

LOYOLA UNIVERSITY CHICAGO

IDENTIFYING ONCOGENIC DRIVERS IN NSCLC CELLS HARBORING EGFR
KINASE DOMAIN MUTATION WITH RESISTANCE TO EGFR TKI AND
MESENCHYMAL PHENOTYPE

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDANCY FOR THE DEGREE OF
MASTER OF SCIENCE

MOLECULAR PHARMACOLOGY AND THERAPEUTICS

BY

YANDI GAO

CHICAGO, IL

DECEMBER 2014

Copyright by Yandi Gao and Takeshi Shimamura, 2014
All rights reserved

ACKNOWLEDGEMENTS

I would like to first express my sincere acknowledgements to my advisor, Professor Takeshi Shimamura, who invested his full effort in guiding me achieving this goal. I cannot say thank you enough for his tremendous help and support. Without his patiently guidance and persistent help this thesis would not have been possible.

I would also like to thank my committee members, Dr. Denning and Dr. Clipstone for the grateful support and critical suggestions on my research. In addition, I would like to thank our laboratory manager Margaret Soucheray and lab technician Jeffery Becker for the technical help of my research, my dearest friend and my fellow Rutu Gandhi for the continually help, care and encouragement.

I wish to take this opportunity to thank all the professors and friends who directly or indirectly have lent their helping hand during my entire study at Loyola. Last but not least, I would like to thank my family for the unceasing support, understanding and love.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iii
LIST OF FIGURES	vi
LIST OF TABLES	viii
LIST OF ABBREVIATIONS	ix
ABSTRACT	xi
CHAPTER ONE: INTRODUCTION	1
Overview of lung cancer	1
EGFR kinase domain mutant NSCLC	2
Resistance to EGFR-target therapy	5
Primary resistance	5
Acquired resistance	7
Overview of EMT	9
EMT and acquired EGFR TKI resistance	10
Involvement of AXL in EGFR TKI resistance in EGFR mutant NSCLC with EMT	11
Chemokine receptor CXCR7 and its potential role in acquired EGFR TKI resistance with epithelial to mesenchymal transition	15
CHAPTER TWO: MATERIALS AND METHODS	19
Cell lines	19
Lentiviral package and production	19
Target gene knockdown using lentiviral pLKO.1 shRNA vector	20
Generation of HCC827 cells stably overexpressing shRNA against Non-target/ EGFR/ CDH1	20
Generation of HCC827 cells stably overexpressing GFP or LacZ or CXCR7	21
Lentiviral knockdown of CXCR7 in HCC827 cells ectopically overexpressing CXCR7	21
Drugs	22
Cell viability assay	22
Western blot	22
Phospho-RTK array analysis	24
siRNA transfection	24
Apoptosis AnnexinV Assay	25
qRT- PCR	25

CHAPTER THREE: RESULTS	27
EGFR or E-Cadherin depletion by lentiviral shRNA in HCC827 cells induces epithelial to mesenchymal transition	27
Mesenchymal-like HCC827 cells with constitutive EGFR or E-Cadherin Knockdown are resistant to EGFR tyrosine kinase inhibitor gefitinib	28
Part I: Tyrosine receptor kinase AXL	30
EGFR TKI resistant mesenchymal like HCC827 shEGFR and HCC827 shCDH1 cells are insensitive to AXL inhibition and AXL inhibition does not restore their gefitinib sensitivity	30
None of the 42 receptor tyrosine kinase listed in the RTK array seem to be driver of EGFR TKI resistant HCC827 shEGFR and HCC827 shCDH1 cells with mesenchymal phenotype.	32
AXL is upregulated in EGFR TKI resistant HCC827 shEGFR and HCC827 shCDH1 cells with mesenchymal phenotype; however its activity is dependent on the activity of EGFR	33
AXL depletion neither induces cell apoptosis nor resensitizes HCC827 shEGFR or HCC827 shCDH1 cells to gefitinib	35
Part II: Chemokine receptor CXCR7	36
CXCR7 is commonly upregulated in mesenchymal-like HCC827 cells	37
CXCR7 is overexpressed in HCC827 shCDH1 cells.	38
Ectopic CXCR7-V5 overexpression in HCC827 NSCLC cells	40
Ectopic CXCR7 overexpression in HCC827 cells promotes mesenchymal phenotype	41
Ectopic CXCR7 overexpression in NCI-H1975 cells promotes mesenchymal transition	43
Ectopic CXCR7 overexpression is sufficient to confer EGFR TKI resistance in HCC827 cells	45
CXCR7 depletion restores gefitinib sensitivity in HCC827 CXCR7-V5 cells	49
CXCR7 knockdown partially reverse mesenchymal HCC827 CXCR7-V5 to epithelial	51
CHAPTER FOUR: DISCUSSION	53
REFERENCES	61
VITA	72

LIST OF FIGURES

Figure	Page
1. Mesenchymal-like HCC827 cells with constitutive EGFR knockdown or E-Cadherin knockdown are resistant to EGFR TKI gefitinib	29
2. AXL inhibition does not restore gefitinib sensitivity in EGFR TKI resistant mesenchymal-like HCC827 cells with constitutive EGFR knockdown or E-Cadherin knockdown	31
3. Analysis using RTK array demonstrates no RTKs are activated in mesenchymal-like EGFR TKI resistant HCC827 cells upon gefitinib treatment	33
4. EGFR inactivation downregulates AXL phosphorylation in mesenchymal-like HCC827 cells with constitutive EGFR or E-Cadherin knockdown	34
5. AXL depletion alone or in combination with EGFR TKI do not induce apoptosis in mesenchymal-like EGFR TKI resistant HCC827 cells with constitutive EGFR or E-Cadherin knockdown	36
6. 49 unique genes commonly upregulated in the mesenchymal-like HCC827 cells	38
7. CXCR7 is overexpressed in HCC827 cells with constitutive E-Cadherin knockdown	39
8. CXCR7-V5 was ectopically overexpressed in HCC827 NSCLC cells	40
9. Phenotype changes in HCC827 cells with ectopic CXCR7-V5 expression	42
10. Ectopic CXCR7-V5 overexpression in HCC827 cells promotes induction of mesenchymal-like phenotype	42
11. Ectopic overexpression of CXCR7 promotes mesenchymal phenotype in NCI-H1975 cells	44
12. Ectopic overexpression of CXCR7 attenuates the sensitivity of HCC827 cells to EGFR TKI	46
13. Assessment of apoptosis by Annexin V-FITC/PI double staining demonstrates that gefitinib treatment does not compromise viability of cells ectopically	

	expressing CXCR7-V5	47
14.	Gefitinib treatment promotes significantly less PARP-cleavage in HCC827 cells with CXCR7-V5 overexpression than in HCC827 GFP control cells	48
15.	EGFR TKI resistance in HCC827 cells by ectopic overexpression of CXCR7 is reversible	50
16.	Depletion of CXCR7 partially restores epithelial phenotype	52
17.	A schematic diagram of a proposed mechanism for CXCR7- mediated acquired EGFR TKI resistance in EGFR mutant NSCLC.	59

LIST OF TABLES

Table	Page
1. Antibody list	23
2. CXCR7 Primer list	26

LIST OF ABBREVIATIONS

AB/ AM	Antibiotic-Antimycotic
ATP	Adenosine Triphosphate
CDH1	E-Cadherin
CMS	Comparative Marker Selection
CXCR7	C-X-C chemokine receptor type 7
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal transition
ERK	Extracellular signal Regulated Kinase
Gas6	Growth arrestin-specific 6
GFP	Green Fluorescent Protein
GPCR	G-Protein Coupled Receptor
HGF	Hepatocyte growth factor
Hsp90	Heat Shock Protein 90
MAPK	Mitogen Activated Protein Kinase
NF-kB	Nuclear Factor Kappa Beta
NSCLC	Non-Small Cell Lung Cancer
PI3K	Phosphatidylinositol 3-Kinase

PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCLC	Small Cell Lung Cancer
shRNA	short hairpin RNA
TGF- β	Transforming Growth Factor beta
TKI	Tyrosine Kinase Inhibitor

ABSTRACT

Lung cancer is the leading cause of cancer death in the world. Across all stages and all types of lung cancer, the overall 5-year survival is less than 15%. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of lung cancer cases. Tumors from approximately 10-15% of NSCLC patients in the U.S. harbor epidermal growth factor receptor (EGFR) kinase domain mutation.

Exon 19 deletion mutation and exon 21 substitution mutations are the two most prevalent mutations in EGFR tyrosine kinase domain. These kinase domain mutations lock EGFR in a constitutively active conformation, leading to hyper-activation of pro-survival and anti-apoptotic signaling pathways in NSCLC. In addition, NSCLC harboring the EGFR kinase domain mutations exhibits a typical oncogene addiction model, where the cells are exquisitely dependent on mutant EGFR for cell survival and proliferation. In this model, successful inhibition of the oncogene results in oncogene shock; which promotes cell cycle arrest and/or cell death. Structurally, EGFR with exon 19 deletion and exon 21 substitution mutations shows higher affinity to small molecule-reversible EGFR tyrosine kinase inhibitors (EGFR TKIs), such as gefitinib and erlotinib than ATP. Consequently, the reversible EGFR TKIs preferentially inhibit EGFR tyrosine kinase activity to promote oncogene shock in the subset of NSCLC with EGFR kinase domain activating mutations. In the clinic, patients with the EGFR kinase domain mutations initially respond well to the EGFR inhibitors; however, acquired drug resistance will mainly emerge after 6-18 months of treatment.

Besides the well-established EGFR TKI resistant mechanisms such as the EGFR T790M secondary mutation and *MET* gene amplification, 30% of resistant mechanisms are still unknown. Systematic genetic and histological analyses of tumor biopsies from patients show that about half of the patients present an evidence of epithelial to mesenchymal transition (EMT). EMT is characterized by the loss of epithelial cell junction marker E-Cadherin and gain of mesenchymal cell marker such as N-Cadherin and Vimentin. EMT is associated with many cellular changes, however, the molecular mechanisms by which mesenchymal-like cells attenuate their dependency on EGFR with kinase domain mutations are still unknown. We hypothesize that the induction of mesenchymal transition in NSCLC cells harboring EGFR kinase domain mutation promotes aberrant upregulation of bypass receptor tyrosine kinases (RTKs) or other oncogenic drivers to replace the oncogenic mutant EGFR.

While this hypothesis was being tested, several groups have demonstrated by Zhang's group that receptor tyrosine kinase AXL is upregulated in EGFR-mutant NSCLC cell lines with acquired resistance to erlotinib with mesenchymal features. It has been shown that AXL activates downstream pathways to bypass the mutant EGFR downstream signaling in NSCLC cells with the mesenchymal phenotype. Despite this finding, we failed to demonstrate that AXL is the oncogenic driver in our mesenchymal NSCLC models. Instead, we observed overexpression of chemokine receptor CXCR7 *in vitro* and NSCLC models of acquired resistance to EGFR TKI with mesenchymal phenotype. Consequently, we hypothesized that CXCR7 is an oncogenic driver in EGFR mutant NSCLC cells with acquired resistance to EGFR TKI to offset the activation of downstream signaling by mutant EGFR. To test this hypothesis, we ectopically

overexpressed V5 tagged CXCR7 in EGFR mutant NSCLC cells. Our studies demonstrate that ectopically overexpressing CXCR7 in EGFR mutant NSCLC cells is sufficient to promote EGFR TKI resistance and EMT. Furthermore, we demonstrated that EGFR TKI resistance induced by ectopic CXCR7 overexpression in mutant EGFR NSCLC cells is reversible. We also demonstrated that CXCR7 knockdown not only restored sensitivity to EGFR TKI but also partially reversed mesenchymal phenotype to epithelial phenotype. In summary, our data suggested CXCR7 as a promising therapeutic target for preventing or overcoming EGFR TKI resistance in a subset of EGFR mutant NSCLC patients.

CHAPTER ONE

INTRODUCTION

Overview of lung cancer

Lung cancer is the leading cause of cancer death in the United States (1). About 1.35 million new patients are diagnosed with lung cancer every year (1) and the 5-year-survival rate is less than 15% (1). Lung cancer originates in the tissue of lungs or cells lining the bronchi and cancer cells continually accumulate mutations which promote aberrant cell growth and proliferation.

Lung cancers are typically divided into two main categories: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). About 85% to 90% of lung cancers are NSCLC and 10 percent of cases are SCLC (2). Some patients are diagnosed with a combined SCLC/NSCLC cancer as well (2). Based on whether the cancer is local or has spread from the lungs to the lymph nodes or other organs, lung cancer has been defined as different stages (3). In Stage I, the cancer is located only in the lungs and has not spread to any lymph nodes. In stage II, the cancer is in the lung and nearby lymph nodes. In the stage III, cancer is found in the lung and in the lymph nodes in the middle of the chest, also described as locally advanced disease. Stage IV is the most advanced stage of lung cancer, and is also described as advanced disease. This is when the cancer has spread to both lungs, to fluid in the area around the lungs, or metastasized to another part of the body. The early symptoms like coughing and fatigue are not easily

noticed, even 7-10% of patients with lung cancer are asymptomatic, lung cancer has a poor prognosis and most people with lung cancer are not diagnosed when the disease is well advanced.

After the initial diagnosis of NSCLC, it is necessary to determine the patients' stages accurately in order to make the right therapeutic decision (3). Treatment of lung cancer mainly involves surgery, chemotherapy, radiation therapy and more recently targeted therapy-alone, or in combination (4). Surgery to remove the tumor is the most potentially curative therapeutic options for the NSCLC in the stage I and early stage II periods. Surgery in combination with chemotherapy and radiation could also provide great efficacy than surgery alone and could reduce the chance of recurrence. Because most lung cancer cannot be completely cured by the current therapy, appropriate palliative care is also a very important part in the lung cancer treatment (5).

One of the most exciting developments in lung cancer therapy is the introduction of targeted therapies. Targeted therapies are designed specifically to attack the abnormal molecules responsible for the specific tumor so that they are considered to be more effective and have fewer side effects as compared to traditional chemotherapies (6). The identification of oncogenic drivers that are known to play essential roles in cancer growth and survival in NSCLC cells is necessary for the efficacy of targeted therapies. Patients who have advanced lung cancer with certain molecular biomarkers may benefit from targeted drug alone or in combination with chemotherapy (7). Among these, epidermal growth factor (EGFR) kinase domain mutations in NSCLC strongly predict the improved response rate and progression-free survival by EGFR inhibition (6).

EGFR kinase domain mutant NSCLC

EGFR is a trans-membrane cell surface receptor tyrosine kinase (8). It is a member of ErbB family of receptors (8). ErbB family is composed of four structurally related receptor tyrosine kinases: ErbB1 (EGFR), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4). These receptors consist of an extracellular ligand-binding domain, a trans-membrane lipophilic domain and an intracellular tyrosine kinase domain. EGFR is activated by binding of its ligands including epidermal growth factor (EGF) and transforming growth factor- α (TGF- α) (8). Upon binding by its ligands, EGFR undergoes conformational changes which promote homo-dimerization with EGFR or hetero-dimerization with other ERBB family members such as ErbB3. EGFR dimerization stimulates the auto-phosphorylation of its tyrosine residues in the intracellular domain through transferring γ -phosphates from adenosine triphosphate (ATP) (9). This phosphorylations lead to: 1) increased catalytic efficiency and furthering phosphorylation of other downstream substrates; 2) phosphorylated tyrosine carboxyl-terminals constitute docking sites and recruit other downstream signaling proteins containing SRC homology 2 (SH2), protein tyrosine-binding domains (PTB), or other phosphor-tyrosine-binding domains (10, 11) EGFR together with its downstream substrates form signaling complexes, and subsequently initiate downstream signaling cascades controlling multiple cellular processes, including phosphatidylinositol 3-kinase (PI3K)/AKT pathway, Ras/Raf/mitogen-activated protein kinase pathway (MAPK) pathway, which contribute to cell survival and proliferation (12, 13).

In 2004, two groups from the Dana-Farber/ Harvard Cancer Center identified somatic mutations in the tyrosine kinase (TK) domain of EGFR in most NSCLC patients who respond well to a small molecule EGFR tyrosine kinase inhibitor (TKI) (14, 15). As EGFR TKI preferentially inhibits mutant EGFR, the efficacy of EGFR TKI is profound in NSCLC tumors that express mutant EGFR but not in normal cells that express wild type EGFR. EGFR kinase domain mutations are present in approximately 30-35 percent of cases in East Asian NSCLC patients and 10 percent of cases in Caucasian NSCLC patients, especially non-smoking young Asian female patients (16, 17), and are more associated with adenocarcinomas with bronchioalveolar features (18). The most prevalent mutations in the EGFR tyrosine kinase domain are located within EGFR exon 18-21, which are around the ATP-binding pocket of enzyme (18) . 45 percent of the cases are exon 19 deletion, amino-acids between residues 747-750 of EGFR polypeptide. L858R point substitution mutation in exon 21 also comprises approximately 40-45 percent of EGFR mutations. Nucleotide substitution in exon 18 or exon 20 insertion individually accounts for 5 percent of EGFR mutations (18). Kinase domain mutations increase the kinase activity of EGFR, leading to hyper-activation of PI3K and MAPK signaling pathways and consequently confer oncogenic effects (14, 18). Therefore, cancer cells, which harbor EGFR kinase domain mutations become addicted to EGFR signaling for their survival and proliferation. In addition, mutant EGFR changes its kinase domain structure, decreases its affinity to ATP and increases its affinity for small molecule EGFR TKI such as gefitinib (Iressa) and erlotinib (Tarceva) (15). EGFR TKI competes with ATP for binding to the catalytic binding site (19) . In the clinic, NSCLC

patients harboring EGFR kinase domain mutations are associated with increased sensitivity to EGFR TKI. One of the major effects of EGFR TKI in sensitive EGFR-mutant NSCLC cells is the induction of intrinsic apoptosis pathways (20, 21). PI3K and MAPK signaling pathways have been shown to cooperate to promote cell survival and proliferation on NSCLC (22). EGFR TKI inhibits both PI3K and MAPK pathways and kills EGFR addicted NSCLC. MAPK inhibition after EGFR TKI treatment leads to rapidly increased activity of proapoptotic protein BIM in EGFR mutant NSCLC by increasing the production and dephosphorylation of BIM (20, 21). PI3K inhibition after gefitinib treatment leads to downregulation of anti-apoptotic protein myeloid leukemia cell differentiation 1 (MCL1) (23). The upregulation of BIM cooperates with the downregulation of MCL1 to promote cell death. Despite the initial response to EGFR TKI, the development of resistance inevitably occurs after 6-18 months treatment (24).

Resistance to EGFR-target therapy

Primary resistance: Most EGFR kinase domain mutations confer sensitivity to EGFR TKI; however, some EGFR kinase domain mutations predict the primary resistance to EGFR TKI. There are insertions or duplications mutations that occur in exon 20 in *EGFR* gene, resulting in amino acid insertion between D770 and N771 or substitution in amino acid position 771, render the receptor 100-fold less sensitive to gefitinib or erlotinib as compared to tumors harboring the exon 21 substitution mutations or exon 19 deletions in EGFR (25, 26). Although in most cases, exon 20 T790M mutations are associated with acquired resistance to EGFR TKI, presence of T790M also links to the primary resistant mechanism (27).

NSCLC tumor harboring EGFR mutation could be insensitive to EGFR TKI because of the presence of other genetic alterations that co-occur with EGFR mutations. EGFR downstream signaling pathways, PI3K and MAPK, are critical for cell survival and proliferation in EGFR mutant NSCLC (22). Tumor cells sensitive to EGFR TKI are characterized by remarkable down-regulation of AKT phosphorylation (28, 29). Consequently, activating mutation in phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) is found to rescue the cells from sensitivity to EGFR TKI (30). Also, loss of phosphatase and tensin homologue (PTEN) expression, which indirectly regulate the activation of AKT, correlates with decreased sensitivity of EGFR mutant NSCLC to EGFR TKI (31). Insulin-like growth factor receptor 1 (IGFR1), has been implicated as a potential resistant mechanism through crosstalk with EGFR to continually activate PI3K signaling pathway in EGFR overexpressed glioblastoma cells (32). Recently, Bivona *et al.* identified the hyperactivation of NFkB pathway as a new mechanism of primary resistance to gefitinib or erlotinib (33). This group observed 18 genes that are involved in NFkB signaling were directly or indirectly amplified in EGFR TKI resistant EGFR mutant NSCLC cells. Activation of NF-kB through overexpression of c-FLIP or IKK, or silencing of Ikb, rescued EGFR-mutant lung tumor cells from EGFR TKI treatment. On the contrary, inhibition of FAS and several components of the NF-kB pathway specifically enhanced EGFR TKI induced cell death in EGFR-mutant lung cancer (33).

It has been increasingly clear that cancer cells that express the canonical mesenchymal markers have intrinsic resistance to EGFR TKI (34, 35). Independent

research groups have processed gene expression and proteomic profiles in an attempt to identify epithelial and mesenchymal cell biomarkers that related to sensitivity to EGFR TKI (34, 35). They found that the cell lines which were sensitive to the EGFR inhibition expressed the canonical epithelial markers and displayed the typical epithelial phenotype. On the contrary, cell lines which were resistant to EGFR inhibition expressed the canonical mesenchymal cell markers and displayed the typical mesenchymal phenotype (34, 35). In addition, restoration of the canonical epithelial cell marker E-Cadherin increases the sensitivity to EGFR TKI in NSCLC cell lines (36).

Acquired Resistance: Although NSCLC patients harboring EGFR mutations respond well to EGFR TKI, the drug resistance mainly develops within 6-18 months of treatment (24). The emergence of acquired resistance to EGFR TKIs greatly limits the efficacy of EGFR TKI to prolong patient survival (18). In order to overcome the acquired EGFR TKI resistance, it becomes more important to study the molecular and cellular mechanisms underlying the acquired EGFR TKI resistance.

About 50 percent of the acquired resistant cases are attributed to the emergence of the second-site gatekeeper mutation in EGFR exon 20, which is a change of a threonine to a methionine in amino acid position 790 (T790M) (37). The mechanism by which the T790M mutation confers resistance to gefitinib and erlotinib has been demonstrated that T790M secondary mutation restores the affinity of EGFR to ATP again, as a result, restore enzymatic activity of EGFR (38). T790M secondary mutation reduces the therapeutic effect of gefitinib or erlotinib to the patients.

MET, which is a transmembrane receptor tyrosine kinase encoded by an oncogene, has been observed to be amplified in up to 20 percent patients who develop EGFR TKI resistance after several month treatments (39). MET amplification contributes to EGFR TKI resistance through a mechanism called 'receptor kinase switch' and it can occur independently or can coexist with EGFR T790M mutation among EGFR TKI secondary resistance (40). MET dimerization and activation can occur in either ligand-dependent or ligand-independent way. MET ligand was identified as hepatocyte growth factor (HGF), also named as scatter factor (SF) (39, 41). Published studies have verified that EGFR mutant NSCLC cells activate the PI3K pathway through dimerization of EGFR with ERBB3 and down-regulation of PI3K signaling is the marker of sensitivity to gefitinib or erlotinib (42, 43). MET amplification replaces the function of EGFR sustaining the activation of PI3K signaling by coupling to ERBB3 in gefitinib resistant EGFR mutant NSCLC cells (39). In addition, the MET ligand HGF has been reported to induce gefitinib resistance by independently activating PI3K signaling pathway (41). Different from the MET receptor amplification that promotes EGFR TKI resistance in EGFR mutant NSCLC cells through coupling to ERBB3 and activating ERBB3/PI3K axis, HGF induces drug resistance through activating MET/PI3K signaling axis (41). MET amplification was also observed, although rare, in EGFR mutant NSCLC patients before gefitinib treatment and associated with EGFR TKI resistance (44).

Among all EGFR TKI resistant mechanisms in EGFR mutant NSCLC, EGFR T790M secondary mutation and MET amplification mutations account for 60-65 percent of all cases. Tumors from remaining EGFR TKI resistant cases neither harbor secondary

T790M mutation nor MET amplification. Sequist *et al.* performed a comprehensive genetic and histological analysis of 37 NSCLC patients before and after EGFR TKI treatment (45). All patients either had EGFR exon 19 deletion mutations or exon 21 L858R substitution mutations and initially respond to EGFR TKI, but subsequently developed drug resistance. Besides T790M secondary mutations and MET amplification, 5 percent of patients develop mutations in PIK3CA (45). Sequist *et al.* also found phenotypic transformation from NSCLC to SCLC leads to EGFR TKI resistance in 14 percent of patients with acquired resistance (45). The study reported that about 30% of the resistant mechanisms remain elusive. Among these unknown resistant mechanisms, about half of the NSCLC patients present evidence of epithelial to mesenchymal transition (EMT).

Overview of EMT

EMT is an evolutionarily conserved process by which cells undergo morphological changes from the epithelial polarized cell-cell adhesion phenotype to a more migratory invasive and fibroblast-like mesenchymal phenotype (46). The molecular markers of EMT are associated with transcriptional reprogramming such as decreased expression of proteins involved in cell junction such as E-Cadherin, and increased expression of mesenchymal cell markers such as Vimentin and N-Cadherin (47). EMT was first recognized as a feature of embryogenesis (46). Epithelial and mesenchymal cells are different not only in phenotype but also in functions. During embryonic development, differentiated epithelial cells could lose their cell-cell junction and basolateral polarity and undergo morphogenetic changes to migratory invasive mesenchymal cells, referred to

as EMT. After reaching specific sites, mesenchymal cells are recruited and differentiate to epithelial tissue and organs through mesenchymal to epithelial transition (46).

Reduced E-Cadherin expression disrupts the cell-cell adherence junction and polarized cell shape, so that the loss of E-Cadherin is considered to be an essential biomarker of EMT (48, 49). E-Cadherin loss has been implicated with activation of β -catenin and its nuclear translocation (50, 51). β -catenin is a critical intracellular protein associated with the E-Cadherin cytoplasmic tail and has been much reported to play an important role in the induction of EMT (51). The loss of E-Cadherin result in the dephosphorylating and activating of β -catenin molecule that is otherwise phosphorylated and targeted for ubiquitylation and degradation. Activated β -catenin forms a nuclear-localized transcriptional complex with other transcription factors, including Snail, ZEB1, and Twist, and promotes complex gene expression changes that contribute to the EMT process (51).

Several extracellular signals such as transforming growth factor- β (TGF- β) signaling (52), Wnt signaling (53), Notch pathway (53), receptor tyrosine kinases (53, 54) also trigger EMT by complex signaling networks. The consequences of these signaling are upregulation of transcription factors, including Snail, Slug, and Twist which have high affinity for the E-box elements in the E-Cadherin promoter and inhibit E-Cadherin expression (55).

EMT and acquired EGFR TKI resistance

Much effort has been focused on understanding how EMT regulates the progression of NSCLC cells to be more invasive and metastatic; however, relatively less

effort has been made on elucidating how EMT promotes resistance to EGFR target-therapies in NSCLC harboring mutant EGFR.

EMT has been shown to promote acquired resistant mechanism to EGFR TKI in the NSCLC cell line models harboring EGFR kinase domain mutations. EMT was observed in about 15 percent cases among post-gefitinib treatment tumors (45). Suda *et al.* reported that chronic exposure of EGFR mutant HCC4006 cells to increasing concentration of erlotinib induced epithelial to mesenchymal transition and confer the cells resistance to erlotinib (56). Additionally, restoration of canonical epithelial cell marker E-Cadherin using histone deacetylase inhibitor MS-275 restored the sensitivity of erlotinib in the HCC4006 cells with acquired resistance to erlotinib (56). EMT is associated with many cellular changes; however, the molecular mechanisms by which mesenchymal-like cells attenuate their dependency on EGFR for survival and proliferation are still unknown. One explanation is that EMT promotes the cells to acquire alternative pathways so that the survival of the cells is independent of EGFR signaling. As a result, cells with a mesenchymal phenotype are resistant to EGFR TKI (57). It has been shown that the cancer cells can shift the cellular dependence in the response of EGFR TKI treatment (42, 58). PI3K and MAPK signaling pathways are critical for the survival and proliferation in NSCLC cells with mesenchymal phenotype (34), therefore, the molecular determinants of activation of PI3K and MAPK pathways which promote cellular resistance to EGFR TKI induced apoptosis have been actively investigated.

Involvement of AXL in EGFR TKI resistance in EGFR mutant NSCLC with EMT

AXL is a tyrosine phosphorylated 140kDa protein which belongs to a member of Tyro3-Axl-Mer (TAM) receptor tyrosine kinase (RTK) subfamily (59) and was first isolated from patients with chronic myelogenous leukemia (CML) and chronic myeloproliferative disorder (60). Like all RTKs, AXL consists of an extracellular domain, a transmembrane domain and a cytoplasmic domain with kinase activity. The extracellular domain of AXL is composed of a combination of two immunoglobulin-like (Ig) motifs and dual fibronectin type III (FN III) repeats (59). AXL transduces signals by binding to its specific ligands. Protein S and vitamin K-dependent protein growth arrest-specific 6 (Gas6) are ligands for AXL, whereas only the Gas6 is confirmed as high affinity to AXL (61, 62). Like other RTKs, conventional activation of the receptor involves ligand binding to the extracellular domain. Ligand binding induces receptor dimerization and transphosphorylates tyrosine residues in the cytoplasmic kinase domain (63). Phosphorylated tyrosine recruits other downstream signaling proteins binding to it through their phosphotyrosine-binding SH2 domain. The primary signaling downstream of AXL appears to be the PI3K and MAPK signaling pathways (64, 65). AXL dimerization and activation has been shown to occur in a ligand-independent manner- homophilic binding of extracellular domains between neighboring cells (66) and AXL overexpression (67). AXL activation and signaling have been linked to multiple cellular responses, including cell survival, proliferation, adhesion and cytokine production, as well as immune-regulatory (68).

The role of AXL in cancer biology has attracted attention. AXL has been implicated to be upregulated in variety of cancers including breast (69), gastric (70), prostate (71), lung (72). Overexpression of AXL has been reported as relative to increased tumor

growth rate, metastasis and poorer prognosis (72, 73). Recently, AXL has also been considered as a potential candidate for overcoming EGFR TKI resistance in EGFR mutant NSCLC cells through activation of PI3K and MAPK signaling by its overexpression or binding to its ligand GAS6 (74). AXL activation enables the cells to bypass the mutant EGFR signaling in a manner similar to the resistant mechanism mediated by MET amplification in mutant EGFR NSCLC. The phenomenon is termed as 'receptor tyrosine kinase switch'. Zhang *et al.* group has developed tumor model with acquired resistance to erlotinib *in vitro* using HCC827 NSCLC cell line with EGFR kinase domain exon 19 deletion mutation and *in vivo* model with HCC827-derived xenograft tumors (74). They found that AXL was highly overexpressed in the EGFR TKI resistant groups with mesenchymal phenotype, as compared to the vehicle-treated tumors. Inhibition of AXL by chemical or genetic methods restored erlotinib sensitivity in HCC827 cells. They validated the finding in matched pre- and post-EGFR TKI treatment clinical specimens from 35 NSCLC patients with either exon 19 deletion or exon 21 point mutation of EGFR and concluded that AXL or GAS6 overexpression was associated with resistance to EGFR TKI (74). Furthermore, AXL promotes the expression of anti-apoptotic BCL-2 family members and to inhibit the expression of pro-apoptotic BAD family proteins which are responsible for the resistance of target therapy (75).

In addition to its role as an oncogenic driver in the EGFR TKI resistant NSCLC cells, AXL is also associated with the process of EMT (76). In pancreatic cancer, Koorastra *et al.* observed that AXL expression is necessary to maintain EMT transcription factors expression, such as Snail, Slug and Twist (73). AXL is also

demonstrated as a downstream effector of the EMT program in many tumor types since the activation of EMT program promotes the upregulation of AXL and downstream signaling (53, 76, 77). A recent published paper from *Asiedu* group revealed a novel function of AXL in acting upstream to induce EMT in normal epithelial breast cancer cells (78). Moreover, it has been shown that downregulation of AXL promotes mesenchymal to epithelial transition in mesenchymal breast cells (78).

Byers *et al.* performed a comprehensive gene expression signature analysis of 76-gene EMT signatures and demonstrated that epithelial and mesenchymal phenotypes determine EGFR TKI sensitivity in NSCLC harboring mutant EGFR (79). Among the genes which are upregulated in the mesenchymal groups, they found high level of AXL. AXL overexpression in mesenchymal like NSCLC cells was further confirmed by proteomics approach and reverse-phase protein array (RPPA). The author then validated the correlations between EMT and poor erlotinib response. NSCLC patients were subjected to Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE-1) trial. Patients with an EMT signature including increased level of AXL and its ligand GAS6 predicted poor clinical outcome to erlotinib as compare to the patients without EMT signature. Furthermore, *in vitro*, they found AXL inhibition together with EGFR inhibition had a striking synergistic effect compare to EGFR inhibition alone in AXL overexpressing mesenchymal NSCLC cells.

Collectively, these data suggest that AXL / GAS6 and its downstream signaling pathways serve as a potential mechanism associated with EGFR TKI resistance in

EGFR mutant NSCLC with mesenchymal phenotype. Detection and targeted therapy to AXL may be a new avenue to circumvent this resistance.

Chemokine receptor CXCR7 and its potential role in acquired EGFR TKI resistance with epithelial to mesenchymal transition

C-X-C chemokine receptor type 7 (CXCR-7), formerly known as RDC1, was first cloned from a dog thyroid cloned DNA (cDNA) library (80). CXCR7 is a member of the G-protein-coupled receptor family and has the canonical structural features of GPCRs with a seven-transmembrane helices connected by extracellular and intracellular loops. It is known that chemokine ligands CXCL12 (or stromal-derived growth factor-1, SDF1) and CXCL11 (or IFN-inducible T cell α -chemoattractant, I-TAC) transmit their signals by binding to the receptor (81, 82). Expression of CXCR7 plays a role in cardiac development as well as with tumor growth and progression (82, 83).

Unlike classical G-protein coupled receptors which are initiated by binding with heterotrimeric G proteins, CXCR7 is activated by association with β -arrestin (84). In the absence of ligand, CXCR7 constitutively interacts with G α i, but does not activate them. Ligand binding to CXCR7 recruits G protein receptor kinase (GRK) which in turn phosphorylates and activates the receptor. Phosphorylated receptor then increases its affinity to β -arrestin and results in activation of MAPK and PI3K signaling pathways (84). The process of CXCR7 activation is a model of ligand initiated, G-protein-independent, β -arrestin-dependent signaling pathways (84-86).

Signal transduction initiated by CXCR7 is controlled at the membrane by the process of CXCR7 trafficking. The level of receptor expression at the cell membrane

directly affects the magnitude of the cellular response initiated by the ligand binding (87). Apart from the β -arrestin-dependent manner, reversible ubiquitination plays an essential role in the regulatory mechanism of CXCR7 internalization (87). Ubiquitination is a post-translational process that results in the covalent bond of the small protein ubiquitin to a lysine in the modified protein with profound consequences for endocytic cycles of the receptor (88). This modification of ubiquitination is mediated by three distinct enzymes. Ubiquitin is processed by an E1-activating enzyme, forming a thioester bond with ubiquitin that is required for transfer to E2-conjugating enzymes, and E3 ubiquitin ligases facilitate the covalent attachment of ubiquitin to substrate proteins (88). A recent study suggests that CXCR7 is constitutively ubiquitinated and such ubiquitination is important in modification of receptor trafficking (87). CXCR7 ubiquitination is necessary for cell surface delivery of the receptor. The absence of this ubiquitination leads to a constitutively internalized receptor. Upon ligand stimulation, CXCR7 phosphorylation at serine/threonine in the C-terminus promotes β -arrestin recruitment. β -arrestin interacts with a de-ubiquitinating enzyme (DUB) causing CXCR7 de-ubiquitination and endocytosis. On the contrary, disassociation of β -arrestin and interacting proteins would render the receptor to undergo ubiquitination and ubiquitinated receptors are recycled back to the cell surface (87).

Accumulating evidence suggests that CXCR7 plays an essential role in the survival, proliferation, metastasis of tumor cell lines including breast, lung, glioma (82, 89, 90). Thus, CXCR7 has attracted more and more attention in the cancer research field as a potential pharmacological target for cancer therapy. Higher CXCR7 expression

is observed in neoplastically transformed cells as compared to their non-transformed control group supporting the notion that CXCR7 is important during tumorigenesis (82). It is also verified that CXCR7 signaling increased growth rate of sarcoma cells in nude mice (91). In addition, higher expression of CXCR7 in NSCLC and prostate cancer cells show a positive correlation in tumor metastatic potential and development (92, 93). It is reported that CXCR7-transduced murine human breast or lung cancer cell lines grow larger tumors in immunodeficient mice, whereas decreasing the expression level of CXCR7 using siRNA shrink the lung tumor size in the *vivo* study (89). The roles of CXCR7 in different types of tumor cells are not entirely consistent. For example, Burns *et al.* demonstrated that CXCR7 was remarkably overexpressed in breast cancer cell line MDA-MB-435a and it was required to inhibit apoptosis rather than increasing proliferation in the cells (82), whereas Meijer *et al.* reported CXCR7 played an important role in cell proliferation in mouse colon and pancreatic cancer cells (94).

In addition, the involvement of CXCR7 in neoangiogenesis and formation of the tumor vasculature has been postulated since higher expression of CXCR7 has been found in breast cancer-related blood vessels compared to the blood vessel in normal breast tissue (89). CXCR7 overexpression has also been observed in blood vessels of other clinical carcinoma biopsy samples such as lung, kidney, prostate (95, 96). Moreover, CXCR7 overexpression in prostate cancer correlates with the increased production of vascular endothelial growth factor (VEGF) and IL-8 which have been shown to be important factors involved in the development of tumor blood supply in the progression of solid tumor (95).

Of note, recent studies proposed that high expression of CXCR7 correlates with tumor metastatic potentials. One prostate cancer study from Wang J *et al.* suggested that high expression of CXCR7 predicted highly aggressive metastasis (95). They found CXCR7 expression regulated CD44 antigen (CD44) as well as cadherin 11 (CDH11) levels. CD44 was found to play a critical role for metastatic behavior of tumor cells including pancreatic cancer (97), prostate cancer (98), head and neck cancer (99). Similarly, expression of CDH11 was also regulated by CXCR7. CDH11 was identified to be expressed in mesenchymal cells and in a subset of highly invasive cell lines and aggressive breast carcinomas (100). In the study, they observed that expression level of CXCR7 also regulated TGF- β expression. TGF- β is reported to induce mesenchymal transition in epithelial cells (101), and exposure to TGF- β is associated with reduced sensitivity to gefitinib in NSCLC (102). Another research group reported that higher expression of CXCR7 was found in early and metastatic recurrence in pathological stage I NSCLC (92). Moreover, the level of CXCR7 is found to be significantly higher in the highly metastatic hepatocellular carcinoma cells and inhibition of CXCR7 by siRNA remarkably reduced tumor proliferation and metastatic invasion (103).

In conclusion, all the evidence demonstrate the importance of CXCR7 in cell signaling pathways and cancer metastatic behavior and also raise the exciting possibility that CXCR7 potentially facilitates EGFR TKI resistance in EGFR mutant NSCLC with mesenchymal phenotype.

CHAPTER TWO

MATERIALS AND METHODS

Cell lines: HCC827, HCC4006, NCI-H1975 cell lines were obtained from the American Type Culture Collection (ATCC) and were maintained in RPMI-1640 growth media (Life Technologies, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA), 1X anti-biotic/anti-mycotic agent (AB/AM) (Life Technologies, Carlsbad, CA). HCC4006, NCI-H1975 cell lines were maintained in RPMI-1640 supplemented with 10% FBS, 1X AB/AM. 293LTV cells were obtained from Cell Biolabs (San Diego, CA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA) supplemented with 10% FBS and 1X AB/AM. Cells were cultured at 37°C in a humidified incubator of 5% CO₂. All clones generated using HCC827 cells used in the experiments were authenticated by short tandem repeat (STR) profiling.

Lentiviral package and production: For virus packaging, the 2.5X 10⁵ 293LTV cells were transfected with 4 µg cDNA cloned in pEXP304-blasticidin vectors or shRNA sequence cloned in pLKO.1-puromycin vectors together with 4 µg Δ8.2 and 0.6 µg VSVG lentiviral packaging vectors using 14 µl TransIT-LT1 transfection reagent (MIRUS, Madison, WI). Transfection mixture was prepared in the proper volume of optiMEM (Life Technologies, Carlsbad, CA) to make up the total volume to 250 µl. After incubated for 20 minutes added to the cells suspended in RPMI-1640 supplemented with 10% FBS without AB/AM and the cells were incubated in the transfection mix for 24 hours. 24

hours post transfection, culture media was replaced with virus production media that is made of DMEM supplement with 10% FBS, 0.2 g/ml BSA, 1X AB/AM. The supernatant was harvested 72 and 96 hours post-transfection and the viral supernatant was filtered using Nalgene Syringe Filter 0.45 μ m (Thermo Scientific, Hanover Park, IL) and stored in 4°C or -80°C until use.

Target gene knockdown using lentiviral pLKO.1shRNA vector: Bacterial stock of short hairpin RNA (shRNA) clones in pLKO.1 designed by The RNA Consortium (MIT/Harvard Broad Institute, Cambridge, MA) were purchased from Open Biosystem (Pittsburgh, PA) or Sigma (St. Louis, MO). In the shRNA experiments, lentiviral encoding shRNA was packaged in 293LTV cells as described above. Target NSCLC cells were transduced at least twice with viral supernatants. Cells containing the target expression were selected with 5 μ g/ml puromycin after infection.

Generation of HCC827 cells stably overexpressing shRNA against Non-Target/ EGFR/ CDH1: HCC827 cells with stably expressing shRNA against non-target (NT), EGFR and CDH1 cells were generated by transducing exponentially growing HCC827 cells with lentivirus coding for shRNA against NT, EGFR and CDH1, respectively. Following infection, the cells were subjected to the antibiotic selection to ensure the expression of shRNA against target. The cells were cultured in RPMI-1640 supplemented with 5% FBS, 1X AB/AM and 5 μ g/ml puromycin. Mass culture was established for HCC827 stably overexpressing shRNA against NT sequence. Western blot was performed using antibody against EGFR or CDH1 to ensure that there is no deletion of EGFR or CDH1 in the mass culture. For the generation of HCC827 cells

stably expressing shRNA against EGFR or CDH1, single clones were established following puromycin selection. Single clones were screened for knockdown efficiency and clone with best EGFR or CDH1 knockdown were used for experiments.

Generation of HCC827 or NCI-H1975 cells stably overexpressing GFP or LacZ or

CXCR7: Exponentially growing HCC827 cells or NCI-1975 cells were seeded on 60x 15 mm dishes at the density of 6×10^5 cells and allowed to adhere to the plate overnight. The cells were then transduced for overnight with lentivirus coding for GFP or LacZ, CXCR7 tagged with V5 tag. This process was repeated three times to ensure that the cells express the genes. HCC827 or NCI-H1975 cells were then subjected to antibiotic selection with 20 $\mu\text{g}/\text{ml}$ blasticidin. Following the selection, mass culture was established and the expression of LacZ-V5 and CXCR7-V5 were confirmed with Western blot using antibodies against V5 tag. Ectopic CXCR7-V5 expression is also confirmed with Western blot using antibodies against CXCR7. The expression of GFP was confirmed using EVOS-XL fluorescent microscope.

Lentiviral knockdown of CXCR7 in HCC827 cells ectopically overexpressing

CXCR7: HCC827 cells ectopically overexpressing GFP or CXCR7-V5 were grown in RPMI-1640 supplemented with 5% FBS, 1X AB/AM and 20 $\mu\text{g}/\text{ml}$ blasticidin and were transduced with lentivirus coding for NT shRNA or CXCR7 shRNA. To ensure knockdown of the targets, the cells were transduced twice. The cells were then subjected to antibiotic selection with 20 $\mu\text{g}/\text{ml}$ blasticidin and 5 $\mu\text{g}/\text{ml}$ puromycin. Mass culture resistant to both blasticidin and puromycin were generated and knockdown efficiency was confirmed with Western blot using antibody against V5 or CXCR7. The

cells were maintained in RPMI-1640 supplemented with 5% FBS, 1X AB/AM, 20 µg/ml blasticidin and 5 µg/ml puromycin.

Drugs: Gefitinib and XL-184 (ChemieTek, Indianapolis, IN) were prepared as a 10mM stock solution in dimethyl sulfoxide (DMSO) and stored in -20°C until use and used at concentrations ranging from 0.001 to 10 µM.

Cell viability assay: Cells were seeded at a density ranging from 2000-3000 depending on cell lines per well on 96-well plates and allowed to adhere overnight in the appropriate growth media mentioned in the cell line section. Subsequently, cells were treated with increasing concentrations of drugs serially diluted in growth media for 72 hours, and subjected to cell viability assay with Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technology, Inc., Rockville, Maryland). Samples were prepared in duplicates. After the incubation with CCK-8, plates were read on a microplate reader (Bio-Tek, Winooski, VT). The data were collected and analyzed by the graphpad prism version 5.00 for Windows (GraphPad Software, La Jolla, CA). The results were expressed as mean viable cells relatively to RPMI-1640 alone (considered as 100% viability) ± SD.

Western blot: The cells were cultured in the tissue culture plates (BD Falcon, San Jose, CA), allowed to attach overnight. To assess cell signaling, cells were incubated with the indicated drug concentration or control DMSO and incubated for 24 or 48 hours. The cells were washed twice with ice-cold 1X modified DPBS with Calcium and Magnesium and whole-cell lysates were prepared in the lysis buffer containing 20 mM Tris-HCl (PH7.5), 15 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1µg/ml leupeptin (Cell

Signaling Technology, Danvers, MA) supplemented with Halt protease and phosphatase single-use inhibitor cocktail (Pierce, Rockford, IL). To detect CXCR7, we use lysis buffer containing 62.5 mM Tris-HCL (pH 6.8), 2% SDS, 10% sucrose, supplemented with halt protease and phosphatase single-use inhibitor cocktail (Pierce, Rockford, IL). Protein concentrations were determined using Pierce bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) and equivalent amount of protein were subjected to mini-protean TGX 4-20% gradient gels (Bio-Rad, Hercules, CA) and blot with different antibodies (Table 1.) Blot detection was done by ECL Western Blot Detection Reagents (PerkinElmer, Waltham, MA). After Western blot, films were developed and scanned. Loading were corrected with ImageJ software (NIH Image, Bethesda, MD).

Table 1. Antibody list

Company Location	Invitrogen Carlsbad, CA	Santa Cruz Biotechnology Santa Cruz, CA	Abcam Cambridge, MA	Cell Signaling Technology Danvers, MA
Antibodies Cat no.	V5 tag (R96125)	EGFR (sc-03)	CXCR7 (ab38089)	E-Cadherin (3195), N-Cadherin (4061), ZO1(5406), p-EGFR (3777), AXL (4939) / p-AXL(5724) AKT (4691) / p-AKT(4060) ERK (9102) / p-ERK(9101) β-Actin (4967), GAPDH (5174)

Phospho-RTK array analysis: Proteome Profiler human phospho-RTK antibody array (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions to measure the relative level of tyrosine phosphorylation of 42 distinct receptor tyrosine

kinases (RTKs). Cells were seeded in 10x20mm dishes at a density of 2.0×10^6 and the cells were allowed to adhere to the plates overnight. Cells were then treated with either control DMSO or 1 μ M gefitinib for 24 hours. The cells lysates were prepared by solubilizing the cells at 1×10^7 cells/ml in RTK array specific lysis buffer (R&D Systems, Minneapolis, MN). 100 μ g of fresh protein lysates were incubated with array membranes overnight at 4°C. The membranes were then incubated with anti-phosphotyrosine-HRP antibody for 2 hours at room temperature to detect tyrosine phosphorylated RTKs. Western blot was done by ECL Western Blotting Detection Reagents (PerkinElmer, Waltham, MA). Then the membrane was exposed to X-ray film to detect activated receptors.

siRNA transfection: Silencer validated small interfering RNAs (siRNAs) for AXL, CXCR7 and control NT were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 2.0×10^5 cells were seeded into 6-well plates and allowed to attach overnight. Concentration of 3.5 μ l of 10 μ M siRNA and 6 μ l of TransIT- siQUEST transfection reagent (MIRUS, Madison, WI) were each mixed with 250 μ l of optiMEM medium. Solution was incubated for 20 minutes in the room temperature before added to the cells. siRNA mediated knockdown efficiency was measured by Western blot after 96 hours of transfection.

Apoptosis AnnexinV Assay: 3×10^5 cells were seeded in duplicates in the 6-well tissue culture plates, allowed attaching to the plate overnight. To assess drug effect to the cell apoptosis, Annexin V staining was proceeding after 48 hours drug treatment. Annexin V binding buffer (Cell Signaling Technology, Danvers, MA) was used at the concentration

range from 1×10^5 to 1×10^6 cells/ml after washing with ice-cold growth media. 96 μ l of cell suspension was transferred to a 5 ml flow tube, stained with AnnexinV/FITC (Cell Signaling Technology, Danvers, MA) and PI (Cell Signaling Technology, Danvers, MA) and incubated on ice in the dark for 10 minutes. The stained cell suspension was then subjected to the Fluorescently Activated Cell Sorting (FACS) analysis using BD canto II machine (BD Biosciences, San Jose, CA). Results were analyzed by using Flowjo software (Tree Star Inc. Ashland, OR).

qRT-PCR: To validate changes in gene expression identified in gene expression profiling, RT-PCR was performed to quantify the amount of target mRNA. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Germantown, MA) according to the manufacturer's instructions. The quantity of RNA and purity were determined using NanoDrop ND-1000 (Thermo Scientific, Hanover Park, IL) and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). cDNA was synthesized from 500 ng total RNA using high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). For qRT-PCR analysis of RNA expression, the RT-PCR was performed in a 20 μ l reaction volume containing TaqMan Universal master mix (Applied Biosystems, Foster City, CA). Cycling conditions were set as 50°C 2 minutes- 95°C 10 minutes- 95°C 15 minutes- 60°C 1min. RT-qPCR was performed and the values were obtained of the threshold cycle for the CXCR7 and normalized using the housekeeping gene GusB.

Table 2. CXCR7 Primers list

Gene	Primer Sequence
CXCR7	Forward: 5'-GACTACAAAGACGATGACGACAAGCTTA Reverse: 5'-AGTAAACCACGAGACGAGGTTCCGTCT

CHAPTER THREE

RESULTS

EGFR or E-Cadherin depletion by lentiviral shRNA in HCC827 cells induces epithelial to mesenchymal transition. HCC827 NSCLC cells overexpress EGFR with the exon 19 deletion mutation and are exquisitely sensitive to EGFR TKI (14). Usually, acquired resistance to EGFR TKI is studied by exposing HCC827 cells to increasing doses of EGFR TKI for up to 6 months in tissue culture system (37). The problem of this approach is that off-target effects of EGFR TKI might contribute to the development of acquired resistance to EGFR TKI. To eliminate the possibility of off-target effects, Dr. Shimamura stably knocked down EGFR using lentiviral shRNA against EGFR in HCC827 cells (HCC827 shEGFR) and acquired resistant clones with mesenchymal phenotype were isolated (acquired resistant clone with MET amplification was also observed, data not shown). Robert Weinberg and his colleagues have demonstrated that the depletion of E-Cadherin in breast epithelial cells is sufficient to promote mesenchymal transition (51). Motivated by this finding and to investigate if inducing a mesenchymal phenotype in HCC827 cells is sufficient to confer EGFR TKI resistance in HCC827 cells, HCC827 cells were transduced with lentivirus coding for shRNA against E-Cadherin (CDH1) and stable clones were isolated by Dr. Shimamura. HCC827 with constitutive E-Cadherin knockdown cells (HCC827 shCDH1) were irreversibly transitioned to the mesenchymal state. HCC827 cells transduced with lentiviral shRNA against non-target served as control (HCC827 shNT). We observed that canonical

epithelial cell marker E-Cadherin is significantly downregulated whereas canonical mesenchymal cell marker N-Cadherin is upregulated in HCC827 shEGFR and HCC827 shCDH1 cells as compared to control HCC827 shNT cells (Figure 1B).

Mesenchymal-like HCC827 cells with constitutive EGFR or E-Cadherin knockdown

are resistant to EGFR tyrosine kinase inhibitor gefitinib. NSCLC cells that display mesenchymal phenotype are resistant to EGFR TKI (34, 48). To determine if HCC827 cells with mesenchymal phenotype are resistance to EGFR TKI, HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells were subjected to cell viability assay with EGFR TKI- gefitinib for 72 hours. As shown in Figure 1A, epithelial HCC827 shNT cells are exquisitely sensitive to gefitinib with the IC₅₀ 0.008 μ M, while mesenchymal HCC827 shEGFR and HCC827 shCDH1 cells were resistant to gefitinib with the IC₅₀ 1.35 μ M and 0.24 μ M respectively. The IC₅₀ of HCC827 shEGFR and HCC827 shCDH1 cells to gefitinib increase more than 30 fold. In addition, we challenged the cells with DMSO or 1 μ M gefitinib for 48 hours. The cells were lysed at the end of 48 hour treatment and lysates were subjected to Western blot with the antibodies indicated (Figure 1B and 1C). Exposure of HCC827 shEGFR to gefitinib did not promote PARP cleavage and exposure of HCC827 shCDH1 cells to gefitinib promoted resulted in decreased level of the PARP cleavage, which is a late apoptosis marker (104), as compared to the control HCC87 shNT cells (Figure 1B). EGFR downstream signaling pathways, PI3K and MAPK, are critical for cell survival and proliferation in EGFR mutant NSCLC (22) and cells sensitive to EGFR TKI are characterized with remarkable down-regulation of phosphorylated AKT (28, 29). Gefitinib treatment effectively inhibited

activity of EGFR (Figure 1C) in HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells. We observed remarkably decreased phosphorylation of AKT, ERK in HCC827 shNT cells (Figure 1C) which indicated that gefitinib treatment effectively depleted the downstream signaling in control cells. In contrast, phosphorylating AKT and ERK were maintained in HCC827 shEGFR cells after gefitinib treatment. Although gefitinib effectively inhibit the phosphorylating ERK, we observed the maintained level of phosphorylating AKT in HCC827 shCDH1 cells (Figure 1C). Our observation indicates that in mesenchymal like HCC827 cells, when the activity of EGFR is completely inhibited, cells sustained their downstream signaling pathway.

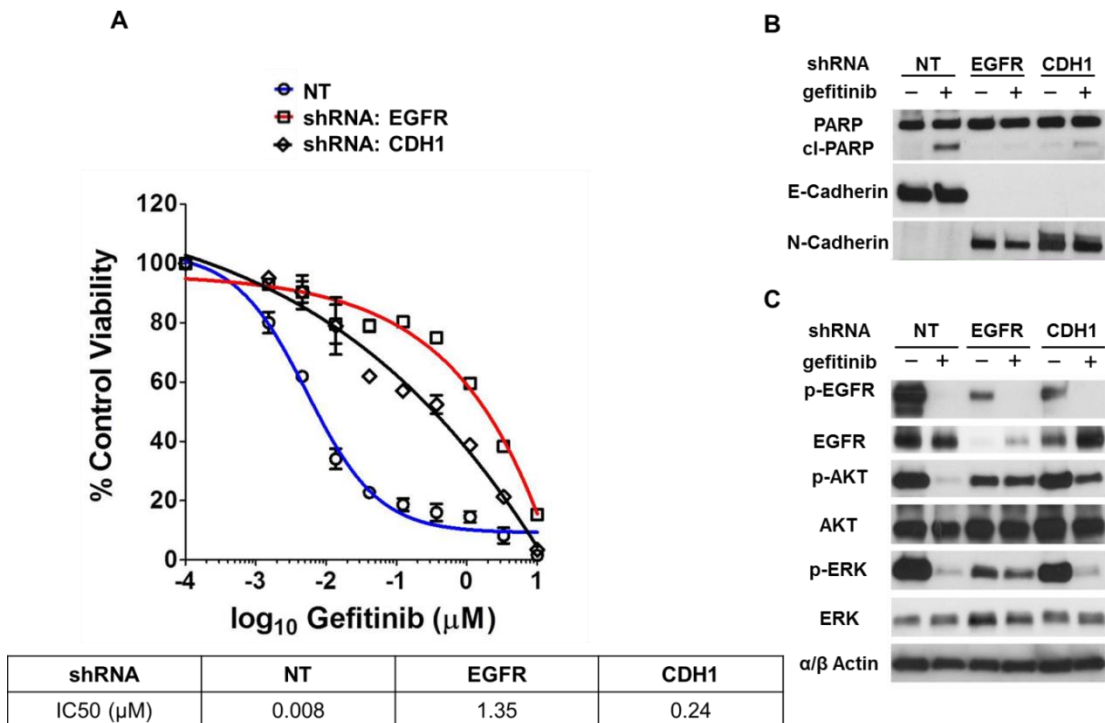
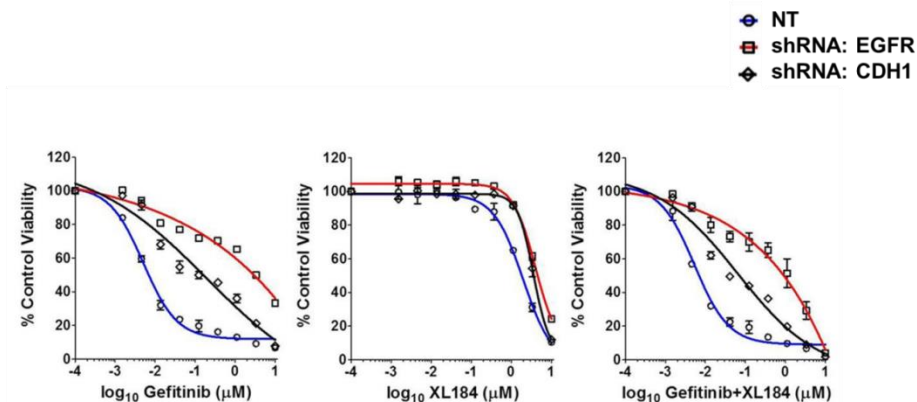


Figure 1. Mesenchymal-like HCC827 cells with constitutive EGFR knockdown or E-Cadherin knockdown are resistant to EGFR TKI gefitinib. A, Exponentially growing HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells were treated with indicated concentration of gefitinib for 72 hours in duplicate and cell viability were accessed using Cell Counting Kit-8. The viability of each sample was normalized to that of DMSO-treated cells. Points, average of normalized values for experiments in duplicates; error bars, SD. B, Exponentially growing HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells were treated with 1 μ M control DMSO (-) or gefitinib (+) for 48hrs and lysates were subjected to Western blot with indicated antibodies. HCC827 shEGFR and HCC827 shCDH1 cells are mesenchymal phenotype with downregulation of E-Cadherin and upregulation of N-Cadherin expression and resistant to gefitinib treatment as evidenced by no or significantly reduced PARP cleavage. C, Exponentially growing HCC827 shNT, HCC827sh EGFR and HCC827 shCDH1 cells were treated with 1 μ M control DMSO (-) or gefitinib (+) for 48hrs and lysates were subjected to Western blot with indicated antibodies. Gefitinib treatment promoted depletion of phospho-EGFR, phospho-AKT and phospho-ERK in HCC827 shNT cells. In contrast, mesenchymal HCC827 shEGFR and HCC827 shCDH1 cells are resistant to gefitinib and EGFR inhibition failed to completely inhibit phospho-AKT.

Part I: Tyrosine receptor kinase AXL

EGFR TKI resistant mesenchymal like HCC827 shEGFR and HCC827 shCDH1 cells are insensitive to AXL inhibition and AXL inhibition does not restore their gefitinib sensitivity. Recently, published studies indicate that upregulation of receptor tyrosine kinase AXL replaced the function of EGFR and sustained PI3K signaling pathway in EGFR TKI resistant NSCLC cells with mesenchymal phenotype when the activity of EGFR is completely inhibited (74, 79). In order to quickly determine if the survival of mesenchymal like HCC827 cells are dependent on AXL activation, we treated the cells with an inhibitor of AXL alone or in combination with EGFR inhibition and determined

percent control viability of the cells after 72 hours of drug treatment. Of note, there was no potent and selective AXL inhibitor at the time when the study was carried out. XL-184 is a MET inhibitor which also inhibits AXL receptor with low concentration (7 nM) (105). Extremely high concentration of XL-184 was required to compromised the cell viability of HCC827 shNT, HCC827 shEGFR or HCC827 shCDH1 cells. In line with the previous observation, HCC827 shEGFR and HCC827 shCDH1 cells increase their resistance to gefitinib more than 17 fold with the IC₅₀ 2.4 μ M and 0.14 μ M, respectively. In contrast, HCC827 shNT cells were sensitive to the gefitinib treatment with the IC₅₀ 0.008 μ M. In addition, XL184 did not restore gefitinib sensitivity in HCC827 shEGFR and HCC827 shCDH1 cells (Figure 2).



IC ₅₀ (μ M)	Gefitinib	XL184	Gefitinib+XL184
NT	0.008	1.76	0.007 + 0.007
E9	2.4	5.2	0.7 + 0.7
CDH1	0.14	3.85	0.06 + 0.06

Figure 2. AXL inhibition does not restore gefitinib sensitivity in EGFR TKI resistant mesenchymal-like HCC827 cells with constitutive EGFR knockdown or E-Cadherin knockdown. Exponentially growing HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells were treated with indicated concentrations of gefitinib, XL-184 or combination of gefitinib and XL-184 at ratio 1:1 for 72 hours in duplicate and cell viability were accessed using Cell Counting Kit-8. The viability of each cell was normalized to that of DMSO-treated cell. Points, average of normalized values for experiments in duplicates; error bars, SD.

None of the 42 receptor tyrosine kinases listed in the RTK array seem to be driver of EGFR TKI resistant HCC827 shEGFR and HCC827 shCDH1 cells with mesenchymal phenotype. The data from Figure 1C showed the persistence of phospho-AKT when the inhibition of EGFR activity was completed in the mesenchymal-like HCC827 cells, and raised the possibility that AXL or any other potential receptor tyrosine kinase was activated to offset the function of EGFR. To address this possibility, we performed profiling of phospho-receptor tyrosine kinase (RTK) array in HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells with or without EGFR TKI treatment. 42 receptor tyrosine kinases including AXL and other known receptors linked to drug resistance in EGFR mutant NSCLC were tested. EGFR TKI treatment completely inhibited phosphorylation of EGFR and the analysis did not identify the activation of any RTK in mesenchymal-like HCC827 cells (Figure 3).

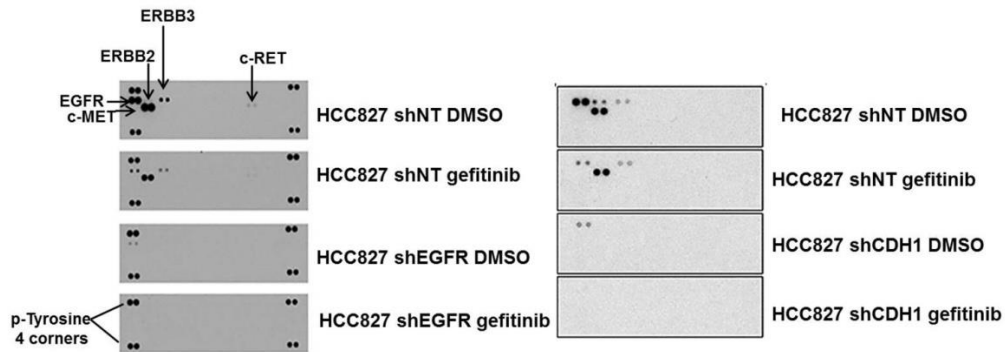


Figure 3. Analysis using RTK array demonstrates no RTKs are activated in mesenchymal-like EGFR TKIs resistant HCC827 cells upon gefitinib treatment. Lysates from HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells treated with either DMSO or 1 μ M gefitinib for 24 hours were subjected to profiling of active RTKs- using RTK array that detects 42 phosphorylated RTKs. In each array, RTK is assayed in duplicates. The dark dots represent activated RTKs. Right figure are made by Dr. Shimamura.

AXL is upregulated in EGFR TKI resistant HCC827 shEGFR and HCC827 shCDH1 cells with mesenchymal phenotype; however its activity is dependent on the activity of EGFR. Considering the limited sensitivity of RTK array, we confirmed expression and activation levels of AXL in HCC827 shEGFR and HCC827 shCDH1 cells treated with or without gefitinib by Western blot. We observed higher levels of AXL in HCC827 shEGFR and HCC827 shCDH1 cells. However, gefitinib reduced AXL phosphorylation (Figure 4), suggesting that AXL might be activated by EGFR-mediated trans-phosphorylation and AXL activity was dependent on the activation of EGFR. This

makes AXL less likely confer resistance to EGFR TKI treatment in mesenchymal-like HCC827 cells.

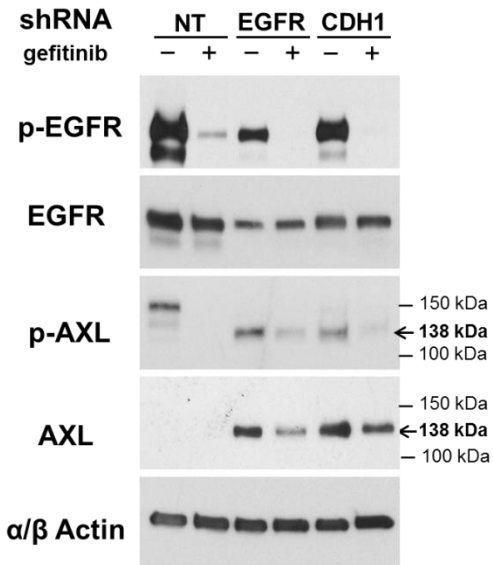


Figure 4. EGFR inactivation downregulates AXL phosphorylation in mesenchymal-like HCC827 cells with constitutive EGFR or E-Cadherin knockdown. Exponentially growing HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells were treated with DMSO or 1 μ M gefitinib for 48hrs and lysates were subjected to Western blot with indicated antibodies. Note that AXL is overexpressed and activated in HCC827 shEGFR and HCC827 shCDH1 cells; however, the phosphorylation of AXL is decreased upon treatment of HCC827 shEGFR and HCC827 shCDH1 cells with gefitinib. Expression of AXL/p-AXL indicated as 138kDa

AXL depletion neither induces cell apoptosis nor resensitizes HCC827 shEGFR or HCC827 shCDH1 cells to gefitinib. To further explore the role of AXL in acquired EGFR TKI resistance in HCC827 shEGFR and HCC827 shCDH1 cells and to avoid off-target effect of XL-184, we transfected HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells with siRNA coding for non-target or AXL and following either treated the cells with DMSO or gefitinib. In line with what we observed before, gefitinib induced the cell apoptosis in control HCC827 shNT cells with increased PARP cleavage. However, AXL depletion alone or in combination with gefitinib did not promote cell apoptosis in HCC827 shEGFR or HCC827 shCDH1 cells as evidenced by no PARP cleavage (Figure 5).

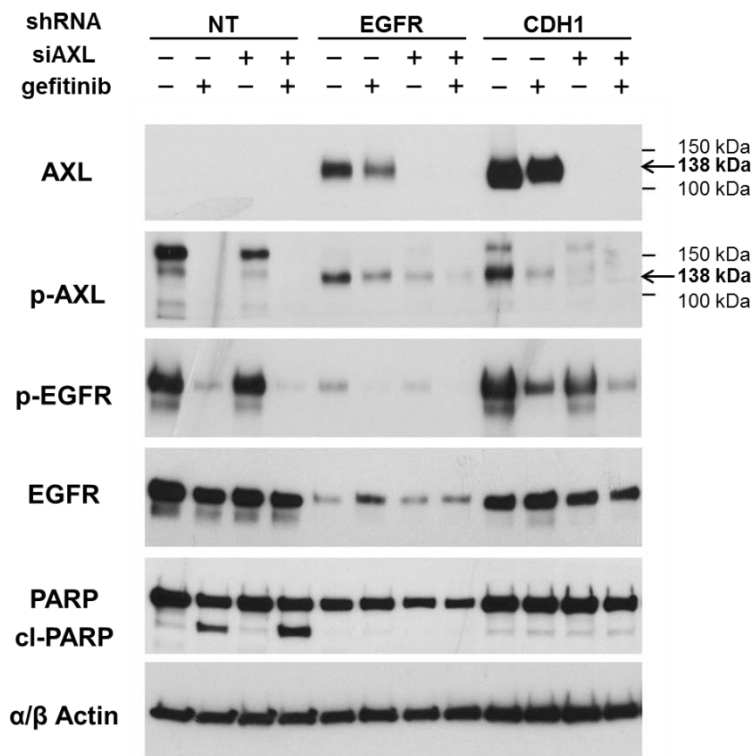


Figure 5. AXL depletion alone or in combination with EGFR TKI do not induce apoptosis in mesenchymal-like EGFR TKI resistant HCC827 cells with constitutive EGFR or E-Cadherin knockdown. HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells were transfected with control siRNA (-) or siRNA targeting AXL (+). Transfected cells were maintained in growth medium supplemented with 5% FBS. 48hours post-transfection, cells were then treated with DMSO (-) or 500nM gefitinib (+) for additional 24 hours. Then the whole cell lysates were collected and subjected to Western blot. Neither depletion of AXL alone nor combination of AXL depletion and EGFR inhibition promotes PARP cleavage in HCC827 shEGFR and HCC827 shCDH1 cells. Expression of AXL/p-AXL indicated as 138kDa.

Part II: Chemokine receptor CXCR7

CXCR7 is commonly upregulated in mesenchymal- like HCC827 cells. Since we eliminated the possibility of AXL as an oncogenic driver replacing the function of EGFR in mesenchymal like HCC827 cells, in order to elucidate other possible candidates that confer EGFR TKI resistance, Dr. Shimamura performed comparative marker selection (CMS) for mesenchymal like HCC827 cells and parental HCC827 cells. Genes which are exclusively upregulated more than 3-fold in mesenchymal like HCC827 cells but not HCC827 parental cells were captured (Figure 6). Of note, besides HCC827 shEGFR and HCC827 shCDH1 cells, our lab also generated mesenchymal like HCC827 cells by chronically exposing the cells to TGF- β which is a potent inducer of EMT in various epithelial cells (101). 49 genes are commonly upregulated in mesenchymal like HCC827 cells as compared to parental HCC827 cells including chemokine receptor CXCR7.

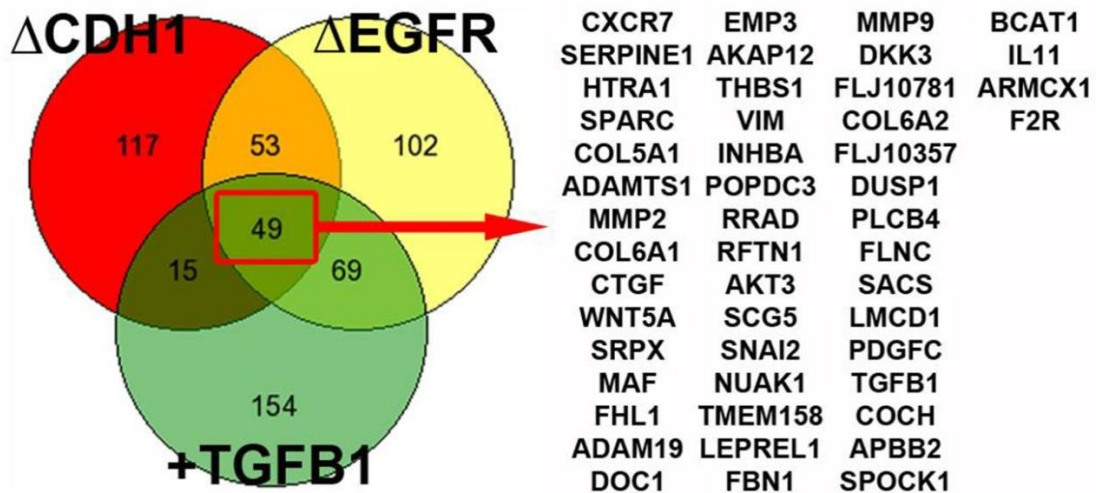


Figure 6. 49 unique genes commonly upregulated in the mesenchymal-like HCC827 cells. Comparative marker selection (CMS) was performed to identify genes uniquely upregulated in each mesenchymal-like HCC827 cells. Genes upregulated more than 3 fold over control at 95% confidence interval was identified for each mesenchymal-like cells. 49 unique genes were found commonly upregulated in HCC827 TGF- β , HCC827 shEGFR and HCC827 shCDH1 cells. Figure was provided by Dr. Shimamura.

CXCR7 is overexpressed in HCC827 shCDH1 cells. In order to confirm the CXCR7 expression level identified in gene expression profiling, we performed RT-PCR to quantify the amount of mRNA of CXCR7 in HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells. Total RNA was isolated and analyzed for CXCR7 transcript in HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells treated with DMSO or gefitinib. CXCR7 was significantly enhanced in EGFR TKI resistant mesenchymal like HCC827 shCDH1 cells as compared to the control HCC827 shNT cells. Acute gefitinib treatment increased the level of CXCR7 in all HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells (Figure 7A). CXCR7 overexpression in mesenchymal like HCC827 shCDH1 cells was also detected in Western blot with DMSO or gefitinib treatment (Figure 7B).

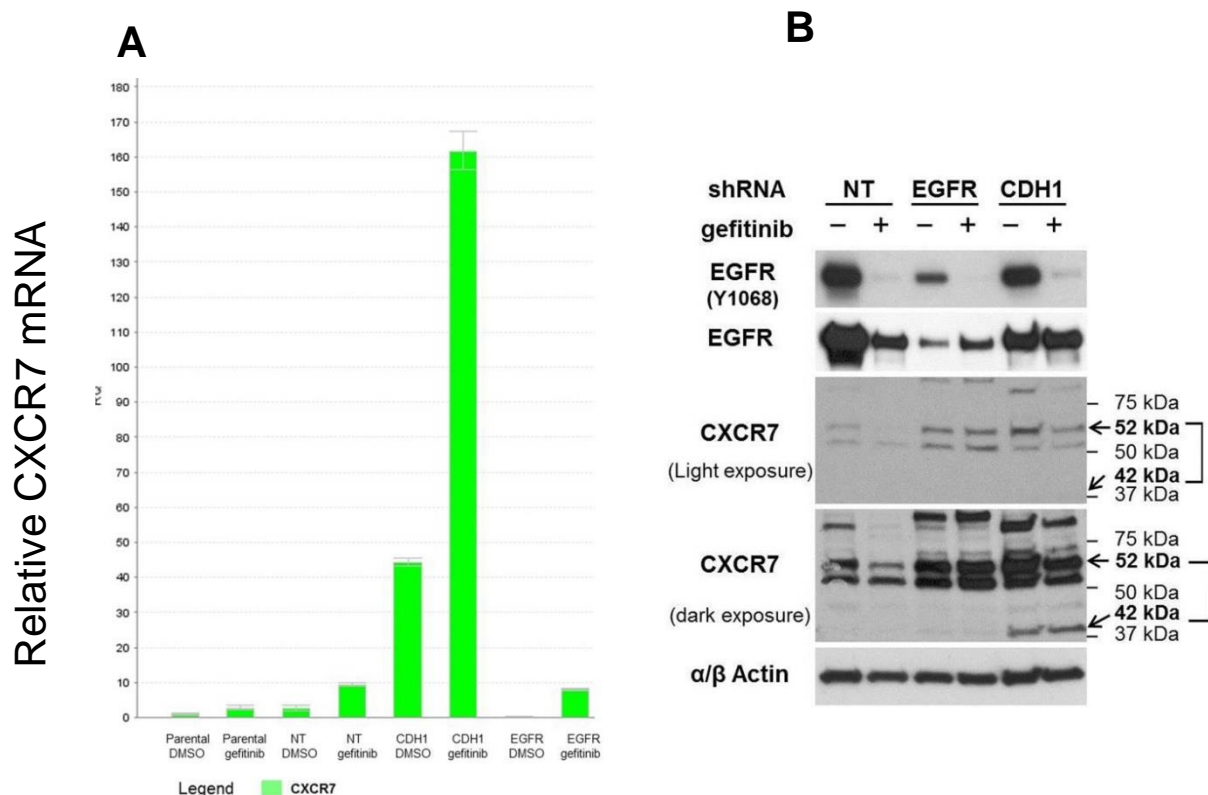


Figure 7. CXCR7 is overexpressed in HCC827 cells with constitutive E-Cadherin knockdown. A, Quantitative RT-PCR analysis of CXCR7 transcript induction in HCC827 shNT, HCC827 shEGFR and HCC827 shCDH1 cells treated with DMSO or gefitinib for 24 hours. Total mRNA was extracted from HCC827shNT, HCC827 shEGFR and HCC827 shCDH1 cells treated with DMSO or gefitinib. Each sample was amplified in duplicate for quantification of CXCR7 and GusB transcripts. Mean expression level of CXCR7 was analyzed by relative quantitation using $\Delta\Delta C_t$ method and normalization to GusB. Each bar represents the relative quantity of mRNA in one sample. Error bars, SD. CXCR7 transcripts were abundantly expressed in HCC827 with constitutively E-Cadherin knockdown. B, To confirm that increase in the expression of CXCR7 transcripts leads to the overexpression of CXCR7 protein, exponentially growing cells were treated with DMSO or 1 μ M gefitinib for 24 hours and lysates were subjected to Western blot with indicated antibodies. CXCR7 in HCC827 cells with constitutive E-Cadherin knockdown is higher compare to the HCC827 shNT cells. Expression of CXCR7 protein indicated as 42 kDa (immature type) and 52 kDa (post-translational ubiquitination type)

Ectopic CXCR7-V5 overexpression in HCC827 NSCLC cells. To systematically assess the role of CXCR7 in EGFR TKI resistant NSCLC cells, we ectopically overexpressed CXCR7 tagged with V5 in HCC827 cells (Figure 8).

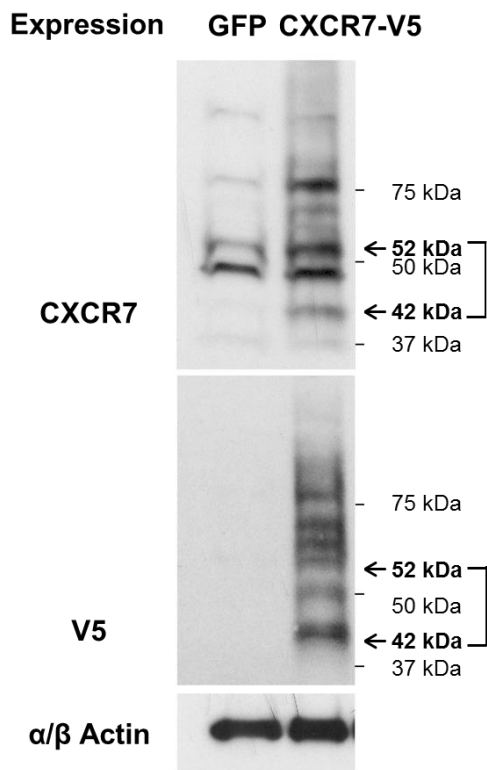


Figure 8. CXCR7-V5 was ectopically overexpressed in HCC827 cells. HCC827 cells were transduced with lentivirus coding for V5 tagged CXCR7 (CXCR7-V5) expression vectors. HCC827 cells transduced with lentivirus coding for GFP expression vector were used as a control. Cell lysates were made from exponentially growing HCC827 GFP cells or HCC827 CXCR7-V5 cells and the lysates were subjected to Western blot with antibodies against CXCR7, V5 tag and control β -Actin. CXCR7 and V5 overexpression

are confirmed in the HCC827 CXCR7-V5 cells. Expression of CXCR7 protein indicated as 42 kDa (immature type) and 52 kDa (post-translational ubiquitination type)

Ectopic CXCR7 overexpression in HCC827 cells promotes mesenchymal phenotype. Unexpectedly, we observed the mesenchymal-like morphological change after ectopic CXCR7-V5 overexpression in HCC827 cells. HCC827 CXCR7-V5 cells exhibited spindle, elongated and scattered mesenchymal-like morphology while the control HCC827 GFP exhibited cobblestone and patching morphology (Figure 9). Since cells that transition into a mesenchymal state undergo cell canonical markers change, we investigated the canonical epithelial and mesenchymal markers for HCC827 GFP and HCC827 CXCR7-V5 cells. In consistent with what we observed in morphological changes, we detected that canonical epithelial marker E-Cadherin was significantly downregulated while canonical mesenchymal marker vimentin was upregulated in HCC827 CXCR7-V5 as compared to HCC827 GFP cells (Figure 10).

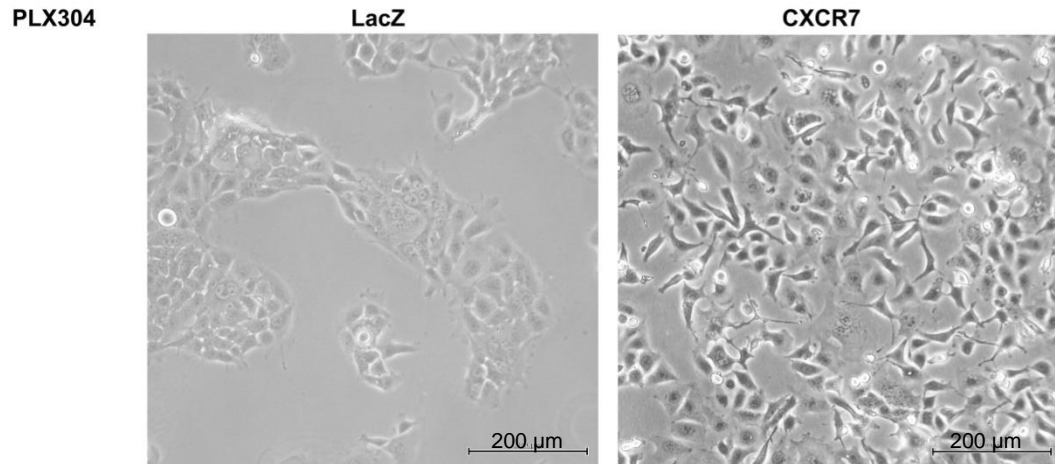


Figure 9. Phenotype changes in HCC827 cells with ectopic overexpression of CXCR7-V5. Bright field microscopic images (10x magnification) demonstrates ectopic expression of CXCR7-V5 in HCC827 promotes elongated mesenchymal-like cell morphology. HCC827 GFP cells show epithelial morphology which are cobblestone and flattend whereas HCC827 CXCR7-V5 cells show mesenchymal-like morphology which are scattered and elongated.

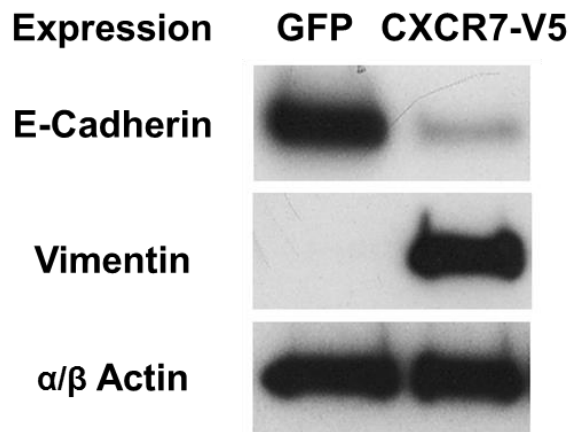


Figure 10. Ectopic CXCR7-V5 overexpression in HCC827 cells promotes induction of mesenchymal-like phenotype. Whole cell lysates were made from exponentially growing HCC827 GFP or HCC827 CXCR7-V5 cells. The whole cell lysates were subjected to Western blot using indicated antibodies. HCC827 CXCR7-V5 cells display mesenchymal-like transition demonstrated by E-Cadherin downregulation and Vimentin upregulation compared to HCC827 GFP cells.

Ectopic CXCR7 overexpression in NCI-H1975 cells promotes mesenchymal transition. In order to investigate if ectopic CXCR7 expression is sufficient to promote epithelial to mesenchymal transition in other EGFR mutant NSCLC cells, we overexpressed CXCR7 in NCI-H1975 cells which express L858R/T790M EGFR kinase domain mutations and are sensitive to irreversible EGFR TKI. In consistent with what we observed in HCC827 cells, ectopic CXCR7 overexpression in NCI-H1975 cells significantly decreased the level of canonical epithelial marker E-Cadherin and upregulated the canonical mesenchymal marker N-Cadherin (Figure 10). In addition, in NCI-H1975 CXCR7-V5 cells, we observed upregulation of Twist and Snail which are important transcription factors to induce mesenchymal phenotype in cultured cells (106) (Figure 10).

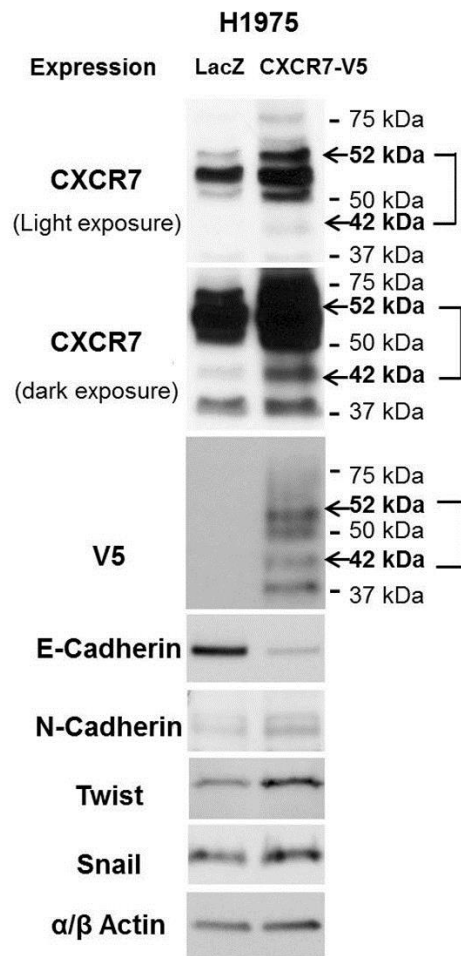


Figure 11. Ectopic overexpression of CXCR7 promotes mesenchymal phenotype in NCI-H1975 cells. NCI-H1975 cells were transduced with lentivirus coding for LacZ or CXCR7-V5 expression vectors. Cell lysates of NCIH1975 LacZ and NCI-H1975 CXCR7-V5 cells were collected and subjected to the Western blot with antibodies against CXCR7, V5 tag and canonical epithelial or mesenchymal cell markers. Expression of CXCR7 protein indicated as 42 kDa (immature type) and 52 kDa (post-translational ubiquitination type).

Ectopic CXCR7 overexpression is sufficient to confer EGFR TKI resistance in HCC827 cells. Epithelial to mesenchymal transition has been reported to confer drug resistance in EGFR mutant NSCLC (45, 56). Moreover, we hypothesized that HCC827 cells with ectopic CXCR7 overexpression attenuates their EGFR TKI sensitivity through 'receptor switch'. To determine whether HCC827 CXCR7-V5 cells with mesenchymal phenotype are resistant to EGFR TKI, HCC827, HCC827 GFP and HCC CXCR7-V5 cells were subjected to cell viability assay with gefitinib for 72 hours. By comparing IC₅₀, we observed that HCC827 CXCR7-V5 cells with mesenchymal phenotype were 14 times resistant to gefitinib treatment as compared to control HCC827. We quantified percentage apoptosis induced by gefitinib using Annexin V-FITC/PI double staining and flow cytometric analysis. Data shown in Figure 13 shows that gefitinib treatment induced apoptosis in 46% of control HCC827 LacZ cells. However, gefitinib treatment induced apoptosis increased about 1 % of HCC827 CXCR7-V5 cells that is in different from percent of apoptotic cells found in DMSO treated HCC827 CXCR7-V5 cells (3.42%). In Western blot, gefitinib treatment induced significant increase in PARP cleavage in control HCC827 GFP cells while same treatment failed to induce PARP cleavage in HCC827 CXCR7-V5 cells (Figure 14). In addition, exposure of HCC827 GFP to gefitinib resulted in significantly decreased level of phospho- AKT and phospho-ERK indicating that gefitinib inhibited the downstream signaling in control cells. However, exposure of HCC827 CXCR7-V5 to gefitinib resulted in decreased the level of phospho-ERK; however, the level of phospho-AKT was maintained (Figure 14).

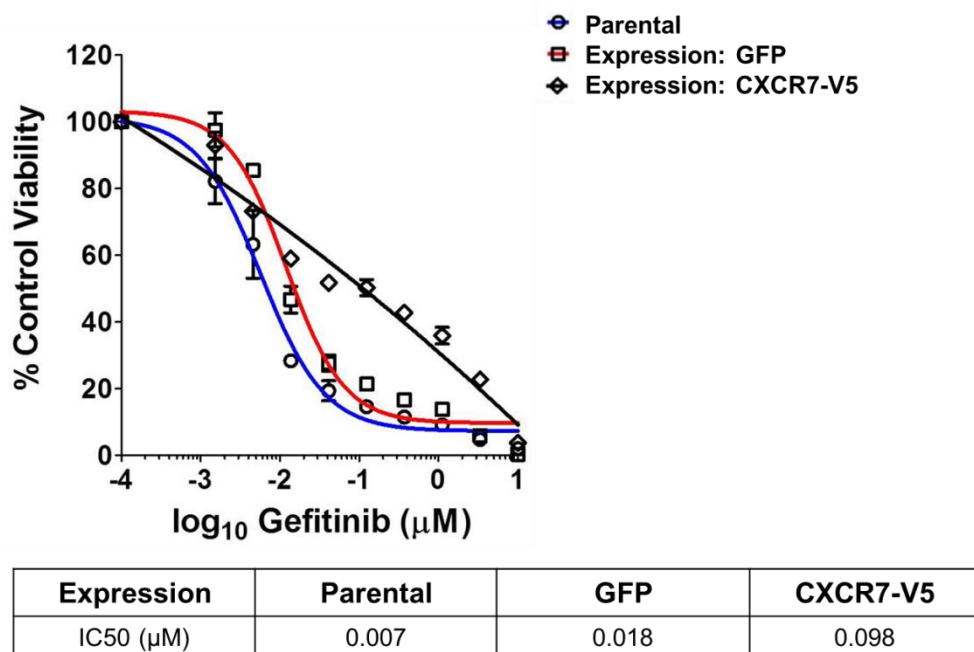


Figure 12. Ectopic overexpression of CXCR7 attenuates the sensitivity of HCC827 cells to EGFR TKI. Exponentially growing HCC827, HCC827 GFP, HCC827 CXCR7-V5 cells were treated with indicated concentration of gefitinib for 72 hours in duplicates and cell viability was assessed using Cell Counting Kit-8. The viability of each cell line was normalized to that of DMSO-treated cell. Points, average of normalized values for experiment in duplicates; error bars, SD. The table list calculated IC₅₀ values against gefitinib.

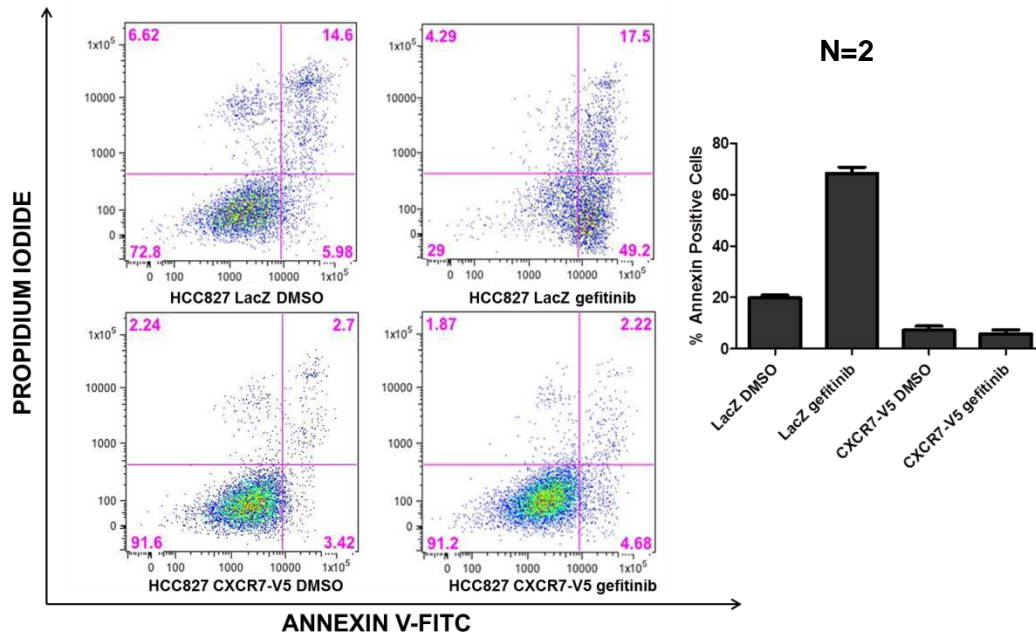


Figure 13. Assessment of apoptosis by Annexin V-FITC/ PI double staining demonstrates that gefitinib treatment does not compromise viability of cells ectopically expressing CXCR7-V5. Quantification of flow cytometric analysis using Annexin V-FITC and propidium iodide staining indicates that 48 hours of 1 μ M gefitinib treatment does not induce apoptosis in CXCR7 overexpressing HCC827 cells. gefitinib treatment induced almost 10 fold more cell death in HCC827 LacZ cells (49.2% apoptosis) when compared with DMSO treated HCC827 LacZ cells (5.98% apoptosis) while there was no change in percentage apoptosis in HCC827 CXCR7-V5 cells treated with (4.68%) or without (3.42%) gefitinib. Right column indicates average of normalized percent Annexin positive cells for two independent experiments; error bars, SD.

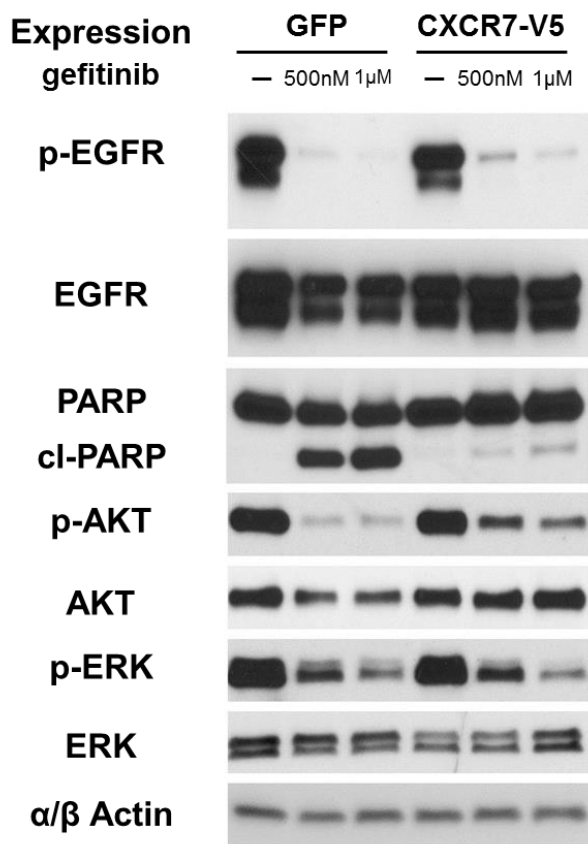


Figure 14. Gefitinib treatment promotes significantly less PARP-cleavage in HCC827 cells with CXCR7-V5 overexpression than in HCC827 GFP control cells. HCC827 GFP and HCC827 CXCR7-V5 cells were treated with DMSO or indicated concentration of gefitinib for 24 hours. The cells were lysed and the lysates were subjected to Western blot using indicated antibodies. HCC827 GFP cells are sensitive to gefitinib treatment as evidenced by the PARP cleavage and significant depletion of phospho-AKT, phospho-ERK after gefitinib treatment. In contrast, exposure to gefitinib does not promote PARP cleavage in HCC827 CXCR7-V5 cells and PI3K signal persists despite the inhibition of EGFR as evidenced by phospho-AKT.

CXCR7 depletion restores gefitinib sensitivity in HCC827 CXCR7-V5 cells. Our observations with stable CXCR7 expression in HCC827 cells demonstrated that ectopic CXCR7 expression confers EGFR TKI resistance in EGFR mutant NSCLC (Figure 12,13,14). To further confirm that CXCR7 is sufficient to confer resistance to EGFR TKI, we depleted CXCR7 from HCC827 CXCR7-V5 cells to see if CXCR7 depletion would resensitize the HCC827 CXCR7-V5 cells to gefitinib. We stably knocked down CXCR7 using lentiviral shRNA against CXCR7 in HCC827 CXCR7-V5 cells. CXCR7 was effectively depleted by shCXCR7 in HCC827 CXCR7-V5 cells as indicated in Figure 15A. Exposure of HCC827 CXCR7-V5 cells with CXCR7 knockdown to gefitinib restored the level of cell apoptosis marker- PARP cleavage while HCC827 CXCR7-V5 cells transduced with shRNA against NT maintained the low level of PARP cleavage after treated with gefitinib (Figure 15B). In addition, depletion of ectopic CXCR7 expression in HCC827 CXCR7-V5 cells resulted in diminishing phospho-AKT and phosph-ERK after gefitinib treatment which was the manner similar as HCC827 GFP cells treated with gefitinib. In line with the previous observation, HCC827 CXCR7-V5 cells transduced with shRNA against NT maintained PI3K and MAPK signaling after treated with gefitinib (Figure 15C).

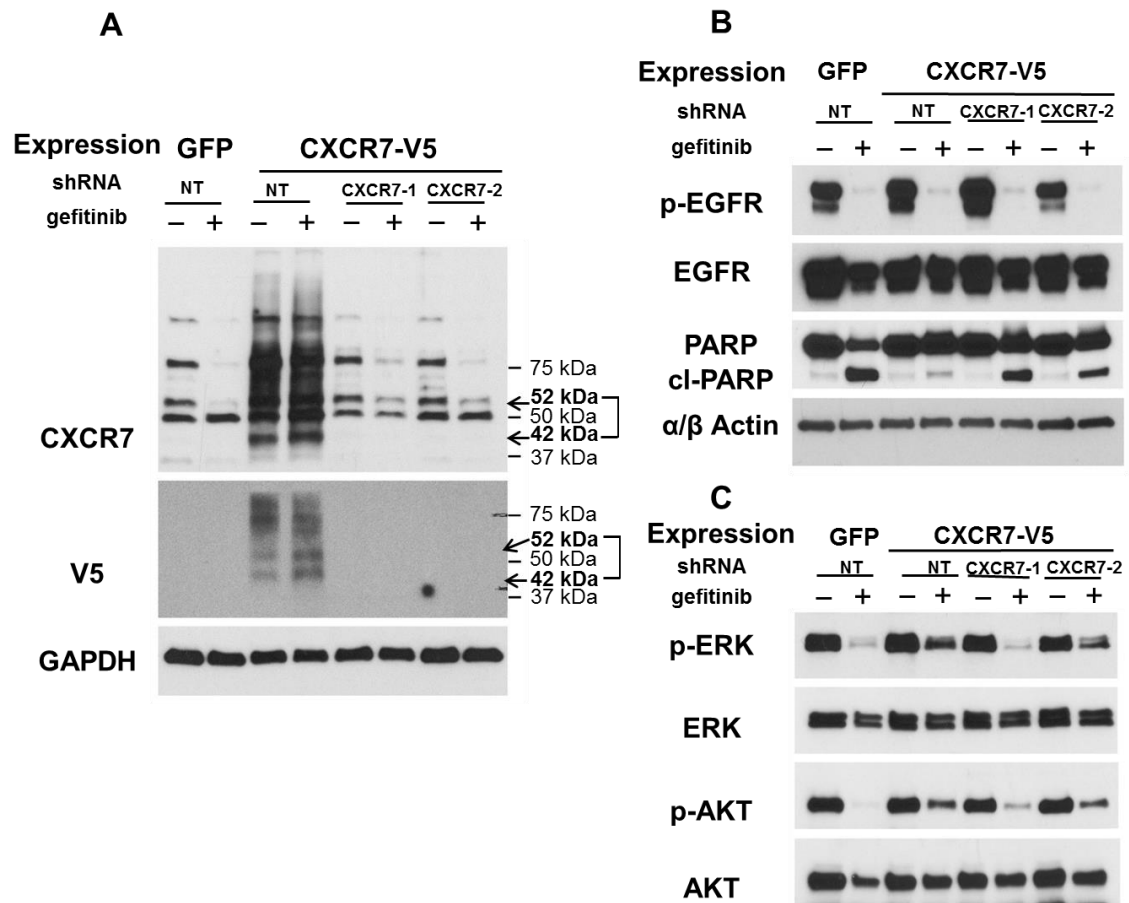


Figure 15. EGFR TKI resistance in HCC827 cells by ectopic overexpression of CXCR7 is reversible. A, HCC827 GFP (GFP) cells stably expressing non-target shRNA (NT) were established and treated with DMSO (-) or 500nM gefitinib (+) for 48 hours. HCC827 CXCR7-V5 (CXCR7-V5) cells stably expressing non-target shRNA (NT) or two best shRNA sequence targeting CXCR7 (CXCR7-1 and CXCR7-2) were established and treated with 1 μ M DMSO (-) or gefitinib (+) for 48 hours. Whole cell lysate were prepared and the lysate were subjected to the Western blot using indicated antibodies. Expression of CXCR7 protein indicated as 42 kDa (immature type) and 52 kDa (post-translationally modified type). B, Using the same lysates, Western blots with indicated antibodies were performed to confirm inactivation of EGFR and PARP-cleavage by gefitinib treatment. Note PARP-cleavage was not observed in gefitinib treated CXCR7-V5 overexpressing cells (4th lane), whereas knockdown of CXCR7 in CXCR7-V5 overexpressing cells restored sensitivity to gefitinib as evidenced by PARP-cleavage (lanes 6 and 8). C,

Using the same lysates, Western blots with indicated antibodies were performed to access PI3K and MAPK pathway activities by interrogating AKT (S473) and ERK (T202/Y204), respectively. Representational figure from two independent experiments shown.

CXCR7 knockdown partially reverse mesenchymal HCC827 CXCR7-V5 to

epithelial. We observed stable CXCR7 overexpression in epithelial HCC827 cells induced the mesenchymal-like transition in EGFR mutant NSCLC (Figure 9,10). EMT is reported to be a reversible process (48) and CXCR7 knockdown reversed gefitinib sensitivity in HCC827 CXCR7-V5 cells, therefore we next investigated if EMT induced by ectopic CXCR7 overexpression in EGFR mutant NSCLC is also reversible. Depletion of CXCR7 in HCC827 CXCR7-V5 cells did not induce an apparent phenotype change when observed under microscope (data not shown). When investigated with Western blot, we observed increased level of canonical epithelial cell marker, ZO1 and decreased expression of canonical mesenchymal marker, Vimentin. However, the expression of E-Cadherin after CXCR7 knockdown did not change` (Figure 15). The results indicate that CXCR7 depletion partially reverses HCC827 CXCR7-V5 to epithelial phenotype.

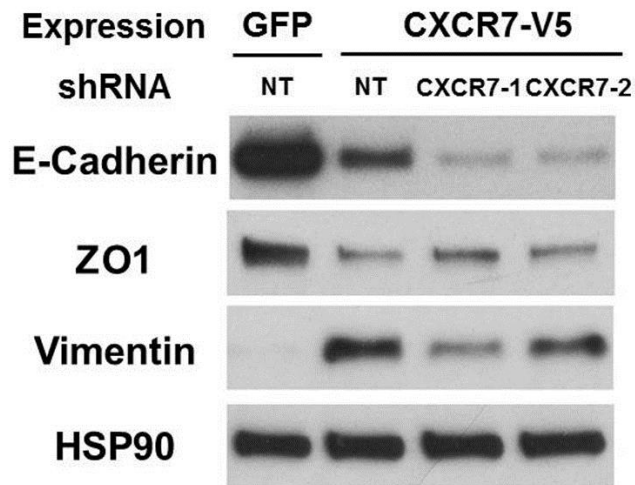


Figure 16. Depletion of CXCR7 partially restores epithelial phenotype. HCC827 GFP (GFP) cells and HCC827 CXCR7-V5 (CXCR7-V5) cells were transduced with lentiviruses coding for non-target sequence (NT) or two best shRNA sequence targeting CXCR7 (CXCR7-1 and CXCR7-2). Stable cell lines were established. Whole cell lysate were prepared and the lysates were subjected to the Western blot using indicated antibodies to interrogate canonical epithelial and mesenchymal markers. HSP90 serves as loading control.

CHAPTER FOUR

DISCUSSION

NSCLC harboring activating EGFR kinase domain mutations is a unique subtype of NSCLC. NSCLC patients harboring EGFR kinase domain mutations respond well to EGFR TKI. However, the emergence of acquired resistance to EGFR TKI greatly limits the efficiency of targeted therapy (24).

Besides the well-established resistant mechanisms including EGFR T790M secondary mutation, and MET amplification mutation, 30 percent of the resistant mechanisms remain elusive. Among these unknown resistant mechanisms, about half of these resistant NSCLC present an evidence of EMT (45). However, it remains unclear which components of EMT directly contribute to the EGFR TKI resistance. Based on the mechanisms for known EGFR TKI acquired resistance, we hypothesized that EMT program associates with the upregulation of the novel cellular signaling mechanisms that the cell can depend on when EGFR activity is inhibited by EGFR TKI.

In order to study EGFR TKI resistant mechanisms in EGFR mutant NSCLC cells with mesenchymal phenotype, we induced mesenchymal phenotype in HCC827 cells by knocking down CDH1. We also made mesenchymal and EGFR TKI resistant HCC827 cells by stably expressing shRNA against EGFR. It has been reported that AXL is overexpressed in mutant EGFR NSCLC cells with mesenchymal phenotype, therefore AXL has been considered as a potential candidate to confer resistance to EGFR TKI

through 'receptor tyrosine kinase switch' (74). In our study we observed the overexpression of AXL in EGFR TKI resistant HCC827 shEGFR cells and HCC827 shCDH1 cells. Furthermore, our experimental results show that AXL phosphorylation is dependent on EGFR activity (Figure 4) as inhibition of EGFR with gefitinib dephosphorylated not only EGFR but also AXL. The results suggest that AXL activity cannot compensate the loss of EGFR activity in our mesenchymal HCC827 shEGFR and HCC827 shCDH1 cells. In addition, inhibition of AXL activity using XL-184 or depletion of AXL using siRNA did not compromise cell viability or restore their gefitinib sensitivity in HCC827 shEGFR and HCC827 shCDH1 cells (Figure 2,5). Our data suggests that AXL is less likely to confer resistance to EGFR TKI in HCC827 cells with mesenchymal phenotype. We speculated if overexpression of AXL is a biomarker of mesenchymal phenotype rather than an oncogenic driver that replaces mutant EGFR.

In order to identify oncogenic driver for mesenchymal EGFR mutant HCC827 cells, Dr. Shimamura performed comparative marker selection (CMS) on differential gene expression data for HCC827 shEGFR, HCC827 shCDH1 and HCC827 cells chronically exposed to TGF- β . G-protein coupled receptor- CXCR7 is significantly upregulated in mesenchymal-like HCC827 cells including HCC827 shEGFR, HCC827 shCDH1 and HCC827 cells chronically exposed to TGF- β (Figure 6). The subsequent analysis of mRNA expression and Western blot confirmed the upregulation of CXCR7 in the mesenchymal-like HCC827 cells (Figure 7). Of note, our collaborative laboratory at Dana-Farber Cancer Institute generated genetically engineered mouse model harboring EGFR kinase domain mutant NSCLC tumor and demonstrated that the induction of the

mutant EGFR in type II clara cells induce lung adenocarcinoma. In the model, adenocarcinoma with acquired resistance to EGFR TKI was observed upon 12 weeks of continuous EGFR TKI treatment. Histological analyses of the resistant tumors showed a significant upregulation of CXCR7 expression and a significant upregulation of canonical mesenchymal markers (data not shown). Collective evidence lead us to further explore the role of CXCR7 in EGFR mutant EGFR TKI resistant NSCLC cells.

The role of CXCR7 with respect to EGFR TKI resistance in EGFR mutant NSCLC cells has never been reported. In order to establish the relationship between CXCR7 and EGFR TKI resistance, we established an *in vitro* model that ectopically express V5-tagged CXCR7 in EGFR mutant HCC827 cells. We observed that ectopic CXCR7 overexpression attenuates EGFR TKI sensitivity in HCC827 cells (Figure 12, 13, 14). In addition, we observed that AKT phosphorylation was maintained after acute EGFR inhibition by gefitinib treatment in HCC827 CXCR7 cells (Figure 14). Similarly, EGFR inhibition did not attenuate AKT phosphorylation in HCC827 CDH1 cells with mesenchymal phenotype (Figure 1C). In NSCLC, AKT (S473) phosphorylation serves as a biomarker of PI3K pathway activity (28, 29). A considerable number of literature has suggested that CXCR7 activates PI3K pathway through beta-arrestin (84, 95). To our surprise, CXCR7 is sufficient to upregulate canonical mesenchymal markers and to downregulate epithelial markers(Figure 10, 11). Importantly, depletion of the ectopically expressed CXCR7 in HCC827 cells (HCC827 CXCR7-V5) resensitized the cells to EGFR TKIs and partially restored epithelial phenotype (Figure 15, 16). The experiment confirmed the notion that the ectopically expressed CXCR7 is responsible for the EGFR

TKI resistance in HCC827 cells. We observed that CXCR7 depletion- induced a partial phenotype revision in HCC827 CXCR7-V5 cells may suggest that CXCR7 induced mesenchymal-like cells may activate varieties of autocrine signaling loops that may be maintained even after CXCR7 is depleted. The activation of EMT programs involves a series of transitions and a spectrum of multiple intermediated states lying between these two endpoints (107). The epigenetic regulations of EMT describe the mechanisms that impose cellular phenotype with covalent modification of DNA, specially the methylation of certain DNA residues, as well as the gain of repressive histone modifications that form DNA-associated nucleosomes (107). Therefore, it is possible that the reversibility of EMT process requires comprehensive reprogramming of gene expression even though the protein that initiates mesenchymal transition is depleted. These evidence may explain why depletion of CXCR7 would not completely reverse the process of EMT.

While overexpression of CXCR7 in HCC827 cells was sufficient to promote EMT and to confer EGFR TKI resistance, it remains elusive if ligand stimulation is involved in the process. In general, the activation of CXCR7 requires the presence of its ligands SDF-1 α / CXCL12 or ITAC/CXCL11 (82). Therefore it is speculated if CXCR7 can be self activated without the presence of appropriate ligand if it is sufficiently overexpressed. Alternatively, it is possible that ectopic expression of CXCR7 promotes the expression of CXCR7 ligand to establish autocrine loop. Nonetheless, further investigations to elucidate CXCR7 activation mechanisms are necessary.

Although several reports have shown that G protein coupled receptor promotes EMT, there is no report demonstrating that CXCR7 promotes EMT and EGFR TKI

resistance. Our observations may suggest a novel link between chemokine receptor CXCR7 and EMT. However, the mechanisms by which CXCR7 induces EMT remain elusive. Additionally, we observed CXCR7 induced upregulation of EMT transcription factor Twist and Snail in H1975 cells (Figure 11) but not in HCC827 cells (data not shown) which indicates that the mechanisms by which CXCR7 induces EMT may be cell lineage specific.

Interestingly, we observed upregulation and phosphorylation of AXL in HCC827 CXCR7-V5 cells (data not shown). However, in consistent with what we observed in HCC827 shEGFR and HCC827 shCDH1 cells, gefitinib treatment of HCC827 CXCR7-V5 cells resulted in decreased AXL phosphorylation and AXL inhibition or depletion by siRNA did not restore gefitinib sensitivity in the cells (data not shown). This observation suggests that AXL upregulation associates with epithelial to mesenchymal transition but AXL activity is not sufficient to replace mutant EGFR activity in mutant EGFR mutant NSCLC with mesenchymal phenotype.

The potential problem of our system with ectopic CXCR7-V5 overexpression in HCC827 cells is that we did not confirm if CXCR7 is activated. Since CXCR7 is activated by association with β -arrestin, future studies with co-immunoprecipitation or Spell FRET (FRET) is required to test if CXCR7 interacts with β -arrestin in HCC827 CXCR7-V5 cells.

Further investigation revealed that HCC827 shCDH1 overexpress CXCR7 (Figure 7) while 42 major RTKs were not phosphorylated (Figure 3). Based on the result, we hypothesized that CXCR7 activates PI3K pathway in HCC827 shCDH1 cells when they are treated with EGFR TKI. In order to determine if CXCR7 is necessary to confer EGFR

TKI resistance in HCC827 shCDH1 cells, we knockdown CXCR7 in EGFR TKI resistant mesenchymal-like HCC827 shCDH1 cells using CXCR7-specific siRNA and determined if we could restore their sensitivity to EGFR TKI in HCC827 shCDH1 cells. Unfortunately, we were not able to effectively deplete CXCR7 using siRNA in HCC827 shCDH1 cells (data not shown). Considering the possibility that siCXCR7 we purchased were not effective to knockdown CXCR7, we also use lentiviral shRNA against CXCR7 in HCC827 Snail-V5 cells that ectopically overexpress EMT-inducing transcription factor Snail. HCC827 Snail-V5 cells exhibit mesenchymal phenotype and the cells are resistant to EGFR TKI. The data suggested that CXCR7 knockdown restored the EGFR TKI sensitivity of HCC827 Snail-V5 cell (data not shown). In conclusion, our data suggests that CXCR7 is sufficient and necessary to confer EGFR TKI resistance in HCC827 cells.

Based on the data we have observed, we proposed that chronic EGFR TKI treatment in EGFR mutant NSCLC promotes overexpression of CXCR7 and CXCR7 maintains survival and proliferation pathway when mutant EGFR is unable to activate the pathways under the EGFR TKI treatment (Figure 16).

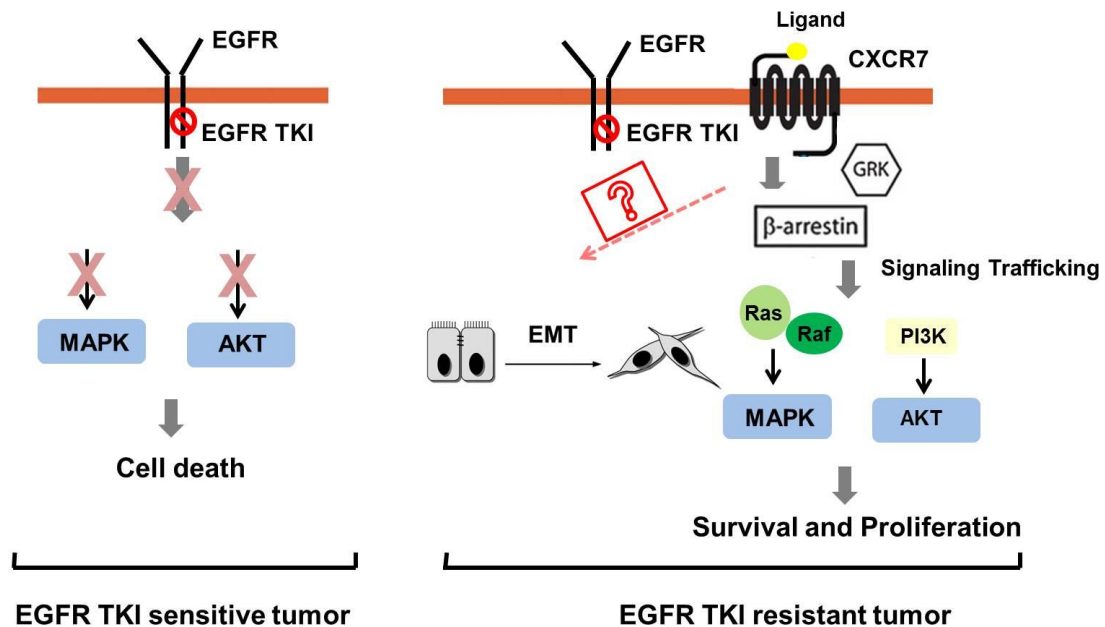


Figure 17. A schematic diagram of a proposed mechanism for CXCR7-mediated acquired EGFR TKI resistance in EGFR mutant NSCLC. Our results suggest that CXCR7 is overexpressed in EGFR mutant NSCLC with mesenchymal phenotype. The activation of CXCR7 augments the mutant EGFR signaling when mutant EGFR receptor is inhibited. CXCR7 signaling promotes cell survival, proliferation and mesenchymal phenotype in EGFR TKI resistant EGFR mutant NSCLC cells.

In conclusion, we discovered that ectopic CXCR7 expression in EGFR-TKI sensitive HCC827 and NCI-H1975 cells confers EGFR TKI resistance and induces the mesenchymal phenotype. Taken together, our data suggests that CXCR7 might have its potential therapeutic implications for preventing or overcoming EGFR TKI resistance in EGFR mutant NSCLC patients with mesenchymal phenotype. Future studies will be necessary to determine if CXCR7 is an oncogene in NSCLC and the mechanisms of CXCR7 upregulation in NSCLC with EGFR TKI resistance. The

investigation will provide insights into the developing the therapeutic intervention of CXCR7 mediated EGFR TKI resistance in EGFR mutant NSCLC.

REFERENCES

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* 2014;64(1):9-29.
2. Pal SK, Figlin RA, Reckamp K. Targeted therapies for non-small cell lung cancer: An evolving landscape. *Mol Cancer Ther* 2010;9(7):1931-44.
3. Rami-Porta R, Chansky K, Goldstraw P. Updated lung cancer staging system. *Future Oncol* 2009;5(10):1545-53.
4. Non-small cell lung cancer treatment. . Available from: http://www.lungcancer.org/find_information/publications/163-lung_cancer_101/269-non-small_cell_lung_cancer_treatment.
5. Parikh RB, Kirch RA, Smith TJ, Temel JS. Early specialty palliative care--translating data in oncology into practice. *N Engl J Med* 2013;369(24):2347-51.
6. Katzel JA, Fanucchi MP, Li Z. Recent advances of novel targeted therapy in non-small cell lung cancer. *J Hematol Oncol* 2009;2:2,8722-2-2.
7. Chen P, Wang L, Liu B, Zhang HZ, Liu HC, Zou Z. EGFR-targeted therapies combined with chemotherapy for treating advanced non-small-cell lung cancer: A meta-analysis. *Eur J Clin Pharmacol* 2011;67(3):235-43.
8. Herbst RS. Review of epidermal growth factor receptor biology. *Int J Radiat Oncol Biol Phys* 2004;59(2 Suppl):21-6.
9. Downward J, Parker P, Waterfield MD. Autophosphorylation sites on the epidermal growth factor receptor. *Nature* 1984;311(5985):483-5.
10. Pawson T, Gish GD, Nash P. SH2 domains, interaction modules and cellular wiring. *Trends Cell Biol* 2001;11(12):504-11.
11. Huang H, Li L, Wu C, Schibli D, Colwill K, Ma S, Li C, Roy P, Ho K, Songyang Z, Pawson T, Gao Y, Li SS. Defining the specificity space of the human SRC homology 2 domain. *Mol Cell Proteomics* 2008;7(4):768-84.

12. Hynes NE, Lane HA. ERBB receptors and cancer: The complexity of targeted inhibitors. *Nat Rev Cancer* 2005;5(5):341-54.
13. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;2(2):127-37.
14. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350(21):2129-39.
15. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M. EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. *Science* 2004;304(5676):1497-500.
16. Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II, Fong KM, Lee H, Toyooka S, Shimizu N, Fujisawa T, Feng Z, Roth JA, Herz J, Minna JD, Gazdar AF. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst* 2005;97(5):339-46.
17. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: Biological and clinical implications. *Cancer Res* 2004;64(24):8919-23.
18. Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. *Nat Rev Cancer* 2007;7(3):169-81.
19. Weinstein IB. Cancer. addiction to oncogenes--the achilles heel of cancer. *Science* 2002;297(5578):63-4.
20. Gong Y, Somwar R, Politi K, Balak M, Chmielecki J, Jiang X, Pao W. Induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors in mutant EGFR-dependent lung adenocarcinomas. *PLoS Med* 2007;4(10):e294.
21. Cragg MS, Kuroda J, Puthalakath H, Huang DC, Strasser A. Gefitinib-induced killing of NSCLC cell lines expressing mutant EGFR requires BIM and can be enhanced by BH3 mimetics. *PLoS Med* 2007;4(10):1681,89; discussion 1690.
22. Lee HY, Srinivas H, Xia D, Lu Y, Superty R, LaPushin R, Gomez-Manzano C, Gal AM, Walsh GL, Force T, Ueki K, Mills GB, Kurie JM. Evidence that phosphatidylinositol 3-kinase- and mitogen-activated protein kinase kinase-4/c-

- jun NH2-terminal kinase-dependent pathways cooperate to maintain lung cancer cell survival. *J Biol Chem* 2003;278(26):23630-8.
23. Faber AC, Wong KK, Engelman JA. Differences underlying EGFR and HER2 oncogene addiction. *Cell Cycle* 2010;9(5):851-2.
 24. Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. *Nat Rev Cancer* 2007;7(3):169-81.
 25. Greulich H, Chen TH, Feng W, Jänne PA, Alvarez JV, Zappaterra M, Bulmer SE, Frank DA, Hahn WC, Sellers WR, Meyerson M. Oncogenic transformation by inhibitor-sensitive and -resistant EGFR mutants. *PLoS Med* 2005;2(11):e313.
 26. Yasuda H, Park E, Yun CH, Sng NJ, Lucena-Araujo AR, Yeo WL, Huberman MS, Cohen DW, Nakayama S, Ishioka K, Yamaguchi N, Hanna M, Oxnard GR, Lathan CS, Moran T, Sequist LV, Chaft JE, Riely GJ, Arcila ME, Soo RA, Meyerson M, Eck MJ, Kobayashi SS, Costa DB. Structural, biochemical, and clinical characterization of epidermal growth factor receptor (EGFR) exon 20 insertion mutations in lung cancer. *Sci Transl Med* 2013;5(216):216ra177.
 27. Inukai M, Toyooka S, Ito S, Asano H, Ichihara S, Soh J, Suehisa H, Ouchida M, Aoe K, Aoe M, Kiura K, Shimizu N, Date H. Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer. *Cancer Res* 2006;66(16):7854-8.
 28. Anderson NG, Ahmad T, Chan K, Dobson R, Bundred NJ. ZD1839 (iressa), a novel epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, potentially inhibits the growth of EGFR-positive cancer cell lines with or without erbB2 overexpression. *Int J Cancer* 2001;94(6):774-82.
 29. Moasser MM, Basso A, Averbuch SD, Rosen N. The tyrosine kinase inhibitor ZD1839 ("iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res* 2001;61(19):7184-8.
 30. Kawano O, Sasaki H, Endo K, Suzuki E, Haneda H, Yukiue H, Kobayashi Y, Yano M, Fujii Y. PIK3CA mutation status in japanese lung cancer patients. *Lung Cancer* 2006;54(2):209-15.
 31. Sos ML, Koker M, Weir BA, Heynck S, Rabinovsky R, Zander T, Seeger JM, Weiss J, Fischer F, Frommolt P, Michel K, Peifer M, Mermel C, Girard L, Peyton M, Gazdar AF, Minna JD, Garraway LA, Kashkar H, Pao W, Meyerson M,
 32. Thomas RK. PTEN loss contributes to erlotinib resistance in EGFR-mutant lung cancer by activation of akt and EGFR. *Cancer Res* 2009;69(8):3256-61.

33. Chakravarti A, Loeffler JS, Dyson NJ. Insulin-like growth factor receptor I mediates resistance to anti-epidermal growth factor receptor therapy in primary human glioblastoma cells through continued activation of phosphoinositide 3-kinase signaling. *Cancer Res* 2002;62(1):200-7.
34. Bivona TG, Hieronymus H, Parker J, Chang K, Taron M, Rosell R, Moonsamy P, Dahlman K, Miller VA, Costa C, Hannon G, Sawyers CL. FAS and NF-kappaB signalling modulate dependence of lung cancers on mutant EGFR. *Nature* 2011;471(7339):523-6.
35. Thomson S, Buck E, Petti F, Griffin G, Brown E, Ramnarine N, Iwata KK, Gibson N, Haley JD. Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition. *Cancer Res* 2005;65(20):9455-62.
36. Yauch RL, Januario T, Eberhard DA, Cavet G, Zhu W, Fu L, Pham TQ, Soriano R, Stinson J, Seshagiri S, Modrusan Z, Lin CY, O'Neill V, Amler LC. Epithelial versus mesenchymal phenotype determines in vitro sensitivity and predicts clinical activity of erlotinib in lung cancer patients. *Clin Cancer Res* 2005;11(24 Pt 1):8686-98.
37. Witta SE, Gemmill RM, Hirsch FR, Coldren CD, Hedman K, Ravdel L, Helfrich B, Dziadziuszko R, Chan DC, Sugita M, Chan Z, Baron A, Franklin W, Drabkin HA, Girard L, Gazdar AF, Minna JD, Bunn PA Jr. Restoring E-Cadherin expression increases sensitivity to epidermal growth factor receptor inhibitors in lung cancer cell lines. *Cancer Res* 2006;66(2):944-50.
38. Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005;352(8):786-92.
39. Yun CH, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong KK, Meyerson M, Eck MJ. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc Natl Acad Sci U S A* 2008;105(6):2070-5.
40. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC, Jänne PA. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007;316(5827):1039-43.
41. Bean J, Brennan C, Shih JY, Riely G, Viale A, Wang L, Chitale D, Motoi N, Szoke J, Broderick S, Balak M, Chang WC, Yu CJ, Gazdar A, Pass H, Rusch V, Gerald W, Huang SF, Yang PC, Miller V, Ladanyi M, Yang CH, Pao W. MET

amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. *Proc Natl Acad Sci U S A* 2007;104(52):20932-7.

42. Yano S, Wang W, Li Q, Matsumoto K, Sakurama H, Nakamura T, Ogino H, Kakiuchi S, Hanibuchi M, Nishioka Y, Uehara H, Mitsudomi T, Yatabe Y, Nakamura T, Sone S. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res* 2008;68(22):9479-87.
43. Engelman JA, Janne PA, Mermel C, Pearlberg J, Mukohara T, Fleet C, Cichowski K, Johnson BE, Cantley LC. ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. *Proc Natl Acad Sci U S A* 2005;102(10):3788-93.
44. Engelman JA, Mukohara T, Zejnullahu K, Lifshits E, Borrás AM, Gale CM, Naumov GN, Yeap BY, Jarrell E, Sun J, Tracy S, Zhao X, Heymach JV, Johnson BE, Cantley LC, Jänne PA. Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. *J Clin Invest* 2006;116(10):2695-706.
45. Turke AB, Zejnullahu K, Wu YL, Song Y, Dias-Santagata D, Lifshits E, Toschi L, Rogers A, Mok T, Sequist L, Lindeman NI, Murphy C, Akhavanfard S, Yeap BY, Xiao Y, Capelletti M, Iafrate AJ, Lee C, Christensen JG, Engelman JA, Jänne PA. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. *Cancer Cell* 2010;17(1):77-88.
46. Sequist LV, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, Bergethon K, Shaw AT, Gettinger S, Cospers AK, Akhavanfard S, Heist RS, Temel J, Christensen JG, Wain JC, Lynch TJ, Vernovsky K, Mark EJ, Lanuti M, Iafrate AJ, Mino-Kenudson M, Engelman JA. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med* 2011;3(75):75ra26.
47. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 2003;15(6):740-6.
48. Maeda M, Johnson KR, Wheelock MJ. Cadherin switching: Essential for behavioral but not morphological changes during an epithelium-to-mesenchyme transition. *J Cell Sci* 2005;118(Pt 5):873-87.
49. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009;119(6):1420-8.

50. Semb H, Christofori G. The tumor-suppressor function of E-cadherin. *Am J Hum Genet* 1998;63(6):1588-93.
51. Kim K, Lu Z, Hay ED. Direct evidence for a role of beta-catenin/LEF-1 signaling pathway in induction of EMT. *Cell Biol Int* 2002;26(5):463-76.
52. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* 2008;68(10):3645-54.
53. Eger A, Stockinger A, Park J, Langkopf E, Mikula M, Gotzmann J, Mikulits W, Beug H, Foisner R. Beta-catenin and TGFbeta signalling cooperate to maintain a mesenchymal phenotype after FosER-induced epithelial to mesenchymal transition. *Oncogene* 2004;23(15):2672-80.
54. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009;139(5):871-90.
55. Singh A, Settleman J. EMT, cancer stem cells and drug resistance: An emerging axis of evil in the war on cancer. *Oncogene* 2010;29(34):4741-51.
56. de Herreros AG, Peiro S, Nassour M, Savagner P. Snail family regulation and epithelial mesenchymal transitions in breast cancer progression. *J Mammary Gland Biol Neoplasia* 2010;15(2):135-47.
57. Suda K, Tomizawa K, Fujii M, Murakami H, Osada H, Maehara Y, Yatabe Y, Sekido Y, Mitsudomi T. Epithelial to mesenchymal transition in an epidermal growth factor receptor-mutant lung cancer cell line with acquired resistance to erlotinib. *J Thorac Oncol* 2011;6(7):1152-61.
58. Barr S, Thomson S, Buck E, Russo S, Petti F, Sujka-Kwok I, Eyzaguirre A, Rosenfeld-Franklin M, Gibson NW, Miglarese M, Epstein D, Iwata KK, Haley JD. Bypassing cellular EGF receptor dependence through epithelial-to-mesenchymal-like transitions. *Clin Exp Metastasis* 2008;25(6):685-93.
59. Guix M, Faber AC, Wang SE, Olivares MG, Song Y, Qu S, Rinehart C, Seidel B, Yee D, Arteaga CL, Engelman JA. Acquired resistance to EGFR tyrosine kinase inhibitors in cancer cells is mediated by loss of IGF-binding proteins. *J Clin Invest* 2008;118(7):2609-19.
60. Linger RM, Keating AK, Earp HS, Graham DK. TAM receptor tyrosine kinases: Biologic functions, signaling, and potential therapeutic targeting in human cancer. *Adv Cancer Res* 2008;100:35-83.

61. Janssen JW, Schulz AS, Steenvoorden AC, Schmidberger M, Strehl S, Ambros PF, Bartram CR. A novel putative tyrosine kinase receptor with oncogenic potential. *Oncogene* 1991;6(11):2113-20.
62. Stitt TN, Conn G, Gore M, Lai C, Bruno J, Radziejewski C, Mattsson K, Fisher J, Gies DR, Jones PF, et al. The anticoagulation factor protein S and its relative, Gas6, are ligands for the tyro 3/axl family of receptor tyrosine kinases. *Cell* 1995;80(4):661-70.
63. Varnum BC, Young C, Elliott G, Garcia A, Bartley TD, Fridell YW, Hunt RW, Trail G, Clogston C, Toso RJ, et al. Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. *Nature* 1995;373(6515):623-6.
64. O'Bryan JP, Frye RA, Cogswell PC, Neubauer A, Kitch B, Prokop C, Espinosa R 3rd, Le Beau MM, Earp HS, Liu ET. Axl, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. *Mol Cell Biol* 1991;11(10):5016-31.
65. Gallicchio M, Mitola S, Valdembri D, Fantozzi R, Varnum B, Avanzi GC, Bussolino F. Inhibition of vascular endothelial growth factor receptor 2-mediated endothelial cell activation by axl tyrosine kinase receptor. *Blood* 2005;105(5):1970-6.
66. Collett GD, Sage AP, Kirton JP, Alexander MY, Gilmore AP, Canfield AE. Axl/phosphatidylinositol 3-kinase signaling inhibits mineral deposition by vascular smooth muscle cells. *Circ Res* 2007;100(4):502-9.
67. Bellosta P, Costa M, Lin DA, Basilico C. The receptor tyrosine kinase ARK mediates cell aggregation by homophilic binding. *Mol Cell Biol* 1995;15(2):614-25.
68. Burchert A, Attar EC, McCloskey P, Fridell YW, Liu ET. Determinants for transformation induced by the axl receptor tyrosine kinase. *Oncogene* 1998;16(24):3177-87.
69. Hafizi S, Dahlback B. Signalling and functional diversity within the axl subfamily of receptor tyrosine kinases. *Cytokine Growth Factor Rev* 2006;17(4):295-304.
70. Berclaz G, Altermatt HJ, Rohrbach V, Kieffer I, Dreher E, Andres AC. Estrogen dependent expression of the receptor tyrosine kinase axl in normal and malignant human breast. *Ann Oncol* 2001;12(6):819-24.

71. Wu CW, Li AF, Chi CW, Lai CH, Huang CL, Lo SS, Lui WY, Lin WC. Clinical significance of AXL kinase family in gastric cancer. *Anticancer Res* 2002;22(2B):1071-8.
72. Jacob AN, Kalapurakal J, Davidson WR, Kandpal G, Dunson N, Prashar Y, Kandpal RP. A receptor tyrosine kinase, UFO/axl, and other genes isolated by a modified differential display PCR are overexpressed in metastatic prostatic carcinoma cell line DU145. *Cancer Detect Prev* 1999;23(4):325-32.
73. Shieh YS, Lai CY, Kao YR, Shiah SG, Chu YW, Lee HS, Wu CW. Expression of axl in lung adenocarcinoma and correlation with tumor progression. *Neoplasia* 2005;7(12):1058-64.
74. Koorstra JB, Karikari CA, Feldmann G, Bisht S, Rojas PL, Offerhaus GJ, Alvarez H, Maitra A. The axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target. *Cancer Biol Ther* 2009;8(7):618-26.
75. Zhang Z, Lee JC, Lin L, Olivas V, Au V, LaFramboise T, Abdel-Rahman M, Wang X, Levine AD, Rho JK, Choi YJ, Choi CM, Kim SW, Jang SJ, Park YS, Kim WS, Lee DH, Lee JS, Miller VA, Arcila M, Ladanyi M, Moonsamy P, Sawyers C, Boggon TJ, Ma PC, Costa C, Taron M, Rosell R, Halmos B, Bivona TG. Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. *Nat Genet* 2012;44(8):852-60.
76. Linger RM, Keating AK, Earp HS, Graham DK. Taking aim at mer and axl receptor tyrosine kinases as novel therapeutic targets in solid tumors. *Expert Opin Ther Targets* 2010;14(10):1073-90.
77. Gjerdrum C, Tiron C, Hoiby T, Stefansson I, Haugen H, Sandal T, Collett K, Li S, McCormack E, Gjertsen BT, Micklem DR, Akslen LA, Glackin C, Lorens JB. Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. *Proc Natl Acad Sci U S A* 2010;107(3):1124-9.
78. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: Acquisition of malignant and stem cell traits. *Nat Rev Cancer* 2009;9(4):265-73.
79. Asiedu MK, Beauchamp-Perez FD, Ingle JN, Behrens MD, Radisky DC, Knutson KL. AXL induces epithelial-to-mesenchymal transition and regulates the function of breast cancer stem cells. *Oncogene* 2014;33(10):1316-24.
80. Byers LA, Diao L, Wang J, Saintigny P, Girard L, Peyton M, Shen L, Fan Y, Giri U, Tumula PK, Nilsson MB, Gudikote J, Tran H, Cardnell RJ, Bearss DJ, Warner

- SL, Foulks JM, Kanner SB, Gandhi V, Krett N, Rosen ST, Kim ES, Herbst RS, Blumenschein GR, Lee JJ, Lippman SM, Ang KK, Mills GB, Hong WK, Weinstein JN, Wistuba II, Coombes KR, Minna JD, Heymach JV. An epithelial-mesenchymal transition gene signature predicts resistance to EGFR and PI3K inhibitors and identifies axl as a therapeutic target for overcoming EGFR inhibitor resistance. *Clin Cancer Res* 2013;19(1):279-90.
81. Libert F, Parmentier M, Lefort A, Dumont JE, Vassart G. Complete nucleotide sequence of a putative G protein coupled receptor: RDC1. *Nucleic Acids Res* 1990;18(7):1917.
82. Balabanian K, Lagane B, Infantino S, Chow KY, Harriague J, Moepps B, Arenzana-Seisdedos F, Thelen M, Bachelier F. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J Biol Chem* 2005;280(42):35760-6.
83. Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z, Penfold ME, Sunshine MJ, Littman DR, Kuo CJ, Wei K, McMaster BE, Wright K, Howard MC, Schall TJ. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J Exp Med* 2006;203(9):2201-13.
84. Sierro F, Biben C, Martinez-Munoz L, Mellado M, Ransohoff RM, Li M, Woehl B, Leung H, Groom J, Batten M, Harvey RP, Martínez-A C, Mackay CR, Mackay F. Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc Natl Acad Sci U S A* 2007;104(37):14759-64.
85. Rajagopal S, Kim J, Ahn S, Craig S, Lam CM, Gerard NP, Gerard C, Lefkowitz RJ. Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proc Natl Acad Sci U S A* 2010;107(2):628-32.
86. Levoye A, Balabanian K, Baleux F, Bachelier F, Lagane B. CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood* 2009;113(24):6085-93.
87. Kumar R, Tripathi V, Ahmad M, Nath N, Mir RA, Chauhan SS, Luthra K. CXCR7 mediated galpha independent activation of ERK and akt promotes cell survival and chemotaxis in T cells. *Cell Immunol* 2012;272(2):230-41.
88. Canals M, Scholten DJ, de Munnik S, Han MK, Smit MJ, Leurs R. Ubiquitination of CXCR7 controls receptor trafficking. *PLoS One* 2012;7(3):e34192.

89. Graham GJ. D6 and the atypical chemokine receptor family: Novel regulators of immune and inflammatory processes. *Eur J Immunol* 2009;39(2):342-51.
90. Miao Z, Luker KE, Summers BC, Berahovich R, Bhojani MS, Rehemtulla A, Kleer CG, Essner JJ, Nasevicius A, Luker GD, Howard MC, Schall TJ. CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. *Proc Natl Acad Sci U S A* 2007;104(40):15735-40.
91. Boldajipour B, Mahabaleswar H, Kardash E, Reichman-Fried M, Blaser H, Minina S, Wilson D, Xu Q, Raz E. Control of chemokine-guided cell migration by ligand sequestration. *Cell* 2008;132(3):463-73.
92. Rago C, Ruhl R, McAllister S, Koon H, Dezube BJ, Früh K, Moses AV. Novel cellular genes essential for transformation of endothelial cells by kaposi's sarcoma-associated herpesvirus. *Cancer Res* 2005;65(12):5084-95.
93. Iwakiri S, Mino N, Takahashi T, Sonobe M, Nagai S, Okubo K, Wada H, Date H, Miyahara R. Higher expression of chemokine receptor CXCR7 is linked to early and metastatic recurrence in pathological stage I nonsmall cell lung cancer. *Cancer* 2009;115(11):2580-93.
94. Hou KL, Hao MG, Bo JJ, Wang JH. CXCR7 in tumorigenesis and progression. *Chin J Cancer* 2010;29(4):456-9.
95. Meijer J, Ogink J, Roos E. Effect of the chemokine receptor CXCR7 on proliferation of carcinoma cells in vitro and in vivo. *Br J Cancer* 2008;99(9):1493-501.
96. Wang J, Shiozawa Y, Wang J, Wang Y, Jung Y, Pienta KJ, Mehra R, Loberg R, Taichman RS. The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer. *J Biol Chem* 2008;283(7):4283-94.
97. Madden SL, Cook BP, Nacht M, Weber WD, Callahan MR, Jiang Y, Dufault MR, Zhang X, Zhang W, Walter-Yohrling J, Rouleau C, Akmaev VR, Wang CJ, Cao X, St Martin TB, Roberts BL, Teicher BA, Klinger KW, Stan RV, Lucey B, Carson-Walter EB, Laterra J, Walter KA. Vascular gene expression in nonneoplastic and malignant brain. *Am J Pathol* 2004;165(2):601-8.
98. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. Identification of pancreatic cancer stem cells. *Cancer Res* 2007;67(3):1030-7.
99. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 2005;65(23):10946-51.

100. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, Weissman IL, Clarke MF, Ailles LE. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci U S A* 2007;104(3):973-8.
101. Hajra KM, Fearon ER. Cadherin and catenin alterations in human cancer. *Genes Chromosomes Cancer* 2002;34(3):255-68.
102. Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. *Cell Res* 2009;19(2):156-72.
103. Kitamura K, Seike M, Okano T, Matsuda K, Miyanaga A, Mizutani H, Noro R, Minegishi Y, Kubota K, Gemma A. MiR-134/487b/655 cluster regulates TGF-beta-induced epithelial-mesenchymal transition and drug resistance to gefitinib by targeting MAGI2 in lung adenocarcinoma cells. *Mol Cancer Ther* 2014;13(2):444-53.
104. Xue TC, Chen RX, Han D, Chen J, Xue Q, Gao DM, Sun RX, Tang ZY, Ye SL. Down-regulation of CXCR7 inhibits the growth and lung metastasis of human hepatocellular carcinoma cells with highly metastatic potential. *Exp Ther Med* 2012;3(1):117-23.
105. Oliver FJ, de la Rubia G, Rolli V, Ruiz-Ruiz MC, de Murcia G, Murcia JM. Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis. lesson from an uncleavable mutant. *J Biol Chem* 1998;273(50):33533-9.
106. You WK, Sennino B, Williamson CW, Falcón B, Hashizume H, Yao LC, Aftab DT, McDonald DM. VEGF and c-met blockade amplify angiogenesis inhibition in pancreatic islet cancer. *Cancer Res* 2011;71(14):4758-68.
107. Yang J, Weinberg RA. Epithelial-mesenchymal transition: At the crossroads of development and tumor metastasis. *Dev Cell* 2008;14(6):818-29.
108. Tam WL, Weinberg RA. The epigenetics of epithelial-mesenchymal plasticity in cancer. *Nat Med* 2013;19(11):1438-49.

VITA

Yandi Gao was born in Qingdao, China, on October 28, 1987. She received Bachelor of Pharmaceutical Science degree from Henan University of Chinese Traditional Medicine (Zhengzhou, China) in June 2010.

In August of 2011, Yandi joined the department of Molecular Pharmacology & Therapeutics at Loyola University Chicago (Maywood, IL). She joined the laboratory of Dr. Takeshi Shimamura in 2012 where she studied EGFR mutant non-small cell lung cancer with resistant to EGFR TKI and mesenchymal phenotype.

After finishing her M.S., Yandi plans to continue her research in pharmaceutical company or in other cancer institute.