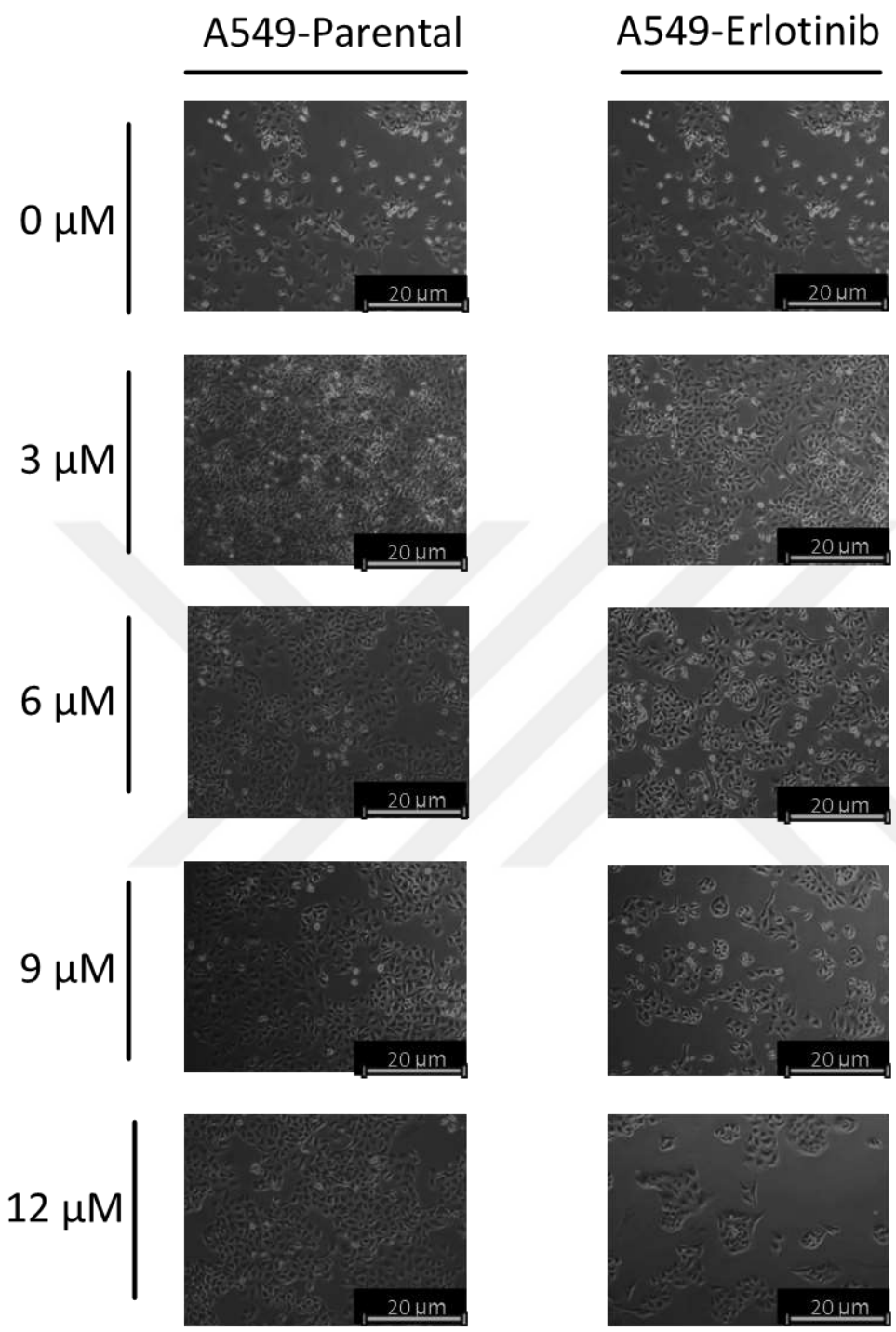


Figure 4.1. Cell morphology differences in A549-PT and A549-ER sublines. Cell morphology was monitored through microscopic examination. A549-PT cells have regular epithelial shape and tissue integrity; on the other hand, A549-ER cells were observed to form clusters in mesenchymal appearance that indicated loss of epithelial polarity. Photographs were taken at the end of each Erlotinib treatment (concentrations indicated on the left) period. Scale bar indicates 20 µm.

4.2. Sensitivity of A549-PT and A549-ER Sublines to Erlotinib

A549-PT and A549-ER sublines were tested for their sensitivity against Erlotinib. Cell viability of A549-PT and A549-ER was assessed by using MTT test after 24 and 48 hours of Erlotinib treatments with indicated concentrations on Figure 4.2. Cell viability changes were more or less equal with slight increase in A549-ER values after 24 hours of treatments with differing Erlotinib concentrations. An exception was under 9 μM Erlotinib concentration that A549-ER cell viability was increased significantly when compared to A549-PT (t-test; $p<0.05$). Results were similar with no statistically significant changes in cell viabilities after 48 hours of Erlotinib treatment with differing concentrations. These results have shown that resistance against Erlotinib does not change cell viability potential of A549-ER significantly but rather it may have been affected other phenotypic properties of this subline.

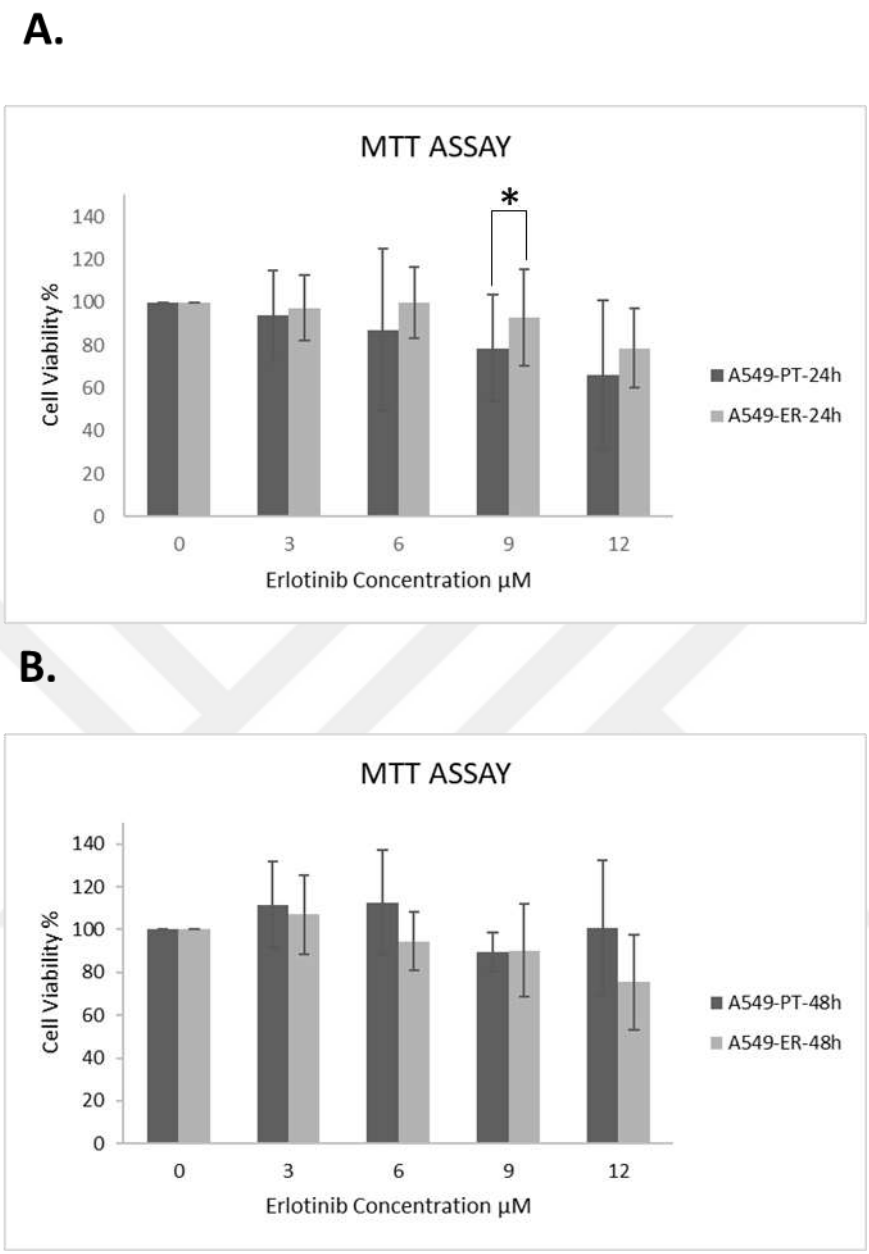


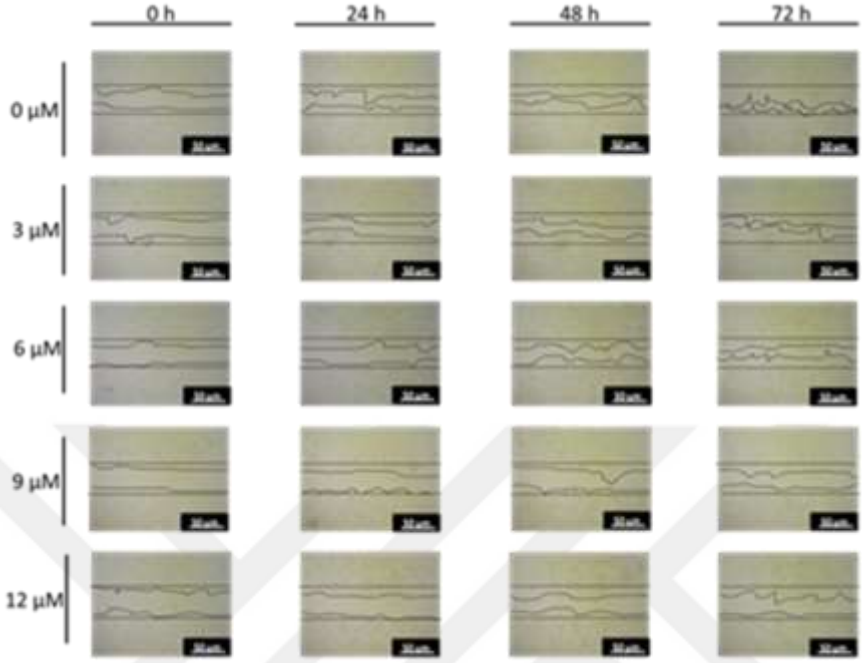
Figure 4.2. Erlotinib sensitivity levels in A549-PT and A549-ER sublines. Cell viabilities were measured A) 24 hours and B) 48 hours after Erlotinib addition by using MTT reagent. According to A549-PT subline, under 9 µM Erlotinib A549-ER subline showed statistically significant increase in cell viability (t-test; $p<0,05$). Data were represented as means of 6 biologically independent experiments. Standard deviations (±) included as error bars on top of the data columns. (*statistically significant data)

4.3. Cell Migration Ability of A549-PT and A549-ER Sublines

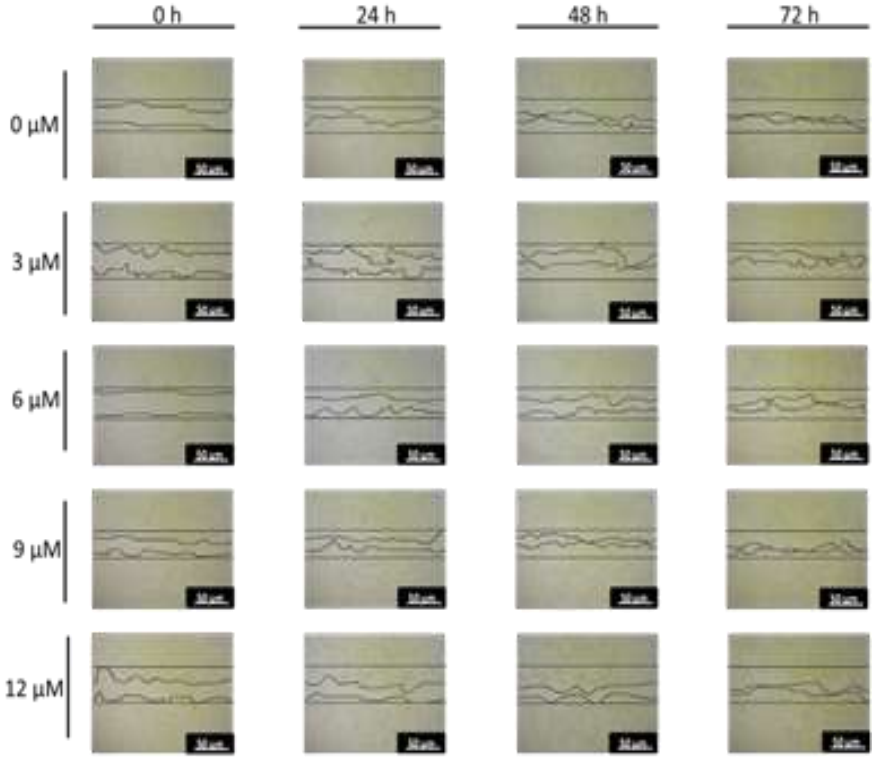
Cell migratory capacities of A549-PT and A549-ER sublines were assessed through cell wound healing assay. For this purpose, images were taken at 24, 48 and 72 hours after exposing cells to differing Erlotinib concentrations as indicated in Figure 4.3A. Wound healing area was calculated by Image software. The percentage of wound closure was calculated using these areas and plotted against Erlotinib concentrations differing to time as shown in Figure 4.3B. It was found that the cell migration capacity of A549-ER subline was higher than parental A549-PT.

A.

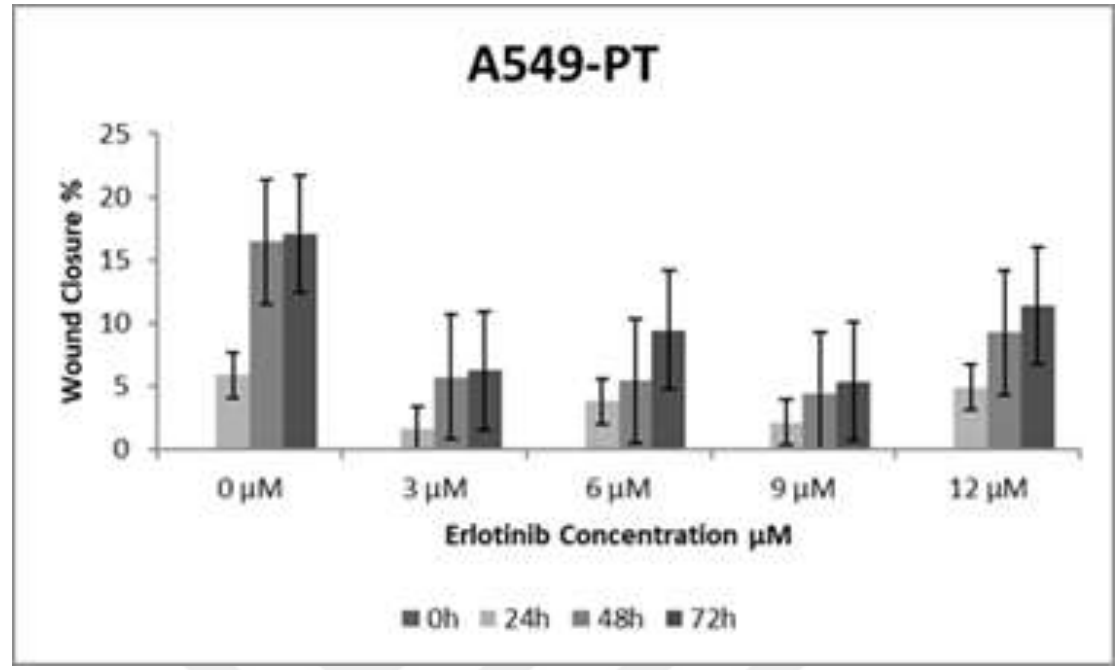
A549-PT



A549-ER



B.



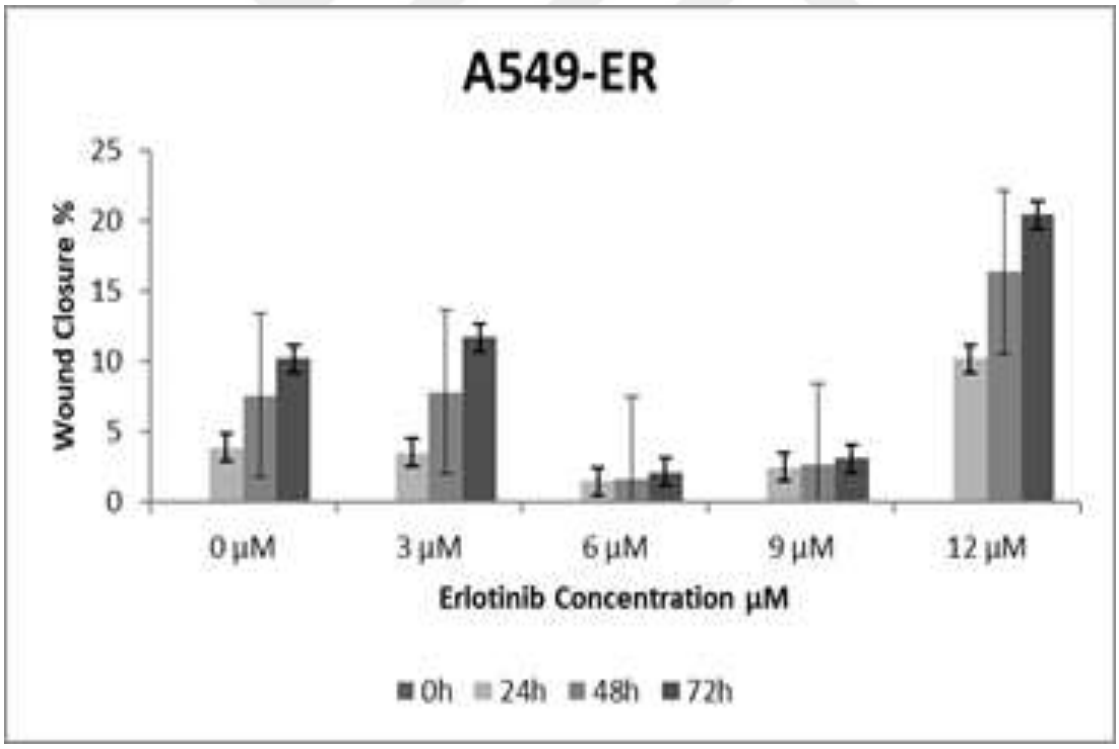


Figure 4.3: Comparison of cell migration abilities and wound healing capacities of A549-ER and A549-PT sublines. A) The speed of wound closure was monitored by taking pictures with inverted microscope at 24, 48, and 72 hours after Erlotinib treatment. Concentrations of the drug was included on the left. B) Wound healing area was calculated by using Image J programme. The percentage of wound closure of the increased concentration of Erlotinib over time was graphed. Scale bar indicates 50 µm.

4.4. Analysis of Tumor Formation Potential of A549-PT and A549-ER Sublines

Colony formation capacity of a tumor cell *in vitro* can be related to its metastatic potential and aggressiveness *in vivo*. Based on this information, soft agar colony formation assay was designed to assess the ability of a single cell to grow into a colony. Cells from A549-ER and A549-PT sublines were suspended in low concentration agar containing media. After 3 weeks of growth with or without 12 μM of Erlotinib treatment, cells were stained by crystal violet dye and their microscopic images were photographed. Figure 4.4A shows that in the presence of Erlotinib, A549-ER subline formed more detectable colonies than both A549-PT and A549 cells. On the other hand, there were no significant differences between colony formation abilities of A549-ER, A549-PT or A549 in the absence of Erlotinib (Figure 4.4B). These results demonstrated that Erlotinib exposure may have been improved colony formation potential and probably agressiveness of A549-ER subline.

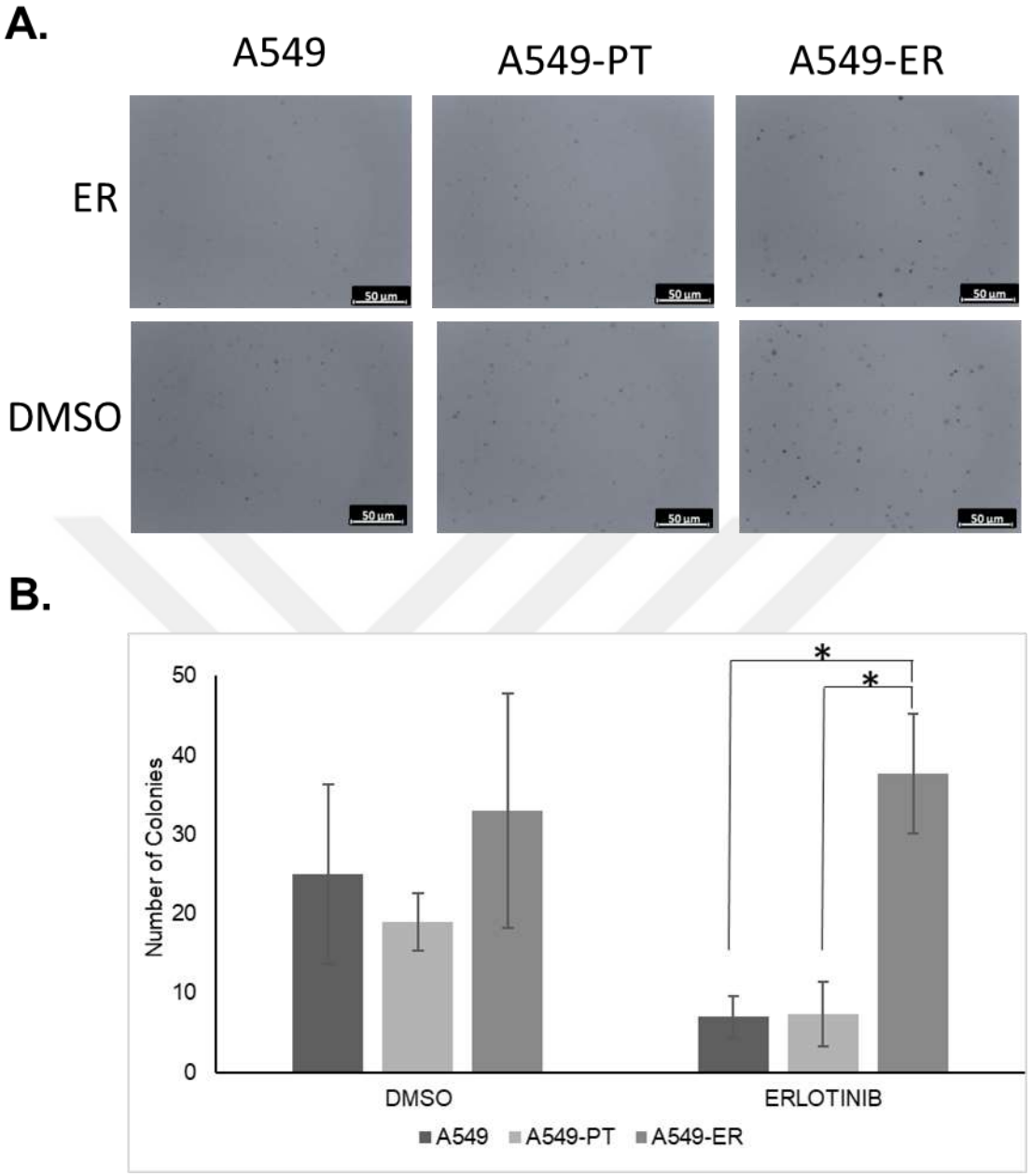


Figure 4.4. Tumor formation capacity in A549, A549-PT and A549-ER. Soft agar colony formation assay was performed to compare the tumor formation potential between A549-ER, A549-PT and A549 cells. Cells were seeded in the 0.3% top agarose layer to form colonies. DMSO was used as a control agent. A) Colonies were photographed under dissecting microscope after crystal violet staining. B) Colonies > 0,2 mm were counted on these images by using Image J software. Statistical analysis of colony numbers was performed by t-test ($p<0.05$ for A549-ER and A549, $p<0.01$ for A549-ER and A549-PT). Stars (*) indicate statistically significant differences.

4.5. Gene Expression of EMT Markers in A549-PT and A549-ER Sublines

Because A549-ER cells with mesenchymal appearance were found to be more aggressive with high migration capacity and because metastatic potential of lung cancer cells are associated with the EMT, we decided to investigate and compare the expression of epithelial and mesenchymal markers in A549-PT and A549-ER sublines. For this purpose, the level of mRNA expression levels of EMT markers in both sublines were measured by using qRT-PCR assay. Although mRNA levels of most markers did not change significantly, there was a notable difference between the levels of CDH2 expressions in A549-PT and A549-ER (Figure 4.5.) It was found that A549-ER cells with acquired mesenchymal phenotype, exhibited an upregulation in CDH2 expression. These results demonstrated that increased migration potential and aggressiveness in A549-ER subline may be attributed to increased mesenchymal CDH2 expression and thus to loss of intercellular connection and tissue integrity.

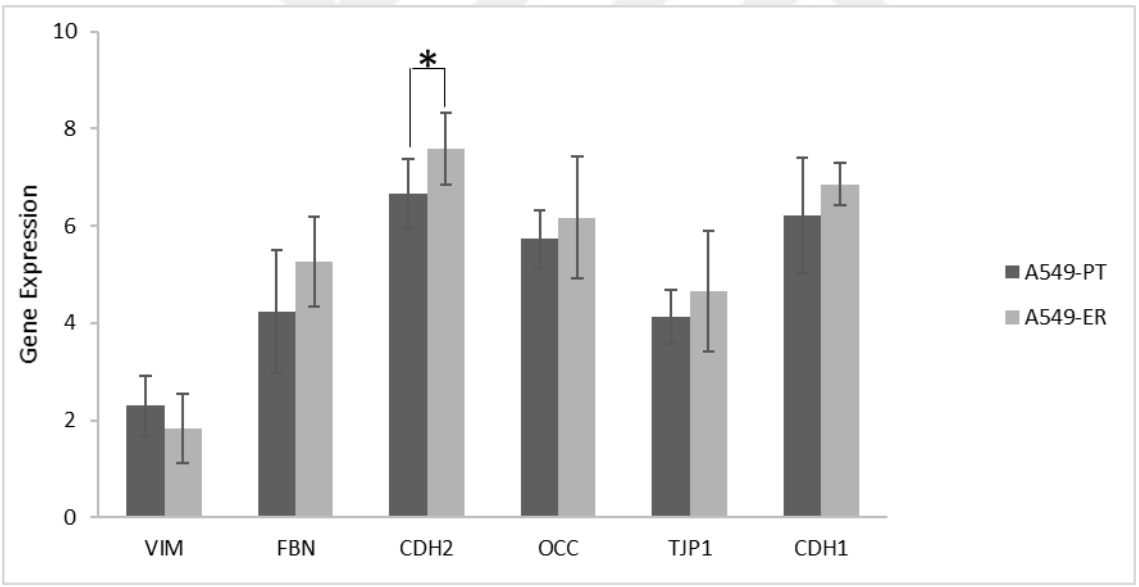


Figure 4.5. Comparison of mRNA expression levels of epithelial and mesenchymal markers. Gene expression of epithelial occludin (OCC), tight junction protein 1 (TJP1), E-cadherin (CDH1) and mesenchymal vimentin (VIM), fibronectin (FBN), N-cadherin (CDH2) markers in A549-ER and A549-PT cells were evaluated by qRT-PCR. The ACTB gene expression was used as a reference. EMT marker expressions were tested 4 times with 2 replicates for each primer and mean values are represented. The bars indicate standard deviation. Significant gene expression differences are indicated with stars (*) (t-test, p value<0.05).

5. DISCUSSIONS

Lung cancer is one of the most deadliest diseases worldwide. Due to limited efficiency of chemotherapy, 5-year disease free survival rate in patients with the most common form of lung cancer, NSCLC is still low (Naumov et al., 2009). This is attributed to high tumor heterogeneity and intrinsic resistance to drugs in NSCLC (Wu et al., 2014). In spite of advances in molecular targeted therapies such as those with new generation EGFR-TKIs (i. e. Erlotinib), low survival rates in patients with NSCLC still remains problematic in clinic due to the development of acquired resistance against drug in use (Tang et al., 2013). In this study, Erlotinib resistant subline of NSCLC cell line A549 was established *in vitro* by mimicking treatment strategy of NSCLC patients to investigate the causes of drug resistance in detail. Then, molecular characterization of resistant cell phenotype was investigated. A549 parental subline (A549-PT) was maintained in culture parallel to A549 Erlotinib resistant subline (A549-ER), for morphological and molecular comparisons.

We monitored morphology of A549-PT and A549-ER sublines for 3 months including 2 weeks consecutive treatments with Erlotinib at 3, 6, 9, and 12 μM concentrations. Morphological analysis demonstrated that normally epithelial A549-ER sublines gained mesenchymal appearance after this treatment. In addition, resistant subline was found to form more cell clusters than the parental subline. A549-ER cells were appeared as spingle-shaped cells, probably due to their loss of attachment from primary tumor mass.

Suda et al., (2011) had shown that an EGFR-mutant cell line which was made resistant to Erlotinib has represented EMT phenotype. On the other hand, Ikeda et al. indicated that A549-ER cells were more resistant to Erlotinib than parental A549 cells and A549-ER resistance do not correspond with EGFR mutations (Ikeda et al., 2011). Because underlying EGFR-TKI resistance mechanism in wild type EGFR bearing NSCLC was not clear, we investigated anti-tumor activity of Erlotinib on A549 (wild type EGFR but with KRAS mutation) cells. Expectedly, Erlotinib treatment was slightly effective on A549 cells in a concentration-dependent manner.

Tumor cells which leave the primary tumor mass migrate from one point to another within the body through the blood and lymph circulatory systems. Ghosh et al. (2012) was generated Erlotinib resistant H1650ER1 cell line harboring deletion in exon 19 of EGFR gene and performed cell migration assay to measure the ability of healing a defect in a cell monolayer. Of note, results showed that H1650ER1 cells have more tendency to move from one point to another. Starting from this, to measure migration capacity of A549-ER cells, which were established by exposing Erlotinib to A549 EGFR wild type cells at increasing concentrations, we performed cell wound healing assay. Our results showed that A549-ER cells had gained more migratory ability and heal the wound on cell culture plate's surface faster than the parental subline. This may show that enhanced migratory capacities of NSCLC cells under Erlotinib treatment, in fact, are independent from EGFR status in these cells.

Soft agar colony formation assay was carried out to analyze tumorigenic potential of A549-ER cells. HCC827 harboring activating EGFR mutation was exposed to Erlotinib and investigated anchorage independent growth by soft agar assay. Xie et al. (2015) reported that HCC827 cells formed colonies, but colonies disappeared after treatment with 1 μM erlotinib. HCC827ER cells on the other hand, were found resistant to Erlotinib because of increasing in number of colonies. We performed soft agar colony formation assay to monitor tumor progression by measuring the tumor formation potential. We found that long time exposure to Erlotinib had increased colony formation ability of A549 cells and they had become more aggressive and more prone to metastasis.

Because I observed a more aggressive phenotype with higher migratory capacity in A549-ER subline, I decided to check whether if the expression of epithelial to mesenchymal transition (EMT) markers had been affected during resistance development. EMT process has been shown to have important roles in tumor invasion, metastasis and recurrence of different cancers (Heerboth et al. 2015). EMT appears in loss of epithelial morphology and cell shapes as well as with loss of cell-cell adhesions (Wu et al., 2014). Drug resistance with poor prognosis has been associated with EMT in NSCLC patients in several studies (Otsuki et al., 2018; Wang et al., 2019; Chae et al., 2018). EMT has been suggested as a target in therapy of NSCLC patients, but its molecular mechanisms are still not clear. To shed light on any possible expressional changes in EMT markers, I carried out qRT-PCR assay. In this process, the mRNA expressions of E-cadherin, N-cadherin, fibronectin, vimentin, occludin ve TJP-1

which are reported to exhibit alterations during EMT were evaluated in Erlotinib resistant and parental sublines. Zhou et al. (2018) established two gefitinib-resistant cell lines PC-9/ZD (T790M mutation) and PC-9/GR (EMT phenotypes) using the human lung adenocarcinoma cell line PC-9 harboring the delE746-A750 mutation in the EGFR gene. They observed that PC-9/GR cells had demonstrated mesenchymal morphology and PC-9/GR cells undergoing EMT indicated higher migration and invasion abilities than PC-9 cells without EMT. However, they have not found any remerkable differences between PC-9/ZD and PC-9 cells and whether decreased sensitivity of the PC-9/GR cell line to various chemotherapeutics was associated with EMT. Yamauchi et al. (2011) reported significant upregulation of N-cadherin in gefitinib-resistant PC9/ZD cells (EGFR T790M). Furthermore, it was demonstrated that N-cadherin regulated survival signal in acquired or inherently gefitinib-insensitive lung cancer cells. Zhang et al. (2013) noticed that molecular changes developed in Erlotinib resistant H1650 cell line consistent with the EMT due to N-cadherin overexpression. Of note, they reported that EMT was associated with erlotinib resistance in NSCLC. N-cadherin increases the metastatic capacity of tumor cells. Therefore, it causes to epithelial cancer metastasis and adverse effects on disease progression. As a conclusion, N-cadherin is demonstrated as a target to prevent metastasis and overcome drug resistance. (Mrozik et al., 2018). In accordance with these studies, the mRNA expression level of N-cadherin increased significantly in A549-ER resistant subline in my study.

6. RECOMMENDATIONS

To find out the reasons why cells acquire resistance to drugs and to overcome the drug resistance, we should understand the resistance mechanisms. A549-ER sub-cell lines generated in here can be used to investigate the effects of 2nd and 3rd generation EGFR-TKIs or other chemotherapautics used in the treatment of NSCLC. Novel combinations of drugs can be tried or effects of TKIs on critical cell signalling pathways can be investigated. For this purpose, signals which contributed to resistance can be detected by RTK analysis. Caspase activity can be investigated in cells to observe whether TKIs induce apoptosis or not. Cell cycle analysis can be carried out to find whether Erlotinib has an influence on cell cycle arrest. DNA content cell cycle analysis of A549-ER sublines could be carried out to investigate underlying resistance mechanism. Another reason which seems as a cause of resistance to drugs is EMT. Finally cDNA microarray analysis may help to confirm significant EMT gene expression changes during drug resistance development.

REFERENCES

Antoni, D., Burckel, H., Josset, E., Noel, G. (2015). '' Three-Dimensional Cell Culture: A Breakthrough in Vivo.'' Int. J. Mol. Sci. 2015, 16, 5517-5527.

Arai, S., Kita, K., Tanimoto, A., Takeuchi, S., Fukuda, K., Sato, H., Yano, S. (2017). "In Vitro and in Vivo Anti-Tumor Activity of Alectinib in Tumor Cells with NCOA4-RET." 8(43): 73766–73.

Azuma K., Nishio M., Hayashi H., Kiura K., Satouchi M., Sugawara S., Hida T., Iwamoto Y., Inoue A., Takeda K., Ikeda S., Nakagawa T., Takeda K., Asahina S., Komatsu K., Morita S., Fukuoka M., Nakagawa K. (2018). "ASP8273 Tolerability and Antitumor Activity in Tyrosine Kinase in Japanese Patients with EGFR Mutation-Positive Non-Small-Cell Lung Cancer." (February): 2532–38.

Barnes, T., A., Leighl, N., B., O'Kane, G., M. (2018). "Resistance to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors , T790M , and Clinical Trials." 25(June): 28–37.

Bhatia, K., Ellis, S., (2006)."Unusual lung tumours: an illustrated review of CT features suggestive of this diagnosis."Cancer Imaging. (2006); (6)1:72-82.

Bleehen, N., M. (1985). "Staging and Treatment of Small Cell Lung Cancer." Journal belge de radiologie 68(3): 185–89.

Cagle, P., T., Allen, T., C., and Olsen, R., J. (2013). "Lung Cancer Biomarkers: Present Status and Future Developments." Archives of pathology & laboratory medicine 137(9): 1191–98.

Chae, Y., K., Chang, S., Ko, T., Anger, J., Agte, S., Iams, W., Choi, WM., Lee, K., Cruz, M. (2018). "Epithelial-Mesenchymal Transition (EMT) Signature Is Inversely Associated with T-Cell Infiltration in Non-Small Cell Lung Cancer (NSCLC)." Scientific Reports 8(1): 2–9.

Cortellini, A., Buttitta, F., Marchetti, A., and Ficorella, C. (2018). "Journal of Molecular and Genetic Practical Advices About How to Handle Disease Progression During Osimertinib in EGFR -Mutant NSCLC Patients : Is It The Same Old Story ?" 12(1): 1–4.

Cosaert, J., Quoix, E. (2002). "Platinum Drugs in the Treatment of Non-Small-Cell Lung Cancer." British journal of cancer 87: 825–33.

Cruz CS., D., Tanoue, L., T., Matthay, R., A. (2011). "Lung Cancer: Epidemiology, Etiology and Prevention." Clin Chest Med 32(4): 1–61.

Edmondson, R., Broglie, J., J., Adcock, A., F., Yang, L. (2014). "Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery and Cell-Based Biosensors." ASSAY and Drug Development Technologies 12(4): 207–18.

Facchinetti, F., Friboulet, L. (2018). "Lorlatinib in ALK- and ROS1-Positive NSCLC: The Future Has a Start." Translational Lung Cancer Research 7(S2): S103–6.

Gainor, J., F., Dardaei, L.,Yoda, S., Friboulet, L., Leshchiner, I., Katayama, R., Dagogo-Jack, I., Gadgeel, S., Schultz, K., Singh, M., Chin, E., Parks, M., Lee, D., DiCecca, R.,H., Lockerman, E., Huynh, T., Logan, J., Ritterhouse, L., L, Le, L., P., Muniappan, A., Digumarthy, S., Channick, C., Keyes, C, Getz, G, Dias-Santagata, D, Heist, RS, Lennerz, J, Sequist, L., V., Benes, C.,H., Iafrate, A., J., Mino-Kenudson, M. Engelman, J., A., Shaw, A., T. (2016). "Molecular Mechanisms of Resistance to First- and Second-Generation ALK Inhibitors in ALK -Rearranged Lung Cancer." Cancer Discov.2016 Oct.;6(10):1118-1133, Epub2016 Jul 18.

Gazdar, A., F. (2009). "Activating and Resistance Mutations of EGFR in Non-Small-Cell Lung Cancer: Role in Clinical Response to EGFR Tyrosine Kinase Inhibitors." Oncogene 28(Suppl 1): 1–14.

Ghosh, G., Lian, X., Kron, S., J., and Palecek, S., P. (2012). "Properties of Resistant Cells Generated from Lung Cancer Cell Lines Treated with EGFR Inhibitors." BMC Cancer 12(1): 95.

Gou, L., Y., Li, A., N., Yang, J., J., Zhang, X., C., Su, J., Yan, H., H., Xie, Z., Lou, N., N., Liu, S., Y., Dong, Z., Y., Gao, H., F., Zhou, Q., Zhong, W., Z., Xu, C., R., Wu, Y., L. (2016). "The Coexistence of MET Over-Expression and an EGFR T790M Mutation Is Related to Acquired Resistance to EGFR Tyrosine Kinase Inhibitors in Advanced Non-Small Cell Lung Cancer." Oncotarget 7(32).

Heerboth, S., Housman G, Leary M, Longacre M, Byler S, Lapinska K, Willbanks A, Sarkar S. (2015). "EMT and Tumor Metastasis." Clinical and Translational Medicine 4(1).

Hirsh, V. (2015). "Next-Generation Covalent Irreversible Kinase Inhibitors in NSCLC: Focus on Afatinib." BioDrugs 29(3): 167–83.

Hopper-Borge, E., Wangari-Talbot, J. (2014). "Drug Resistance Mechanisms in Non-Small Cell Lung Carcinoma." J Can Res Updates 2(4): 265–82.

Huang, L., and Fu, L. (2015). "Mechanisms of Resistance to EGFR Tyrosine Kinase Inhibitors." Acta Pharmaceutica Sinica B 5(5): 390–401.

Huber, H. (2004). "Drug Resistance in Lung Cancer: Future Perspectives." Lung Cancer 21: S28.

Iglesias, S., V., Giuranno, L., Dubois, L., J., Theys, J., Vooijs, M. (2018). "Drug Resistance in Non-Small Cell Lung Cancer: A Potential for NOTCH Targeting?" Frontiers in Oncology 8(July).

Ikeda, R., Vermeulen, L., C., Lau, E., Jıang, Z., Kavanaugh, S., M.,Yamada, K., Kolesar J., M. (2011). "Isolation and Characterization of Erlotinib-Resistant Human Non-Small Cell Lung Cancer A549 Cells." Oncology Letters 2(1): 91–94.

Imamura, Y., Mukohara, T., Shimono, Y., Funakoshi, Y., Chayahara, N., Toyoda, M., Kıyota, N., Takao, S., Kono, S., Nakatsura, T., Minami, H. (2015). ''Comparison of 2D- and 3D-culture models as drug-testing platforms in breast cancer.'' Oncology Reports 33: 1837-1843.

Inamura, K. (2017). ‘’Lung Cancer: Understanding Its Molecular Pathology and the 2015 WHO Classification.’’ Front. Oncol. 7:193.

Isozaki, H., Takigawa, N., Kiura, K. (2015a). “Mechanisms of Acquired Resistance to ALK Inhibitors and the Rationale for Treating ALK-Positive Lung Cancer.” Cancers 7(2): 763–83.

Isozaki, H., Takigawa, N., Kiura, K. (2015b). “Mechanisms of Acquired Resistance to ALK Inhibitors and the Rationale for Treating ALK-Positive Lung Cancer.” Cancers 7(2): 763–83.

Jo, Y., Choi, N., Kim, K., Koo, H., K., Choi, J., Kim, H., N. (2018). ‘’Chemoresistance of Cancer Cells: Requirements of Tumor Microenvironment-mimicking In Vitro Models in Anti-Cancer Drug Development.’’ Theranostics 2018; 8(19):5259-5275.

Joyce, H. (2014). “Molecular Mechanisms of Drug Resistance and Invasion in a Human Lung Carcinoma Cell Line.” PhD thesis, Dublin City University.

Kapałczyńska, M., Kolenda, T., Przybyła, W., Zajączkowska, M., Teresiak, A., Filas, V., Ibbs, M., Bliźniak, R., Łuczewski, L., Lamperska, K. (2016). ‘’ 2D and 3D cell cultures – a comparison of different types of cancer cell cultures.’’ Arch Med Sci, 4:910-919.

Kılıç, A. (2012). “Epidermal Büyüme Faktör Reseptör İnhibitörleri ve Dermatolojik Yan Etkiler Inhibitors of Epidermal Growth Factor Receptor and Dermatological.” Derleme / Review (3): 168–74.

Kim, E., S. (2016). ‘’ Olmutinib: First Global Approval.’’ Drugs. 2016 Jul;76(11):1153-7.

Kosaka, T., Yamaki, E., Mogi, A., Kuwano, H. (2011). “Mechanisms of Resistance to EGFR TKIs and Development of a New Generation of Drugs in Non-Small-Cell Lung Cancer.” Journal of Biomedicine and Biotechnology 2011.

Landi, L., Cappuzzo, F. (2013). “Irreversible EGFR-TKIs: Dreaming Perfection.” Translational lung cancer research 2(1): 40–49.

Lee, Y., Choi, Y., R., Kim, K., Y Shin, D., H. (2016).’’ The impact of intermittent versus continuous exposure to EGFR tyrosine kinase inhibitor on selection of EGFR T790M-mutant drug-resistant clones in a lung cancer cell line carrying activating EGFR mutation.’’ Oncotarget. (2016). Jul 12; 7(28):43315-43323.

Liao, B., C., Lin, C., C., Lee, J., H., Yang, J., C., H. (2016). “Update on Recent Preclinical and Clinical Studies of T790M Mutant-Specific Irreversible Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors.” Journal of Biomedical Science 23(1): 1–10.

Lopes G., L., de Queiroz Vattimo E., F., Gilberto, de C. (2015). “Identifying Activating Mutations in the EGFR Gene: Prognostic and Therapeutic Implications in Non-Small Cell Lung Cancer.” The Jornal Brasileiro de Pneumologia 41(4): 365–75.

Mahipal, A., Kothari, N., Gupta, S. (2014). “Epidermal Growth Factor Receptor Inhibitors: Coming of Age.” Cancer control : journal of the Moffitt Cancer Center 21(1): 74–79.

McDermott, M., Eustace, A., J., Breen, L., Busschats, S., Crown, J., Clynes, M., O'Donovan, N., Stardal, B. (2014). "In Vitro Development of Chemotherapy and Targeted Therapy Drug-Resistant Cancer Cell Lines: A Practical Guide with Case Studies." Frontiers in Oncology 4(March).

Morgillo, F., Corte, C., M., D., Ciardiello, F. (2016). "Mechanisms of Resistance to EGFR-Targeted Drugs : Lung Cancer." : 1–10.

Mrozik, K., M., Blaschuk, O., W., Cheong, C., M., Zannettino, A., C., W., Vandkye, K. (2018). "N-Cadherin in Cancer Metastasis, Its Emerging Role in Haematological Malignancies and Potential as a Therapeutic Target in Cancer." BMC Cancer 18(1): 1–16.

National Cancer Institute. (1995). "Lung Cancer." Lung Cancer 12(3): 326–27.

Naumov, G., N., Nilsson, M., B., Cascone, T., Briggs, A., Straume, O., Akslen, L., A., Lifshits, E., Byers, L., A., Xu, L., Wu, H., Jänne, P., Kobayashi, S., Halmos, B., Tenen, D., Tang, X., M., Engelman, J., Yeap, B., Folkman, J., Johnson, B., E., Heymach, J., V. (2009). "Combined Vascular Endothelial Growth Factor Receptor and Epidermal Growth Factor Receptor (EGFR) Blockade Inhibits Tumor Growth in Xenograft Models of EGFR Inhibitor Resistance." Clinical Cancer Research 15(10): 3484–94.

Nicholson, S., A., Beasley, M., B., Brambilla, E., Hasleton, P., S., Colby, T., V., Sheppard, M., N., Falk, R., Travis, W., D. (2002). ''Small Cell Lung Carcinoma'' The American Journal of Surgical Pathology 26(9): 1184–1197, 2002.

Nikounezhad, N., Nakhjavani, M., Shirazi, F., H. (2016). ''Generation of Cisplatin-Resistant Ovarian Cancer Cell Lines.'' Iranian Journal of Pharmaceutical Sciences 2016: 12 (1): 11-20.

Nursal, A., F. (2017). "Importance of Anaplastic Lymphoma Kinase Gene Re-Arrangements on Non-Small Cell Lung Cancer." Kafkas Journal of Medical Sciences 7(2): 169–73.

Okamoto, K., Okamoto, I., Hatashita, E., Kuwata, K., Yamaghuchi, H., Kita, A., YAmanaka, K., Ono, M., Nakagawa, K. (2011). "Overcoming Erlotinib Resistance in EGFR Mutation-Positive Non-Small Cell Lung Cancer Cells by Targeting Survivin." Molecular Cancer Therapeutics 11(1): 204–13.

Otsuki, Y., Saya, H., Arima, Y. (2018). "Prospects for New Lung Cancer Treatments That Target EMT Signaling." Developmental Dynamics 247(3): 462–72.

Paraschiv, B., Diaconu, C., C., Dumitrache- Rujinski, S., Belaconi, I., N., Constantinescu, T., Stroescu, C., C., Dantes, E., Fildan, A., P., Bogdan, M., A., Toma, C., L. (2016). "Treatment Options in Stage III Non-Small Cell Lung Cancer - Short Review." Pneumologia 65(2): 67–70.

Pathak, A., K., Bhutani, M., Mohan, A., Gularia, A., Bal, S., Kochupillai, V. (2004). "Non Small Cell Lung Cancer (NSCLC): Current Status and Future Prospects." The Indian journal of chest diseases & allied sciences 46(3): 191–203.

Prim, N., Legrain, M., Guerin, E., Mennecier, B., Weingertner, B., Voegeli, A., C., Guenot,

D., Maugard, C., M., Quoix, A., E., Beau-Faller, M. (2014). “Germ-Line Exon 21 EGFR Mutations, V843i and P848l, in Nonsmall Cell Lung Cancer Patients.” European Respiratory Review 23(133): 390–92.

Remon, J., Morán, T., Majem, M., Reguart, N., Dalmau, E., Márquez-Medina, D., Lianes, P. (2014). “Acquired Resistance to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors in EGFR-Mutant Non-Small Cell Lung Cancer: A New Era Begins.” Cancer Treatment Reviews 40(1): 93–101.

Rho, J., K., Choi, Y., J., Lee, J., K., Ryoo, B., Y., Na, I., I., Yang, S., H., Kim, C., H., Lee, J., C. (2009). Epithelial to mesenchymal transition derived from repeated exposure to gefitinib determines the sensitivity to EGFR inhibitors in A549, a non-small cell lung cancer cell line. Lung Cancer 2009;63:219-26

Riely, G., J., Pao, W., Pham, D., Li, A., R., Rizvi, N., Venkatraman, E., S., Zakowski, M., F., Kris, M., G., Ladanyi, M., Miller, V., A. (2006). “Cancer Therapy : Clinical Clinical Course of Patients with Non ^ Small Cell Lung Cancer and Epidermal Growth Factor Receptor Exon 19 and Exon 21 Mutations T Reated with Gefitinib or Erlotinib.” 12(3): 839–45.

Sève, P., Dumontet, C. (2005). “Chemoresistance in Non-Small Cell Lung Cancer.” : 73–88.

Sgambato, A., Casaluce, F., Maione, P., Rossi, A., Ciardiello, F., Gridelli, C. (2014). “Cetuximab in Advanced Non-Small Cell Lung Cancer (NSCLC): The Showdown?” Journal of Thoracic Disease 6(6): 578–80.

Škarda, J., Hajdúch, M., Flodr, P., Fridman, E., Kopolovic, J., Kolek, V., Klein, J., Kolář, Z. (2007). “Drug Resistance in Lung Cancer.” 570: 545–70.

Smythe, W., R. (2001). “Treatment of Stage I and II Non-Small-Cell Lung Cancer.” Cancer Control 8(4): 318–25.

Stewart, E., L., Tan, S., Z., Liu, G.,Tsao, M., S. (2015). “Known and Putative Mechanisms of Resistance to EGFR Targeted Therapies in NSCLC Patients with EGFR Mutations — a Review.” Translational Lung Cancer Research 4(16): 67–81.

Stone, A., Rajanna, S., Rastogi, I., Cruz, J., Harrington, K., Blank, K., Frakes, M., Puri, N. (2014). “C-Met: A Potential Target for Current Non-Small-Cell Lung Cancer Therapeutics.” Chemotherapy: Open Access 3(3): 1000136.

Suda, K., Tomizawa, K., Fujii, M., Murakami, H., Osada, H., Maehara, Y., Yatabe, Y., Sekido, Y., Mitsudomi, T. (2011). “Epithelial to Mesenchymal Transition in an Epidermal Growth Factor Receptor-Mutant Lung Cancer Cell Line with Acquired Resistance to Erlotinib.” Journal of Thoracic Oncology 6(7): 1152–61.

Suda, K., Onozato, R., Yatabe, Y., Mitsudomi, T. (2015). “Small Cell Lung Cancer Transformation and T790M Mutation: Complimentary Roles in Acquired Resistance to Kinase Inhibitors in Lung Cancer.” Scientific Reports 5(1): 14447.

Suda, K., Onozato, R., Yatabe, Y., Mitsudomi, T. (2009). "EGFR T790M Mutation: A Double Role in Lung Cancer Cell Survival?" Journal of Thoracic Oncology 4(1): 1–4.

Sullivan, I., Planchard, D. (2017). "Next-Generation EGFR Tyrosine Kinase Inhibitors for Treating EGFR-Mutant Lung Cancer beyond First Line." Frontiers in Medicine 3(January): 1–13.

Sun, W., Yuan, X., Tian, Y., Wu, H., Xu, H., Hu, G., Wu, K. (2015). "Non-Invasive Approaches to Monitor EGFR-TKI Treatment in Non-Small-Cell Lung Cancer." Journal of Hematology and Oncology 8(1): 1–9.

Tan, C., S., Kumarakulasinghe, N., B., Huang, Y., Q., En Ang, Y., L., Rou-En Choo, J., Goh,B.,C., Soo, R., A. (2018). "Third Generation EGFR TKIs : Current Data and Future Directions." : 1–14.

Tang, J., Salama, R., Gadgeel, S., M., Sarkar, F., H., Ahmad, A. (2013). "Erlotinib Resistance in Lung Cancer: Current Progress and Future Perspectives." Frontiers in Pharmacology 4 FEB(February): 1–9.

Thomson, S., Petti, F., Sujka-Kwok, I., Epstein, D., Haley, J., D. (2008). "Kinase Switching in Mesenchymal-like Non-Small Cell Lung Cancer Lines Contributes to EGFR Inhibitor Resistance through Pathway Redundancy." Clinical and Experimental Metastasis 25(8): 843–54.

Timm, A, Kolesar, J., M. (2013). "Crizotinib for the Treatment of Non-Small-Cell Lung Cancer." American Journal of Health-System Pharmacy 70(11): 943–47.

Tsvetkova, E, Goss, G., D. (2012). "Drug Resistance and Its Significance for Treatment Decisions in Non-Small-Cell Lung Cancer." Current oncology (Toronto, Ont.) 19 (Suppl 1): S45-51.

Verma, M. (2012). ''Personalized Medicine and Cancer.'' J. Pers. Med. 2012, 2, 1-14; doi:10.3390/jpm2010001

Vyse, S. (2019). "Targeting EGFR Exon 20 Insertion Mutations in Non-Small Cell Lung Cancer." Signal Transduction and Targeted Therapy (January): 1–10.

Wang, J., Wang, B., Chu, H., Yao, Y. (2016). "Intrinsic Resistance to EGFR Tyrosine Kinase Inhibitors in Advanced Non-Small-Cell Lung Cancer with Activating EGFR Mutations." OncoTargets and Therapy 9: 3711–26.

Wang, S., Cang, S., Liu, D. (2016). "Third-Generation Inhibitors Targeting EGFR T790M Mutation in Advanced Non-Small Cell Lung Cancer." Journal of Hematology & Oncology 9(1): 34.

Wang, X., Goldstein, D., Crowe, P., Yang, J., L. (2016). "Next-Generation EGFR/HER Tyrosine Kinase Inhibitors for the Treatment of Patients with Non-Small-Cell Lung Cancer Harboring EGFR</Em> Mutations: A Review of the Evidence." OncoTargets and Therapy Volume 9: 5461–73.

Wang, X., Zhang, H., Chen, X. (2016). “Drug Resistance and Combating Drug Resistance in Cancer.” Cancer Drug Resistance.

Wongvaranon, P., Pongrakhananon, V., Chunhacha, P., Chanvorachote, P. (2013). “Acquired Resistance to Chemotherapy in Lung Cancer Cells Mediated by Prolonged Nitric Oxide Exposure.” Anticancer Research 33(12): 5433–44.

Westover, D., Zugazagoitia J., Cho BC, Lovly CM, Paz-Ares L. (2018). “Mechanisms of Acquired Resistance to First-and Second-Generation EGFR Tyrosine Kinase Inhibitors.” Annals of Oncology 29(Supplement 1): i10–19.

Wu F., Li J., Jang C., Wang J., Xiong J. (2014). “The Role of Axl in Drug Resistance and Epithelial-to-Mesenchymal Transition of Non-Small Cell Lung Carcinoma.” International Journal of Clinical and Experimental Pathology 7(10): 6653–61.

Wu, J., Savooji, J., Liu, D. (2016). “Second- and Third-Generation ALK Inhibitors for Non-Small Cell Lung Cancer.” Journal of Hematology & Oncology 9(1): 19.

Wu, S., G., Liu, Y., N., Tsai, M., F., Chang, Y., L., Yu, C., J., Yang, P., C., Yang, J., C., Wen, Y., F., Shih, J., Y. (2016). “The Mechanism of Acquired Resistance to Irreversible EGFR Tyrosine Kinase Inhibitor-Afatinib in Lung Adenocarcinoma Patients.” Oncotarget 7(11): 12404–13.

Xavier, C., P., R., Pesic, M., Vasconcelos, M., H. (2016). “Understanding Cancer Drug Resistance by Developing and Studying Resistant Cell Line Models.” Current Cancer Drug Targets 16(3): 226–37.

Xie, C., Jin, J., Bao, X., Zhan, W., H., Han, T., Y., Gan, M., Zhang, C., Wang, J. (2015). “Inhibition of Mitochondrial Glutaminase Activity Reverses Acquired Erlotinib Resistance in Non-Small Cell Lung Cancer.” Oncotarget 7(1).

Xu, J., Wang, J., Zhang, S. (2017). “Mechanisms of Resistance to Irreversible Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors and Therapeutic Strategies in Non-Small Cell Lung Cancer.” Oncotarget 8(52): 90557–78.

Yamauchi, M., Yoshino, I., Yamaguchi, R., Shimamura, T., Nagasaki, M., Imoto, S., Niida, A., Koizumi, F., Kohno, T., Yokota, J., Miyano, S., Gotoh, N. (2011). “N-Cadherin Expression Is a Potential Survival Mechanism of Gefitinib-Resistant Lung Cancer Cells.” American journal of cancer research 1(7): 823–33.

Yu, C., Chou, C., Tu, H., Huang, W., Ho, Y., Khoo, K., Lee, M., Changu, G. (2018). “Antibody-Assisted Target Identification Reveals Afatinib, an EGFR Covalent Inhibitor, down-Regulating Ribonucleotide Reductase.” Oncotarget 9(30): 21512–29.

Yu, J., Xiao, J., Yang, Y., Cao, B. (2015). “Oxaliplatin-Based Doublets versus Cisplatin or Carboplatin-Based Doublets in the First-Line Treatment of Advanced Nonsmall Cell Lung Cancer.” Medicine (United States) 94(27): e1072.

Zappa, C., Mousa, S., A. (2016). “Non-Small Cell Lung Cancer: Current Treatment and Future Advances.” Translational Lung Cancer Research 5(3): 288–300.

Zhang, X., Liu, G., Kang, Y., Dong, Z., Qian, Q., Ma, X., (2013). “N-Cadherin Expression Is Associated with Acquisition of EMT Phenotype and with Enhanced Invasion in Erlotinib-Resistant Lung Cancer Cell Lines.” PLoS ONE 8(3).

Zhang, N., Wei, X., Xu, L. (2013). ‘’miR-150 promotes the proliferation of lung cancer cells by targeting P53’’. FEBS Letters 587 (2013) 2346–2351

Zheng, M. (2016). “Classification and Pathology of Lung Cancer.” Surgical Oncology Clinics of North America 25(3): 447–68.

Zhou, J., Hu, Q., Zhang, X., Zheng, J., Xie, B., Xu, Z., Zhang, W. (2018). “Sensitivity to Chemotherapeutics of NSCLC Cells with Acquired Resistance to EGFR-TKIs Is Mediated by T790M Mutation or Epithelial-Mesenchymal Transition.” Oncology Reports 39(4): 1783–92.

Zou, Q., Zhan, P., Lv, T., Song, Y. (2015). “The Relationship between BIM Deletion Polymorphism and Clinical Significance of Epidermal Growth Factor Receptor-Mutated Non-Small Cell Lung Cancer Patients with Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitor Therapy: A Meta-Analysis.” Translational lung cancer research 4(6): 792–96.

CURRICULUM VITAE

AYLİN GÜNDÜZ

Address: Yenikent Mah, Irmak Sitesi, 4. Blok, Daire 27
Gebze/KOCAELİ
Phone number: +905394638496
E-mail: aylingunduz14@gmail.com

CAREER GOAL

Become professional in the field of biotechnology using my academic knowledge and experiences.

EDUCATION

September 2016-June.2019	Adana Alparslan Türkeş Science and Technology University
	Graduate School of Natural and Applied Sciences
	Department of Nanotechnology and Engineering Sciences
	Master of Science
	Adana/TURKEY
September 2008- August 2014	Ege University, Faculty of Engineering
	Department of Bioengineering, Bachelor's Degree
	Izmir / TURKEY
August 2012-February 2013	University of Minho
	Academic Semester As An Exchange Student
	Braga / PORTUGAL

WORK/INTERNSHIP

April 2019-Contunie	**N**ovakim Pharmaceuticals Co.Inc/Novakim Biotech Research and Development Specialist
August 2015-October 2015	Bioneks Biotechnology Responsible for Microbiology Department
July 2011-August 2011	Ege University, Department of Bioengineering Cell Tissue Culture Laboratory (Voluntary Internship)
June 2012-July 2012	Tüpraş, Kocaeli Izmit Refinery Izmit Refinery Management Research And Development Laboratory (Internship)

CERTIFICATES

August 2014	Iema Educatıon Consultancy Good Hygiene, Laboratory and Manufacture Practices GHP, GLP, GMP
April 2013	Ege Unıversıty Environment Awareness Symposium
March 2011	Istanbul Unıversıty Student Wınter School International VIII. Iugen Molecular Biology and Genetic
September 2010-June 2011	Turkısh Educatıon Volunteers Foundatıon Education of Communication, Çiğli / IZMIR

LANGUAGES

Turkish : Native Speaker

Arabic : Advance

English : Advance

Portuguese : Basic

COMPUTER KNOWLEDGE

Excellent Knowledge : Internet , Microsoft Office (Word, Power Point , Excel)

SKILLS

- To accord easily with working team
- Communicate effectively with people
- Pleasant to work in process line and laboratory
- Work long hours without physical stress and annoyance

INTERESTS

- Travel
- Foreign Language
- Reading of newspaper and books
- Reading of scientific papers
- Cooking

REFERENCES

Name: Prof. Dr. Murat ELİBOL
Address: Ege University, Bioengineering
Research interests:

- Chemical Reaction Engineering
- Chemical Process Design
- Bioengineering
- Biotechnology

E-mail: murat.elibol@ege.edu.tr

Name: Prof. Dr. Rengin ELTEM
Address: Ege University, Bioengineering
Research interests:

- Industrial Biotechnology
- Microbial Biotechnology

E-mail: renginеltem@gmail.com

Name: Doç. Dr. Aylin Şendemir ÜRKMEZ
Address: Ege University, Bioengineering
Research interests:

- Biomaterials
- Tissue Engineering
- Bioengineering

E-mail: aylin.sendemir@ege.edu.tr

Name: Asst. Prof. Dr. Özgür Cem ERKİN
Address: Adana Alparslan Türkeş Science and Technoloy University Bioengineering

Research interests:

- Cancer Genetic
- Cancer Signal Pathways
- Drug Resistance Mechanisms in Cancer

E-mail: ocemerkin@adanabtu.edu.tr