FUNCTIONAL ANALYSES OF PATIENT-DERIVED TUMOR CELLS REVEAL POTENTIAL TARGETED THERAPIES FOR HNSCC

By

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ADCC	Antibody-dependent cellular cytotoxicity
AKT	Protein Kinase B
ALCL	Anaplastic large-cell non-Hodgkin's lymphoma
ALK	Anaplastic lymphoma kinase
BCRP	Breast cancer resistant protein
ChIP	Chromatin immunoprecipitation
C_{max}	Maxima plasma concentration
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
EGFR	Epidermal growth factor receptor
EML4-ALK	Echinoderm microtubule-associated protein-like 4-anaplastic lymphoma
kinase	
ER	Endoplasmic reticulum
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
IGF1R	Insulin-like growth factor receptor 1
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
mAbs	Monoclonal antibodies
MAPK	Mitogen-activated protein kinases
mTOR	Mechanistic target of rapamycin

nEGFR	Nuclear EGFR
NPM-ALK	Nucleophosmin- anaplastic lymphoma kinase
NSCLC	Non-small cell lung cancer
PI3K	Phosphoinositide 3-kinase
PROVEAN	Protein variation effect analyzer
RAPID	RNAi-assisted protein target identification
RNAi	RNA-mediated interference
RT	Radiation therapy
SMMART	Serial measurement of molecular and architectural responses to therapy
STAT3	Signal transducers and activators of transcription 2
TCGA	The Cancer Genome Atlas
TKI	Tyrosine kinase inhibitor
WES	Whole exome sequencing

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer worldwide, with high morbidity, high mortality, and few therapeutic options outside of surgery, standard cytotoxic chemotherapy, and radiation. Epidermal growth factor receptor (EGFR) is overexpressed in up to 90% of HNSCC and is associated with poor outcome. An EGFR monoclonal antibody, cetuximab, is the only FDA-approved cancer intrinsic molecular targeted therapy for HNSCC; however, resistance eventually occurs in all patients. In my graduate studies, functional screens, including a small-molecule kinase inhibitor screen and siRNA screening panels, were used to identify agents that synergized with EGFR inhibitors in reducing viability in HNSCC patient-derived tumor cells. Bioinformatics analysis was performed to determine the coverage by the drugs on the inhibitor assay of genomic alterations in the HNSCC The Cancer Genome Atlas (TCGA) cohort. Fourteen out of 122 drugs on the inhibitor assay panel showed synergistic effects with EGFR inhibitors in at least one patient's tumor cells, including phosphoinositide 3-kinase (PI3K) and the mechanistic target of rapamycin (mTOR) inhibitors. Two anaplastic lymphoma kinase (ALK) inhibitors on the drug screen panel showed synergistic effects with EGFR inhibitors in 4/8 HNSCC patients' tumor cells, despite ineffectiveness of single drug. The most effective combination therapies from inhibitor assays were validated in scale-up experiments, and their true targets were evaluated using siRNAs to rule out off-target effects of the drugs. siRNAs targeting ALK synergized with the EGFR inhibitor gefitinib to reduce cell viability in cases that were sensitive to EGFR and ALK inhibitor combinations but not in a resistant cases, indicating specificity to ALK. Scale-up dose-response experiments confirmed patient cell sensitivity to 4 different ALK inhibitors in combination with

gefitinib, including 2 ALK inhibitors FDA approved for other cancers, ceritinib (LDK378) and brigatinib (AP26113). Co-targeting EGFR and ALK decreased HNSCC patients' tumor cell number and colony formation ability and increased annexin V staining.

We hypothesized that EGFR inhibition induced ALK RNA and protein because of low baseline expression of ALK mRNA in the original tumors and patient tumor-derived cells. Indeed, inhibition of EGFR by gefitinib increased ALK protein expression and phosphorylation in primary tumor cells. Gefitinib treatment also increased ALK protein expression in patient-tumor derived spheroids. Xenograft models were established and ALK RNA and protein levels were elevated in tumors from mice treated with gefitinib compared to vehicle controls. These findings suggest induction of ALK expression and activation as a novel mechanism of EGFR inhibitor resistance in HNSCC amenable to combination therapy. In addition, nuclear EGFR was increased by gefitinib in an EGFR and ALK inhibitor combination sensitive case but not in a relatively resistance case, suggesting a role of nuclear EGFR in ALK inhibitor sensitivity in the sensitive cells.

Overall, we identified EGFR and ALK inhibitor combinations as a potential combination therapeutic strategy for treating EGFR inhibitor resistant HNSCC, and induction of ALK by EGFR inhibitor as one novel mechanism potentially relevant to resistance to EGFR inhibitor in HNSCC.

Chapter 1 – Background and Introduction.

Head and neck squamous cell carcinoma (HNSCC) overview.

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer worldwide, affecting ~600,000 patients per year (Ferlay et al. 2010). In the United States, 50,000 cases are diagnosed each year and nearly 10,000 deaths are attributable to this disease (Rothenberg and Ellisen 2012). HNSCCs comprise a heterogeneous group of malignancies arising from the mucosal surfaces of the paranasal sinuses, the oral and nasal cavities, the pharynx, and the larynx (Hooper et al. 2014) (Figure 1- 1). HNSCC develops mostly via one of the two primary carcinogenic routes, namely the chemical carcinogenesis through exposure to tobacco and alcohol abuse, which are known to be synergistic, and high-risk human papillomavirus (HPV) induced carcinogenesis (Andre et al. 1995; Bose, Brockton, and Dort 2013; Kang, Kiess, and Chung 2015; C. René Leemans, Braakhuis, and Brakenhoff 2011). Smoking is implicated in the rise of HNSCC in developing countries, and the role of human papillomavirus (HPV) is emerging as an important factor in the rise of oropharyngeal tumors affecting non-smokers in developed countries (Ang et al. 2010a). HPV-positive HNSCCs, most-commonly occurring in the oropharynx, have a better prognosis than HPV-negative tumors and thus HPV-positive status has served as the only biomarker in HNSCC to de-intensify therapy in a subset of patients with a favorable prognosis (Kang, Kiess, and Chung 2015). HPV-negative HNSCC patients exhibit worse outcomes to the current treatment options compared to the HPV-positive HNSCC (Ang et al. 2010a; Fakhry et al. 2008a; Lassen et al. 2009a; O'Sullivan et al. 2012a). Currently, the most common HNSCC therapeutic modalities include the use of nonselective treatments (surgery, radiation and chemotherapy) with very high systemic toxicities and associated morbidity and

mortality. Efforts to improve the outcome of disease in HPV-negative patients by intensifying treatment (through induction chemotherapy (E. E. W. Cohen et al. 2014; Haddad et al. 2013) or addition of the anti-EGFR antibody cetuximab (Ang et al. 2014) to concurrent chemoradiotherapy), have not resulted in a significant survival benefit. Identification of novel therapeutic targets and development of effective targeted agents would greatly benefit this patient population ("The Molecular Basis of Cancer - 4th Edition").

In the last two decades, immunotherapies showed promising results in preclinical studies and clinical trials in multiple cancers including HNSCC. In August 2016, FDA granted accelerated approval of an anti-programmed cell death protein-1 (anti-PD-1) mAb, pembrolizumab, for the treatment of patients with recurrent or metastatic HNSCC with disease progression on or after platinum-containing chemotherapy based on results from the open-label phase Ib KEYNOTE-012 trial. However, the response using this agent was very limited. According to the FDA approval summary, the objective response rate was only 16% for the patients treated with pembrolizumab (Economopoulou et al., 2016). Full approval was contingent on results from confirmatory trials. However, it was reported in July, 2017 that pembrolizumab did not meet the primary endpoint of overall survival (OS) in patients with previously-treated recurrent or metastatic in the phase III KEYNOTE-040 trial compared to standard chemotherapy. In November 2016, FDA approved another anti-PD-1 mAb nivolumab for HNSCC. However, the median overall survival of patients in the nivolumab group was only 2.4 months longer than standard therapy group (methotrexate, docetaxel, or cetuximab) (7.5 vs 5.1 months) (Ferris et al. 2016). Low and non-reproducible response and low survival rates underlie the urgent need for more treatment options for HNSCC.

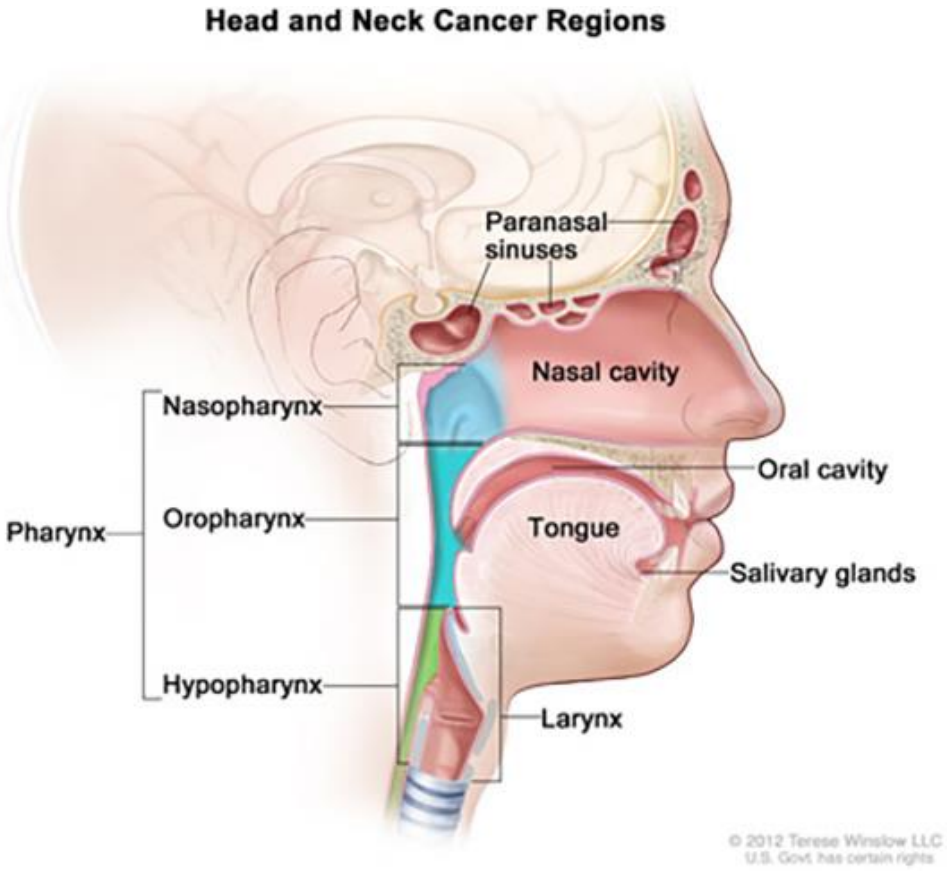


Figure 1-1. Head and neck cancer regions.

The term head and neck carcinoma encompasses all malignancies arising in the nasal and oral cavities, pharynx, larynx and the paranasal sinuses. (National Cancer Institute, Terese Winslow)

Epidermal growth factor receptor (EGFR) inhibitor resistance.

EGFR biology in HNSCC

EGFR is a member of the EGFR tyrosine kinase family, which consists of EGFR (ErbB1/HER1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). All family members contain an extracellular ligand-binding domain (domains I, II, III, IV), a single membrane-spanning region, a juxtamembrane nuclear localization signal, and a cytoplasmic tyrosine kinase domain. These receptors interact with a family of 12 polypeptide growth factors, the binding of which causes a conformational change in the receptor that allows for both homo- and hetero-dimerization with other activated HER family members (Marmor, Skaria, and Yarden 2004). Dimerization activates the intrinsic tyrosine kinase of each receptor, leading to the phosphorylation of tyrosine residues on each receptor's C-terminal tails, leading to the recruitment of downstream effectors and the activation of proliferative and cell-survival signals (Dassonville et al. 2007).

EGFR is ubiquitously expressed in various cell types, but primarily in those of epithelial, mesenchymal and neuronal origin. Aberrant expression or activity of EGFR has been shown to be an important factor associated in the progression of many human epithelial cancers, including HNSCC, non-small cell lung cancer (NSCLC), colorectal cancer (CRC), breast cancer, pancreatic cancer and brain cancer. EGFR signaling regulates multiple intracellular signaling circuits, including the JAK/STAT3, RAS/MAPK, and PI3K/AKT/mTOR pathways (Dassonville et al. 2007), mediating tumor cell proliferation, survival, and invasion. Wild type EGFR protein is expressed at moderate to high levels in up to 90 percent of HNSCC (Mrhalova et al. 2005; Grandis and Tweardy 1993). Increased EGFR expression has been linked to poor outcomes in HNSCC, including poorer overall survival, increased locoregional relapse, and treatment failure (Ang et al.

2002a; Ganly et al. 2007). Biomarker analysis from a phase III trial demonstrated that high EGFR expression was associated with significantly shorter overall survival and disease-free survival, and higher locoregional relapse rates of HNSCC (Ang et al. 2002a). However, high EGFR expression is not significantly correlated with anti-EGFR therapy response in HNSCC (Aung and Siu 2016b).

So far, there are two well-identified categories of drugs targeting EGFR, monoclonal antibodies and tyrosine kinase inhibitors (TKIs) (Figure 1-2 and Table 1-1). MAbs target receptor-ligand binding at the extracellular domain of the receptor, and TKIs are oral, low-molecular-weight, ATP-competitive inhibitors of the tyrosine kinase located in the intracellular part of the receptor (Dassonville et al. 2007). These two categories of therapies target the same protein, yet have differential mechanisms of action, which will be discussed later in this chapter.

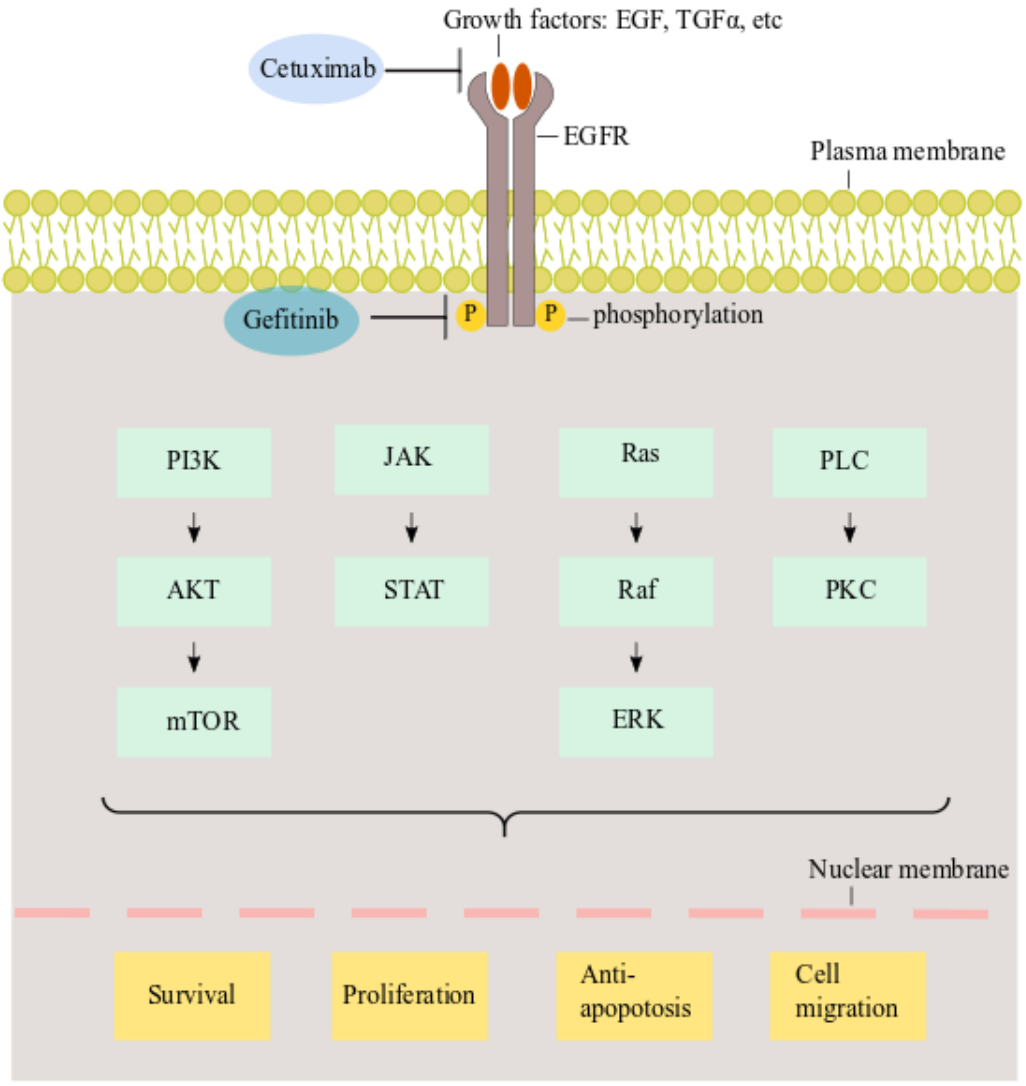


Figure 1-2. Mechanism of action of anti-EGFR therapies in cancer.

Drug (Trade name)	Class	Target	Initial Approval Date	Indication	Dosage	Reference
Cetuximab (Erbitux)	mAb	EGFR	Feb-04	1. In combination with radiation therapy for locally or regionally advanced HNSCC 2. Recurrent or metastatic HNSCC progressing after platinum-based therapy 3. Single agent in metastatic CRC (EGFR-expressing) after failure of irinotecan- and oxaliplatin-based regimens 4. Metastatic CRC (EGFR-expressing) in combination with irinotecan for irinotecan-refractory patients	Intravenous. 400 mg/m^2 initial dose then 250 mg/m^2 weekly	(Pirker et al. 2012; "Cetuximab Approved by FDA for Treatment of Head and Neck Squamous Cell Cancer" 2006)
Erlotinib (Tarceva)	TKI	EGFR	Nov-04	1. Second-line therapy in locally advanced or metastatic NSCLC 2. First line, in combination with gemcitabine in locally advanced or metastatic pancreatic cancer	Oral.150 mg daily	(M. H. Cohen et al. 2005)
Gefitinib (Iressa)	TKI	EGFR	May-03	1. Monotherapy for the treatment of patients with advanced or metastatic NSCLC who are benefiting or have benefited from gefitinib 2. First-line treatment of patients with metastatic NSCLC whose tumors harbor exon 19 deletions or exon 21 L858R substitution gene mutations	Oral. 250 mg daily	(M. H. Cohen et al. 2003; Kazandjian et al. 2016)
Lapatinib (Tykerb)	TKI	EGFR/HER2	Mar-07	1. In combination with capecitabine for the treatment of patients with advanced or metastatic HER2-overexpressing breast	Oral. 1250 mg daily for 21 days then 1 week off	(Ryan et al. 2008)

				cancer who have received prior treatment with an anthracycline, a taxane, and trastuzumab		
Panitumumab (Vectibix)	mAb	EGFR	Sep-06	1. Metastatic colorectal carcinoma (EGFR-expressing) after treatment with fluoropyrimidine-, oxaliplatin-, and irinotecan-containing chemotherapy regimens	Intravenous. 6 mg/kg every 14 days	(Giusti et al. 2007)
Vandetanib (Caprelsa)	TKI	EGFR	Apr-11	1. Symptomatic or progressive medullary thyroid cancer in patients with unresectable locally advanced or metastatic disease	Oral. 300 mg daily	(Thornton et al. 2012)
Necitumumab (Portrazza)	mAb	EGFR	Nov-15	1. First-line treatment of metastatic NSCLC in combination with gemcitabine and cisplatin	Intravenous. 800 mg on days 1 and 8 of each 3-week treatment cycle	(Thatcher et al. 2015)
Osimertinib (TAGRISSO)	TKI	EGFR	Mar-17	1. Metastatic T790M mutation-positive NSCLC	Oral. 80 mg daily	(Greig 2016)
Neratinib (Nerlynx)	TKI	EGFR	Jul-17	1.Extended adjuvant treatment of adult patients with early stage HER2-overexpressed/amplified breast cancer, to follow adjuvant trastuzumab-based therapy	Oral. 40 mg daily	(Center for Devices and Radiological Health n.d.)
Afatinib (gilotrif)	TKI	EGFR, HER2,3 and 4	Apr-13	1. first-line treatment of patients with metastatic non-small cell lung cancer (NSCLC) whose tumors have epidermal growth factor receptor (EGFR) exon 19 deletions or exon 21 (L858R) substitution mutations	Oral. 40 mg daily	(Keating 2014)

Table 1-1. FDA approved anti-EGFR therapies.

TKI = tyrosine kinase inhibitor; mAb = monoclonal antibody; NSCLC= non-small-cell lung cancer; CRC=colorectal cancer.

Anti-EGFR treatments in HNSCC- cetuximab

Based on above findings on the association of EGFR and poor survival in HNSCC patients, efforts had been made to target EGFR in HNSCC. The first efforts to target EGFR in cancer therapy was by Mendelsohn and colleagues, who developed a monoclonal antibody to extracellular epitopes of the EGFR (C225; Cetuximab) (Shawver, Slamon, and Ullrich 2002). Cetuximab is an immunoglobulin (Ig) G1 human-murine antibody with high affinity to EGFR, which prevents receptor-ligand binding thus inhibiting receptor phosphorylation and dimerization, ultimately inducing receptor internalization and down regulation (Sunada et al. 1986). Bonner et al. conducted a multinational phase III trial evaluating the use of cetuximab concurrent with radiation therapy (RT) for patients with late stage-HNSCC (stages III–IVB). Median OS was improved with cetuximab plus RT compared to RT alone by 20 months (Bonner et al. 2006). A subsequent 5-year follow-up study demonstrated an improved OS rate in the patients receiving cetuximab plus RT compared to those receiving RT alone by about 20% (45.6% vs 35.4%) (Bonner et al. 2010). Based on the positive clinical trial results, cetuximab gained approval from the US food and drug administration in 2006 for the treatment of primary HNSCC in combination with radiation in patients that were not responsive respond to platinum-based therapy. For recurrent or metastatic HNSCC, cetuximab was FDA approved in combination with standard chemotherapy in 2011(Bonner et al. 2010). However, despite some evidence of clinical activity, this treatment eventually fails; the responses to EGFR-targeted antibodies are limited to less than 10% of patients; improvements in survival are transient, resistance is acquired in less than 3 months (Chong and Jänne 2013). Although EGFR activating mutations correlate with clinical responsiveness to the tyrosine kinase inhibitor gefitinib in

NSCLC (Lynch et al. 2004), with low EGFR mutation frequency in HNSCC (Hayes, Grandis, and El-Naggar 2013; Pao et al. 2004), there is no response predictor for anti-EGFR therapies for HNSCC patients.

Putative mechanisms of cetuximab in EGFR positive tumors are twofold. The first mechanism is by directly blocking ligand binding, increasing the internalization of receptors for degradation (Harding and Burtness 2005; Maier et al. 1991). The second mechanism of cetuximab is indirect action mediated by the immune system. The elimination of tumor cells using mAbs depends on Ig-mediated mechanisms, including complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), to activate immune-effector cells.

Independent of the phosphorylation status of the receptor, the EGFR-cetuximab complex is subsequently internalized (Fan et al. 1994; Prewett et al. 1996). This receptor internalization was thought to be a mechanism of membrane bound receptor down-regulation and thus a mechanism of action of cetuximab. However, it has been also reported that EGFR nuclear translocation followed internalization with cetuximab is associated with cetuximab resistance by up-regulation of target genes by nuclear EGFR functioning as a transcription factor (Brand et al. 2013).

Anti-EGFR treatments in HNSCC- a history of gefitinib discovery

The mAb approach was first applied to block EGFR-mediated signaling for cancer treatment in the early 1980s. 10 years later, the potential of EGFR-targeted therapy contributed to the development of small-molecule EGFR TKIs (Chong and Jänne 2013) which are less expensive and more convenient to administer than mAbs. Unlike mAbs, small-molecule agents can translocate through plasma membranes and interact with the cytoplasmic domain of cell-surface

receptors and intracellular signaling molecules. One of the most promising EGFR TKI candidates was gefitinib (Iressa, ZD1839, AstraZeneca) due to its relatively high efficacy, selectivity to EGFR and good bioavailability (Wakeling et al. 2002).

In pre-clinical models, selectivity of gefitinib was evaluated both in enzymatic experiments and cell growth assays. Gefitinib showed at least a 100-fold difference in IC50 for EGFR compared with the other RTKs, including HER2 and the VEGF TKs KDR and Flt-1 (Woodburn et al. 1997; Wakeling et al. 2002). Selectivity towards EGF-stimulated growth was also determined by measuring IC50 for cells grown in the presence or absence of EGF, and compared with other mitogens that are not ligands to EGFR such as FGF- or VEGF-stimulated growth and showed over 100-fold difference.

Drug efficacy *in vitro* was evaluated by inhibition of EGFR autophosphorylation by incubating tumor cells with gefitinib before EGF stimulation. In these studies, gefitinib completely blocked EGF-stimulated EGFR phosphorylation at low concentration in prostate, lung, oral squamous and colon tumor cells (Ciardiello et al. 2000; Moasser et al. 2001; Wakeling et al. 2002).

Drug efficacy *in vivo* was tested in tumor xenografts. Gefitinib inhibited the growth of a broad range of human solid tumor xenografts in a dose-dependent manner with marked regressions seen in some tumors (Wakeling et al. 2002; Woodburn et al. 1997).

Then pharmacokinetic/pharmacodynamic studies of gefitinib were tested in xenograft models. *In vitro*, gefitinib was not the most potent compound in the studies, but *in vivo* it showed a good oral bioavailability and achieved high and sustained blood levels over a 24-hour period. These results were used to prioritize candidates for further anti-tumor testing in xenograft models. After

efficacy evaluation, toxicity studies were performed in mice. Treatment for up to 4 months in nude mice was well tolerated (Barker et al. 2001).

In 1998, the first phase I clinical trial on gefitinib revealed favorable tolerability and good responses in NSCLC. This clinical trial also showed biomarker evidence for inhibition of the EGFR signal transduction pathway and anti-tumor activity. Gefitinib was then approved in September 2002 for use in patients with advanced NSCLC based on the result from a phase II clinical trial showing 10% tumor response rate in late-stage non-small-cell lung cancer (M. H. Cohen et al. 2003). However, at that time, it was not known that the effect of gefitinib in NSCLC is restricted to patients who harbor specific mutations in the EGFR gene, since EGFR mutations in cancers were not observed until 2004. Until then, gefitinib was used to treat unselected NSCLC patients irrespective of EGFR mutational status. Not surprisingly, gefitinib failed to show a benefit in the large mandatory confirmatory "Iressa survival evaluation in lung cancer" trial (M. H. Cohen et al. 2003), which resulted in the FDA retraction of its approval in 2005. Independent researchers had managed to identify the EGFR mutation that selects for the 10% of patients with lung cancer who respond to the drug (Lynch et al. 2004; Paez et al. 2004; Pao et al. 2004). AstraZeneca then designed new clinical trials with geftinib for EGFR mutant patients. These new gefitinib studies revealed promising results with better overall response rates in NSCLCs, and exemplify why focusing targeted therapy clinical trials on patients with the specific mutation the drug targets has become the norm for targeted therapies. In 2015, the FDA approved the use of gefitinib specifically for those NSCLC patients who have metastatic cancer, who have not received any treatment, and whose cancers have the most common types of EGFR

mutation: exon 19 deletions or exon 21 L858R substitution gene mutations (Kazandjian et al. 2016).

Based on the approval of gefitinib in NSCLC, 29 clinical trials were opened for HNSCC where around 90% of the patients' tumors are EGFR positive. In Phase I and II studies, gefitinib has demonstrated evidence of anti-tumor activity in patients with head and neck cancer (Saarilahti et al. 2010). However, a multi-center, randomized, partially-blinded, parallel-group phase III trial of gefitinib versus methotrexate in recurrent HNSCC revealed that gefitinib does not demonstrate an improvement in overall survival compared to methotrexate (J. S. W. Stewart et al. 2009). In this study EGFR gene copy number was evaluated by FISH. However, EGFR gene copy number was not shown to be statistically significantly correlated with survival. EGFR protein expression was measured by immunohistochemistry staining, and given that the proportion of EGFR expression–positive patients at the predefined ≥ 10% positive cells cutoff level was 99.6%, no formal analyses were performed. It is appropriate to note that the cutoff values used to define EGFR FISH-positive patients and protein expression were based on historical data from similar analyses among patients with NSCLC. Further studies are needed to establish whether these cutoff values are appropriate in HNSCC. In addition, tumors with increased EGFR gene copy number appeared to have an increased chance of responding to gefitinib 500 mg (13.8%) compared with EGFR FISH–negative tumors (4.8%) (J. S. W. Stewart et al. 2009). Other phase III studies have demonstrated little activity of gefitinib in HNSCC, and there are therefore no further plans to develop gefitinib for patients with recurrent or metastatic HNSCC (Argiris et al. 2013; Sacco and Worden 2016; J. S. W. Stewart et al. 2009).

Comparison between gefitinib and erlotinib

Both gefitinib and erlotinib are first-generation EGFR TKIs that are FDA approved for advanced NSCLC patients. Gefitinib has been approved only for EGFR mutation bearing patients regardless the line of treatment, while erlotinib is also indicated in patients without EGFR mutation who undergo second- or third-line treatment (Bronte et al. 2014). Although some differences in the trial results for erlotinib and gefitinib led to differences in FDA regulatory policy, no head-to-head randomized controlled trials were published to provide a treatment selection strategy.

Recently, the results of a phase III randomized controlled trial of erlotinib vs gefitinib in advanced NSCLC with EGFR mutations was published. In this study, erlotinib was not significantly superior to gefitinib in terms of efficacy in advanced NSCLC with EGFR mutations in exon 19 or 21, and the two treatments had similar toxicities (J. J. Yang et al. 2017). Gefitinib and erlotinib demonstrated comparable effects on progression-free survival, overall survival, overall response rate, and disease control rate, which did not vary considerably with EGFR mutation status, ethnicity, line of treatment, and baseline brain metastasis status (Z. Yang et al. 2017). In another recently published randomized phase III study comparing gefitinib with erlotinib in patients with previously treated advanced lung adenocarcinoma, no noninferiority of gefitinib compared with erlotinib in terms of progression-free survival in patients with lung adenocarcinoma was observed (Urata et al. 2016). These results suggest that FDA regulatory policies regarding recommendations for patients with these diseases need to be reconsidered.

Difference between EGFR targeting mAbs and TKIs

Anti-EGFR monoclonal antibodies act at the receptor's extracellular domain, whereas TKIs act on the cytosolic adenosine triphosphate-binding domain of EGFR to inhibit autophosphorylation (Bozec et al. 2009; Sacco and Worden 2016). The mAbs and small-molecule inhibitors differ in several pharmacological properties. Anti-EGFR mAbs are large proteins (around 150 kDa) and are generally intravenously administered, whereas EGFR TKIs are orally available, small molecule compounds (approximately 500 Da). The large molecular weight of mAbs is probably the cause of less efficiency for tissue penetration, tumor retention and blood clearance than for small-molecule agents.

Cetuximab and gefitinib have differential efficacy *in vitro* and *in vivo*. In general, cetuximab treatment of tumor cell lines in culture results in a modest inhibition (15–50% range) of tumor cell growth and proliferation (Normanno et al. 1999; Overholser et al. 2000). However, data from tumor xenograft studies suggest that the *in vivo* efficacy of cetuximab is markedly enhanced as compared with *in vitro* effects in cell lines (Bos et al. 1997; Goldstein et al. 1995). Such differences between *in vitro* and *in vivo* noted for cetuximab are not so marked with TKIs such as gefitinib. Gefitinib has shown marked *in vitro* and *in vivo* growth-inhibitory activity in a wide range of cell lines (Ciardiello et al. 2000; Magné et al. 2002; Tortora et al. 2001). Thus the *in vivo* efficacy of cetuximab may be less in a cancer cell-autonomous manner. Cetuximab may act through the inhibition of tumor-related processes such as cell migration and neovascularization. In addition, part of their antitumor activity could be attributed to antibody-dependent cell-mediated cytotoxicity (ADCC) (Lammerts van Bueren et al. 2006). ADCC is an immunological mechanism which involves the interaction of the Fc fragment of mAbs with Fc receptors on immune cells such as NK cells and macrophages. Since the FcγR category is under the influence of a germinal genetic

polymorphism (Carter 2001), there are different genotypes for FcγR on different immune cells which may result in one of the major sources of variability in mAbs efficacy as opposed to TKIs.

The presence of activating mutations within the ATP-binding cleft of the EGFR kinase domain is associated with the sensitivity of NSCLC to gefitinib, but not to cetuximab. By contrast, cetuximab shows a clinical benefit for colorectal cancers that overexpress EGFR in a manner independent of EGFR mutations. In malignant glioma, the sensitivity to gefitinib is closely related to deletions within the ectodomain of EGFR. In contrast to these drug-sensitivity mutations, the appearance of the T790M mutation confers resistance to gefitinib in NSCLC. However, EGFR mutations in HNSCC is relatively rare, only 4% of HNSCC TCGA cohort harbor mutations in EGFR in their tumors. Therefore, there is no response predictor for anti-EGFR treatment for HNSCC.

Mechanisms of resistance of anti-EGFR treatments.

Different mechanisms of EGFR inhibitor resistance defined in various cancers include the following: secondary alterations within EGFR such as EGFRvIII in HNSCC and T790M EGFR mutation in NSCLC (E. L. Stewart et al. 2015); heterodimerization and transactivation of other RTKs such as MET, HER2 and IGF-1R (Erjala et al. 2006; Jameson et al. 2011); upregulation of other parallel pathways such as MET, aurora kinase A and HER3 (Wheeler, Dunn, and Harari 2010; Erjala et al. 2006; Hoellein et al. 2011; Erjala et al. 2006, 2); and nuclear localized EGFR (C. Li et al. 2009b; W.-C. Huang et al. 2011).

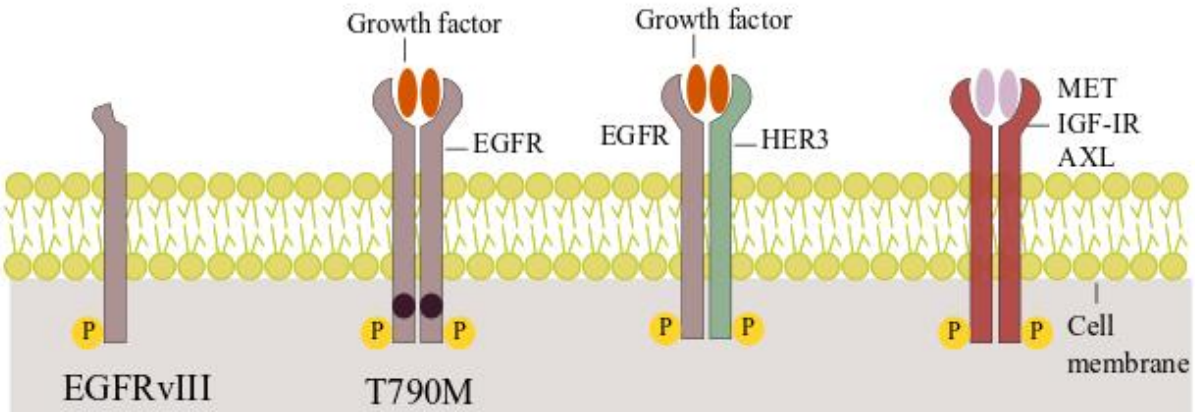


Figure 1-3. EGFR inhibitor resistance mechanisms.

Resistance mechanisms of anti-EGFR treatments include EGFR activating mutations such as a truncating EGFR variant III and a gatekeeper mutation T790M which could block drug binding to the kinase domain. Other mechanisms of resistance include oncogenic shift to parallel pathways by heterodimerization with other HER family members or upregulation and activation of other receptor tyrosine kinases such as MET, IGF1R and AXL that could compensate downstream signaling of EGFR.

First-generation EGFR inhibitors, such as erlotinib and gefitinib, are reversible EGFR TKIs which represented an important treatment option for NSCLC patients with activating EGFR mutations. However, all patients inevitably develop acquired resistance to these agents, primarily due to secondary EGFR mutations such as the T790M EGFR gatekeeper mutation. Preclinical and clinical trials suggested a potential efficacy of irreversible or covalent inhibitor and inhibitors that target more HER family members, in overcome acquired resistance related to T790M. For example, Afatinib, an panHER family inhibitor that can covalently bind and thus irreversible inhibits signaling of all homodimers and heterodimers formed by the EGFR, HER2, HER3, and HER4 receptors. The irreversible inhibition of multiple HER family receptors by afatinib results in more potent and prolonged suppression of kinase activity compared with reversible EGFR inhibitors (Hirsh 2015; Landi and Cappuzzo 2013; Maione et al. 2014). Recently, the third-generation EGFR inhibitors, which are irreversible kinase inhibitor that have a significantly increased potency for EGFR mutants than for wild-type EGFR, including AZD9291 (osimertinib, mereletinib) and PF-06747775, have emerged as potential therapeutics to in treating EGFR T790M-positive tumors (S. Wang, Cang, and Liu 2016).

Although plasma membrane EGFR signaling has been intensely researched over the last thirty years, new functions of the EGFR are now beginning to unravel. One new prominent mode of EGFR signaling has been found in the cell's nucleus (Brand et al. 2011; Han and Lo 2012). Nuclear EGFR (nEGFR) is involved in several biological functions, including DNA replication, DNA repair, transcriptional regulation, and resistance to therapy, through associations with various molecules (C. Li et al. 2009a; S.-C. Wang and Hung 2009; Huo et al. 2010; Chen et al. 2011; W.-C. Huang et al. 2011). Upon entry into the nucleus, the EGFR can function in ways distinct from

its plasma membrane bound counterparts such as a transcription factor. It was first shown in 1994 that a kinase dead EGFR could enhance transcriptional expression of the c-fos gene (Eldredge et al. 1994), later in 2001, a landmark paper provided direct evidence that EGFR could regulate the cyclin D1 promoter as a transcription factor (S. Y. Lin et al. 2001a). Since these initial findings, nuclear EGFR has been shown to regulate a variety of genes, including inducible nitric oxide synthase (iNOS), B-Myb, cyclooxygenase-2 (COX-2), aurora Kinase A, c-Myc, breast cancer resistant protein (BCRP), and Stat1 (Hanada et al. 2006; Hung et al. 2008; Jaganathan et al. 2011; Lo, Hsu, et al. 2005a; Lo et al. 2010). The association of other oncogenic genes' expression with nEGFR and its role in anti-cancer treatment and drug resistance is yet to be determined.

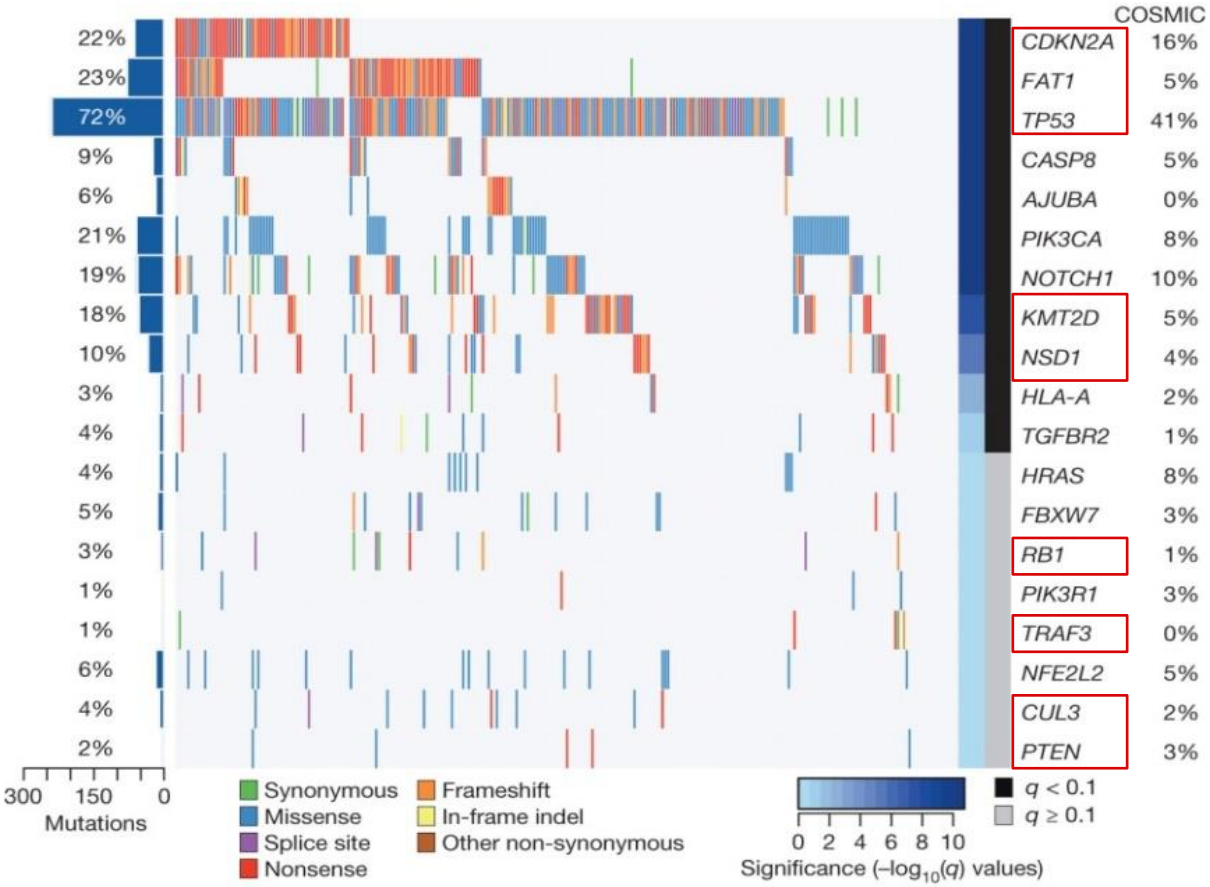
In vitro studies in cancer cells resistant to both gefitinib and cetuximab have demonstrated that resistant cells often contain high levels of nuclear localized EGFR (W.-C. Huang et al. 2011; C. Li et al. 2009a; D. Wang and Lippard 2005). In the case of gefitinib resistance, nuclear EGFR was shown to function as a co-transcriptional activator for breast cancer resistant protein (BCRP/ABCG2), a plasma-membrane bound ATP dependent transporter that can extrude anti-cancer drugs from cells and thereby diminish their effects (W.-C. Huang et al. 2011). Authors hypothesize that this ATP dependent transporter may function to remove gefitinib from cells and thereby enhance resistance (W.-C. Huang et al. 2011).

Cetuximab resistance has also been attributed to nuclear EGFR including in HNSCC (C. Li et al. 2009a). Various researchers have demonstrated that cetuximab treatment can enhance the nuclear localization of EGFR (C. Li et al. 2009a; Chunrong Li et al. 2010; Liao and Carpenter 2009), and that cell lines with intrinsic resistance to cetuximab contain high levels of nuclear EGFR (C. Li et al. 2009a). Collectively, this body of work demonstrates that nuclear EGFR plays a role in

resistance to both gefitinib and cetuximab therapies. Although efforts have been made to target nuclear EGFR, including targeting AKT (W.-C. Huang et al. 2011) and Src family kinases (C. Li et al. 2009a) that promote EGFR nuclear translocation, in order to overcome EGFR inhibitor resistance, no changes were made in the clinic in terms of treating patients with high nuclear EGFR. In addition, whether nuclear EGFR could function as a transcriptional activator for other oncogenic proteins that may result in drug resistance in HNSCC is unknown. EGFR localization in HNSCC patient derived tumor cells will be evaluated in EGFR inhibitor resistant patients in this study.

Combination therapies in HNSCC

Considering different biological pathways are active in different subtypes of HNSCC, single agent activity of targeted agents in non-selected population is likely to be modest. To significantly improve clinical outcomes, rational combination treatment strategies should be tested in selected populations enriched by unique tumor molecular features present in the tumors. Currently, based on available genomic data mainly derived from early stage tumors such as The Cancer Genome Atlas (TCGA), the proportion of HNSCC patients who could benefit from personalized therapy remains relatively small considering the most common genetic events in HNSCC occur in tumor suppressor genes such as TP53, CDKN2A and FAT1 (Figure 1-2). Functional assays, including drug screens and siRNA screens that target a board spectral of anti-cancer targets, using patient-derived specimen, may uncover novel therapeutic targets for HNSCC.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HLA-A 2%
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FBXW7 3%
RB1 1%
PIK3R1 3%
TRAF3 0%
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CUL3 2%
PTEN 3%
300 150 0
Mutations
Synonymous
Missense
Splice site
Nonsense
Frameshift
In-frame indel
Other non-synonymous
0 2 4 6 8 10
Significance (–log10(q) values)
q < 0.1
q ≥ 0.1

Figure 1-4. Significantly mutated genes in HNSCC TCGA cohort.

Genes (rows) with significantly mutated genes (identified using the MutSigCV algorithim; $q < 0.1$) ordered by q value; additional genes with trends towards significance are also shown. Tumor suppressor genes are marked in red squares. Samples (columns, $n = 279$) are arranged to emphasize mutual exclusivity among mutations. Left, mutation percentage in TCGA. Right, mutation percentage in COSMIC ('upper aerodigestive tract' tissue). Color coding indicates mutation type. (Figure adapted, modified and reprinted with permission from Nature Publishing Group, *MS Lawrence et al. Nature 2015)*

Given compensatory cross talk between kinases within cancer cells, the use of combinations of other kinase inhibitors with EGFR inhibitors in HNSCC to overcome resistance to EGFR inhibitors has been investigated and suggested to be beneficial in preclinical models and under active testing in clinical trials (Aung and Siu 2016a). EGFR regulates multiple intracellular signaling circuits, including the JAK/STAT3, RAS/MAPK, and PI3K/AKT/mTOR pathways (Kalyankrishna and Grandis 2006; Marmor, Skaria, and Yarden 2004). Among them, recent findings indicate that genetic and epigenetic alterations in PI3K/AKT/mTOR pathways are among the most frequent alterations in HNSCC (Figure 1-2), resulting in the activation of PI3K/AKT/mTOR signaling in the majority of HNSCC lesions (Z. Wang, Martin, Molinolo, Patel, Iglesias-Bartolome, Degese, et al. 2014; T. L. Yuan and Cantley 2008). Based on above findings, EGFR inhibitors and PI3K/mTOR inhibitors are the most investigated drug combinations in HNSCC both in preclinical models and clinical trials, examples including clinical trials on combination between cetuximab and BKM120, PX-866 and PF-05212384 (clinicaltrials.gov). Other combination therapies that are in active development include drug combinations between EGFR mAb and EGFR TKIs, MET TKI and CDK4/6 inhibitors (Aung and Siu 2016b; C. René Leemans, Braakhuis, and Brakenhoff 2011; Moreira et al. 2017), based on the genomic landscape studies on large cohort of HNSCC patients (Hayes, Grandis, and El-Naggar 2013). Unfortunately, so far, no biomarkers have been identified predictive for any of the therapies for HNSCC patients, leaving patients with limited information for treatment selection. Functional assays using patient-tumor-derived models for individual patients may provide crucial information for drug selection for a given HNSCC patient.

Functional screens using patient-derived tumor models.

Cancer cell lines and patient-derived tumor models.

For decades, immortal cancer cell lines have served as easily accessible biological models for investigating cancer biology and exploring the potential efficacy of anticancer drugs. Efforts such as the cancer cell line encyclopedia (CCLE) (Barretina et al. 2012) comprised detailed genetic characterization of over 1,000 cell lines across more than 36 cancer types. CCLE characterized cell lines for gene expression, copy number variation (CNV), and mutations in select genes. Using the cell lines well characterized by CCLE, the Project Achilles (Cowley et al. 2014) conducted functional screens to identify genetic dependency in hundreds of cancer cell lines by systematic knockdown of genes through shRNAs and CRISPR–Cas 9 (Meyers et al. 2017). The identification of genetic dependency using large cancer cell line panels provides a wealth of information to develop novel therapies.

Studies in breast cancer reported similarities between cell-line models and primary tumors at both the transcript and genome copy-number levels (Neve et al. 2006; Laura M. Heiser et al. 2012). In a test of 77 therapeutic compounds, nearly all drugs showed differential responses across these cell lines, and approximately one third showed subtype-, pathway-, and/or genomic aberration-specific responses. These observations suggest mechanisms of response and resistance and may inform efforts to develop molecular assays that predict clinical response (L. M. Heiser et al. 2011).

However, concerns on cell lines for cancer research include poorly representation of tumor heterogeneity, diversity and drug-resistance of tumors in patients. In the era of precision medicine, the importance of utilizing patient derived primary tumor cells rather than cell lines in cancer research has become apparent for generating high-fidelity data for translating *in vitro*

findings to *in vivo* models and ultimately to clinical settings. The ability to functionally evaluate patient tumors will add the refinement of databases pairing mutational or expression analyses of genes, RNA and proteins to sensitivities to treatments, clinical and histopathologic analyses well beyond the capabilities of the standard tissue bank. Here I review the currently available methods for generating and culturing primary tumor cells and relevant pros and cons (Table 1-1).

Techniques	Advantages	Disadvantages	References
Two dimensional monolayer culture	Easy to culture from single cell suspension of tumor tissues.	Contamination with non-tumorigenic cells, differentiation and genetic drift. Variability in drug responses. Less chances to retain original phenotypes.	(Roskelley, Srebrow, and Bissell 1995; Soule, Maloney, and McGrath 1981)
Explant cell culture	Maintains good morphology of epithelia, initial "feeder layer/structure and soluble factor" of own stroma.	Not good for soft tissues (melanoma). Need serial differential trypsinization subculturing to enrich tumor cells over fibroblasts.	(Pei et al. 2004)
Three dimensional culture	Optimal simulation of *in vivo* condition with minimal genetic drift in long term culture.	Biomimetic scaffolds are expensive. Challenge to generate cyto- architecture.	(Clevers and Bender 2015; Kim, Stein, and O'Hare 2004)

	Tumor cells are able to maintain 3D architecture.		
Chemical reprogramming	No genetic alternation after treatment with Rock inhibitor Y-27632 for several passages. Rapid proliferation of cells. Treatment can be applied to all types of tissues.	It is not clear whether heterogeneous population can proliferate.	(Dairkee et al. 1997)

Table 1-2. Pros and Cons of techniques for establishing primary tumor cultures for solid tumors.

Currently available methods for generating and culturing primary tumor cells and relevant pros and cons.

Utilization of primary tumor cell lines offers great advantages for translational research. We can generate primary cultures from individual patients for high throughput drug screenings at a personalized level. Further, in combination with functional studies, these primary tumor cells are valuable resources for genomic sequencing and transcriptomic studies for identifying the association between functional assay sensitivity and genomic and transcriptional alterations which I will provide some examples in chapter 3.

Small molecule inhibitor screens.

High throughput drug screens emerged around 1955, stimulated by the discovery that chemical agents, such as nitrogen mustard and folic acid antagonists, were found as cytotoxic agents in malignant lymphomas (Gilman and Philips 1946). As a result, the NCI initiated the first large-scale evaluation of synthetic agents and natural products for antitumor activity (Shoemaker 2006).

Current small molecular compound libraries mainly fall into the following three categories: target focused libraries, disease focused libraries and natural product-like compound libraries. Target focused libraries consist of compounds that target a specific target protein or a certain type of proteins such as protein kinases, and they are the most commonly used screen libraries for drug development. One example of a target focused library drug panel is the kinase inhibitor assay developed in the Tyner/Druker laboratories. This drug panel consists of approximately 120 drugs including kinase inhibitors as well as drugs with activity against select families of non-tyrosine kinases including PI3K/AKT, PKC, PKA, IκK, RAF/MEK/ERK, JNK, p38, AMPK, aurora kinases, and cyclin-dependent kinases. It has been used to identify kinase dependence in many cancer models including leukemia, gallbladder cancer and oesophageal squamous cell carcinoma (Jeffrey W. Tyner 2017; Weber et al. 2017; J. Yuan et al. 2017).

A less commonly used drug screen library category is the disease focused library. One strategy of designing this type of library involves large cohort genomic and transcriptional alteration analysis using bioinformatics approaches based on the assumption that those alterations may be responsible for or associated with drug response. These drug panels usually consist of FDA approved drugs as well as drug candidates in development that relevant to this disease based on disease specific genomic alterations or previous reported cancer cell drug sensitivities.

Natural product-like compounds libraries are valuable resource to identify novel therapeutic agents. Natural products usually have a broader spectrum of targets and may target proteins and protein-protein interactions that are otherwise difficult to target by available small molecular compounds or those compounds are hard to synthesize. Therefore, these libraries can be a good complement to compensate for deficiencies in the disease focused library when a large proportion of genomic or transcriptional alterations in a disease are not druggable. Limitations of using natural products include a lack of well annotated drug target databases that are free for the public. So far, numerous natural products that showed promising results in traditional medicine are still being used in the form of plant extract and not isolated as compounds, providing potential opportunities to identify novel compounds and drug chemical structures from natural sources.

Limitations using small molecular drug screens include that compounds usually have multiple targets, and lack of well documented annotation of the spectrum of targets for any give drug could be misleading when trying to identify true target. Efforts on improving annotation of drug targets has been made such as recently published work on stratifying FDA approved anti-cancer drugs based on different levels of drug target evidence (Blucher et al. 2017). In addition,

relatively more specific approaches such as RNA-mediated interference (RNAi) can be used in parallel with drug screens to rule out off-target effects.

siRNA screens.

In the last two decades, RNAi has evolved from a fascinating biological phenomenon into a powerful experimental tool. Progress in genome sequencing and annotation, as well as technological advances that permit biological assays to be executed and analyzed in high throughput, has sparked increasing interest in genome-wide and target specific RNAi screens through which the effects of gene silencing on biological phenotypes can be systematically explored.

One advantage of siRNA screens is that for the numerous genetic and transcriptional alterations that are not druggable, siRNAs provide the possibility to test the roles of targeting those genes and pathways in disease models. One difference between small molecular inhibitors and siRNAs that cannot be overlooked is that while small molecules inhibit the enzymatic activity of their direct targets, siRNAs knock-down their gene expression, rendering different subsequent effects using these two approaches. This is especially important in the cases where the protein target has other activities that can persist after drug inhibition such as non-enzymatic functions of nEGFR (S. Y. Lin et al. 2001a).

Given the usually large scale of siRNA screens, appropriate statistics should be implemented in siRNA screen analysis. For example, negative and positive control siRNA should ideally be included on every plate with experimental siRNA to monitor quality control during screening. ‘Hits’ are typically identified as replicates that are beyond two to three standard deviations (SD) from the mean or beyond a predetermined threshold. Although mean and SD values may be

calculated with control samples (treated with scrambled siRNA), it is recommended that they be calculated with the entire set of experimental siRNA to control for general complications due to RNAi; strong 'hits' (typically >5 SD from mean in replicate) should be excluded so as not to skew the calculated mean values. Arrayed screens should be done at least in duplicate, and preferably in triplicate, to minimize false discovery.

RAPID (RNAi-assisted protein target identification) assay developed in Tyner/ Druker lab at OHSU is a good example of siRNA screen panel. This siRNA screen panel was designed to target each member of the tyrosine kinase gene family. This assay has been tested using primary cells from patients with hematologic malignancies, and can detect therapeutic targets in 4 days on an individual patient basis (J. W. Tyner et al. 2009).

Introduction of ALK

Efforts have been made to overcome EGFR inhibitor resistance in HNSCC using combination therapies. A recent study reported that co-targeting another oncogenic protein anaplastic lymphoma kinase (ALK) enhanced anti-tumor activity of EGFR inhibitor gefitinib in oral squamous cell carcinoma cell lines (Gonzales et al. 2016).

ALK gene was first discovered as a nucleophosmin (NPM)-ALK fusion oncogene in anaplastic large-cell non-Hodgkin's lymphoma (ALCL) in 1994 (Morris et al., 1994), and was in the limelight again in 2007 as the highly potent echinoderm microtubule-associated protein-like 4 (EML4)-ALK fusion oncogene in lung cancer (Soda et al., 2007). The oncogenic roles of ALK have been reported in various cancer types including anaplastic large-cell lymphoma, NSCLC and neuroblastoma (Mologni 2012). The incidence of ALK-rearranged lung cancer is estimated to be approximately 3%–5% for NSCLC, comprising approximately 60,000 individuals every year worldwide (Koivunen et al., 2008; Shaw et al., 2009; Soda et al., 2007; Takeuchi et al., 2008). In HNSCC, the frequency of ALK alterations in TCGA cohort (528 patients) is 6% aggregately, including 2.24% mRNA upregulation, 2.24% missense mutations, 0.94% truncating mutations, 0.37% amplification and 0.18% deep deletion (Cerami et al. 2012).

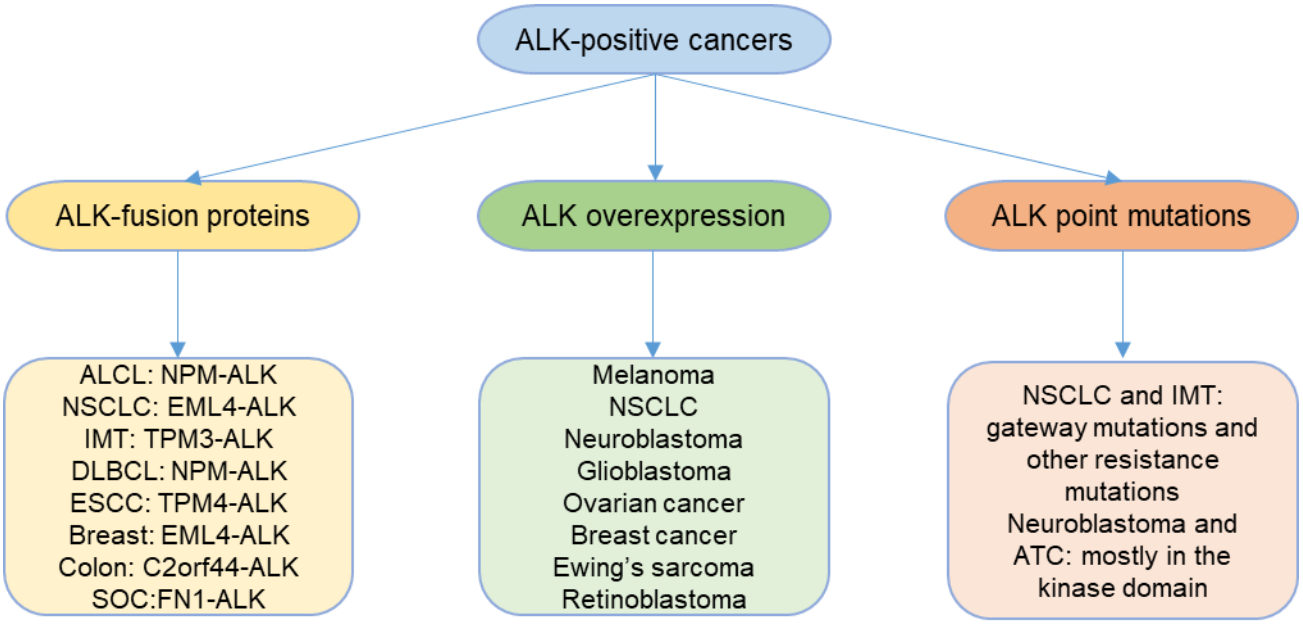


Figure 1-5. Major ALK alterations in human cancers.

ALK alterations have been found in anaplastic large cell lymphoma (ALCL), inflammatory myofibroblastic tumour (IMT), diffuse large B cell lymphoma (DLBCL), non-small-cell lung cancer (NSCLC), breast cancer, colon carcinoma, serous ovarian carcinoma (SOC) and oesophageal squamous cell carcinoma (ESCC) and anaplastic thyroid cancer (ATC). Selected fusion proteins are shown for each cancer type.

One of the characteristics shared by different ALK fusion oncogenes is the constitutive expression of the ALK fusion protein by the active promoter of the fusion partner gene. Additionally, ALK tyrosine kinase is constitutively activated by dimerization or oligomerization (Katayama, Lovly, & Shaw, 2015). As a result, the constitutively active ALK fusion protein strongly induces oncogenic cell growth signaling. Very similar to EGFR signaling, signaling pathways downstream of ALK that contribute to tumor growth mainly consist of PI3K-AKT, RAF-MEK-ERK and JAK-STAT signaling pathways (Chiarle et al. 2008). Multiple ALK inhibitors that were developed to target lung cancers with ALK rearrangements were successful in achieving marked tumor shrinkage (Galkin et al. 2007a; Ceccon et al. 2013). However, ALK rearrangements in HNSCC are very rare (Hayes, Grandis, and El-Naggar 2013; Cancer Genome Atlas Network 2015a), which contradicts the finding that co-targeting EGFR and ALK could be more effective than using gefitinib as single agent in HNSCC (Gonzales et al. 2016). This suggests that further studies of potential benefits of targeting wild type ALK in HNSCC are warranted.

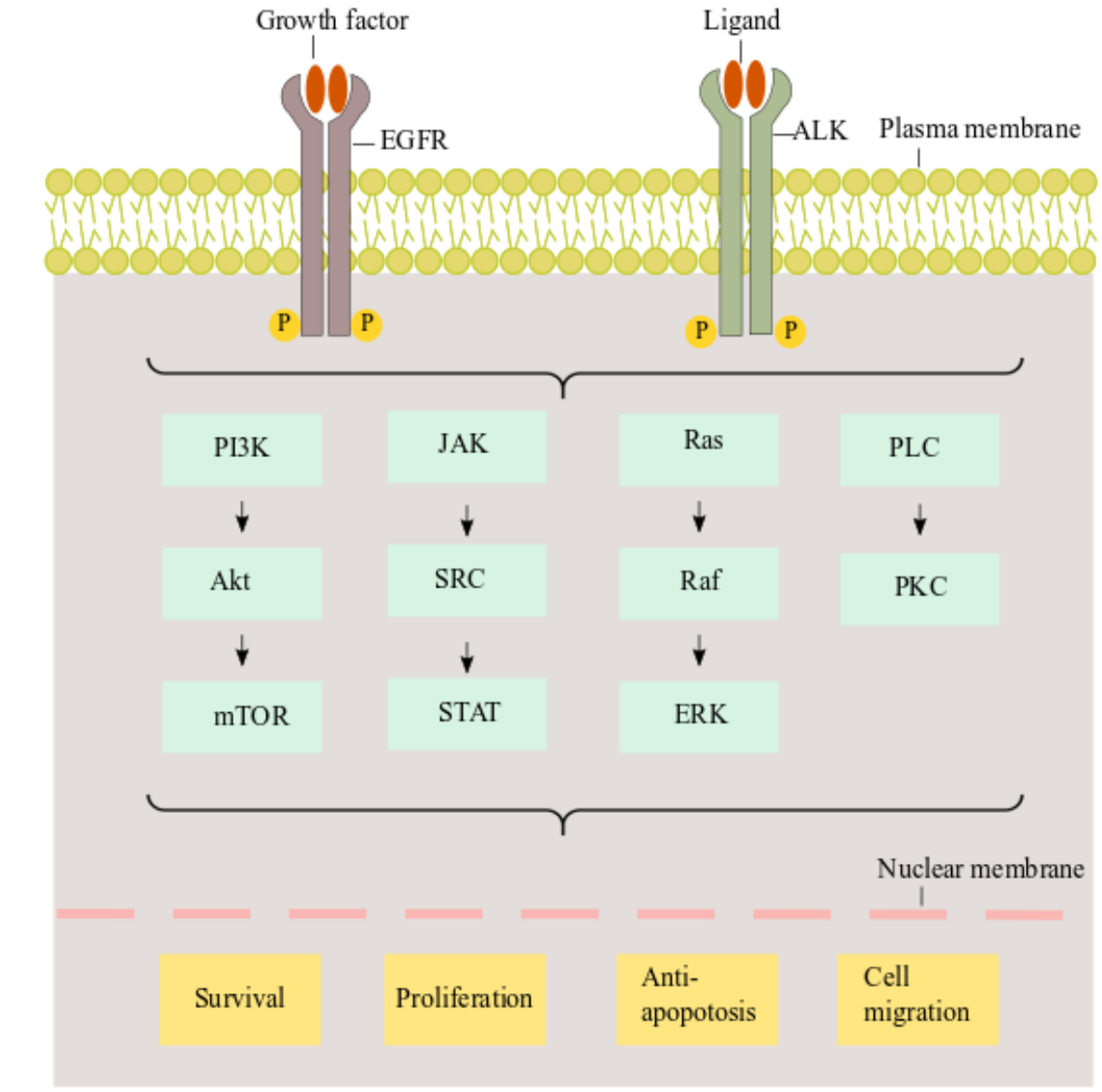


Figure 1-6. **Downstream signaling of EGFR and ALK.**

ALK and EGFR share common downstream signaling pathways that contribute to tumor growth including of PI3K-AKT, RAF-MEK-ERK, JAK-STAT and PLC-PKC (Chiarle et al. 2008; Seshacharyulu et al. 2012).

In my thesis studies, I applied functional screens to patient-derived tumor cells to test the following hypotheses:

1) Combinations of drugs with EGFR inhibition are more effective than EGFR alone,

2) Responses to targeted therapy will differ among individual patient derived HNSCC cases,

3) In the context of knowledge on ALK gained from previous studies of HNSCC cell lines, and from studies of other cancers, tumor cell models derived from individual OHSU HNSCC patients can provide insight into mechanisms of EGFR inhibitor resistance and effectiveness of the combination of EGFR and ALK inhibitors.

The studies that address these hypotheses are described in Chapter 2, and implications and future directions based upon preliminary data are presented in Chapter 3.

Chapter 2 – Inhibiting Anaplastic Lymphoma Kinase (ALK) Overcomes EGFR Inhibitor Resistance in Head And Neck Squamous Cell Carcinoma Patient-Derived Models

Abstract

EGFR is overexpressed in up to 90% of HNSCC and is associated with poor outcome. The only FDA approved cancer intrinsic molecular targeted therapy, an anti-EGFR monoclonal antibody cetuximab, eventually fails in almost all patients. In this study, functional screens, including a small-molecule kinase inhibitor panel of drugs FDA approved or in development for cancer and a siRNA panel, were used to identify agents that synergized with EGFR inhibitors in reducing viability in HNSCC patient-derived tumor cells. Two ALK inhibitors on the drug screen panel showed synergistic effects with EGFR inhibitors in 4/8 HNSCC patients' tumor cells. siRNA targeting ALK synergized with EGFR inhibitor gefitinib in reducing cell viability. Scale-up dose-response experiments confirmed patient cell sensitivity to 4 different ALK inhibitors in combination with gefitinib, including 2 ALK inhibitors FDA approved for other cancers, ceritinib (LDK378) and brigatinib (AP26113). Co-targeting EGFR and ALK decreased HNSCC patients' tumor cell number and colony formation ability and increased annexin V staining. Gefitinib treatment increased ALK protein expression in primary tumor cells, patient-tumor derived spheroids and xenograft models. Further, nuclear EGFR was increased in an EGFR and ALK inhibitor combination sensitive case but not in a relatively resistant case. Overall, we identified EGFR and ALK inhibition combination as a potential therapeutic strategy for treating HNSCC, induction of ALK by gefitinib as a novel mechanism potentially relevant to resistance to EGFR inhibitor, and ALK induction to be potentially mediated by nuclear EGFR in EGFR and ALK inhibitor combination sensitive cells.

Introduction

EGFR has been reported to be upregulated in up to 90% of HNSCC patients and is associated with poor survival (Cassell and Grandis 2010; Ang et al. 2002b). Cetuximab, a humanized monoclonal antibody targeting EGFR, has been the only FDA-approved HNSCC cancer intrinsic targeted therapy since 2006. However, this treatment eventually fails, as patients either have intrinsic resistance to it or acquire resistance in less than 3 months (Chong and Jänne 2013). The use of combinations of other kinase inhibitors with EGFR inhibitors in HNSCC to overcome resistance to EGFR inhibitors has been investigated and suggested to be beneficial in preclinical models and under active testing in clinical trials (Aung and Siu 2016a).

ALK is an oncogenic protein involved in various cancer types including anaplastic large-cell lymphoma, NSCLC and neuroblastoma (Mologni 2012). Signaling pathways are triggered by ALK not only to enhance cell proliferation and survival, but also to induce cell migration (Chiarle et al. 2008). In lung adenocarcinoma, ALK is generally activated by the expression of chimeric proteins containing the ALK kinase domain, whereas in other ALK-positive neoplasms (e.g., neuroblastoma, sarcoma) ALK activation is caused by overexpression of wild-type or mutated transcripts (Minoo and Wang 2012).

Four inhibitors targeting ALK, crizotinib, alectinib, ceritinib (LDK378) and brigatinib (AP26113), have been FDA approved for the treatment of metastatic NSCLC positive for ALK fusions (Blackhall and Cappuzzo 2016; Shaw and Engelman 2014). Patients with metastatic ALK-positive NSCLC receive crizotinib as their initial, or first-line, treatment. Alectinib, ceritinib and brigatinib were later approved for ALK-positive patients no longer responding to or able to tolerate the ALK-targeted drug crizotinib. Ceritinib was

developed as a highly potent and selective ALK inhibitor derived from TAE684, a selective inhibitor of NPM-ALK that blocks the growth of ALK-rearranged ALCL cells (Galkin et al., 2007). IC50 of ceritinib for ALK is 150 pM *in vitro* enzymatic assays, approximately 20–30-fold lower than that of crizotinib. In addition to ALK, ceritinib can inhibit insulin-like growth factor receptor 1 (IGF1R, IC50, 8 nM) and insulin receptor (IC50, 7 nM). Ceritinib was approved in 2014 in the US. On April 28 2017, the FDA granted accelerated approval to a new ALK inhibitor brigatinib (Alunbrig) for patients with metastatic NSCLC and alterations in the ALK gene whose cancer has progressed during their initial therapy. Brigatinib has exhibited activity as a potent dual inhibitor of ALK and EGFR with IC50s of 0.6 nM and 39.9 nM, respectively, in cell free assays.

Although ALK has been well studied in other cancers both in preclinical and clinical settings, the oncogenic roles of ALK and the effect of ALK inhibitors in HNSCC are less clear, due to relatively low expression and frequency of ALK mutations or fusion proteins in naive HNSCC tumors compared to other cancer types (Cerami et al. 2012). Manipulation of ALK in HNSCC has been found capable of regulating invasiveness and metastatic progression in HNSCC cell lines (T.-T. Huang et al. 2013), and ALK is upregulated in advanced disease compared to early-stage tumors (Gonzales et al. 2016), although whether the patients received any prior treatment is unclear. A recent study reported that co-targeting ALK and EGFR using TAE684 and gefitinib significantly reduces HNSCC cell proliferation *in vitro* and decreases tumor volumes of cell line derived xenografts by 30% (Gonzales et al. 2016). However, whether the effectiveness of the combination of gefitinib and TAE684 was due to inhibition of EGFR and ALK was

uncertain, since TAE684 has multiple targets other than ALK (Galkin et al. 2007b). More importantly, the mechanism of synergy between these two agents is unknown. Further, to better predict clinical outcome of using EGFR and ALK inhibitor combinations in treating HNSCC patients, patient-derived models are needed.

The purpose of our study was to interrogate HNSCC patient-derived epithelial tumor cells for response to combinations of drugs with EGFR inhibitor as a basis for repurposing available drugs to HNSCC treatment. We used patient-derived cell models to examine the role of ALK in HNSCC, determine whether co-targeting ALK and EGFR could overcome EGFR resistance in HNSCC cells, and define potential mechanisms of synergy of these agents.

Results.

Establishment of HNSCC tumor cultures

Cell lines have contributed to current knowledge of HNSCC and is routinely used in evaluation of therapies either now in clinical trials or in preclinical development. While Li et al. reported 11 genes exclusively mutated in HNSCC tumors and not in HNSCC established cell lines (H. Li et al. 2014). Patient-derived cultures may reflect clinically relevant heterogeneity found in the human HNSCC tumors better than long-term passaged cell lines. In addition, our cases have the advantage of linkage to patient-specific outcomes critical to placing gene aberrations in clinical context. Further, in other cancers a systems biology evaluation of cell lines has led to clinically relevant subclassifications; e.g. Heiser et al. defined subtypes of breast cancer with predictable sensitivities to chemotherapy through analysis of 50 breast cancer cell lines that have clinical impact (Neve et al. 2006; Laura M. Heiser et al. 2012). Thus, while cell culture has its limitations, it is state-of-the-art as part of a multiplex strategy that includes direct assessments of tumor tissue, animal models, and clinical trials, for bringing new standards of care to HNSCC patients, and for focusing these other approaches.

Since established cell lines, due to long term culture, may have genetic drift and cross-contamination with other cell lines, patient derived models were used in this study in order to correlate functional assays to clinical annotation such as HPV status, tumor site, stage and tobacco and alcohol use. We used the explant culture technique (Figure 2-1) for establishment of HNSCC tumor cultures in this study. Compared to other cell culture techniques such as culture from single cell suspension, this method in our hands

facilitates the retention of native tissue architecture and microenvironment by maintaining good morphology of epithelia and at least initially, a “feeder layer/structure and soluble factors” of the tumor’s own stroma and thus may reflect better representation of molecular interactions *in vivo*.

To establish cell lines from explants, tumor tissue is minced (</=2 mm^3) and distributed into plates that have been coated with collagen I. Other than cell culture plates, roller bottles are also used regularly in our lab for primary culture based on the following advantages: 1. They providing better gas exchange and thus better mimicking the tissue environment in the head and neck area; 2. The rolling prevents gradient formation in the medium which may affect the growth of cells; 3. Large surface area also enables scale up cell culture. Explants are then supplemented with essential nutrients for optimal growth. The explants were observed periodically and explants with fibroblast outgrowth were treated with trypsin for short period of time, usually within 1-3 min, to remove fibroblasts differentially, so that only those with epithelial monolayer outgrowth are preserved.

The cells were allowed to grow to 90% confluence and were subject to passaging. Cells used for experiments had more than 70% epithelial cells by morphology and were of passage 0-2 for inhibitor assays and RAPID assays, and 1-5 for scale-up experiments. Primary culture success rate over the last 5 years in our lab has been approximately 25-50%. Fibroblasts from passage 0 were also collected and cryopreserved for future studies.

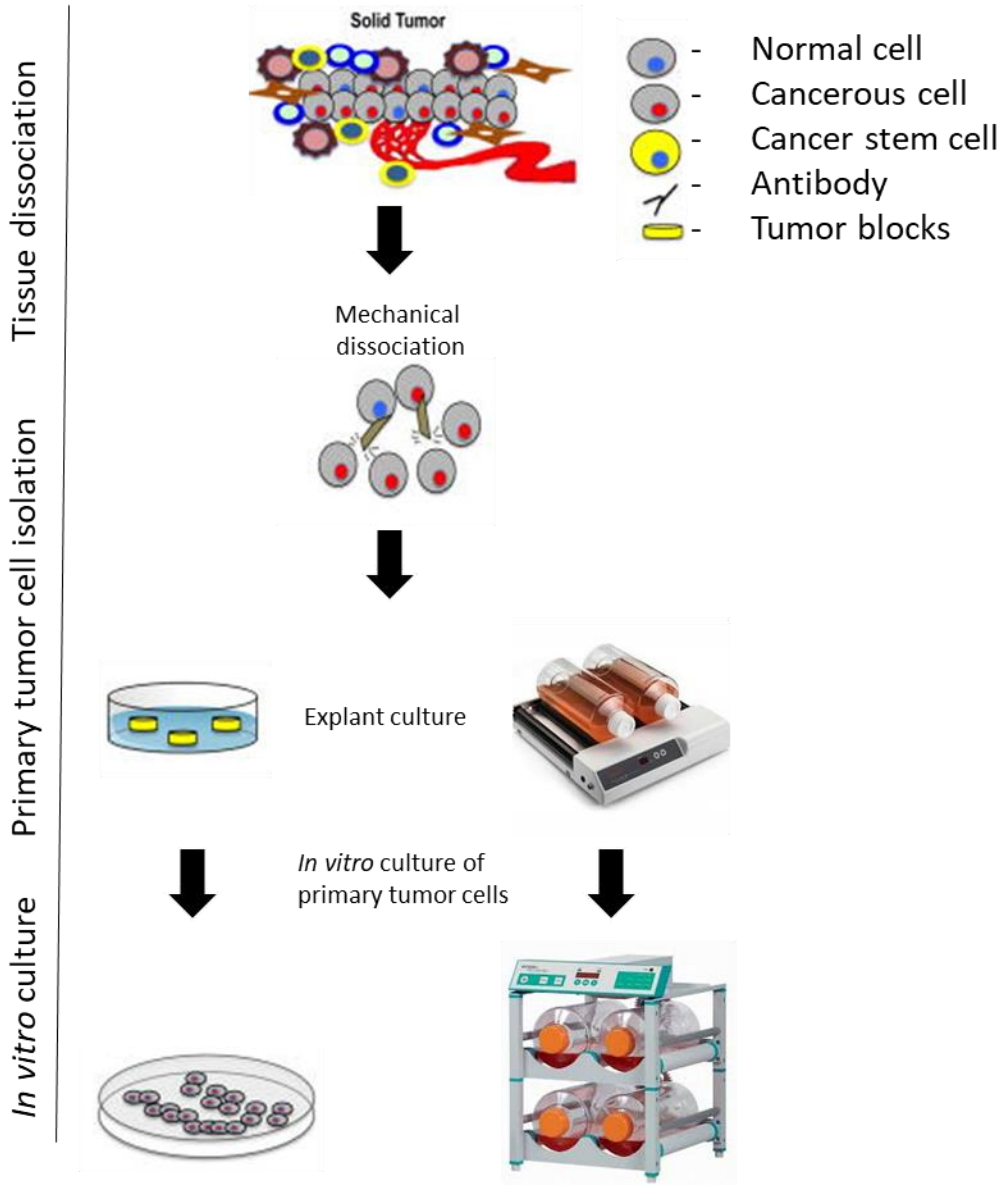


Figure 2-1. A schematic representation of the process of primary tumor cell line establishment.

Primary neoplastic mass is dissociated by mechanical method. Then tumor cells are enriched from explant. These cells are cultured with medium by adding suitable tissue

specific supplements. Explants were incubated either in collagen I coated 60 mm plates or roller bottles. (*Modified from Charles J. Lin, BA, BASIC SCIENCE REVIEW, 2006*)

Original HNSCC tumor samples collected in our lab undergo routine review by pathologists at the OHSU Biolibrary for percentages of tumor, stromal and necrosis tissue. Tumors used in this study have a median epithelial tumor component percentage of 65% (range 40-90%) (Figure 2-2).

To ensure that a high percentage of epithelial cells rather than fibroblast are used in experiments, primary cultures were tested for cell type specific antigenic marker expression by immunofluorescent staining. Since the keratins are the typical intermediate filament proteins of epithelia (Moll, Divo, and Langbein 2008), pan-keratin antibody was used to stain epithelial makers, while vimentin was used for fibroblasts. Primary cells for experiments in this study have a median epithelial cell percentage of 90.5% (range 62-98%) (Figure 2-2, Table 2-1). For 10139, although cell morphology under bright field microscopy showed significant percentage of epithelial cells (Figure 2-2), a large ratio of cells did not show staining of any of the markers and hence other differentiation markers are being tested for this case.

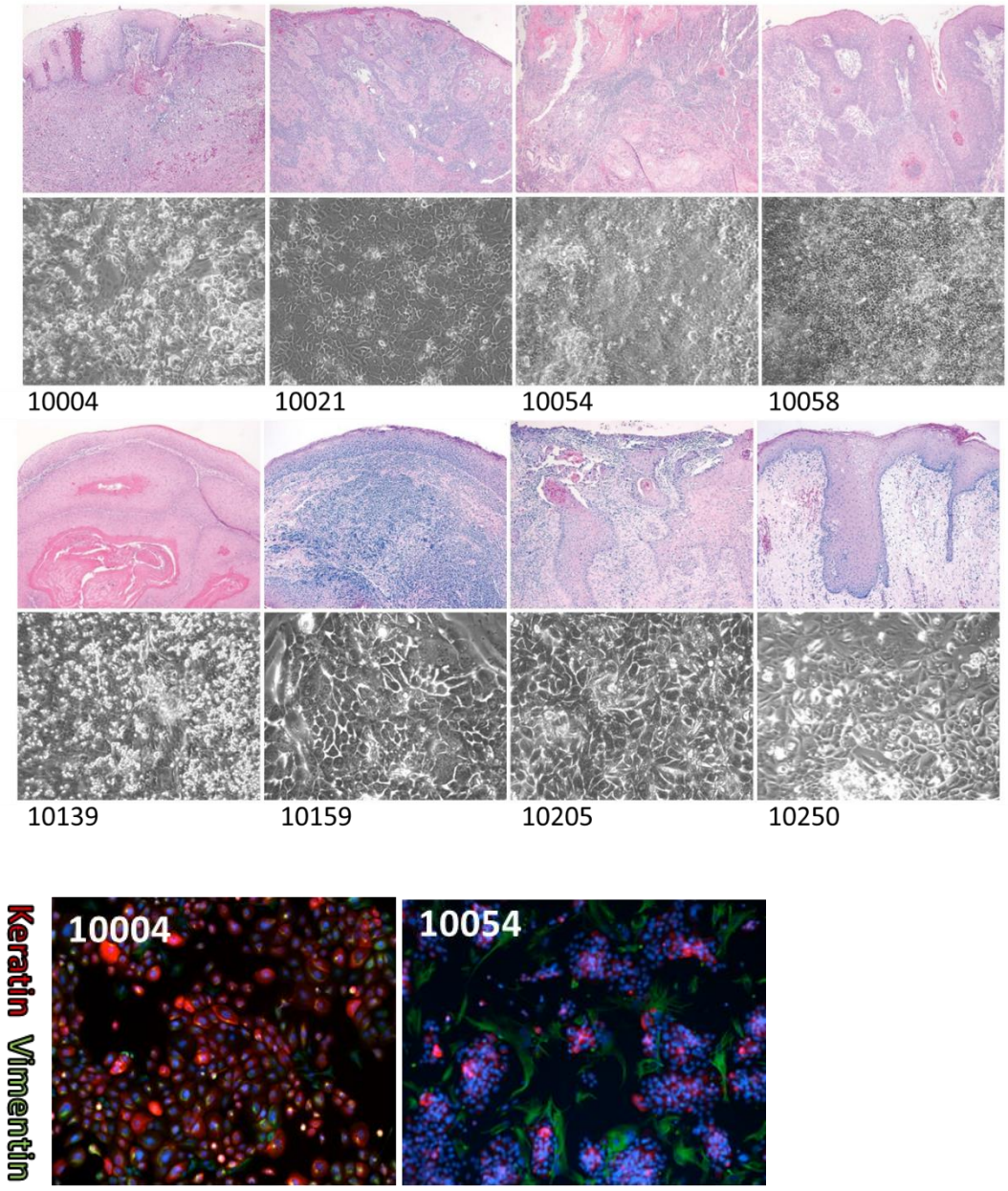


Figure 2-2. Original HNSCC tumor morphology and primary tumor cells.

Original HNSCC tumor morphology by haematoxylin and eosin (H&E) staining and bright field images of primary cultures (A). Representative images of differentiation

makers, pan-keratin (red) and vimentin (green), expression in HNSCC primary cultures by IF staining (B).

Patient	Keratin single positive	Vimentin single positive	Keratin+Vimentin double positive (EMT)	Keratin positive (Epithelial cells)
10004	9%	6%	85%	94%
10021	83%	3%	4%	87%
10054	77%	8%	1%	78%
10058	62%	2%	1%	63%
10139	0%	53%	21%	21%
10159	96%	3%	1%	97%
10205	16%	2%	80%	96%
10250	28%	2%	70%	98%

Table 2-1. Cell type specific markers expression in primary culture.

Quantification of pan-keratin (red) which detects endogenous levels of total keratin 4, 5, 6, 8, 10, 13 and 18, and vimentin (green) expression in HNSCC primary cultures by IF staining.

OHSU HNSCC patient tumor-derived cells functional assay top targets

Hypothesis driven drug screening for HNSCC

One important strategy to overcome EGFR inhibitor resistance is by combining other targeted therapies to anti-EGFR treatments. For example, EGFR inhibitors and PI3K/mTOR inhibitors are the most investigated drug combinations in HNSCC both in preclinical models and clinical trials. Other combination therapies that include drug combinations between EGFR mAb and EGFR TKIs, MET TKI and CDK4/6 inhibitors are in active development, based on the genomic landscape profiling studies on large cohort of HNSCC patients such as TCGA.

Given the ubiquitous role of tyrosine kinases in regulating critical cellular processes and redundant functions of kinases in cancer cells, we hypothesized that co-targeting EGFR and certain other kinase inhibitors would lead to enhanced anti-oncogenic response compared to single treatment of EGFR inhibitors. To test this hypothesis and to identify therapeutic agents that could overcome EGFR inhibitor resistance in HNSCC, we subjected patient-derived tumor cells of less than 2 passages to a small-molecule inhibitor screening assay (J. W. Tyner et al. 2013), with or without an EGFR inhibitor, in order to identify agents that synergize with EGFR inhibitors in reducing HNSCC cell viability.

Bioinformatics analysis on inhibitor assay coverage of HNSCC genomic alterations

To ascertain the relevance of the inhibitor assay drug panel to HNSCC, we examined the drug target coverage of the drug panel in the context of our analysis of HNSCC somatic mutation data from the Cancer Genome Atlas (TGCA). Using a bioinformatics approach (see supplementary methods), we were able to leverage known drug-target data to discover potentially targetable HNSCC pathways. Of 224 pathways judged relevant to HNSCC in analysis of mutation enrichment from 279 TCGA HNSCC cases, 111 pathways (49.4%) were targeted by the combined inhibitor panel and FDA-approved drugs. These were defined as “light” pathways. Each of the light pathways contained 1 to 208 targets of the drugs on the combined panel indicating likely relevance of the panel to HNSCC. In addition, we identified 113 (50.5%) “dark” pathways defined as not currently targeted by the combined drug panel and, therefore, relevant to HNSCC for future development and modification of the panel.

Definition of an effective drug

An effective drug from the inhibitor assay for any given patient was defined as a drug that has a IC50 that is lower than 20% of the median IC50 of all the HNSCC patients tested on this panel, therefore this drug is specific for reducing cell viability of tumor cells from this patient rather than generally toxic to all patients. A drug that was synergistic with EGFR inhibitor was defined as a drug that was not an effective drug as single agent for that patient, but decreased IC50 below 20% of the median IC50 after adding a EGFR inhibitor.

Since different drugs may have different pharmacokinetics in patients, it is likely that even when the IC50 of a drug is below 20% of median, it is still not clinically achievable. Therefore, maximum plasma concentration and tissue concentration need to be considered as another layer of cut-off for effective drugs. A phase III study of gefitinib for recurrent HNSCC reported mean predicted trough plasma concentrations (lowest concentration reached by a drug before the next dose is administered) of gefitinib were 305 ng/mL (683 nM) and 594 ng/mL (1.33 μM) in patients receiving 250 and 500 mg, respectively (J. S. W. Stewart et al. 2009). Although a gefitinib dose-escalation in advanced NSCLC patients has shown that gefitinib 250 mg daily was as effective as, and better tolerated than, gefitinib 500 mg daily (Xue et al. 2015), another study reported that daily oral administration of gefitinib (250 mg) to breast cancer patients resulted in gefitinib concentrations in each tumor sample (mean, 16.7 μM) substantially higher (mean, 42-fold) than the corresponding plasma sample. This extensive tissue distribution of gefitinib is probably related to its physicochemical properties (aqueous solubility, 3.77

μM), which allow the compound to distribute preferentially out of plasma and into tissues (McKillop et al. 2005). Therefore, it is very likely that the tissue concentration of gefitinib, even given 250 mg daily, may be achievable at 1.33 μM, hence 1.33 μM was used as a clinical achievable cut-off for later IC50 studies.

A low dosage (50 nM) of EGFR inhibitor was selected to be tested in combination the of drug on the inhibitor assay panel. This dosage is clinical achievable, and is lower than the IC50s of most HNSCC cell lines reported in the literature (Norio Kondo oncology reports 2008); therefore it was selected as likely to allow detecting improved IC50s of combinations with the drugs on the panel, and to reduce off-target effects from high dosage of drug.

Synergistic drugs with EGFR inhibitor in inhibitor assay in patient-derived tumor cells

Figure 2-3 to 9 show individual patient's responses to the top 15 effective drugs, as well as EGFR inhibitors, as single agents or in combination with a EGFR inhibitor based upon an 8 HNSCC patient OHSU cohort enrolled in this study. Fourteen out of 122 drugs on the panel showed synergy with EGFR inhibitors in the patient-derived tumor cells, supporting my hypothesis in chapter 1 that combinations of drugs with EGFR inhibition are more effective than EGFR alone. Our approach detected PI3K inhibitors PI103, BEZ235 and PP242 as effective combinations with EGFR inhibitor (Table 2-2), which is consistent with previous preclinical HNSCC studies *in vitro* and *in vivo* and the testing of PI3K/mTOR inhibitor combinations with EGFR inhibitors in clinical trials for HNSCC (Z. Wang, Martin, Molinolo, Patel, Iglesias-Bartolome, Sol Degese, et al. 2014; De Felice and Guerrero Urbano 2017; Jimeno et al. 2014, 866).

Of note, in both the inhibitor assay (Figure. 2-9) and in the RAPID assay (Figure. 2-14), patient 10205 was resistant to all the drugs and siRNAs on the panels as single agents; however, after adding a low dosage of EGFR inhibitor on top of the panel, multiple drugs and siRNAs become effective for this patient. This result provides a proof of principle example, that validates our approach using patient-derived tumor cells for functional assays to identify novel therapeutics for individual patients that desperately need treatment options.

More than 50% of the HNSCCs will relapse within 2 years (C. R. Leemans et al. 1994a). The above approach usually takes less than 1-2 months from generation of primary cell culture to finish inhibitor and RAPID assay analysis, allowing researchers to identify or

prioritize therapeutic options at a personalized level before tumor recurrence, even without any previous biological knowledge of this patients' tumor biology.

Synergistic drug with EGFR inhibitor	Target
GSK-1838705A	ALK, IGF1R, InsR
NVP-TAE-684	ALK, IGF1R, InsR
PI-103	PI3K/mTOR
GDC-0941	PI3K
PP242	mTOR
BEZ235	PI3K/mTOR
Lapatinib	ERB2/EGFR reversible
BIBW-2992	ERB2/EGFR irreversibe
HKI-272	ERB2/EGFR irreversibe
GSK-1120212	MEK1/2
VX-680	pan-Aurora
BI-2536	PLK1
XL-880	MET, VEGFR2, KDR
Staurosporine	PKC, wide range of targets

Table 2-2. Synergistic drugs with EGFR inhibitors.

Drugs that synergized with EGFR inhibitors were defined as drugs that have an IC50 below 20% of the median IC50 when combined with EGFR inhibitor and decreased more than 50% compared to the single agent in more than 1 patient. Fourteen out of 122 drugs showed synergy with EGFR inhibitors in inhibitor assays, 2 of which were ALK inhibitors.

Individual patient inhibitor assay results with or without a EGFR inhibitor

Figure 2-3 to 9 show individual patient's responses to the top 15 effective drugs, as well as EGFR inhibitors, as single agents or in combination with a EGFR inhibitor across 8 patients.

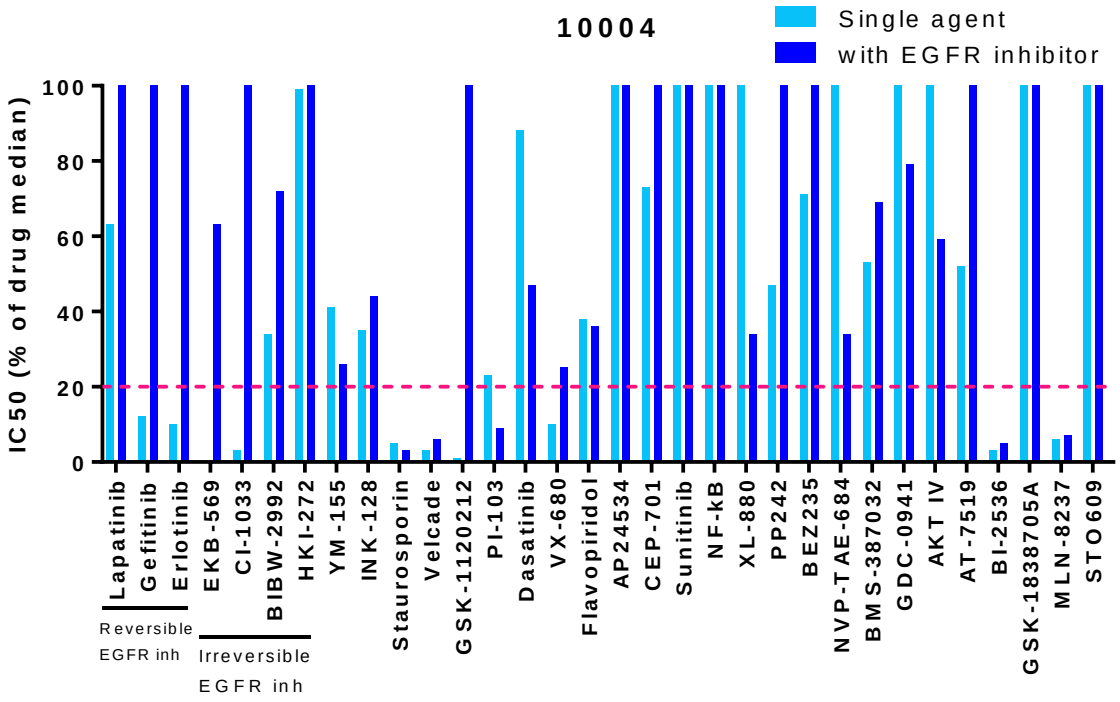


Figure 2-3. Relative IC50s of top 15 effective drugs across 8 patients for primary tumor cells from patient 10004.

10004 was a HPV negative stage T3N2b (IVa) oral tongue SCC patient with previous tobacco use. No previous treatment was received before surgery. No EGFR mutation was detected by exome seq. This patient was sensitive to 4 out of the 7 EGFR inhibitors, although the results were variable in scale-up validation experiments. Other than EGFR

inhibitors, this patient was sensitive to PI3K inhibitor PI-103, pan-aurora inhibitor VX-680 and PKC inhibitor staurosporine.

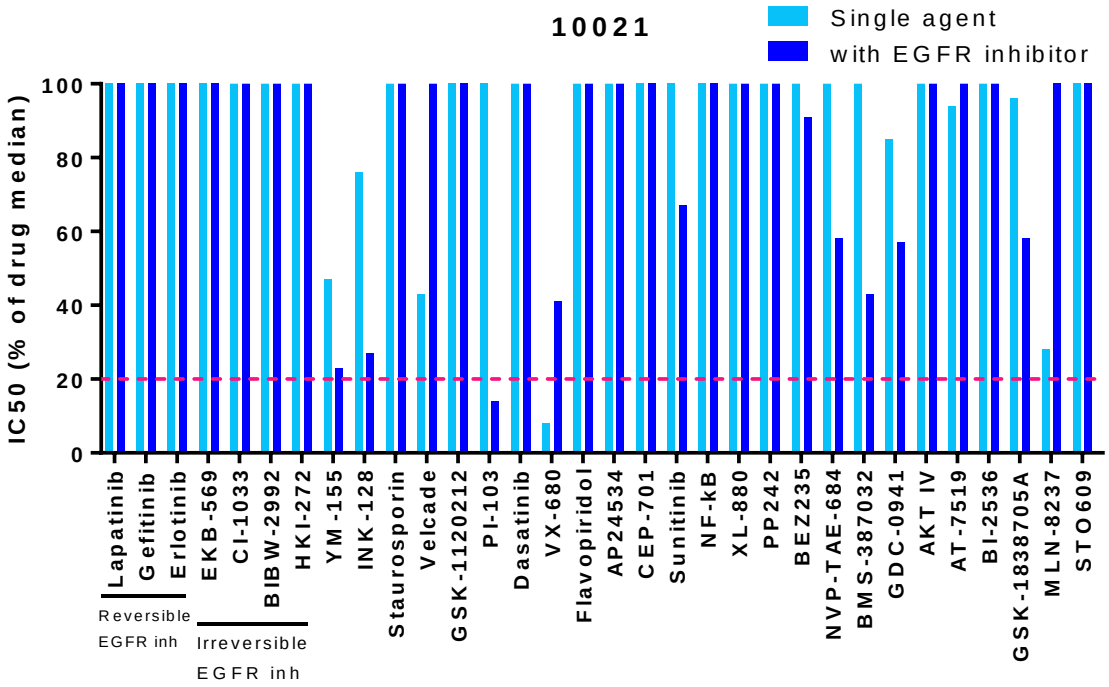


Figure 2-4. Relative IC50s of top 15 effective drugs across 8 patients for primary tumor cells from patient 10021.

10021 was a HPV negative stage T2N0MX (II) lateral tongue SCC patient with previous tobacco and alcohol use. No previous treatment was received before surgery. This patient was resistant to all EGFR inhibitors tested on the panel. As single agent, this patient was only sensitive to pan-aurora inhibitor VX-680. After adding a low dosage of EGFR inhibitor, PI3K inhibitor PI-103 became effective for this patient.

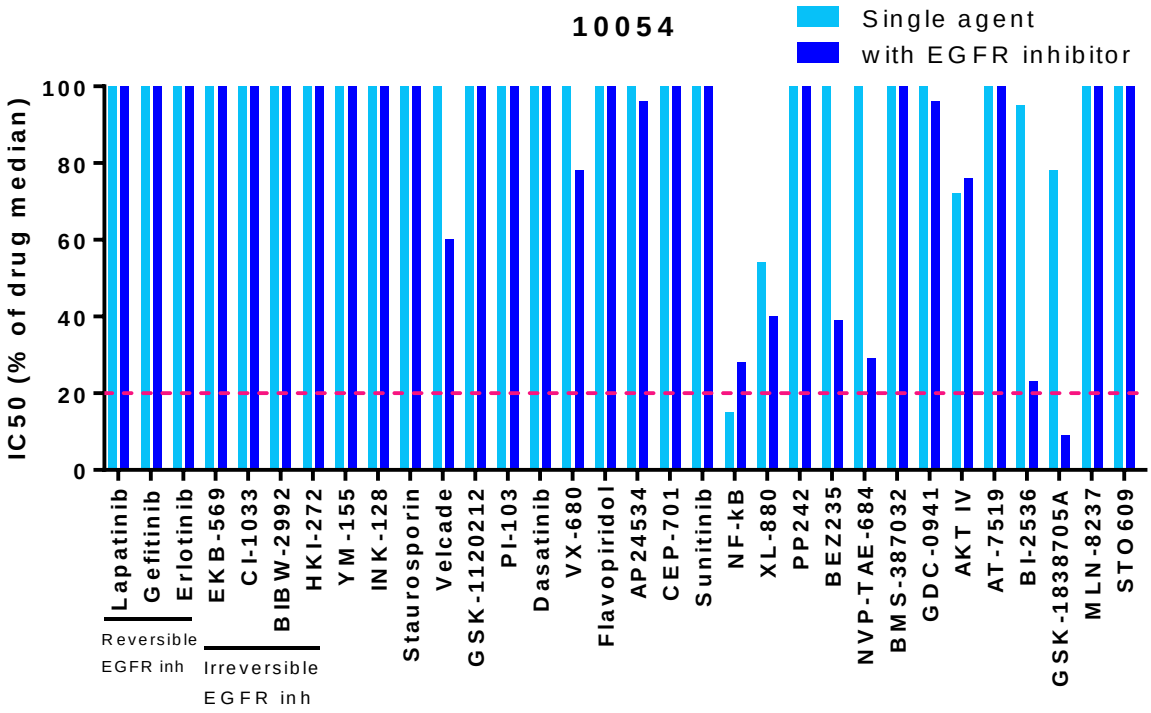


Figure 2-5. Relative IC50s of top 15 effective drugs across 8 patients for primary tumor cells from patient 10054.

10054 was a HPV negative stage T3N0MX (III) subglottis larynx SCC patient with previous tobacco and alcohol use. This patient did not receive any previous treatment before surgery. This patient was resistant to all EGFR inhibitors tested on the panel. As single agents, this patient was only sensitive to a NF-Kb inhibitor. After adding a low dosage of EGFR inhibitor, ALK inhibitor GSK-1838705A became effective for this patient.

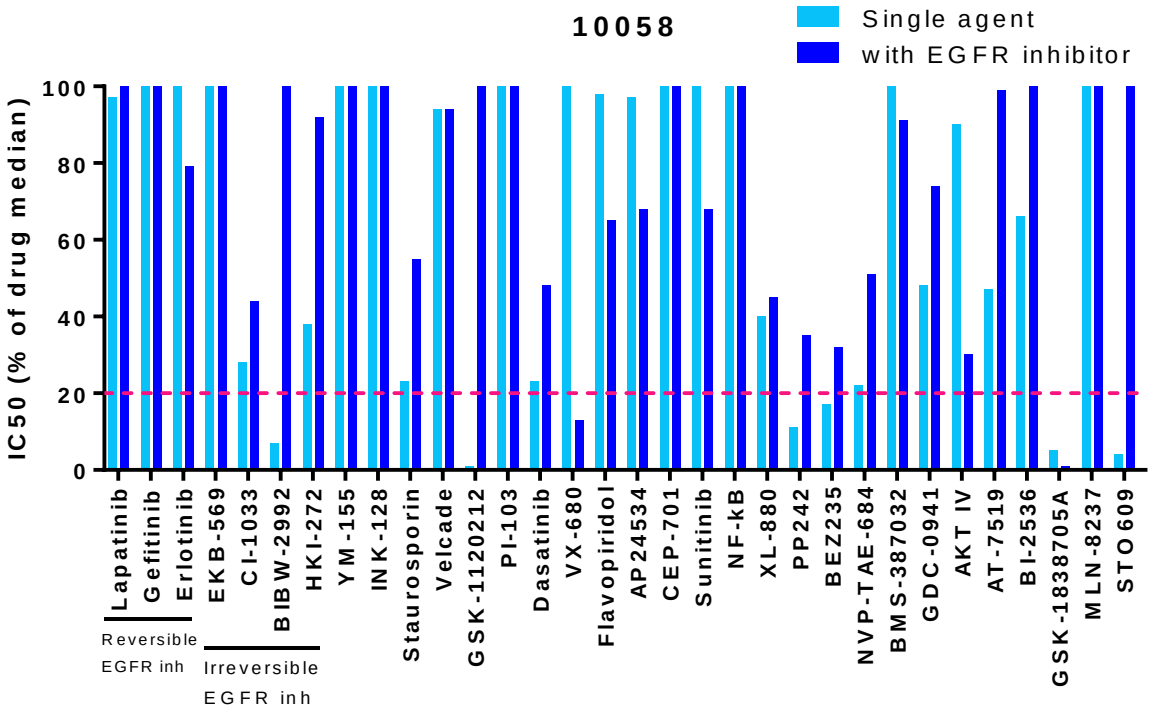


Figure 2-6. Relative IC50s of top 15 effective drugs across 8 patients for primary tumor cells from patient 10058.

10058 was a HPV negative stage T4aN0MX (IVa) bilateral glottic larynx SCC patient with previous tobacco and alcohol use. This patient did not receive any previous treatment before surgery. No EGFR mutation was detected in the tumor by exome seq. This patient was sensitive to 1 out of the 7 EGFR inhibitors as single agent. This patient was sensitive to ALK inhibitor GSK-1838705A regardless of EGFR inhibitor addition, and was also sensitive to PI3K inhibitor PP242, MEK1/2 inhibitor GSK-1120212, and Ca2+/calmodulin-dependent protein kinase kinase inhibitor STO609 as single agents.

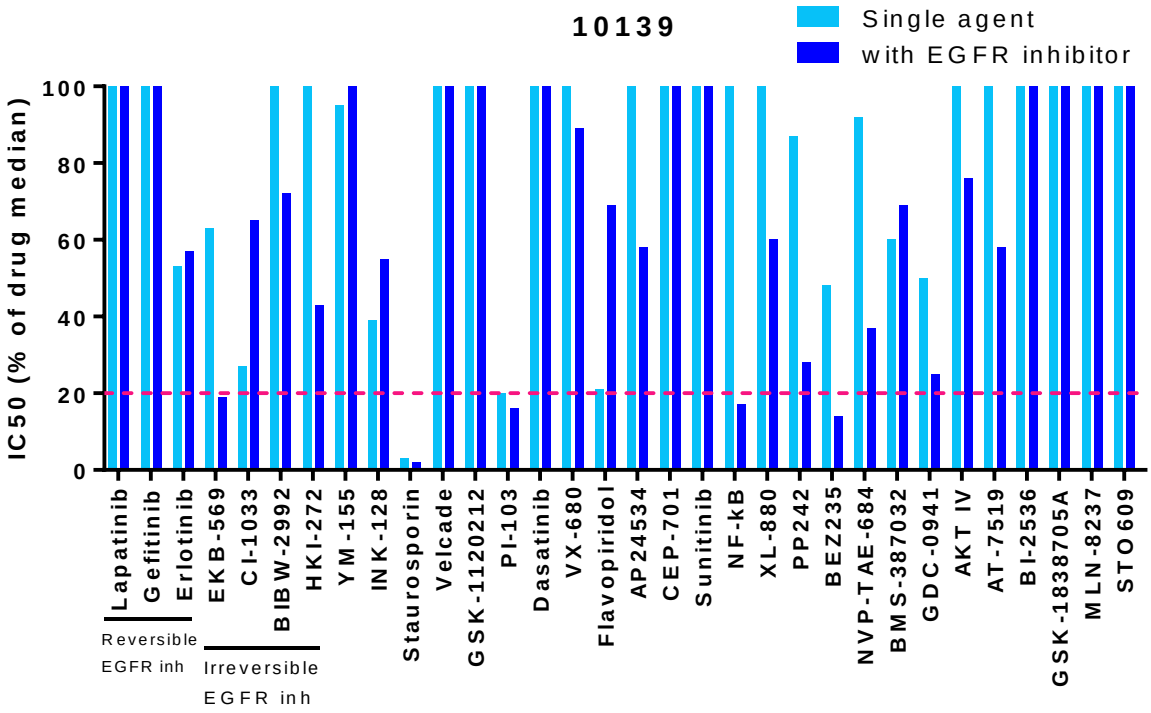


Figure 2-7. Relative IC50s of top 15 effective drugs across 8 patients for primary tumor cells from patient 10139.

10139 was a HPV negative stage T2N0MX (II) right maxilla SCC patient with previous tobacco and alcohol use. This patient did not receive any previous treatment before surgery. No EGFR mutation was detected in the tumor by exome seq. This patient was resistant to all EGFR inhibitors tested on the panel. This patient was sensitive to a PI3K inhibitor PI-103 and PKC inhibitor staurosporin regardless of EGFR inhibitor addition. After adding a low dosage of EGFR inhibitor, PI3K/mTOR inhibitor BEZ235 and an irreversible EGFR inhibitor EKB-569 became effective for this patient.

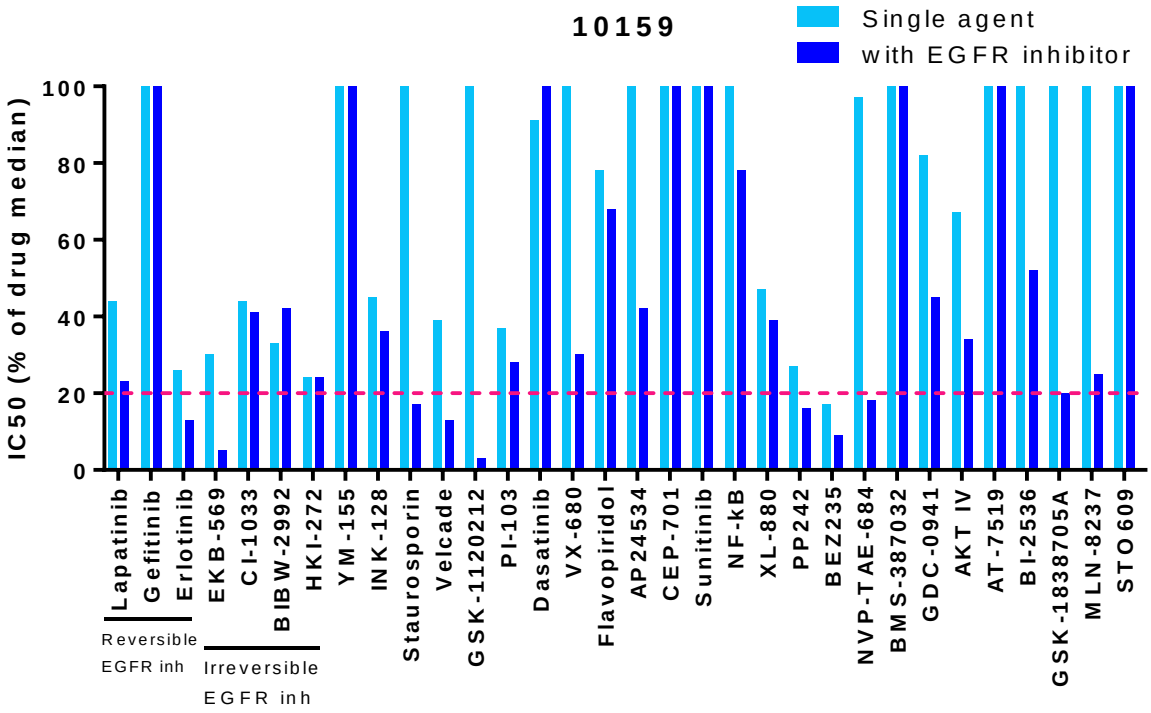


Figure 2-8. Relative IC50s of top 15 effective drugs across 8 patients for primary tumor cells from patient 10159.

10159 was a HPV positive stage T2N2aM0 (IVa) base of tongue SCC patient, which is the most common site for HPV positive cases, with no tobacco and unknown alcohol use. This patient received radiation before surgery which is common for HPV positive patients. No EGFR mutation was detected in the tumor by exome seq. This patient was sensitive to 2 out of the 7 EGFR inhibitors after a low dosage of EGFR inhibitor was added. This patient was sensitive to 1 drug as single agent, however, became sensitive to 9 different inhibitors after EGFR inhibitor was added.

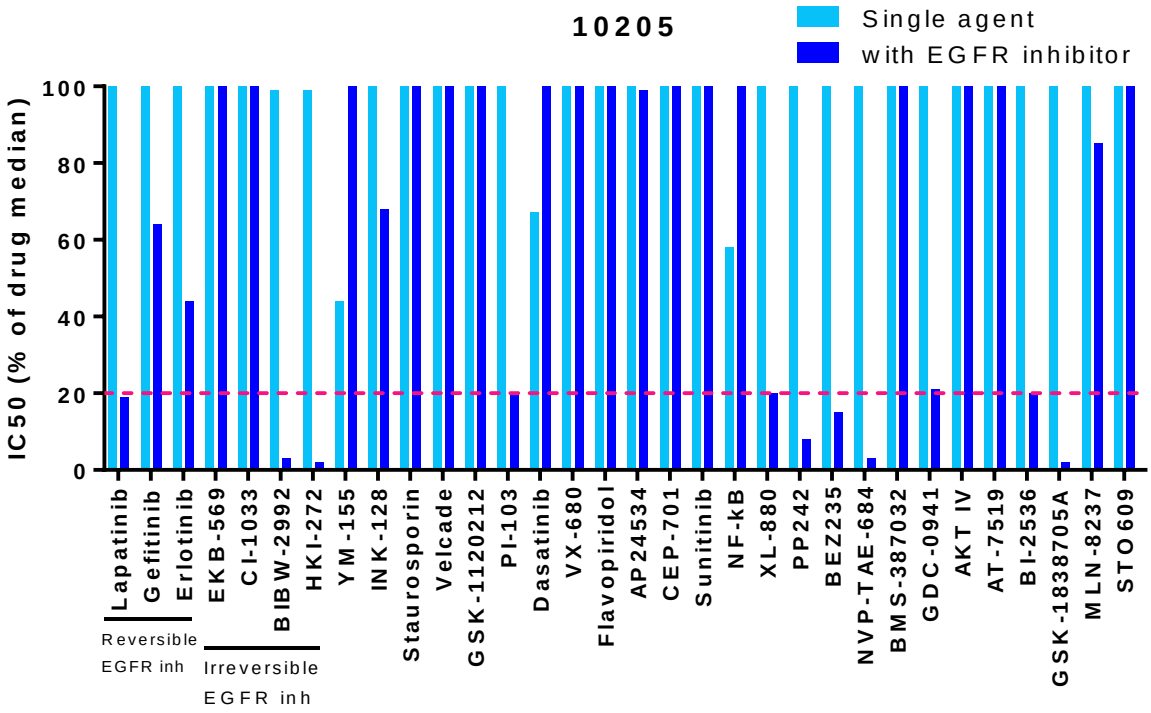


Figure 2-9. Relative IC50s of top 15 effective drugs across 8 patients for primary tumor cells from patient 10205.

10205 was a HPV negative stage T4aN1M0 (IVa) maxillary sinus SCC patient with no previous tobacco use, but a former alcohol user. This patient received chemoradiation before surgery. A EGFR insertion mutation D725DG was detected in the tumor by exome seq. This patient was resistant to all the top 15 effective drugs as single agents. However, after adding a low dosage of EGFR inhibitor on top of the panel, this patient became sensitive to 10 of the 32 drugs. It is unclear that if the EGFR insertion mutation D725DG in the tumor was responsible for the drug sensitivity in combination with EGFR inhibitor.

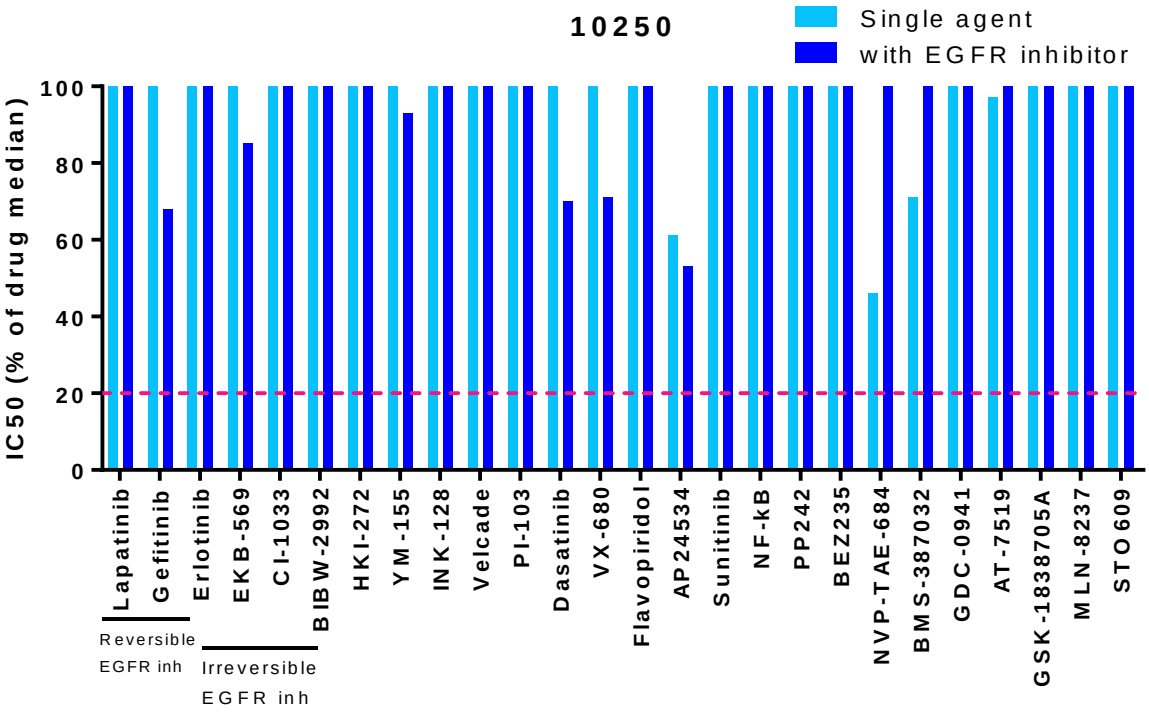


Figure 2-10. Relative IC50s of top 15 effective drugs across 8 patients for primary tumor cells from patient 10250.

10250 was a HPV negative stage T1N0M0 (I) oral tongue SCC patient with previous tobacco and alcohol use. This patient did not receive any previous treatment before surgery. No EGFR mutation was detected in the tumor by exome seq. This patient was resistant to all drugs regardless of addition of a EGFR inhibitor.

Individual patient RAPID assay results with or without a low dosage of EGFR inhibitor gefitinib

RAPID assay was performed in five patients 'tumor cells with genomic mutation and differential expression analysis data. Patient-derived tumor cells from patients were transfected with siRNA pools individually targeting each member of the receptor tyrosine kinome in addition to NRAS and KRAS co-treated with vehicle or with 50 nM gefitinib. In these five patients, 10004, 10054, 10058 and 10250 responded to siRNAs differentially from one another (Table 2-3), supporting my hypothesis in chapter 1 that responses to targeted therapies will differ among individual patient derived HNSCC cases. Notably, in 10205 none of the siRNAs as single agents was effective in reducing cell viability in this case, while FRK, KIT and PTK9L became effective in combination with a low dosage (50 nM) of gefitinib, again supporting my hypothesis in chapter 1 that combination therapies would be more effective than single agents.

Importantly, siRNAs targeting ABL1, AXL, and FRK became effective in 2 out of 5 patients in combination with gefitinib, indicating synergistic effects between gefitinib and targeting these genes in our cases. In particular, AXL has been reported to mediate EGFR inhibitor resistance in NSCLC (Zhang et al. 2012) and HNSCC (Brand et al. 2014), supporting our approach of utilizing RAPID assay to identify combination therapy that could overcome EGFR inhibitor resistance. Further, ALK became effective in 3 out of 5 patients in combination with gefitinib (Table 2-4), suggesting synergistic effects between gefitinib and targeting this gene in HNSCC. I will discuss the effects of co-targeting ALK and EGFR in HNSCC in depth in the rest of chapter. However, reported EGFR inhibitor resistance associated genes such as HER2, 3 and IGF1R (Erjala et al. 2006; Jameson et

al. 2011) were represented in the RAPID assay and did not become effective in combination with gefitinib (Table 2-3). In addition, differential expression analysis did not show an upregulation of these genes in patients' tumors or tumor-derived cells, suggesting these genes were not drivers of gefitinib resistance in these cases.

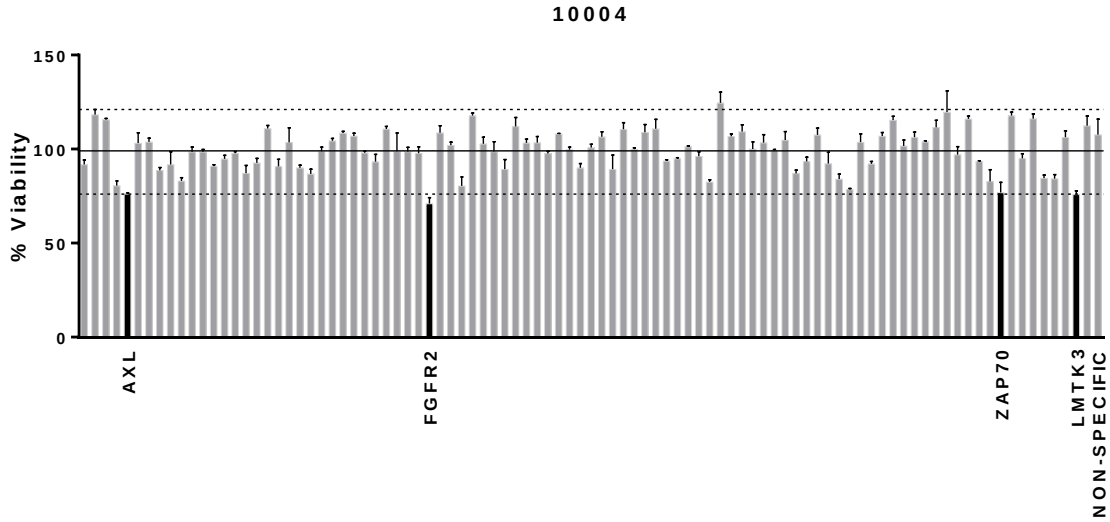
Figure 2-11 to Figure 2-15 are showing individual patient's response to siRNAs (Table 2-3).

Effective siRNAs as single agents					Effective siRNAs with gefitinib				
10004	**10054**	**10058**	**10205**	**10250**	**10004**	**10054**	**10058**	**10205**	**10250**
AXL	TP53RK	PDGFRB		JAK2	ABL1	ALK	ALK	FRK	ABL1
FGFR2	ROR1	SYK		YES1	TNK2	ROR1	SYK	KIT	JAK2
ZAP70		KRAS			ALK		AXL	PTK9L	
LMTK3					AXL				

Table 2-3. Effective siRNAs as single agents and in combination with gefitinib in individual patient-derived tumor cells.

Target	Patient	Number of patient	Target	Patient	Number of patient
AXL	10004	1	**ABL1**	10004, 10250	2
FGFR2	10004	1	**TNK2**	10004	1
ZAP70	10004	1	**ALK**	10004, 10054, 10058	3
LMTK3	10004	1	**AXL**	10004, 10058	2
TP53RK	10054	1	**ROR1**	10054	1
ROR1	10054	1	**SYK**	10058	1
PDGFRB	10058	1	**FRK**	10205, 10250	2
SYK	10058	1	**KIT**	10205	1
KRAS	10058	1	**PTK9L**	10205	1
JAK2	10250	1	**JAK2**	10250	1
YES1	10250	1			

Table 2-4. Summary of effective siRNAs as single agents and in combination with gefitinib in patient-derived tumor cells.


10004
% Viability
150
100
50
0
AXL
FGFR2
ZAP70
LMTK3
NON-SPECIFIC

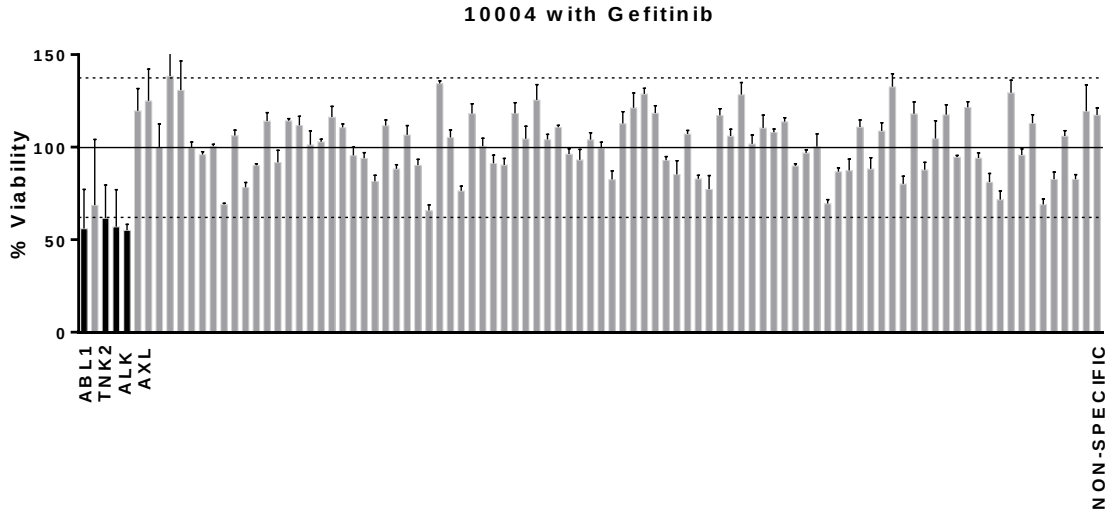

10004 with Gefitinib
% Viability
150
100
50
0
ABL1
TNK2
ALK
AXL
NON-SPECIFIC

Figure 2-11. RAPID assay result for primary tumor cells from patient 10004.

Cell viability was calculated by normalizing absorbance at 490 nM (as determined by the MTS assay) to the median plate value after 96 hours of treatment. Dotted lines indicate mean ± 2SD. Effective siRNAs are defined as those that inhibit cell viability 2SD below the mean-of all siRNAs and were statistically different from the non-specific siRNA controls and are marked in black. Error bar represents the mean ± SEM, each containing three replicates (n = 3).

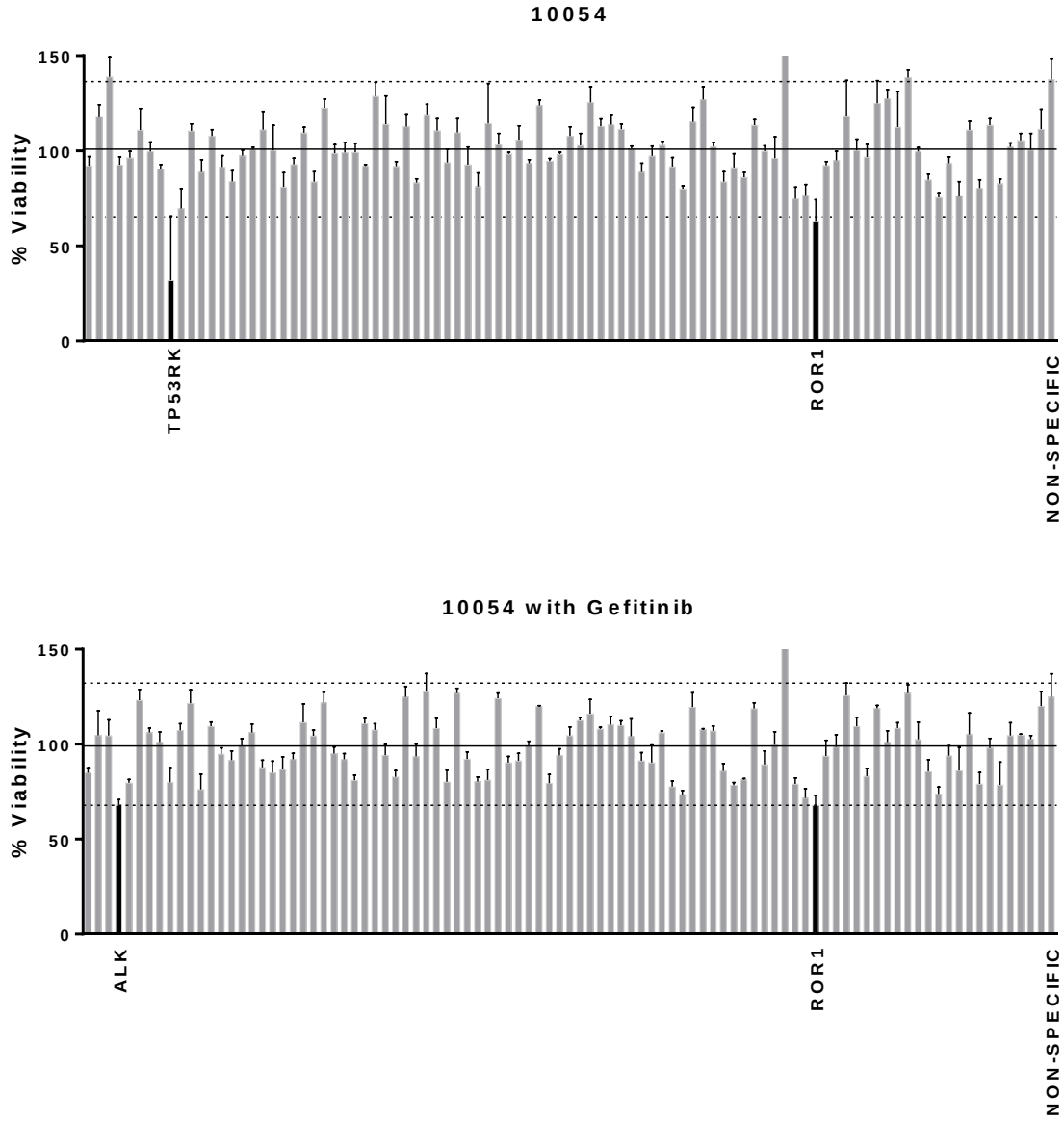


Figure 2-12. RAPID assay result for primary tumor cells from patient 10054.

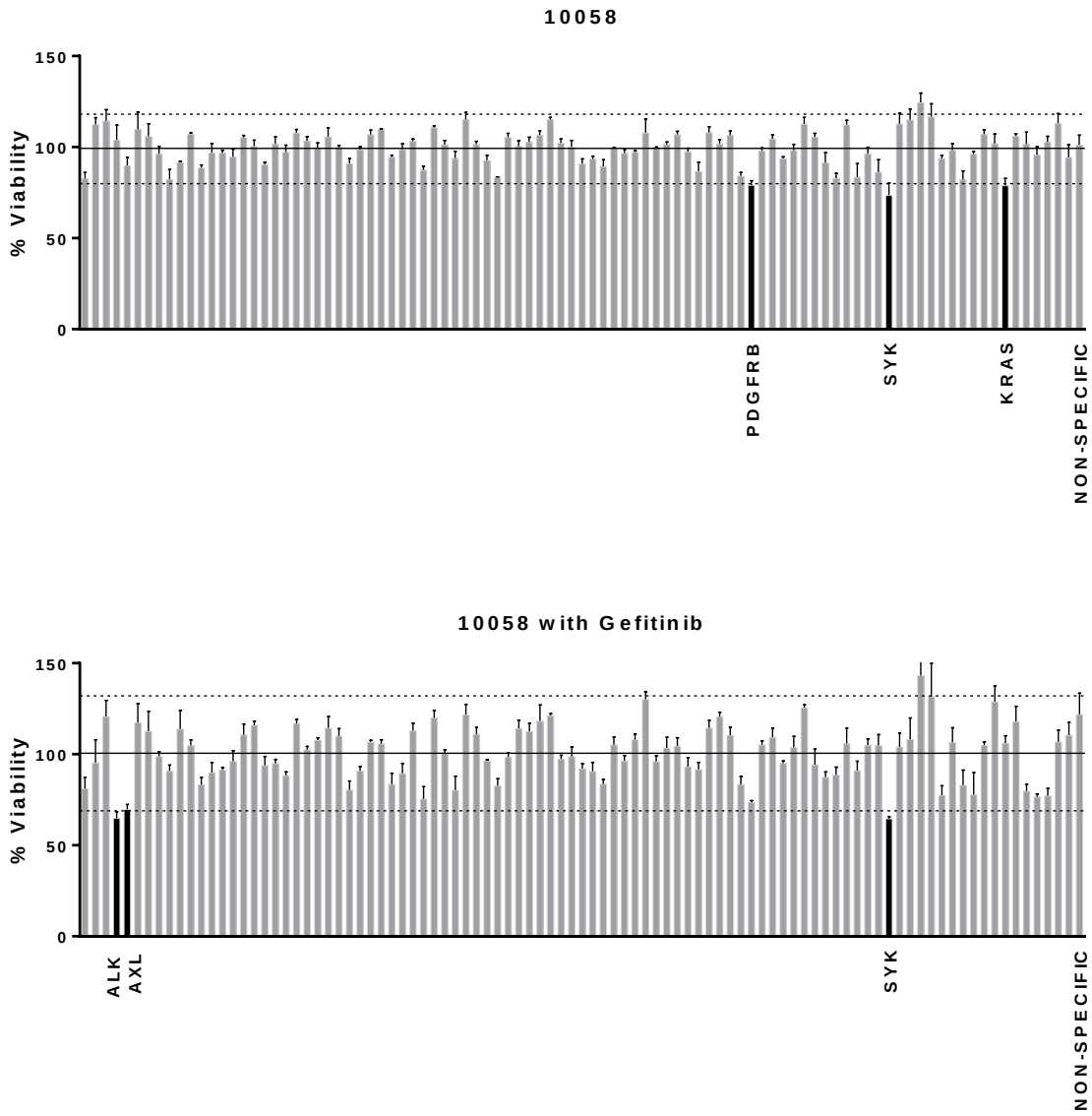


Figure 2-13. RAPID assay result for primary tumor cells from patient 10058.

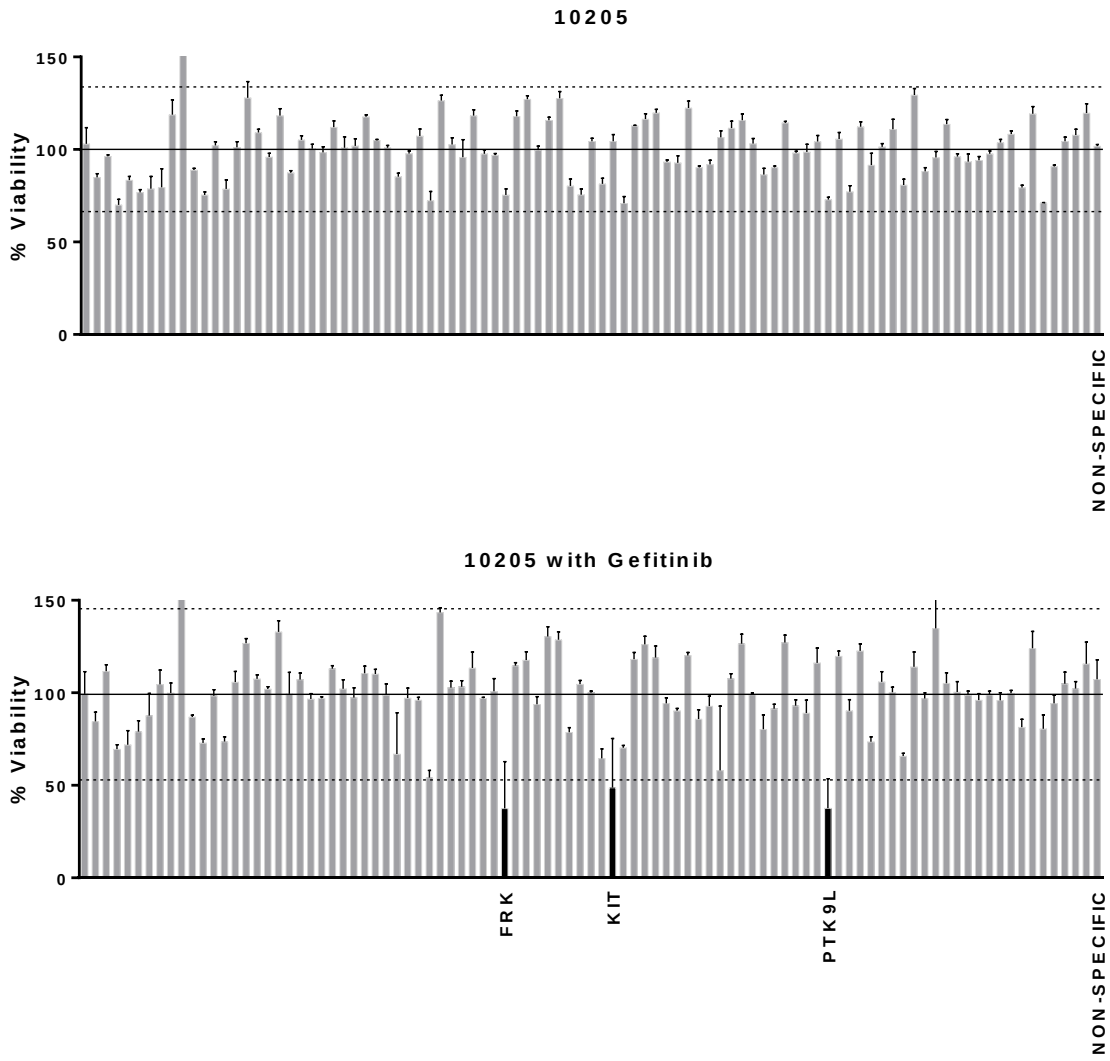


Figure 2-14. RAPID assay result for primary tumor cells from patient 10205.

Consistent with inhibitor assay result, as single agents, none of the siRNAs were effective for this patient; however, after adding 50 nM gefitinib, 3 siRNAs became effective for this patient.

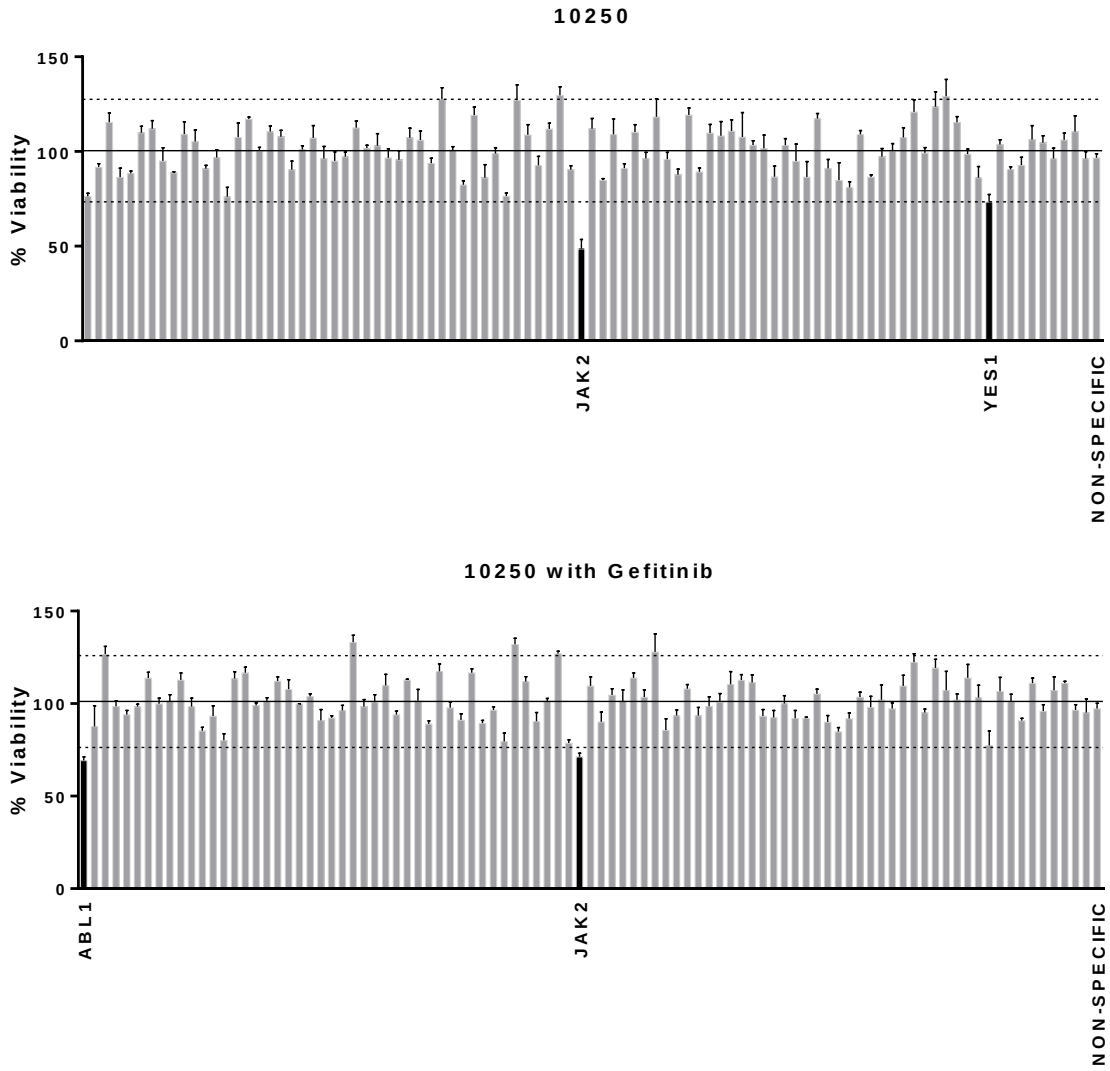


Figure 2-15. RAPID assay result for primary tumor cells from patient 10250.

Inhibitor assays identified ALK and EGFR inhibitors as effective combination therapies in HNSCC patient-derived tumor cells

Notably, 2 out of the 14 drugs that synergized with EGFR inhibitors in inhibitor assays were ALK inhibitors. While ALK inhibitors were effective in only 1 out of 8 patients as single agents (Table 2-5A), with a low dosage of EGFR inhibitor, 4 out of 8 patients' tumor cells became sensitive to ALK inhibitors NVP-TAE-684 and GSK-1838705A (Table 2-5B), suggesting synergistic effects between ALK inhibitors with EGFR inhibitors.

Table 2-5A.

ALK inhibitor alone								
Patient	10004	10021	10054	10058	10139	10159	10205	10250
NVP-TAE-684 IC50 (nM)	3163	3021	1739	364	1550	1623	10000	777
% of median IC50	188%	180%	103%	22%	92%	97%	360%	46%
GSK-1838705A IC50 (nM)	10000	9551	7763	514	10000	10000	10000	10000
% of median IC50	100%	96%	78%	5%	100%	100%	118%	100%

Table 2-5B.

ALK inhibitor with a low dosage of EGFR inhibitor								
Patient	10004	10021	10054	10058	10139	10159	10205	10250
NVP-TAE-684 IC50 (nM)	576	978	491	852	618	296	49	7245
% of median IC50	34%	58%	29%	51%	37%	18%	2%	431%
GSK-1838705A IC50 (nM)	10000	5755	913	133	10000	1996	246	10000
% of median IC50	100%	58%	9%	1%	100%	20%	3%	100%

Table 2-5C.

EGFR inhibitor alone								
Patient	10004	10021	10054	10058	10139	10159	10205	10250
Gefitinib IC50(nM)	1176	10000	10000	10000	10000	10000	10000	10000
% of median IC50	12%	100%	100%	100%	100%	100%	100%	100%
Lapatinib IC50(nM)	6311	10000	10000	9667	10000	4423	10000	10000
% of median IC50	63%	100%	100%	97%	100%	44%	100%	100%
Erlotinib IC50(nM)	1024	10000	10000	10000	5326	2580	10000	10000
% of median IC50	10%	100%	100%	100%	53%	26%	100%	100%

Table 2-5. ALK inhibitors synergize with EGFR inhibitors in inhibitor assays in HNSCC patient-derived tumor cells.

HNSCC patient-derived tumor cells (within 0 to 2 passages) from 8 patients were screened by inhibitor assays. Patient-derived tumor cells were plated in 384-well-plates containing 122 drugs in seven serial dilutions with or without 50 nM of EGFR inhibitor. MTS cell proliferation assay was used to determine the IC50s of the drugs on the panel as single agents or in combination with EGFR inhibitors. IC50s of ALK inhibitors NVP-TAE-684 and GSK-1838705A as single agents (**A**) or in combination with 50nM gefitinib (**B**), as well as IC50s of three EGFR inhibitors as single agents (**C**) are shown.

Effective drugs for an individual patient are defined as the drugs that have IC50s below 20% of the median IC50.

Scale-up experiments confirmed patients' sensitivity to 4 different EGFR and ALK inhibitor combinations in HNSCC patient-derived tumor cells

Patient selection criteria for scale-up experiments

In order to functionally evaluate HNSCC cell responses and their relevance to individual patients, we evaluated patient-derived tumor cells. The demographics and tumor characteristics of patients enrolled in this study include the oral and laryngeal sites predominant in TCGA HNSCC patients and alcohol and/or tobacco use in all but 1 (an HPV positive case), based on our analysis of 279 TCGA HNSCC patients (Table 2-6) (Cancer Genome Atlas Network 2015b).

Patient 10205 was a maxillary sinus SCC case that is anatomically different from other cases; therefore, we focused on oral cavity SCC and larynx SCC which represent the two most prevalent site types in TCGA. Material for sequencing patient 10021 was unavailable; therefore, this case was not followed up in later studies. Case 10159 was a HPV positive case which has different etiology from HPV negative cases and more effective treatment options than HPV negative HNSCC. The HPV positive cases will be expanded and followed-up in separate, later mechanistic studies.

Patient number	10004	10021	10054	10058	10139	10159	10205	10250
Age	64	51	59	83	78	36	54	63
Gender	Female	Female	Male	Male	Female	Female	Female	Male
Stage	T3N2b (IVa)	T2N0MX (II)	T3N0MX (III)	T4aN0MX (IVa)	T2N0MX (II)	T2N2aM0 (IVa)	T4aN1M0 (IVa)	T1N0M0 (I)
Location	Oral Tongue SCC	Lateral tongue SCC	Subglottis larynx SCC	Bilateral glottic larynx SCC	Right maxilla SCC	Base of tongue SCC	Maxillary Sinus SCC	Oral Tongue SCC
HPV	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative
Tobacco	Yes	Yes	Yes	Yes	Yes	No	No	Yes
Alcohol	No	Yes	Yes	Yes	Yes	Unknown	Former Use	Yes
Treatment before surgery	No	No	No	No	No	Radiation	Chemo-radiation	No
EGFR mutation	No	Unknown	Unknown	No	No	No	D725DG	No

Table 2-6. HNSCC patient clinical annotation.

Drug selection for scale-up studies

Based upon these results from inhibitor assays we validated responses in 6 of the patients' tumor cells with 4 ALK inhibitors in total, including 2 FDA approved ALK inhibitors that are not present on the inhibitor panel.

The three EGFR inhibitors, gefitinib, erlotinib and lapatinib, tested in inhibitor assays, all have been tested in clinical trials for HNSCC, with, lapatinib in fewer clinical trials, 11, than gefitinib and erlotinib, 29 and 42, respectively, as of October 2017. There were phase 3 and 4 trials for both gefitinib and erlotinib for HNSCC, starting around the same time, however neither have been approved for HNSCC in any form. Our rationale for using gefitinib in follow-up studies is supported by a study already showing efficacy of gefitinib and a ALK inhibitor TAE684 in HNSCC cell lines and cell line derived xenografts (Gonzales et al. 2016). In addition, gefitinib was reported to have relatively higher concentration in tumor than plasma and longer half-life as follows. A clinical study of NSCLC patients reported that the tumor tissue concentration of gefitinib is markedly higher than the plasma concentration (Haura et al. 2010). By contrast, the tumor/plasma concentration ratio of erlotinib was approximately 63% in a clinical study of lung cancer (Petty et al. 2004). Thus, gefitinib could reach tumor cells more efficiently than erlotinib. In addition, gefitinib has a longer half-life (41 h) than erlotinib (36 h), which indicates there is a more prolonged steady state gefitinib concentration in plasma (Rukazenkov et al. 2009).

Scale-up dose-response experiments

Scale-up dose-response experiments confirmed synergistic effects between the EGFR inhibitor gefitinib used with FDA approved ALK inhibitors ceritinib and brigatinib (Figure 2-16. A–B), and with the ALK inhibitors NVP-TAE-684 and GSK-1838705A (Figure 2-16. C–D) that were used in inhibitor assays. IC50s as single agents and in combination with gefitinib were calculated based on dose-response-curves. In patient 10004's tumor cells, the IC50s of gefitinib and ALK inhibitors as single agents were up to 340 fold and 12 fold higher than as combinations, respectively; In 10058, the IC50s were up to 35 fold and 12 fold higher when used as single agents than when used in combinations, respectively, ranking these cases the highest in the cohort in terms of sensitivity; while in 10250, the IC50s of gefitinib and ALK inhibitors were no greater than 3.9 fold and 1.8 fold higher when used as single agents than when used in combinations, respectively, ranking this case relatively insensitive across all four ALK inhibitors (Figure 2-16. A–D). The IC50s of the combinations between gefitinib and ceritinib, in 10004, 10054 and 10058, and gefitinib and brigatinib in 10058, 10159 and 10004 were within C_{max} (Figure 2-16. A-B) and therefore clinically relevant.

One limitation of the scale-up dose-response experiments in this study is that in 10004, 10054, 10159 and 10139, gefitinib did not inhibit more than 50% of the cell viability even at the highest concentration 20 μM. Concentrations higher than 20 μM will be far beyond clinical achievable dosages, and therefore effects of higher concentrations would have limited clinical relevance. Although a non-linear regression curve fit can predict an IC50 based on the does-response curve, the IC50 lacks accuracy; hence in these four cases 20 μM was shown as the highest IC50.

IC50s showing in Figure 2-16 and combination indices calculated in table 2-8 were based on 3 independent experiments performed at different times and using different cell passage numbers. IC50 calculation is sensitive to the number of cell divisions happening over the course of the assay, which will change when cell division rates vary. As a result, it is possible for IC50 values for a single drug in a single cell line to vary 100-fold or more simply as a consequence of exogenously imposed changes in the rate of division (Hafner et al. 2016; Haibe-Kains et al. 2013). Factors affecting cell division rates include tissue of origin, media composition, culture conditions, and plating density (Hafner et al. 2017). Although we try to control the plating density and culture medium composition to the best of our ability between independent experiments, and we work within 5 passages, division rates of the cancer cells from the same patient may still be different in these 3 independent experiments due to different batches of reagents, different passage numbers and the inherent variability of division rate of epithelial cells. This may explain the variance between independent experiments in scale-up validation cell viability assays (Figure 2-16) and combination index calculations (Table 2-8).

Although ceritinib and brigatinib had similar potency for ALK, with IC50 values of 0.2 nM and 0.6 nM (Table 2-7) (Marsilje et al. 2013; W.-S. Huang et al. 2016), respectively, brigatinib had lower IC50s for all the cases as single agents compared to ceritinib (Figure 2-16. A–B). Interestingly, brigatinib is a ALK/EGFR dual inhibitor (W.-S. Huang et al. 2016; Uchibori et al. 2017), supporting the hypothesis that co-targeting ALK and EGFR

is more effective than inhibiting either alone. Thus, our findings support potential use of this newly FDA approved drug for NSCLC in HNSCC.

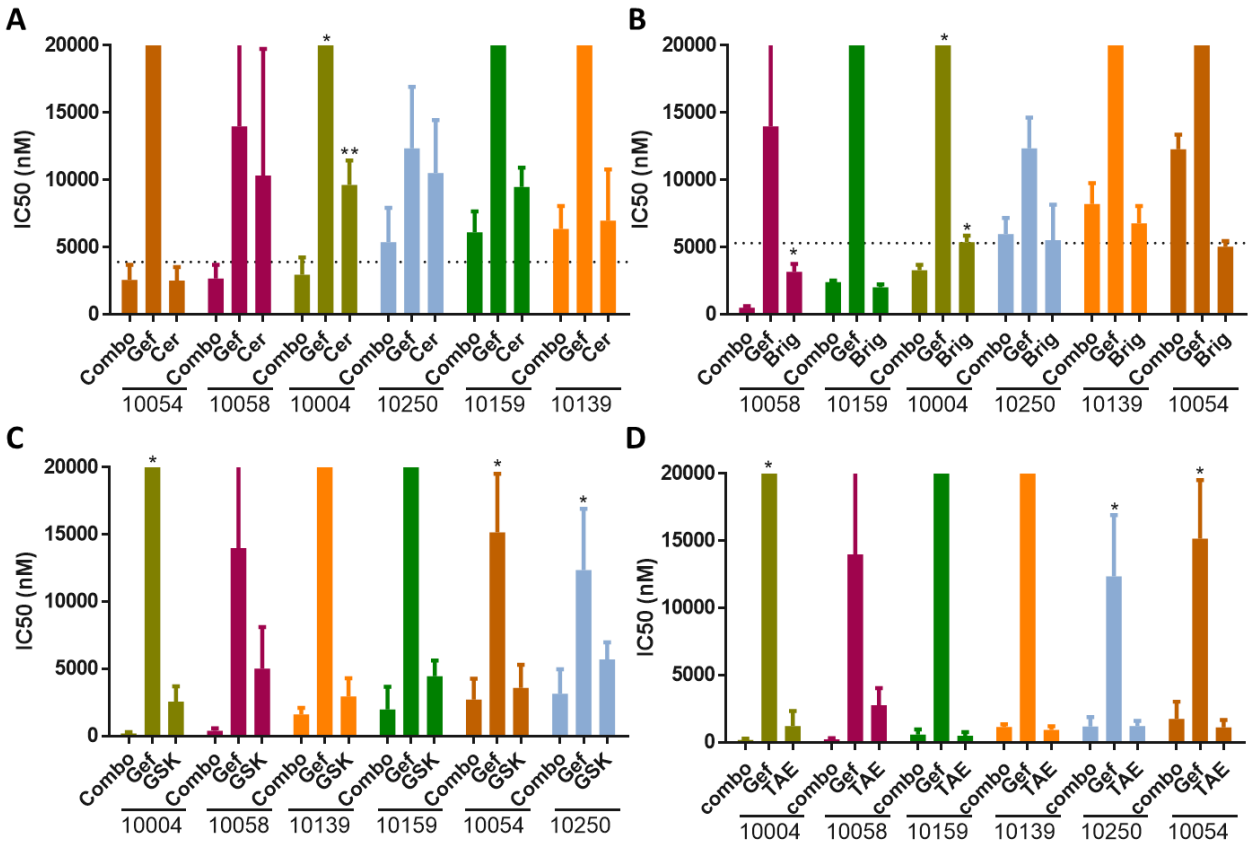

A
IC50 (nM)
20000
15000
10000
5000
0
Combo
Gef
Cer
10054
10058
10004
10250
10159
10139
B
IC50 (nM)
Combo
Gef
Brig
10058
10159
10004
10250
10139
10054
C
IC50 (nM)
Combo
Gef
GSK
10004
10058
10139
10159
10054
10250
D
IC50 (nM)
combo
Gef
TAE
10004
10058
10159
10139
10250
10054

Figure 2-16. Validation of EGFR and ALK inhibitor combinations in patient-derived tumor cells.

Patient-derived tumor cells from patients 10004, 10054, 10058, 10139, 10159 and 10250 were treated with a dose gradient of gefitinib, ALK inhibitors including ceritinib (**A**), brigatinib (**B**), GSK1838705A (**C**) and TAE684 (**D**) or their combinations (**A–D**). Patient maxima plasma concentration (C_{max}) of ALK inhibitors and gefitinib combination were shown as dotted lines in (**A**) and (**B**). After 72 hours, cell viability was assessed using a MTS assay and normalized to vehicle treated cells. Patient numbers are sorted based on the IC50s for each EGFR/ALK drug combination from low to high. Data represents the mean ± SD between three independent experiments (n=3).

	IC50 (nM)					C_{max}	FDA approved	Reference
Drug	ALK	IGF-1R	InsR	ROS1	EGFR			
TAE684	2 - 5[a]	10 - 20b	>1000[a]; 10 - 20[b]	10[a]	Not a target	NA	No	(Galkin et al. 2007b, 684)
GSK1838705A	0.5[b]	2[b]	1.6[b]	NA	>10,000[b]	NA	No	(Sabbatini et al. 2009)
Ceritinib	150[a], 0.2[b]	8[b]	7[b]	72.9[a]	900[b]	2.6 µM	Yes	(Cooper et al. 2015; Khozin et al. 2015; Marsilje et al. 2013)
Brigatinib	0.6[b]	46[b]	45[b]	1.9[b]; 7.4[a]	39.9[a]; 129[b]	4 µM	Yes	(Katayama et al. 2011a, 4)
Gefitinib	NA	NA	NA	NA	33[a]	1.3 µM	Yes	(M. H. Cohen et al. 2003; Brehmer et al. 2005; McKillop et al. 2005; Wakeling et al. 2002)
Note: [a]Cell proliferation assay [b]*In vitro* kinase activity assay								

Table 2-7. IC50s and maxima plasma concentration (C_{max}) of ALK inhibitors and gefitinib.

FDA approved- FDA approved for any disease. NA (Not available)- no data is available on the IC50s, binding affinity, effects on cell proliferation or plasma concentration in the literatures. For Brigatinib and GSK1838705A, *in vitro* kinase activity assays were

performed under optimal ATP concentrations for each enzyme (Km). For ceritinib and TAE684, ATP concentration was not provided in the literature that reported the IC50s.

Combination index

To quantify synergistic effects between gefitinib and ALK inhibitors and to differentiate between synergistic effect versus addictive effects, the level of synergism is measured and quantified using the drug combination index (Chou 2010), a quantitative measure of drug combination effects (Table 2-8). A combination index below 1 indicates synergy between the drug combination, while a combination index above 1 indicates antagonistic effects between the drugs. Thus, the lower the CI the stronger the synergy, and vice versa. Combination indices between gefitinib and all four ALK inhibitors tested in scale-up experiments for tumor cells from patient 10004 and 10058 were below 1, suggesting synergetic effects between gefitinib and these ALK inhibitors in these cases. In 10004, the indices for gefitinib and GSK-1838705A, NVP-TAE-684, ceritinib and brigatinib at IC50 were 0.25, 0.17, 0.13 and 0.13, respectively, suggesting strong synergism between gefitinib and the 4 ALK inhibitors tested.

In 10054, combination indices between gefitinib and all ALK inhibitors but brigatinib were below 1, suggesting synergetic effects between gefitinib and these three ALK inhibitors in these cases. Notably, brigatinib is a ALK and EGFR dual inhibitor, therefore it is logical that adding EGFR inhibitor gefitinib will not result in a synergistic effect in reducing cell viability in these cells. In 10250, although combination indices between gefitinib and all ALK inhibitors but brigatinib were below 1, the absolute IC50s were still

higher than the C_{max} and other cases, therefore this case is still considered as a relatively insensitive case.

Although brigatinib and gefitinib both target EGFR, they also have several other targets (Table 2-7). Aside from EGFR, gefitinib has also been reported to target another EGFR family receptor, Her2 (Moulder et al. 2001), as well as other putative targets including the protein tyrosine kinases BRK, Yes, CSK, and EphB4 and the serine/threonine kinases RICK, GAK, CaMKII, Aurora A, JNK2 and p38 (Brehmer et al. 2005), hence possibly rendering synergistic effects when combined with brigatinib in 10004 and 10058.

Combination Index at IC50:				
Drug	10004	10058	10054	10250
Gefitinib+Brigatinib	0.2±0.05	0.1±0.06	1.3±0.32	0.79±0.02
Gefitinib+Ceritinib	0.16±0.04	0.27±0.24	0.55±0.16	0.5±0.28
Gefitinib+TAE684	0.06±0.03	0.7±0.56	0.61±0.6	0.44±0.47
Gefitinib+ GSK1838705A	0.38±0.2	0.66±0.6	0.33±0.12	0.17±0.11

Table 2-8. Combination index of gefitinib and ALK inhibitors at IC50 for 10004, 10054, 10058 and 10250. Mean combination indices ± 95% confidence intervals that represent 3 independent experiments are shown.

siRNA confirmed a synergistic effect between siALK and EGFR inhibitor in HNSCC patient-derived tumor cells

While anti-cancer drugs have reported targets, nearly all, including those FDA approved drugs have additional targets besides the presumed target (Law et al. 2014) (Table 2-7). To rule out the possibility that the drug combinations' effect on reducing cell viability was due to what is referred to as "off-target" effects, we performed RAPID assays (J. W. Tyner et al. 2009) in the EGFR/ALK inhibitor combination sensitive and insensitive patients' patient-derived tumor cells. In RAPID assays, we exposed tumor cells to pooled siRNAs consisting of 4 different sequences of the siRNAs targeting the same gene in order to validate true targets responsible for reduction in tumor cell viability by the drugs. We transfected HNSCC patient-derived tumor cells from ALK inhibitor sensitive patients (10004, 10054 and 10058) and a relatively insensitive patient (10250) with a panel of siRNAs targeting the entire tyrosine kinase gene family in addition to NRAS and KRAS (93 genes total) (J. W. Tyner et al. 2009) as well as non-specific siRNA controls.

An effective siRNA was defined as a siRNA reduced cell viability below mean viability of the whole panel minus two standard deviations (SD) and statistically different from the non-specific siRNA controls.

siRNA against ALK alone did not reduce relative cell viability significantly in any of the patients' tumor cells. In 10004 and 10054, although reduction in viability by siALKs was statistically significant different from the non-specific siRNA controls, it was not below the pre-defined cut-off mean-2SD in any of the cases, and therefore not considered as effective. However, after adding 50 nM gefitinib to the siRNA panel, siRNA targeting

ALK reduced the relative cell viability 2SD below the mean in tumor cells derived from patients 10004, 10054 and 10058 (Figure 2-17-17. A-E), but not in 10250 (Figure 2-17-17. E–F), suggesting synergistic effects between siALK with gefitinib, and consistent with the effects using EGFR and ALK inhibitor combinations. In addition, based on the results of these siRNA screens, potential alternative targets, such as IGF-1R, insulin receptor (InsR) and ROS1, which are common targets of ALK inhibitors, including NVP-TAE-684, GSK-1838705A, ceritinib and brigatinib, but with lower affinity than ALK (Table 2-7) (Sabbatini et al. 2009; Katayama et al. 2011a; Galkin et al. 2007b, 684; Davare et al. 2015; Marsilje et al. 2013), were ruled out as true targets of the inhibitor combinations. The siRNAs targeting EGFR were not effective regardless of gefitinib addition (Figure 2-17) and hence brigatinib was not effective due to targeting EGFR when in combination with gefitinib.

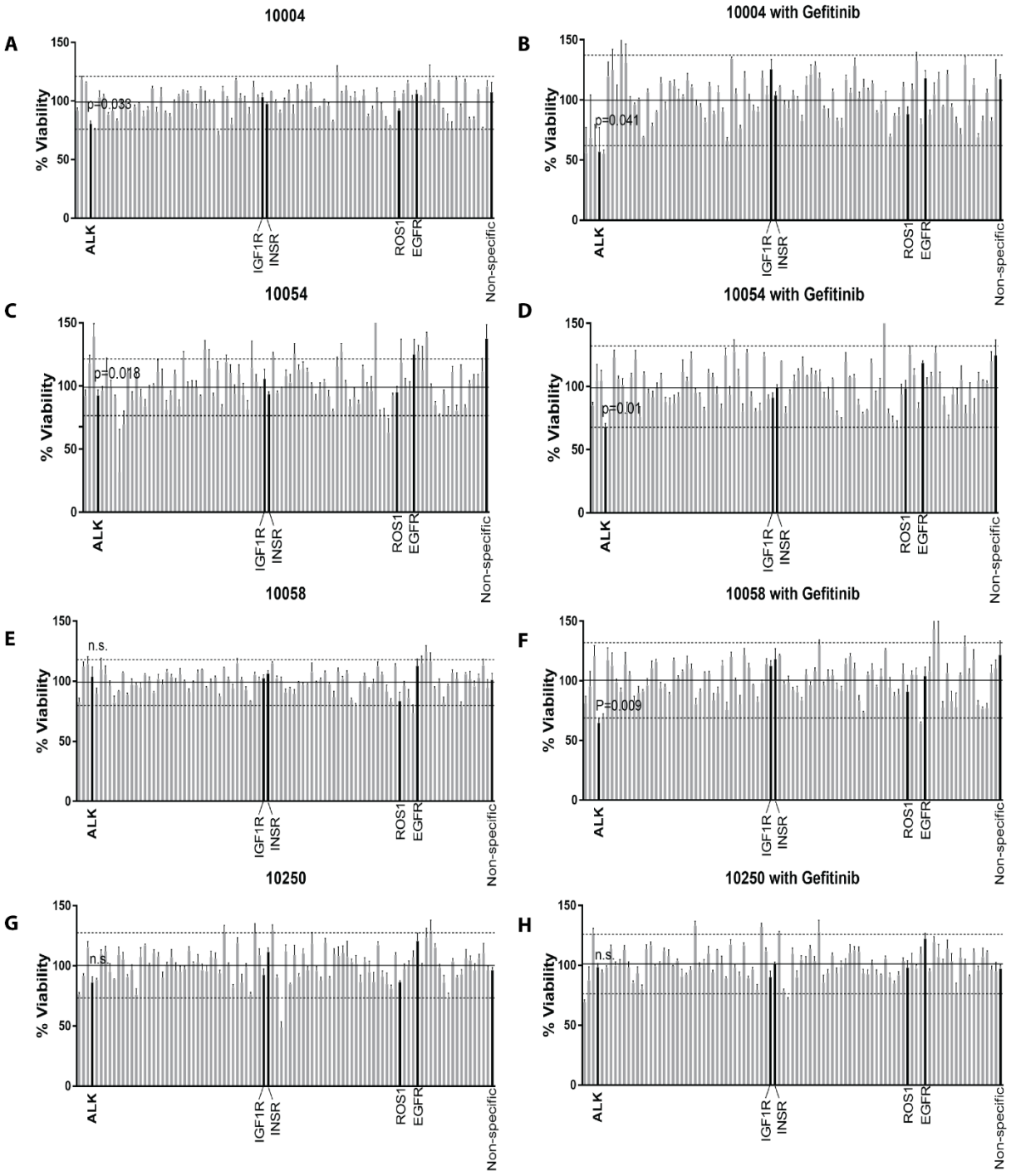

A
10004
% Viability
p=0.038
ALK
IGF1R
INSR
ROS1
EGFR
Non-specific
B
10004 with Gefitinib
p=0.041
C
10054
p=0.018
D
10054 with Gefitinib
p=0.01
E
10058
n.s.
F
10058 with Gefitinib
P=0.009
G
10250
n.s.
H
10250 with Gefitinib
n.s.

Figure 2-17. Synergy between siALK and gefitinib in HNSCC patient-derived tumor cells.

Patient-derived tumor cells from patients indicated were transfected with siRNA pools individually targeting each member of the receptor tyrosine kinome in addition to NRAS and KRAS co-treated with vehicle (**A**, **C**, **E** and **G**) or with 50nM gefitinib (**B**, **D**, **F** and **H**). Cell viability was calculated by normalizing absorbance at 490 nM (as determined by the MTS assay) to the median plate value after 96 hours of treatment. Dotted lines indicate mean ± 2SD. Effective siRNAs are defined as those that inhibit cell viability 2SD below the mean-of all siRNAs and were statistically different from the non-specific siRNA controls. Error bar represents the mean ± SEM, each containing three replicates (n = 3).

Co-targeting EGFR and ALK decreased HNSCC patient tumor cell number, colony formation ability and increased annexin V staining

To further determine the effects of EGFR and ALK inhibition in HNSCC patient- derived tumor cells, cell number, colony formation ability and annexin V staining were evaluated after treatment with EGFR/ALK inhibitor or siRNAs.

In scale-up experiments, IC50s for gefitinib in all patients were higher than C_{max} (1.33 μM). Therefore, we used 1 μM, a concentration of gefitinib that was lower than $C_{max,}$ as single agent in follow-up studies, and this concentration of gefitinib showed the capacity to inhibit phosphorylation of EGFR in our cells. We used 600 nM, a concentration of ceritinib that showed the capacity to inhibit phosphorylation of ALK in the literature (Friboulet et al. 2014), as single agent the following scale-up experiments. Half of the concentration of the single agents of these two drugs were used in combination groups to test the synergetic effects rather than additive effects between the two drugs.

Co-targeting EGFR and ALK by gefitinib and ceritinib significantly reduced patient 10004 and 10054's cell number by nucleus staining (Figure2-18A and Figure 2-19. A), consistent with the cell viability reduction, suggesting EGFR and ALK were not just important in cell metabolism. In 10250, although gefitinib and ceritinib combination significantly decreased cell number compared to vehicle control (Figure 2-19. B) (Figure 2-20. C), the absolute IC50s (Figure 2-1. A-B) were still higher than the C_{max}, therefore this case was considered relatively insensitive. The reduction of cell number by gefitinib and ceritinib combination in tumor cells from 10250 may due to a loss of attachment of cells, which may still be viable, such that the cell viability was not reduced significantly

in the whole population of tumor cells (Figure 2-1. A-B). This hypothesis could be tested by evaluating the cell viability of floating cells across cases.

To determine the effects of co-targeting EGFR and ALK on cell reproductive ability, colony formation assay was used to determine the ability of single cell to grow into a colony. Normal cells are reported to cease to divide after about 50 passages regardless of the density as they reach the Hayflick limit and become senescent (Shay and Wright 2000). The assay measures the extent to which every cell in the population is able to undergo cell division. Colony formation ability in 10004 was statistically significantly reduced when EGFR and ALK were simultaneously co-targeted by siRNAs compared to single siRNAs, suggesting that co-targeting EGFR and ALK could impair cancer cell's reproductive ability.

To determine the effects of EGFR and ALK inhibitor combination on cell apoptosis, annexin V staining followed by flow cytometry analysis was performed. Annexin V is a member of the annexin family of intracellular proteins that binds to phosphatidylserine (PS) in a calcium-dependent manner. PS is normally only found on the intracellular leaflet of the plasma membrane in healthy cells, but during early apoptosis, membrane asymmetry is lost and PS translocates to the external leaflet. Fluorochrome-labeled annexin V can then be used to specifically target and identify apoptotic cells. To help distinguish between the necrotic and apoptotic cells, propidium iodide (PI) was used. Early apoptotic cells will exclude PI, while late stage apoptotic cells and necrotic cells will stain positively, due to the passage of these dyes into the nucleus where they bind to DNA. Because PI positive cells are either late stage apoptotic cells or necrotic cells, and they are not distinguishable, annexin V positive and PI negative cells that were early

apoptotic cells were plotted (Figure 2-28. C). Gefitinib and ceritinib combination statistically significantly increased annexin V positive and PI negative cell population compared to single agents, suggesting that co-targeting EGFR and ALK could increase cell apoptosis (Figure 2-18. A–C).

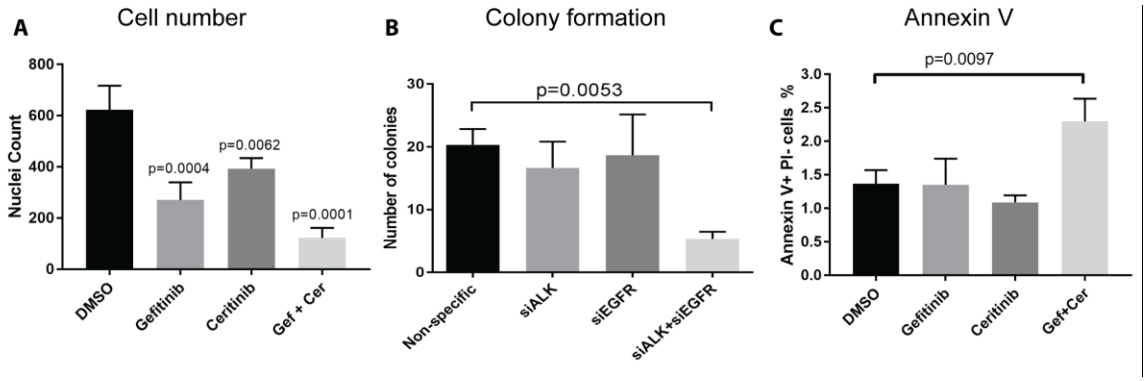


Figure 2-18. Co-targeting EGFR and ALK decreases HNSCC patient's tumor cell number, cell colony formation ability and increases cell apoptosis in patient 10004 derived tumor cells.

A, Patient 10004 derived tumor cells were treated with 1 μM gefitinib, 600 nM ceritinib or their combination for 72h, cells were fixed and stained by DAPI and cell number was counted. **B**, Patient 10004 derived tumor cells were transfected with siRNA pools targeting ALK, EGFR or their combination for 96 hours; cell colony formation ability was determined by crystal violet staining after 12 days. **C**, Patient 10004 derived tumor cells were treated with 1 μM gefitinib, 600 nM ceritinib, or the combination of 500 nM gefitinib and 300 nM ceritinib for 72 hours. Cells were stained by annexin V and PI, and flow cytometry was performed to determine the ratio of annexin V positive and PI negative cells for each group. Data represents the mean ± SD, each containing three replicates (n = 3). A one-way ANOVA was performed for statistical analysis.

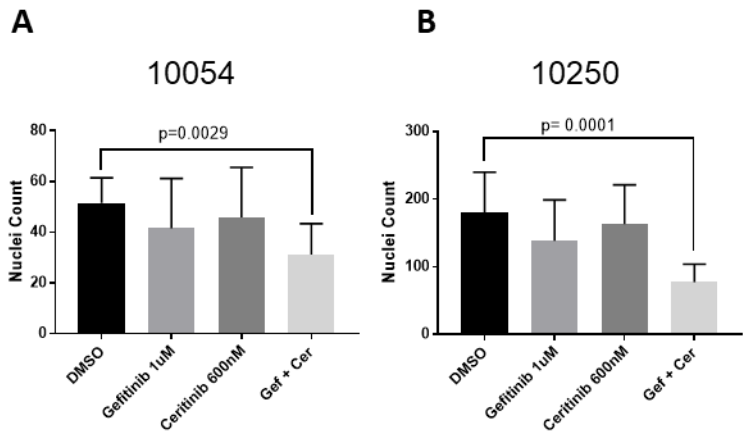


Figure 2-19. Cell nuclear count after gefitinib and ceritinib treatment in tumor cells from 10054 and 10250.

Patient derived tumor cells from 10054 (**A**) and 10250 (**B**) were treated with 1 μM gefitinib, 600 nM ceritinib and their combination for 48h in triplicate 24-well-plate on poly-l-lysine treated coverslips. Cells were fixed and stained with DAPI after treatment. Data represents the mean ± SD, each containing three replicates (n = 3). A one-way ANOVA was performed for statistical analysis.

Synergy between ALK inhibitor and gefitinib is associated with induced phosphorylation and/or expression of ALK after gefitinib treatment in patient tumor-derived 2D cultures, spheroids and xenografts

Hypothesis of ALK induction

Oncogenic ALK has been reported in various cancers as fusions (Mologni 2012). However, full-length ALK expression due to point mutations and constitutive activation were reported in numerous cancers including neuroblastoma, neuroectodermal tumors, melanoma and glioblastoma (Grzelinski et al. 2009; Miyake et al. 2002; Powers et al. 2002; Webb et al. 2009; Wellstein 2012). Further, wild type expression of ALK was also strongly correlated with poor prognosis in cancer patients (Passoni et al. 2009) and a significant correlation was reported between 140 kDa ALK protein levels and ALK inhibitor TAE684 response in wild type neuroblastoma cell lines (Duijkers et al. 2011), suggesting targeting wild type ALK in cancer may also be beneficial.

Multiple ALK inhibitors that were developed to target NSCLCs with ALK rearrangements were successful in achieving marked tumor shrinkage. However, it has been reported that a group of NSCLC patients expressing EML4-ALK fusion protein initially respond very well to the ALK small molecule inhibitor, crizotinib, but eventually develop resistance to ALK inhibition via induction of EGFR bypass signaling of ALK (Katayama et al. 2011b, 2012; Koivunen et al. 2008; Y.-W. Wang et al. 2011). The mechanism underlying the cross-talk between EGFR and ALK is unclear.

ALK alterations are not commonly detected (5% according to TCGA cBioPortal analysis) in HNSCC (Cancer Genome Atlas Network 2015b; Cerami et al. 2012) (Figure.

2-20). Exome sequencing mutation calling analysis showed no ALK mutations in our OHSU cohort patients' tumors. RNAseq analysis of our HNSCC patients' native tumors and patient-derived tumor cells without any treatment showed low expression levels of ALK mRNA. However, this could not explain the response of 4 out of 8 HNSCC patients' tumor cells to ALK inhibitors when EGFR inhibitors was added to the inhibitor assays. One logical explanation would be that EGFR inhibitor upregulated ALK expression and hence EGFR inhibition was thwarted by ALK parallel signaling in tumors. Therefore, only when EGFR and ALK inhibitors were used in combination would a significant cell viability reduction be observed.

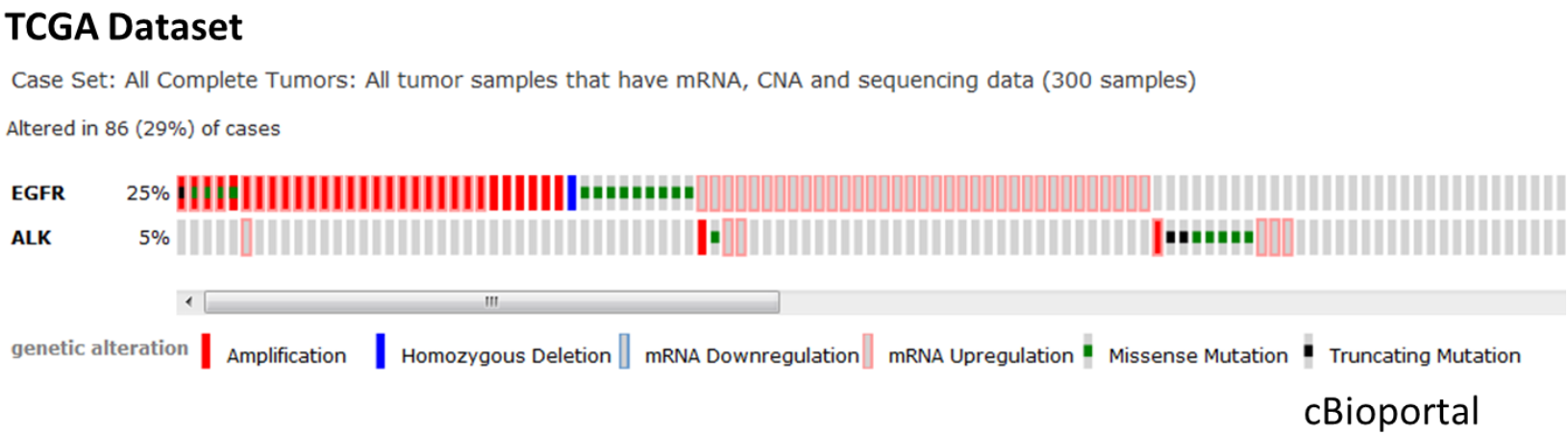


Figure 2-20. EGFR and ALK alterations in the cancer genome atlas (TCGA) HNSCC cohort.

ALK induction in primary cell cultures

Based upon the idea that compensating pathways are activated after monotherapy treatment of anti-cancer drugs, I hypothesized that synergy between EGFR and ALK inhibition was due to induction of ALK activation and expression levels by EGFR inhibitor, as a mechanism to explain why ALK inhibitors were not effective as single agents but showed significant effects when combined with an EGFR inhibitor. I tested this hypothesis by determining ALK phosphorylation and expression levels in patient - derived tumor cells after EGFR inhibition by gefitinib, first examining a wide range of gefitinib concentrations at single time point, then selecting a limited range of concentrations over extended range of times. In patient 10004 tumor derived cells, over the range of 0.03-9 μM of gefitinib for 24h, ALK protein was induced up to 3 fold in a dose-dependent manner from 0.03-1 μM concentration, and plateaued at 1 μM (Figure 2-21). Therefore 0.03-1 μM of gefitinib was used in tumor cells and ALK induction was measured at 4 different time points (6h, 24h, 48h and 72h) in the following time-course experiments.

In the time-course experiment, up to 5.6 fold increases in total levels of ALK protein compared to vehicle controls were detected by western blotting in patient 10004's tumor cells after 48 hours of 0.03 μM gefitinib treatment, with increases in phosphorylation of ALK up to 3.7 fold (Figure 2-22. A–E). Notably, EGFR phosphorylation was inhibited early (at 6 hours) as expected and restored after 48 hours gefitinib treatment.

Although patient 10058's tumor cells were also sensitive to EGFR/ALK inhibitor combinations, increases in ALK protein level were small, up to 1.3 fold (Figure. 2-23). Notably, 10058's tumor cells exhibited a higher basal level of total ALK expression

compared to 10004 (Figure 2-24). This is one explanation for the initial sensitivity of 10058 to ALK inhibitor GSK1838705A and TAE684 as single agents in the inhibitor assay (Table 2-3), possibly due to less reliance upon induction of ALK for ALK inhibitor sensitivity in this case. Although 10054's sensitivity to gefitinib and four ALK inhibitors in scale-up studies is variable (Figure 2-16), RAPID assay (Figure 2-17) and cell nuclear staining (Figure 2-19. A) showed sensitivity to ALK inhibition in combination with gefitinib, and induction of ALK protein after gefitinib treatment up to 2.1 fold was detected (Figure. 2-23). In contrast, ALK protein was not induced above vehicle control in patient 10250's tumor cells, the case that was relatively resistant to the EGFR/ALK inhibitors as shown in Figure 2-16.

Of note, a previously reported (Iwahara et al. 1997; Morris et al. 1997; Moog-Lutz et al. 2005) but less investigated 140 kDa ALK form, rather than the full length 220 kDa form, was induced by gefitinib in these HNSCC patient-derived tumor cells, warranting further investigation of distinct oncogenic roles of ALK forms in HNSCC and other cancers. ALK 140 kDa has been reported as ALK-specific band and is frequently detected in untreated ALK positive cell line, including Jurkat/neo cells (Mourali et al. 2006), a panel of neuroblastoma (NBL) cell lines (Duijkers et al. 2011) and SK-N-SH cell line that we used in our western blots as a ALK positive control. This band most probably results from an extracellular cleavage or processing of ALK, as indicated by other groups (Moog-Lutz et al. 2005; Morris et al. 1994; Mourali et al. 2006). The LC50 (the drug concentration lethal to 50% of the population) of ALK inhibitor TAE684 was highly correlated with ALK mRNA, ALK 220 and 140 kDa protein levels in all NBL cell lines (Duijkers et al. 2011). A significant correlation was present between 140 kDa ALK

protein levels and TAE684 response in only wild type cell lines (Duijkers et al. 2011), suggesting the association between ALK inhibitor sensitivity and 140 kDa ALK. These evidence support the hypothesis that the induction of 140 kDa ALK was responsible for ALK inhibitor sensitivity in combination of gefitinib in ALK wild type HNSCC.

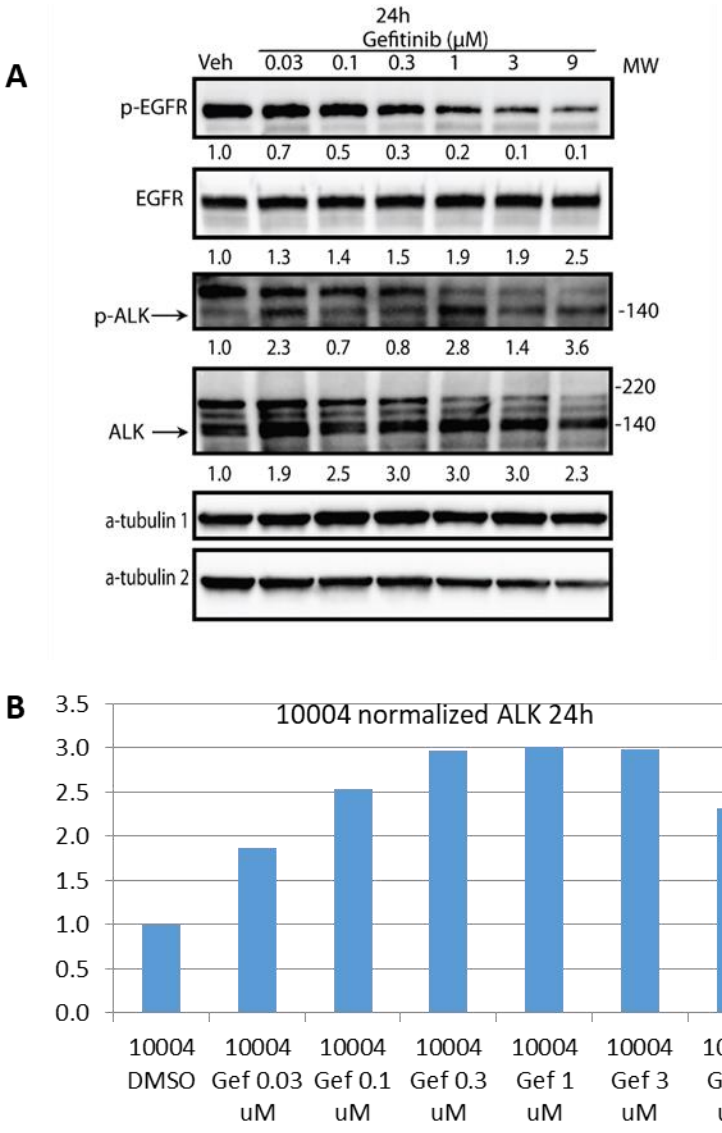


Figure 2-21. ALK protein and phosphorylation levels after gefitinib treatment in HNSCC patient-derived tumor cells.

Patient-derived tumor cells from patient 10004 were treated with gefitinib at 0.03-9 μM or vehicle for 24 hours. Levels of total and phospho-EGFR and ALK as well as alpha-

tubulin were assessed by immunoblot analysis. Total and phospho-ALK bands are indicated by arrows (**A**). Quantification of total levels of ALK is shown in (**B**).

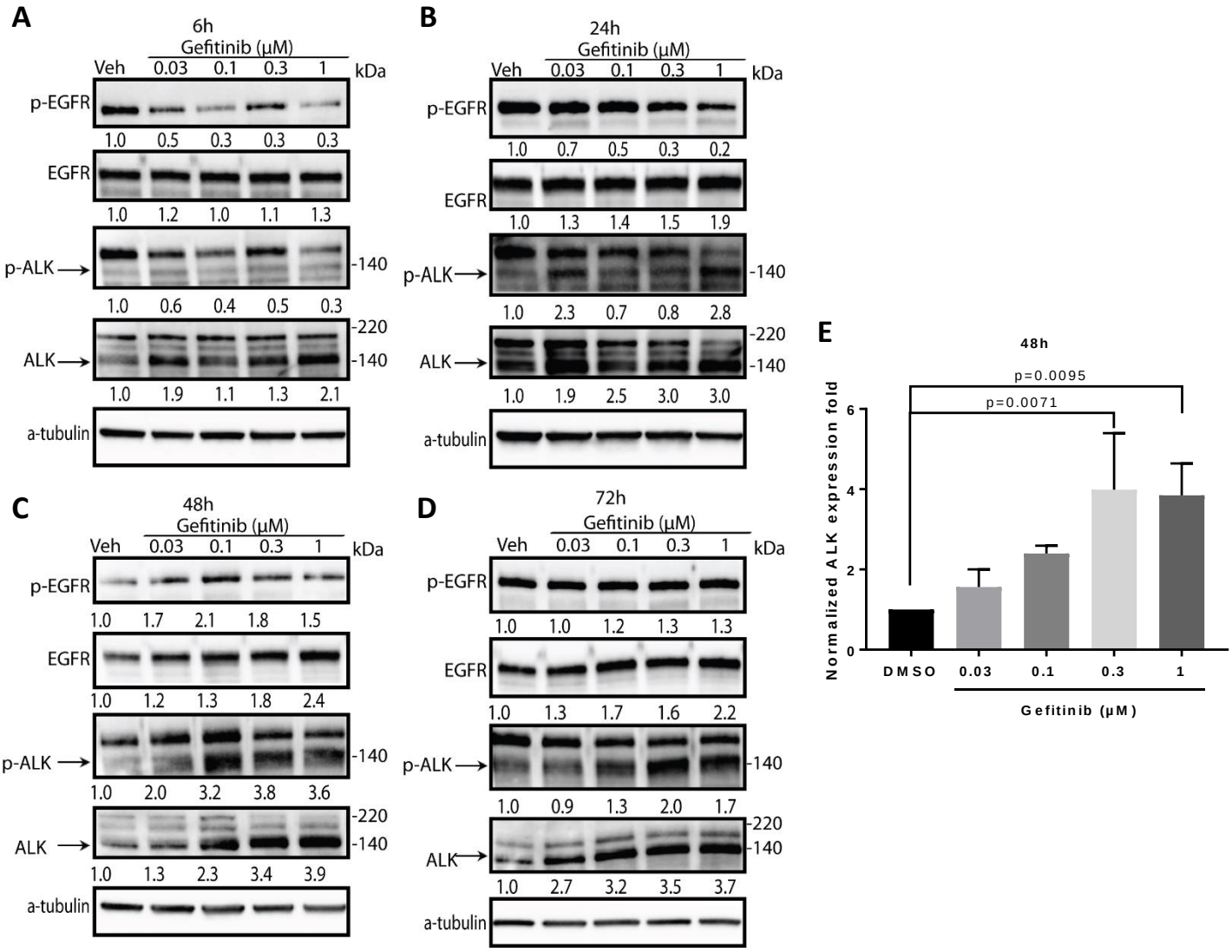

A
6h
Gefitinib (μM)
Veh 0.03 0.1 0.3 1 kDa
p-EGFR
1.0 0.5 0.3 0.3 0.3
EGFR
1.0 1.2 1.0 1.1 1.3
p-ALK
-140
1.0 0.6 0.4 0.5 0.3
-220
ALK
-140
1.0 1.9 1.1 1.3 2.1
a-tubulin
B
24h
Gefitinib (μM)
Veh 0.03 0.1 0.3 1 kDa
p-EGFR
1.0 0.7 0.5 0.3 0.2
EGFR
1.0 1.3 1.4 1.5 1.9
p-ALK
-140
1.0 2.3 0.7 0.8 2.8
-220
ALK
-140
1.0 1.9 2.5 3.0 3.0
a-tubulin
C
48h
Gefitinib (μM)
Veh 0.03 0.1 0.3 1 kDa
p-EGFR
1.0 1.7 2.1 1.8 1.5
EGFR
1.0 1.2 1.3 1.8 2.4
p-ALK
-140
1.0 2.0 3.2 3.8 3.6
-220
ALK
-140
1.0 1.3 2.3 3.4 3.9
a-tubulin
D
72h
Gefitinib (μM)
Veh 0.03 0.1 0.3 1 kDa
p-EGFR
1.0 1.0 1.2 1.3 1.3
EGFR
1.0 1.3 1.7 1.6 2.2
p-ALK
-140
1.0 0.9 1.3 2.0 1.7
-220
ALK
-140
1.0 2.7 3.2 3.5 3.7
a-tubulin
E
48h
p=0.0095
p=0.0071
Normalized ALK expression fold
0
2
4
6
DMSO
0.03
0.1
0.3
1
Gefitinib (μM)

Figure 2-22. ALK protein and phosphorylation levels increase after gefitinib treatment in HNSCC patient-derived tumor cells.

Patient-derived tumor cells from patient 10004 were treated with gefitinib at 0.03-1 μM or vehicle for 6 hours (**A**), 24 hours (**B**), 48 hours (**C**) and 72 hours (**D**). Levels of total and phospho-EGFR and ALK as well as alpha-tubulin were assessed by immunoblot analysis. Total and phospho-ALK bands are indicated by arrows. **E**, Quantification of ALK protein expression after 48 hours of gefitinib treatment. Data represents the mean ± SD, each containing three independent experiments (n = 3).

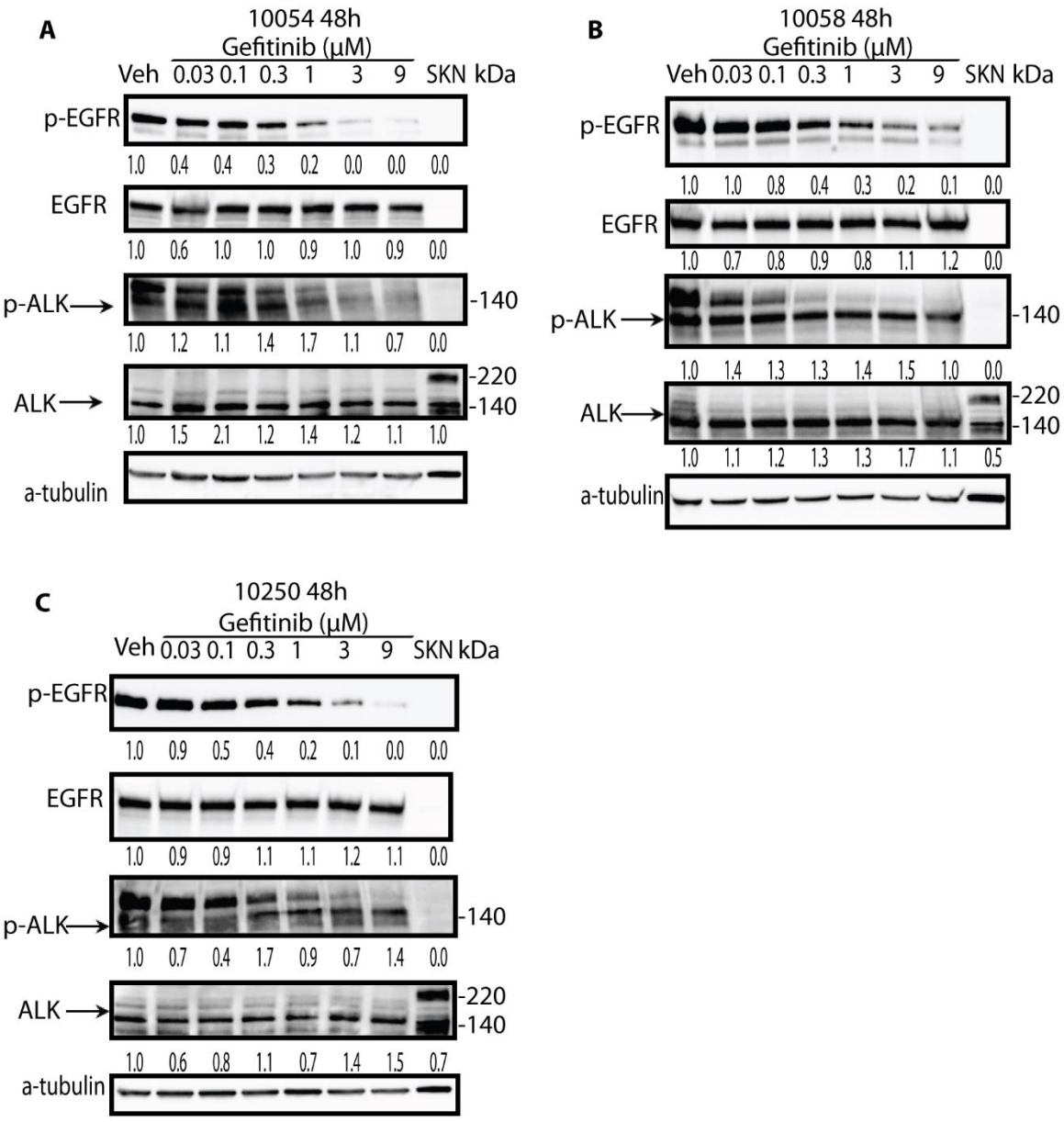

A
10054 48h
Gefitinib (μM)
Veh 0.03 0.1 0.3 1 3 9 SKN kDa
p-EGFR
1.0 0.4 0.4 0.3 0.2 0.0 0.0 0.0
EGFR
1.0 0.6 1.0 1.0 0.9 1.0 0.9 0.0
p-ALK
-140
1.0 1.2 1.1 1.4 1.7 1.1 0.7 0.0
ALK
-220
-140
1.0 1.5 2.1 1.2 1.4 1.2 1.1 1.0
a-tubulin
B
10058 48h
Gefitinib (μM)
Veh 0.03 0.1 0.3 1 3 9 SKN kDa
p-EGFR
1.0 1.0 0.8 0.4 0.3 0.2 0.1 0.0
EGFR
1.0 0.7 0.8 0.9 0.8 1.1 1.2 0.0
p-ALK
-140
1.0 1.4 1.3 1.3 1.4 1.5 1.0 0.0
ALK
-220
-140
1.0 1.1 1.2 1.3 1.3 1.7 1.1 0.5
a-tubulin
C
10250 48h
Gefitinib (μM)
Veh 0.03 0.1 0.3 1 3 9 SKN kDa
p-EGFR
1.0 0.9 0.5 0.4 0.2 0.1 0.0 0.0
EGFR
1.0 0.9 0.9 1.1 1.1 1.2 1.1 0.0
p-ALK
-140
1.0 0.7 0.4 1.7 0.9 0.7 1.4 0.0
ALK
-220
-140
1.0 0.6 0.8 1.1 0.7 1.4 1.5 0.7
a-tubulin

Figure 2-23. ALK protein and phosphorylation levels after gefitinib treatment in 10054, 10058 and 10250 patient-derived tumor cells.

Patient-derived tumor cells from patient 10054 (**A**), 10058 (**B**) and 10250 (**C**) were treated with gefitinib at 0.03-9 μM or vehicle for 48 hours. ALK positive NSCLC cell line SK-N-SH was used as positive controls. Levels of total and phospho-EGFR and ALK as well as alpha-tubulin were assessed by immunoblot analysis. Total and phospho-ALK bands are indicated by arrows.

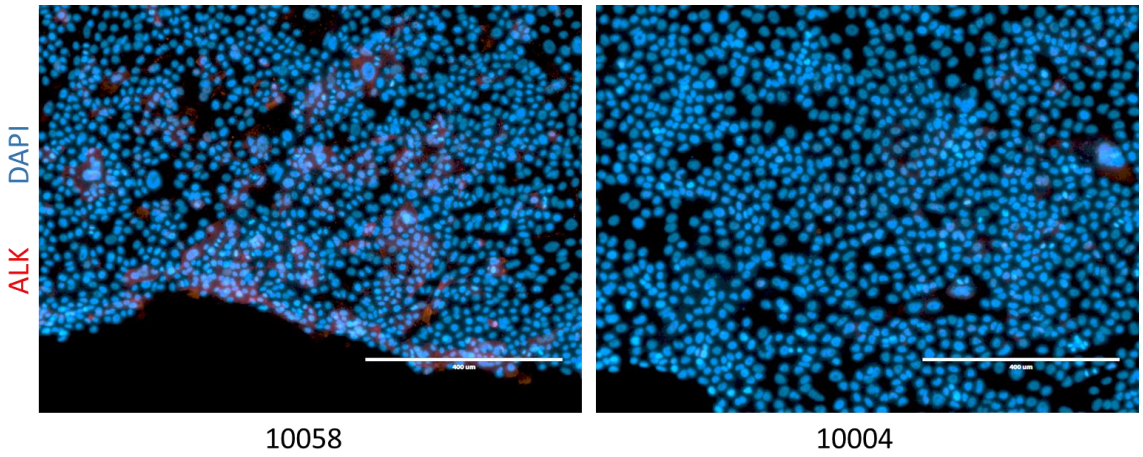


Figure 2-24. Basal levels of ALK protein in 10058 and 10004 patient-derived tumor cells.

Immunofluorescent staining of ALK (red) and nucleus (blue) of patient-derived tumor cells from 10058 and 10004. Scale bar = 400 μm.

ALK induction in patient-derived spheroids

To evaluate the mechanism of EGFR inhibitor resistance under culture conditions considered more relevant to tissue than 2D culture, a spheroid model using patient-derived-tumor cells was established. ALK protein was determined by immunofluorescent staining in gefitinib or vehicle treated spheroids after treatment for 72 hours. Consistent with our prediction, ALK protein levels were induced in 10004 spheroids treated with 1μM gefitinib compared to vehicle controls (Figure 2-25). These results support ALK induction and activation after EGFR inhibition as a mechanism underlying the observed synergistic effect of EGFR and ALK inhibitors in combination. Interestingly, ALK expression was more prominent at the margins of shepherds (Figure 2-25), consistent with the finding that ALK is involved in invasion property of the cancer cells in HNSCC (T.-T. Huang et al. 2013). Establishment of spheroids was attempted in 10054, 10058 and 10250, however, the successful rate of the formation of spheroids varied, and more optimization will be needed to establish spheroid models using these cases.

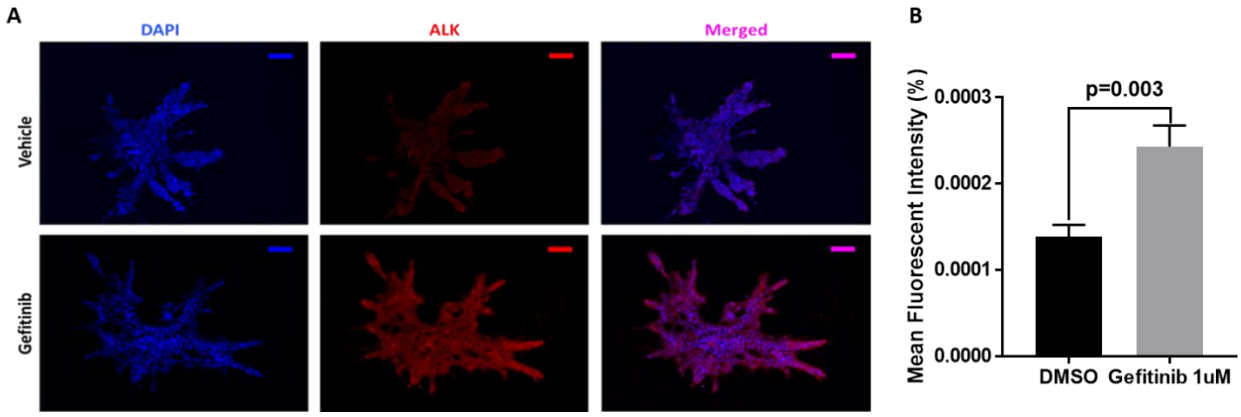


Figure 2-25. ALK induction in patient-derived spheroids.

A, Immunofluorescent staining of ALK protein (red) and the nucleus (blue) on 10004 patient tumor cell derived spheroids treated with gefitinib 1 μM or vehicle. Scale bar = 100 μm. **B**, Data represents the mean fluorescent intensity of ALK normalized to the volume of the gefitinib treated (n=9) and vehicle treated (n=5) spheroids.

ALK induction in patient-derived xenografts

To test the hypothesis of ALK induction by gefitinib *in vivo*, patient-derived xenograft models were generated in our lab. To select a suitable mouse stain for engrafting, three mouse stains, NOD scid gamma (NSG) mice, NSG hairless mice and athymic nude mice, were subcutaneously injected with patient-derived tumor cells from patient 10004. Tumor growth curves from xenografts using these three strains were evaluated (Figure 2-26). Tumors grew significantly slower in NSG hairless mice and athymic nude mice compared NSG mice (Figure 2-26); therefore, NSG mice were chosen for following studies.

Patient-derived tumor cells from patient 10004 were subcutaneously injected into NSG mice. Mice were treated with 100 mg/kg gefitinib or vehicle for 48 hours. Morphology of xenograft tumors and the original patient tumor were evaluated by means of H&E staining. Similar differentiation status was found in xenograft tumors and the original tumor (Figure 2-27A). Levels of ALK RNA and protein were assessed by qRT-PCR and immunofluorescent staining, respectively. ALK RNA expression levels tended to be increased after 48 hours of gefitinib treatment (Figure 2-27. B), with p value of 0.0818, suggesting ALK upregulation, at least partially, was at RNA level. Matched RNA and protein data suggested that a threshold of RNA induction may be required for ALK protein induction, since that the protein was over 10 fold expressed in 4/5 gefitinib treated mice and 0/6 vehicle mice, and the RNA is expressed 1 to 2 fold in all 4 mice with 10 fold protein induction (Figure 2-27. E). Whether the mechanism was via directly increasing transcription can be determined by a nuclear run-on assay, which can detect the genes that are in the process of being transcribed. However, RNA stability, post-

transcriptional, translational and post-translational regulation may also play roles in ALK induction in these tumors and will be discussed in chapter 3.

Immunofluorescent staining showed statistically significant induction of ALK protein in 10004 patient-derived xenograft tumors treated with gefitinib compared to vehicle treated ones, which is consistent with patient-derived cell culture and spheroid results.

Together, phosphorylation and/or expression of ALK after gefitinib treatment were induced in patient tumor-derived 2D cultures, spheroids and xenografts, which may be the mechanism underlying synergy between ALK inhibitors and gefitinib.

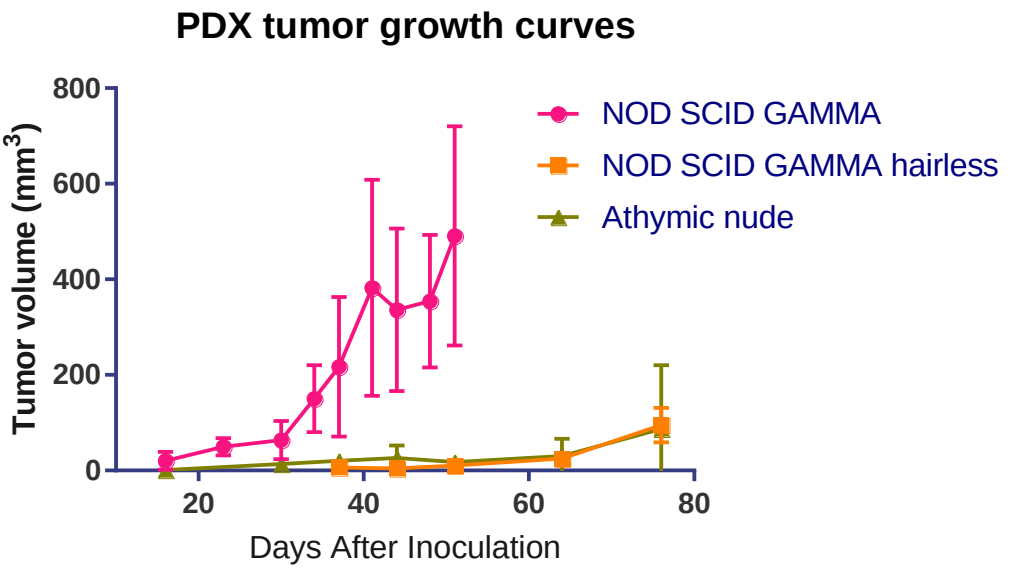


Figure 2-26. Tumor growth curves of patient 10004 tumor derived xenografts using NOD SCID GAMMA (NSG) mice, NOD SCID GAMMA hairless mice and athymic nude mice.

Patient derived tumor cells (2×10^6 in 0.1 ml) from 10004 were inoculated subcutaneously into the right flank of NSG mice (n=9), NSG hairless mice (n=5) and athymic nude mice (n=5) (8-16 weeks old). Tumor dimensions were determined 1-3 times a week, and tumor volumes were calculated from measurements of 3 diameters of individual tumors based on the following formula: tumor volume (mm^3) = 1/2(length × width^2). Gefitinib treatment were started when tumors reached 500 mm^2.

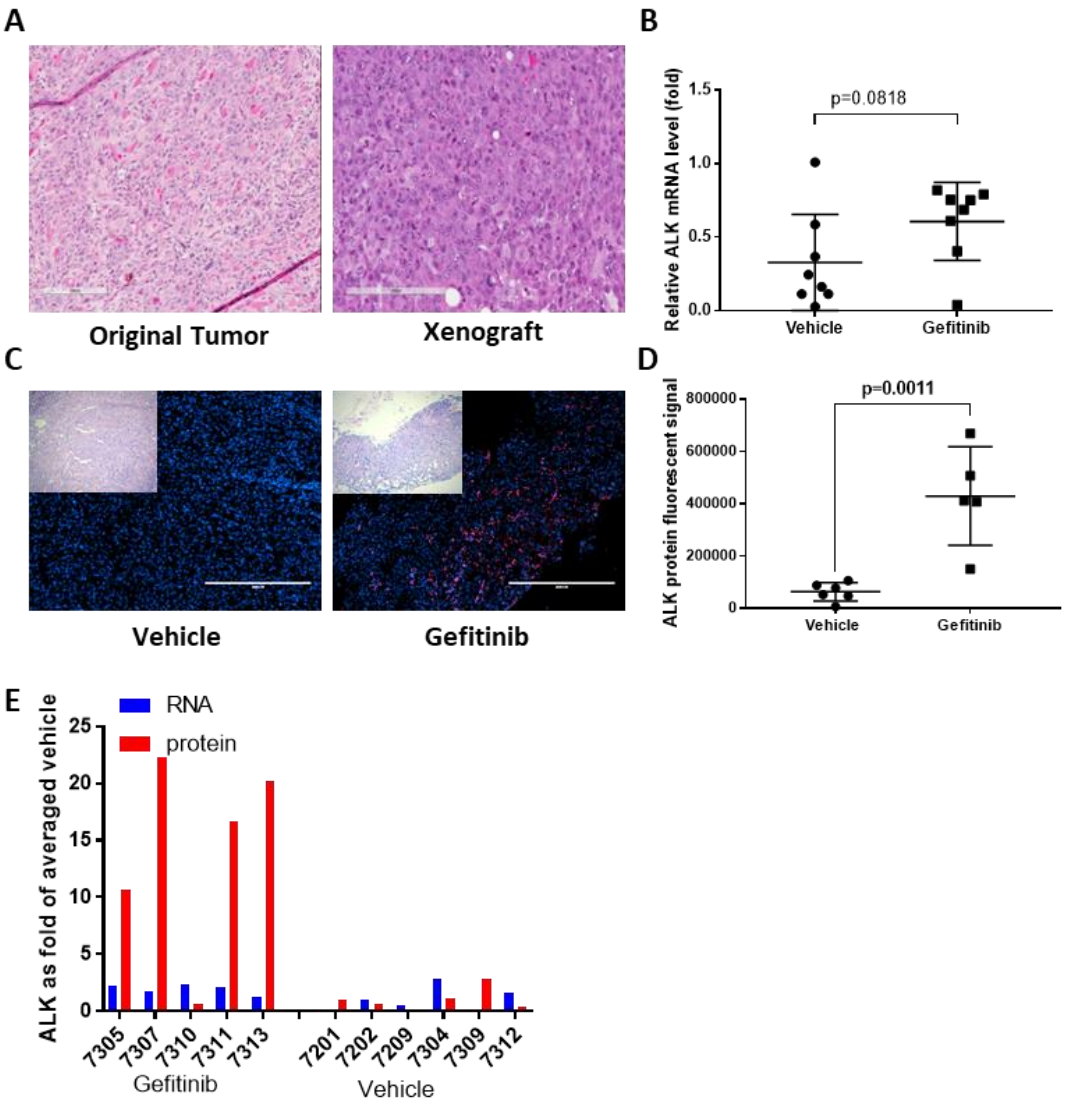


Figure 2-27. ALK RNA and protein levels increase after gefitinib treatment in HNSCC patient-derived xenografts.

Patient-derived tumor cells from patient 10004 were subcutaneously injected into NSG mice. Mice were treated with 100 mg/kg gefitinib or vehicle for 48 hours. **A**, Morphology of a xenograft tumor and the original patient tumor were shown. **B-D**, Levels of ALK RNA and protein were assessed by qRT-PCR and immunofluorescent staining,

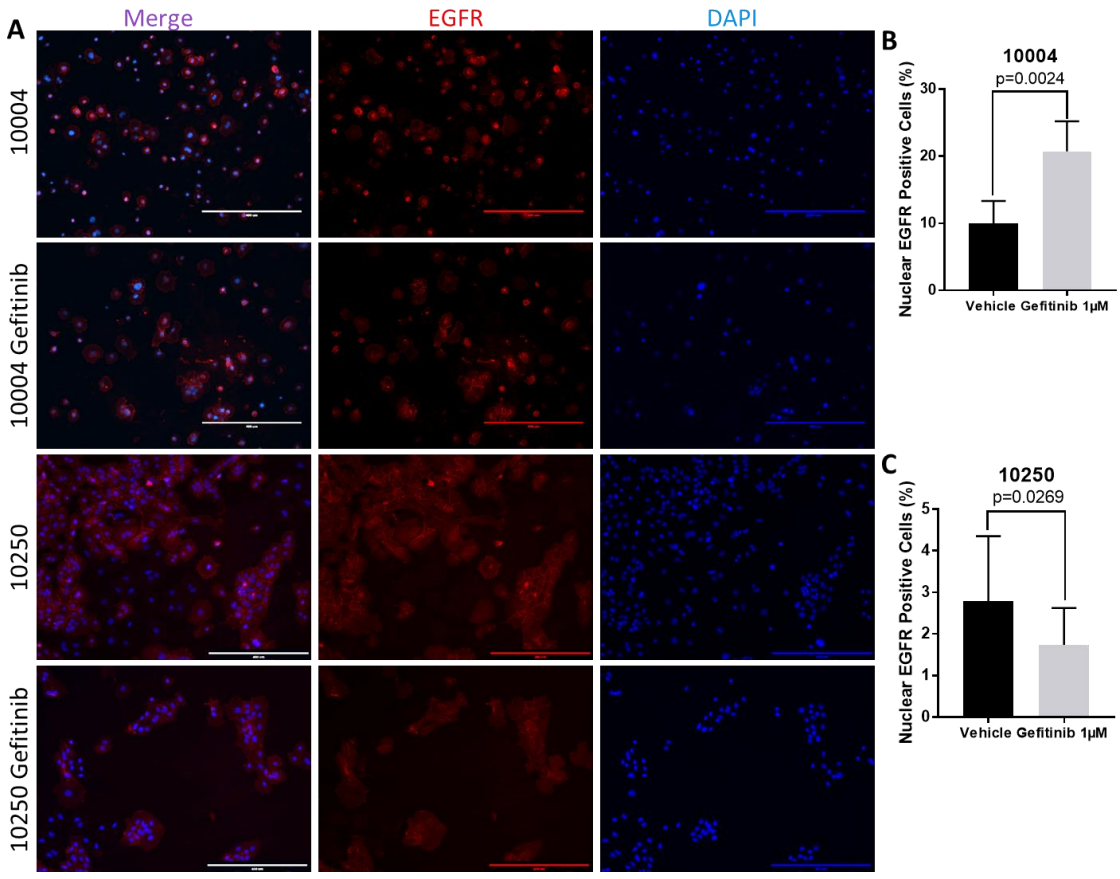
respectively. **B**, Quantification of ALK RNA expression after 48 hours of gefitinib treatment. Data represents the mean ± SD (n = 8). **C**, Immunofluorescent staining of ALK protein (red) and the nucleus (blue) on 10004 patient-derived xenograft treated with gefitinib or vehicle. Scale bar = 400 μm **D**, Data represent the integrated density (ALK positive area times mean fluorescent intensity) of ALK staining in gefitinib treated and vehicle treated mice (n=5-6). **E**, Graphic representation of ALK RNA and protein matched samples from individual mice.

Mechanism underlying ALK induction

Emerging evidence over the last decade has indicated that nEGFR has been detected in highly proliferative tissues and linked with poor clinical outcome in breast cancer, oropharyngeal SCC and ovarian cancer (Lo, Xia, et al. 2005; Psyrri et al. 2005; Xie and Hung 1994). A landmark study of nuclear EGFR has demonstrated that EGFR shares several features with transcription factors: it can be located in the nucleus, it contains a transactivation domain, it associates with genes, and it activates sequence-specific gene expression, supporting a model of nuclear EGFR function as a transcription factor. This study has demonstrated that EGFR can bind to specific DNA sequences to activate gene expression such as cyclin D1(S. Y. Lin et al. 2001a). Other genes regulated by nEGFR include iNOS, B-myb and Aurora kinase A (Cao et al. 1995; Hanada et al. 2006; Hung et al. 2008; S. Y. Lin et al. 2001b; Lo, Hsu, et al. 2005b; Marti et al. 1991). It is likely that ALK is regulated by nuclear EGFR directly or indirectly.

To assess the association between nuclear EGFR and EGFR and ALK inhibitor combination sensitivity, we determined the localization of EGFR in EGFR and ALK inhibitor combination by immunofluorescent staining in patients' tumor cells of a relatively sensitive case 10004 and a relatively insensitive case 10250. The percentage of cells positive with nuclear EGFR was statistic significantly increased by 1 μM gefitinib treatment for 48h in the relatively sensitive case 10004, whereas it was decreased in 10250 (Figure 2-28). Notably, we also observed a significant induction of EGFR around the nucleus (Figure 2-29). This compartment may be the endoplasmic reticulum (ER), based upon a report that in cells treated with EGF, EGF receptor is slowly trafficked from the cell surface to the ER prior to nuclear localization (Liao and Carpenter 2007);

hence it’s likely that these EGFR molecules are en route to the nucleus. These lines of evidence suggest that nuclear EGFR might be associated with the ALK induction by gefitinib in 10004.


A
Merge
EGFR
DAPI
10004
10004 Gefitinib
10250
10250 Gefitinib
B
10004
p=0.0024
Nuclear EGFR Positive Cells (%)
30
20
10
0
Vehicle
Gefitinib 1μM
C
10250
p=0.0269
Nuclear EGFR Positive Cells (%)
5
4
3
2
1
0
Vehicle
Gefitinib 1μM

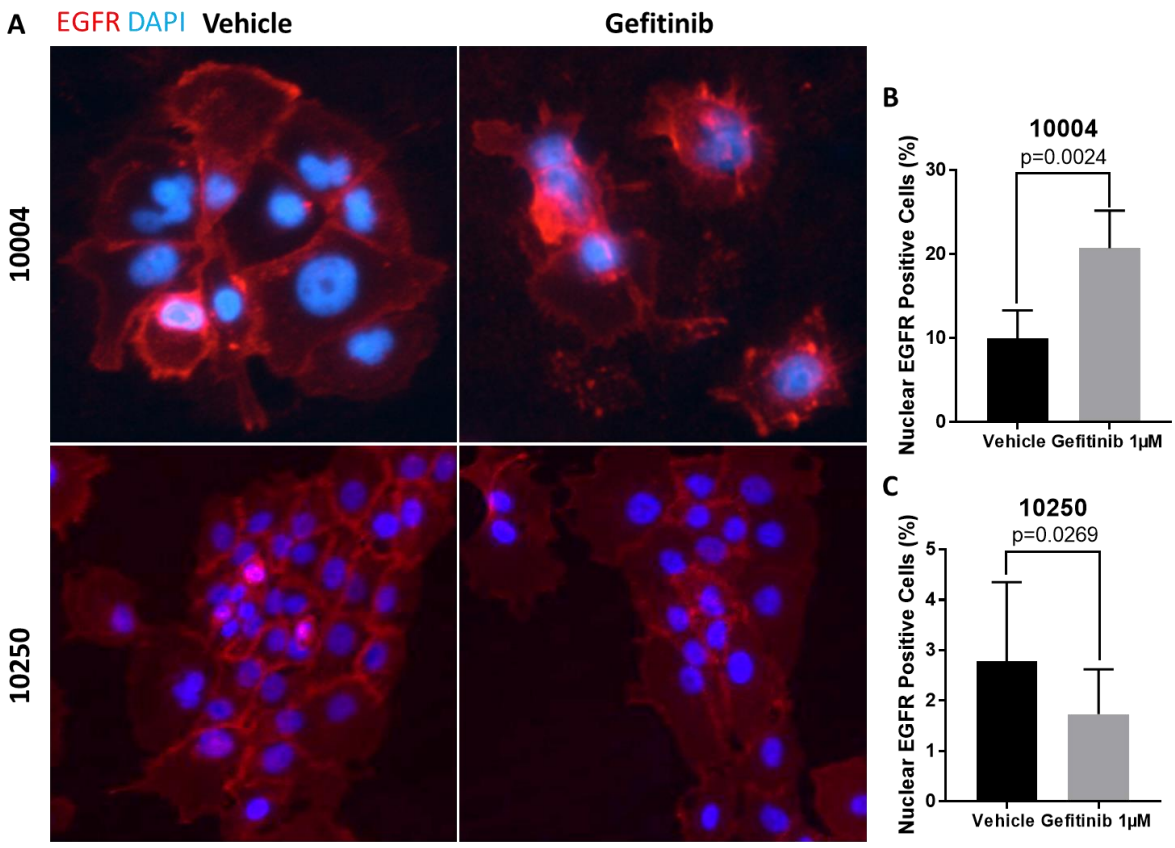

A
EGFR DAPI
Vehicle
Gefitinib
10004
10250
B
10004
p=0.0024
Nuclear EGFR Positive Cells (%)
30
20
10
0
Vehicle Gefitinib 1μM
C
10250
p=0.0269
Nuclear EGFR Positive Cells (%)
5
4
3
2
1
0
Vehicle Gefitinib 1μM

Figure 2-28. **Immunofluorescent staining of EGFR and nuclei in a EGFR and ALK combination sensitive case 10004 and a relatively insensitive case 10250.**

Patient-derived tumor cells from 10004 and 10250 were plated on coverslips and treated with or without 1 μM gefitinib for 48h. Representative images of immunofluorescent staining of EGFR and DAPI are shown (**A**) and nuclear EGFR was quantified (**B-C**). Scale bar= 400 μm.

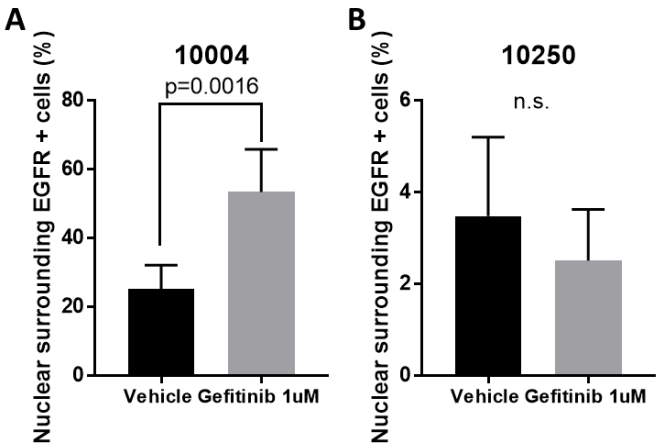


Figure 2-29. **Quantification of immunofluorescent staining of EGFR inside nuclei as well as surrounding nuclei in 10004 and 10250.**

Patient-derived tumor cells from 10004 and 10250 were plated on coverslips and treated with or without 1 μM gefitinib for 48h. EGFR inside nuclei and surrounding nuclei was quantified (**A**-**B**).

Discussion

The current study identifies EGFR and ALK inhibitor combinations as effective combination therapies in EGFR inhibitor resistant HNSCC patient-derived tumor cells. Four different ALK inhibitors, including two that are FDA-approved, ceritinib and brigatinib (Friboulet et al. 2014; Nishio et al. 2015), showed synergistic effects with gefitinib in patient-derived tumor cells, suggesting a potential benefit of using ALK inhibitors in combination with an EGFR inhibitor for treating HNSCC patients.

ALK has been implicated in the development and progression of many malignancies, including anaplastic large-cell lymphoma, NSCLC and neuroblastoma. ALK fusions, mutations and amplifications are the most common alterations in ALK in cancers (Mologni 2012; Cerami et al. 2012), and ALK fusions and other rearrangement have been used as an patient selection criteria for ALK inhibitors in NSCLC patients (Shaw et al. 2013; Kwak et al. 2010). However, ALK alterations are not commonly detected in HNSCC (Figure 2-20), and no expression level changes or mutations in ALK were detectable by RNAseq and whole exome sequencing analysis in our patients' tumors. The functional screens using viable patient-derived tumor cells treated with gefitinib uncovered a mechanism of EGFR inhibitor resistance through ALK induction that was missed by genomic and transcriptomic analysis in naive tumors alone.

Although ALK protein expression is relatively low in naive HNSCC patient tumors (The Human Protein Atlas (Uhlen et al. 2010)), it is possible that ALK is upregulated in cancers after EGFR inhibitor treatment. Support for this view comes from reports of different mechanisms of EGFR inhibitor resistance in various cancers including upregulation of other parallel pathways such as MET, aurora kinase A and HER3

(Wheeler, Dunn, and Harari 2010; Erjala et al. 2006; Hoellein et al. 2011) and heterodimerization and transactivation of other RTKs such as MET, HER2 and IGF-1R (Erjala et al. 2006; Wheeler, Dunn, and Harari 2010). The effectiveness of ALK inhibitors plus gefitinib in patient-derived cells was associated with strong induction of total ALK protein, induction of phosphorylated ALK and/or higher basal levels of ALK protein (Figure 2-21-Figure 2-27). In EGFR and ALK combination sensitive cases 10054 and 10058, up to 2.1 fold ALK induction was observed, while 10004 showed up to 5.6 fold induction. This could due to the limitation of ALK induction experiments that only 24 hours and 48 hours was tested in cases other than 10004. It is possible that it will take longer for gefitinib to induce ALK in 10054 and 10058. It is unclear at this point that ALK induction was an epigenetic regulation of gene expression or an evolutionary drug selection for cells that express ALK scenario. If the latter is the case, ALK expressing tumor cells from 10054 and 10058 may need longer to replenish the population, because 10054 and 10058 appeared to have longer doubling times (approximately 115h for both) compared to 10004 (approximately 68h), based upon observations of time to double in confluence. Further we cannot rule out the possibility that both scenarios are true, that epigenetic regulation of ALK expression happens followed by a drug selection for cells that express ALK. In either case, cell replication time may affect the timing of ALK induction. Therefore, an extended time course for 10054 and 10058 would address this possibility.

Nuclear EGFR has been shown to function as a transcription factor or co-transcription factor alongside STAT3, E2F1 and STAT5 to enhance the transcription of aurora kinase A, c-Myc, cyclin D1, iNOS and B-myb (Brand et al. 2013; Hanada et al. 2006; Hung et

al. 2008; Jaganathan et al. 2011; S. Y. Lin et al. 2001b; Lo, Hsu, et al. 2005b; Lo et al. 2010). Studies have shown that stress conditions such as anti-cancer drug gefitinib and cetuximab could induce EGFR translocation into the nucleus (Liao and Carpenter 2009; Tan et al. 2016). Nuclear EGFR can contribute to acquired resistance to gefitinib and cetuximab by elevating expression of breast cancer resistance protein (BCRP/ABCG2) (W.-C. Huang et al. 2011) and by upregulation of cyclin D1 and B-myb (C. Li et al. 2009a), respectively. Our finding that gefitinib increased nuclear EGFR positive cells in EGFR and ALK inhibitor sensitive case 10004 but not in a relatively resistant case 10250 (Figure 2-27) suggested that nuclear EGFR could be involved in ALK inhibitor sensitivity in combination with gefitinib. These sets of evidence support the hypothesis that gefitinib induces ALK expression through a nuclear EGFR mediated manner. However, to test this hypothesis, inhibition of EGFR nuclear trafficking, for example by inhibiting SFK (C. Li et al. 2009a), and induction of nuclear translocation, for example by introducing nuclear localization sequence-tagged EGFR, will be needed during gefitinib treatment, and ALK levels can be measured to determine the association between ALK induction and EGFR nuclear translocation. In addition, whether ALK is induced through nuclear EGFR as a co-transcription factor has yet to be determined. A nuclear run-on assay could be performed to determine whether nuclear EGFR induced ALK by promoting transcription of ALK RNA. The evaluation of other nuclear target genes of EGFR may enable predictions of other combinations with EGFR inhibitor in order to prevent development of resistance.

Further, suppressing EGFR endocytosis have shown to decrease cell viability and increase apoptotic cell death in gefitinib-insensitive lung cancer with wild type EGFR *in vitro* and *in vivo*, suggesting EGFR internalization may play important roles in gefitinib resistance in wild-type EGFR tumors (Jo et al. 2014). Based on my finding, it is possible that co-targeting EGFR nuclear trafficking could be beneficial in EGFR and ALK inhibitor combination sensitive patients.

Post-transcriptional regulation of ALK could involve the proteolytic cleavage of ALK. ALK is expressed as the 220 kDa full-length transmembrane receptor, and a shorter form of 140 kDa could result from the extracellular proteolytic cleavage of the full-length receptor (Moog-Lutz et al. 2005). 140 kDa ALK has been shown to have kinase activity and its activity could be inhibited by siRNA targeting ALK and ALK inhibitor TAE684 (Motegi et al. 2004; Moog-Lutz et al. 2005). A significant correlation was reported between 140 kDa ALK protein levels and ALK inhibitor TAE684 response in wild type neuroblastoma cell lines (Duijkers et al. 2011), suggesting potential kinase dependence of these cancer cells on 140 kDa ALK protein and the benefit of targeting ALK in ALK wild type cancers. Our finding that 140 kDa ALK protein expression and phosphorylation was induced after EGFR inhibitor treatment in ALK wild type HNSCC provides evidence supporting further study of the oncogenic functions of this form in HNSCC and other cancers and possible specific targeting strategies in these diseases.

Overall, our study reports evidence for ALK induction and activation after EGFR inhibitor treatment in HNSCC, suggesting a novel mechanism for and strategy to address EGFR inhibitor resistance in the clinic. These findings provide rationale to further investigate this phenomenon in other cancer types where EGFR inhibitors are relevant.

Materials and methods

Collection of patient samples and cell culture

Clinical samples were obtained from patients treated at Oregon Health &Science University upon informed consent under approval IRB00010071 by the Oregon Health & Science University Institutional Review Board (Portland, OR). Primary human HNSCC cell lines were developed from excised tumors, using IRB-approved collection techniques. Surgically removing fresh tumor tissue under aseptic conditions specifically for laboratory use from non-necrotic and uninfected areas greatly increases the success rate of primary culture. The fresh specimen (within 6-47 min) were washed with complete medium containing 2x antibiotic solution consisting of penicillin, streptomycin, and amphotericin B. Minced tumor fragments are grown in collagen-I (Gibco Coating Matrix Kit, Hyclone, R-011-K) coated petri dishes and/or 490 cm^2 roller bottles and incubated in minimal culture medium supplemented with amino acids and serum containing 1x penicillin-streptomycin-amphotericin B for 3-5 days. Cell culture medium was developed by James Rheinwald of the Harvard Skin Disease Research Center, Boston, MA. DMEM/F12 Medium (Gibco, 11320082), supplemented with 5% BCS (Hyclone, SH3007203,), 1x antibiotic/antimitotic (Gibco, 15240112), 1.8 x 10^{-4} M adenine (Sigma, A2786), 0.4 µg/mL hydrocortisone (Sigma, H0888), 1 x10^{-10} M cholera enterotoxin (Sigma, C8052), 2 x 10^{-11} M triiodothyronine (Sigma, T6397), 5 µg/mL insulin (Sigma, I9278) and 10 µg/mL epidermal growth factor (Gibco, PHG0311), was used for cell maintenance. When explants start to spread out, start rolling the roller bottles in the incubator to enhance air exchange and better mimicking *in vivo* environment of the mucosal lining of the head and neck area. As the epithelial cells were

growing, fibroblasts were routinely removed by differential trypsinization (0.25% with EDTA, Gibco, 25200114). Since fibroblasts were more sensitive to trypsin than epithelial cells in our cultures, differential trypsinization between 1-3 minute was used as needed to remove fibroblasts under visualization with an inverted microscope and blocking the trypsin activity with serum-containing medium before the epithelial cells detach.

Inhibitor assay

HNSCC patient-derived cells were examined for sensitivity against a panel of 122 small-molecule inhibitors as previously described (J. W. Tyner et al. 2013). Briefly, 6–8 x 10^3 cells per well were treated with a threefold interval dilution series totaling 8 concentrations of each drug (including no-drug control) in 384-well plates. After 72 hours, relative cell viability was determined using a tetrazolium-based MTS assay (Promega, PR-63581), and IC50 values were determined from the dose response curves. A final concentration of 50 nM of one of three EGFR inhibitors used in clinical trials for HNSCC or other cancers was used in combination with the inhibitors on the panels to identify synergistic agents (lapatinib (Selleck, S1028) in patient 10004's and 10021's cells, erlotinib (Selleck, S1023) in patient 10054's and 10058's cells, and gefitinib (Selleck, S1025) in patient 10139's, 10159's, and 10250's cells). No significant differences in IC50s were detected using these three EGFR inhibitors in inhibitor assays as single agents (Table 2-3C). Gefitinib and TAE684 combination was reported to have efficacy in treating HNSCC cells lines and cell line derived xenografts (Gonzales et al. 2016); therefore gefitinib was used in all follow-up validation experiments.

Scale-up Inhibitor validation studies

Patient-derived tumor cells were distributed in 96-well plates with dilution series totaling 10 concentrations of each drug. Cells were plated at a density of 8 x 10^3 cells/well and treated with the following inhibitors or combination of inhibitors for 72 hours: gefitinib, NVP-TAE-684, GSK-1838705A, ceritinib and brigatinib. For the drug combinations, gefitinib and ALK inhibitors were used at a ratio of 2:1. All inhibitors were purchased from Selleck Chemicals. All conditions were plated in triplicates. Cell viability was measured using MTS assay, and absorbance (490 nm) was read at 1 to 4 hours after adding reagent using a BioTek Synergy 2 plate reader. MTS absorbance values of inhibitor-treated wells were normalized to those of untreated cells. IC50 values were determined by CalcuSyn (BioSoft).

RAPID assay

The RNAi-assisted protein target identification (RAPID) assay has been previously described. All siRNAs were SMARTpool: siGENOME siRNA pools (GE Dharmacon). Lipofectamine RNAiMAX (ThermoFisher, 13778075) was used as transfection reagent. RAPID assays were performed with or without 50 nM gefitinib on top of the panels.

Quantification of annexin V/propidium iodide staining

Cells were stained using annexin V Apoptosis Detection Kit APC (eBioscience, 88-8007) according to the manufacturer's protocol. Cells were treated with 1 μM gefitinib, 600 nM ceritinib or 500 nM gefitinib, and 300 nM ceritinib, or vehicle control (DMSO) for 72h prior to analysis. Cells were re-suspended in annexin V binding buffer containing APC conjugated annexin V, followed by addition of propidium iodide (PI). Subsequently, cells

were analysed by flow cytometry, using a Canto II. Data was analysed using FlowJo_v10. Unstained cells as well as annexin V, and PI single stained cells were used as negative controls.

Colony formation assay

After exposure to the pooled siRNAs for 12 days, cells were washed with PBS, then fixed and stained with a mixture of 6.0% glutaraldehyde (Sigma, 340855-25ml) and 0.5% crystal violet (Sigma, C0775-25G) for 30 minutes. Plates were rinsed with tap water and dried in normal air at room temperature (20 °C). Colonies were counted manually.

Nucleated cell number analysis

Patient-derived tumor cells in triplicate wells were treated with either 1 uM gefitinib, 600 nM ceritinib, the combination of 1 uM gefitinib+ 600 nM ceritinib or vehicle for 48 hours. After treatment, cells were fixed using 4% paraformaldehyde (Electron Microscopy Science, 15710), blocked using 5% goat serum (Abcam, AB138478) and 0.3% Triton X-100 (Sigma, T8787), and stained with Hoechst (Thermo Fisher, 62249). After subsequent washing with PBS and 0.5% Tween20 (Sigma, P7949), coverslips were mounted using prolong diamond antifade (Fisher, P36961). Images were taken using an EVOS FL microscope (Thermo Fisher) with a 10x objective. Five images were taken per coverslip, resulting in 15 images for each treatment group. Nuclei were counted using Image J (Schindelin et al. 2012).

Immunoblotting

Patient-derived tumor cells were treated with cell lysis buffer (Cell Signaling Technologies, 9803S) with complete mini protease inhibitor mixture tablets (Roche,

11836153001), and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche, 4906845001). Lysates were spun at 8,000 xg for 10 minutes at 4°C to pellet cell debris, mixed 3:1 with 4x Laemmli Sample Buffer (Bio-Rad, 1610747) with β-ME, and heated at 95°C for 5 minutes. Lysates were run on 4% to 15% Criterion TGX Precast Midi Protein Gel (Bio-Rad, 5671083), transferred to a polyvinylidene difluoride membrane (Bio-Rad, 1704157), and blocked for 1 hour in TBS-T with 5% BSA. Blots were probed overnight at 4°C with anti-ALK rabbit antibody (1:300, 3333), Phospho-ALK (Tyr1604) Antibody (1:300, 3341), EGF Receptor (D38B1) XP® Rabbit mAb (1:1000, 4267), Phospho-EGF Receptor (Tyr1068) (1H12) Mouse mAb (1:1000, 2236) or α-Tubulin (DM1A) Mouse mAb (1:1000, 3873), followed by anti-rabbit or anti-mouse IgG HRP conjugate secondary antibodies. All primary antibodies are from Cell Signaling. Blots were developed using Clarity™ or Clarity Max™ Western ECL Substrate (Bio-Rad, 1705060 and 1705062) and imaged using a Bio-Rad ChemiDoc touch MP Imaging System. Optimal exposure time was automatically determined by ChemiDoc touch. The SK-N-SH cell line which expresses both 220 kDa and 140 kDa ALK was used as an ALK positive control to confirm molecular weight in all western blot experiments for ALK and p-ALK detection.

Patient-tumor-cell-derived spheroids formation, treatment and immunofluorescent staining

Patient-derived tumor cells were grown into spheroids using a hanging drop method as previously described (Foty 2011). Spheroids were embedded in Collagen I (Corning, 354236) and were cultured for four days. 1 μM gefitinib diluted in DMEM/F12 (Gibco, 11330057) supplemented with 5% bovine calf serum (Hyclone, SH3007203) or vehicle

was applied to the cells for 72 hours. The experimental procedure for embedding and staining was as previously reported with minor modifications. Spheroids were fixed in 4% paraformaldehyde and 1% Triton X 100 (Sigma, T8787), and then washed with PBS and 10mM Glycine (Bio-Rad, 1610717). Spheroids were dehydrated in an ascending series of methanol, then rehydrated before blocking overnight at 4°C with 3% normal goat serum (Abcam, AB138478) in PBS. ALK antibody (1:100, Cell Signaling, 3633) was diluted in 3% normal goat serum and 0.1% Triton X 100 in PBS and incubated for two nights at 4°C. Alexa Goat Anti-Mouse Texas Red antibody (Invitrogen, T2767) and Hoechst (Thermo Fisher, 62249) was applied at 1:1000 and 1:2000 dilution overnight at 4°C. Cells were imaged using a Nikon/Yokogawa CSU-W1 Spinning Disk Confocal. Replicate spheroids of each treatment condition were completed and imaged. Mean fluorescent intensity was quantified using Fiji (ImageJ, NIH, Bethesda, MD) at five separate levels in the z stack, consistent across all images and was normalized to the volume of the spheroid, calculated using Bitplane Imaris (Oxford Instruments). calculated using Bitplane Imaris (Oxford Instruments).

Xenograft mouse model for ALK induction after Gefitinib treatment

NOD SCID GAMMA (NSG) mice (8-16 weeks old) were used for this study. Mice were divided into 2 groups (n = 8 mice per group), 1) vehicle control; 2) 100 mg/kg gefitinib. Patient derived tumor cells (2 × 106 in 0.1 ml) from 10004 were inoculated subcutaneously into the right flank of all mice. Treatments were initiated when tumors reached 500 mm^2. Body weights and tumor dimensions were determined 3 times a week, and tumor volumes were calculated from measurements of 3 diameters of individual

tumors based on the following formula: tumor volume (mm3) = 1/2(length × width 2). Treatment with gefitinib or vehicle control (dimethyl sulfoxide 5% and corn oil 95%) administered daily by oral gavage for 2 days. Volume of liquid for oral gavage was 0.1 ml / 10 g. Mice were sacrificed and the tumors were harvested 2 days after the last gefitinib treatment. All studies were performed according to guidelines approved by OHSU Institutional Animal Care and Use Committee.

Statistical analyses for bench experiments

For the nucleated cell count assay, colony formation assay, and annexin V/PI staining assay, a one-way ANOVA test with Dunnett's multiple comparison was carried out for each treatment condition compared with vehicle treated cells or appropriate controls. For western blot quantification and spheroid staining, student's t tests were performed for treatment groups compared with vehicle groups. Combination indices were calculated using CalcuSyn (Biosoft), whereby data points for combinations with upper confidence limits below 1 are considered synergistic (Chou 2010).

Light and dark pathway analysis

The design of the light and dark pathway analysis included somatic mutation data, pathway membership, and drug targeted genes. We analyzed Level 2 TCGA curated mutations that used Illumina Hiseq 2000 to sequence primary HNSCC tumor samples and mapped to the genome build GRCh37/hg19 (time stamped February 10, 2015). We queried the Hugo Symbols mutated in our cohort within the gene-pathway membership

from the human Reactome Pathways database. The Reactome database uses a hierarchical pathway system; therefore, in addition to considering the pathway membership, we evaluated whether each dark pathway was nested within light pathways. Hugo Symbols were cleaned by using the approved or synonym nomenclature.

We applied a bioinformatics approach that combines drug-target interaction and bioactivity data from FDA-approved antineoplastic drugs across four public databases. The drug targets were extracted from prediction of small-molecule kinase inhibitor targets and aggregated results from drug-target data in public sources. The kinase inhibitor targets were predicted using a bioinformatics approach to identify gene targets underlying inhibitor sensitivity profiles and overlapping with known gene products that are targeted by each drug. The FDA approved drug set contained 141 drugs and was used to gather target information from the National Cancer Institute's Cancer Drugs website. The drug-target interactions were collected from a number of public sources and were categorized into three levels of evidence: 1) Interactions with unsupported evidence; 2) Interactions with supporting literature evidence; and 3) interactions with both supporting literature evidence and assay values. We used all levels of evidence for this study to identify any potentially targeted pathways for further exploration.

Statistical analysis for light and dark pathways

In-house workflows in the R Statistical Programming environment were used for all QA/QC and pathway analysis. We filtered out silent mutations in order to query only the potentially damaging somatic mutations including missense, nonsense, nonstop, splice site, frame shift deletion, and frame shift insertions. There were 279 out of 528 TCGA HNSCC patients that were annotated for 51,799 mutations, and 1,650 Reactome

pathways were evaluated for aberration enrichment based on hypergeometric statistical analysis. This statistical test takes into account the number of genes belonging to a pathway and, of those genes, the number of genes that are aberrational in the patient cohort. Pathways were considered aberrationally enriched based on the FDR adjusted p-value < 0.05. The light and dark pathways were categorized based on whether or not these aberrationally enriched pathways contained gene members that are drug targeted genes on the panel.

Author contributions

Xiaoming Ouyang and Molly Kulesz-Martin developed the hypothesis, conceptual and experimental designs, and are responsible for data analysis and interpretation, and participated in integration of bioinformatics with experimental design and hypothesis testing. Xiaoming Ouyang performed experiments including inhibitor assay for 10205 and 10250, all scale-up validation studies, all siRNA screens, combination index calculation, Annexin V staining followed by flow cytometry, colony formation assay, all western blot experiments, establishment of patient-tumor-derived spheroid and xenograft, qRT-qPCR for ALK expression in PDX model, and isolation of DNA and RNA for next generation sequencing. Ashley Barling performed cell nuclear number staining experiment, nuclear EGFR quantification, generated patient-tumor-derived spheroids followed by immunofluorescent staining, helped with generation of PDX and performed immunofluorescent staining on PDX tumors. Aletha Lesch established patient-derived cell cultures and performed inhibitor assays for 10004, 10054, 10139 and 10159. Jeffrey W. Tyner provided inhibitor assay panels, siRNA screen panels and analysis support. Gabrielle Choonoo performed dark and light pathway analysis. Christina Zheng, Sophia Jeng and Shannon K. McWeeney performed RNAseq and exome seq data analysis and contributed helpful discussion. Sara Courtneidge provided technical guidance for spheroid model establishment, helpful suggestions of experimental design and manuscript preparation. Daniel Clayburgh provided support for patient sample collection.

Chapter 3 – Discussion and Future Directions.

Summary of key findings.

My graduate studies have focused on the identification of novel targeted therapeutics for HNSCC. My focus has been identifying combination therapies to overcome EGFR inhibitor resistance in HNSCC patient derived models. By utilizing functional screens, including small-molecule kinase inhibitor panels, we identified agents that synergized with EGFR inhibitors in reducing viability in HNSCC patient-derived tumor cells. Our approach detected PI3K inhibitors PI103, BEZ235 and PP242 (Table 1) as effective combinations with EGFR inhibitor, which is consistent with previous preclinical HNSCC studies *in vitro* and *in vivo* and the testing of PI3K/mTOR inhibitor combinations with EGFR inhibitors in clinical trials for HNSCC (Z. Wang, Martin, Molinolo, Patel, Iglesias-Bartolome, Sol Degese, et al. 2014; De Felice and Guerrero Urbano 2017; Jimeno et al. 2014, 866).

Other than drugs that target the above previously reported important pathways in HNSCC, I found two ALK inhibitors on the drug screen panel that showed synergistic effects with EGFR inhibitors. As single agents, these two ALK inhibitors were only effective in 1/8 patient derived tumor cell cultures. However, they became effective inhibitors in 4/8 HNSCC patients' tumor cells when used in combination with a low dosage of an EGFR inhibitor (Table 2-5), suggesting synergistic effects between these ALK inhibitors with EGFR inhibitors.

To rule out off-target effects of small molecule inhibitors, a relatively specific approach, RAPID assay, was used to determine the true targets of EGFR and ALK inhibitor

combinations. siRNA targeting ALK as a single agent were not effective in reducing patients' tumor cell vitality, however, they became effective when a low dosage of EGFR inhibitor gefitinib was added in combination with each siRNA in tumor cells from the patients that were relatively sensitive to EGFR and ALK drug combinations, but not in a relatively insensitive case (Figure 2-17).

To validate high-throughput drug screening results, I performed scale-up dose-response experiments and confirmed patients' tumor cell sensitivity to total of 4 different ALK inhibitors in combination with gefitinib in reducing cell viability, including 2 ALK inhibitors FDA approved for other cancers, ceritinib and brigatinib (Figure 2-16). Combination indexes were calculated for patients and confirmed synergistic effects rather than additive effects between gefitinib and ALK inhibitors (Table 10).

To evaluate the effects of ALK and EGFR inhibitor combinations on patient-derived tumor cells other than cell viability, additional end points including cell number, colony formation ability and annexin V positive-PI negative staining by flow cytometry were assessed in single agent treated cells as well as ALK and EGFR inhibitor combination treated ones. Co-targeting EGFR and ALK decreased HNSCC patients' tumor cell number and colony formation ability and increased annexin V staining (Figure 2-18).

To dissect the mechanism underlying synergy between EGFR and ALK inhibitor combinations, we performed RNAseq and exome seq analysis for differential expression and somatic mutations of EGFR and ALK in relative sensitive and insensitive HNSCC cases. Surprisingly, ALK expression levels were very low in the patients' native tumors, and no somatic mutations in the ALK gene were detected. Further, in the TCGA cohort, frequency of alterations in ALK was only 5% (Figure 2-20). These data do not support

inherent overexpression or activation of ALK as responsible for my finding that 4/8 HNSCC patients' tumor cells were sensitive to EGFR and ALK inhibitor combinations. However, exome seq and RNAseq were all performed in HNSCC tumors and tumor-derived cells without EGFR inhibitor treatment. This led to my hypothesis that EGFR inhibitor could induce ALK expression and activation in tumor cells, allowing ALK signaling to bypass signaling of EGFR.

To test this hypothesis, I determined ALK expression and phosphorylation levels by western blot after gefitinib treatment in patient-tumor derived primary cultures, spheroids and xenografts. Gefitinib treatment increased ALK protein expression in tumor cells, spheroids and xenograft tumors established from cases that were EGFR and ALK inhibitor combination sensitive, suggesting induction of ALK by EGFR inhibitor as a potential novel mechanism relevant to resistance to EGFR inhibitor (Figure 2-21 to 2-27). In addition, nuclear EGFR was induced by gefitinib in an EGFR and ALK inhibitor combination sensitive case but not in a relatively resistant case (Figure 2-28 to 2-29).

In summary, I identified EGFR and ALK inhibitor combinations as effective combination therapies in HNSCC patient-derived tumor cells. Four different ALK inhibitors, including two that are FDA-approved, ceritinib and brigatinib, showed synergistic effects with gefitinib in patient-derived tumor cells. Further, I found ALK could be induced and/or activated after EGFR inhibitor treatment in HNSCC, suggesting a novel mechanism for and strategy to address EGFR inhibitor resistance. These findings provide rationale to further investigate this phenomenon in other cancer types where EGFR inhibitors are relevant.

Future perspectives and new questions.

Understanding mechanisms underlying ALK induction after EGFR inhibitor treatment

I started my thesis work by identifying combination therapies to overcome EGFR inhibitor resistance in HNSCC patient derived models, and then turned my focus to the ALK and EGFR inhibitor combinations, and determined a novel mechanism underlying synergy between ALK and EGFR inhibitors. I found that ALK was induced and activated after EGFR inhibitor treatment in HNSCC patient-derived models, as a potential mechanism underlying EGFR inhibitor resistance. However, how ALK induction occurs after EGFR inhibitor treatment is not clear. In patient-derived xenograft tumors from an EGFR and ALK inhibitor combination sensitive case, both RNA and protein levels of ALK were induced in tumors, reaching 1.8 fold and 15.6 fold (Figure 2-26), respectively ($p=0.0818$ and 0.0008, respectively). The possible explanations for increases at the RNA and protein level include: promote transcription directly or indirectly, enhance RNA stability, and/or promote translation and/or post-translational modifications. The tendency to increase in steady state of RNA levels, and the 24 to 72 hour time course of ALK induction are consistent with ALK regulation by gefitinib, at least partially, at the RNA level.

Evidence has shown that gefitinib and cetuximab could promote EGFR translocation into the nucleus (Tan et al. 2016). My finding that gefitinib increased nuclear EGFR in tumor cells from 10004, a case that was sensitive to EGFR and ALK inhibitor combination, but not in 10250, a case that was relatively resistant to the combination (Figure 2-27), is

consistent with the hypothesis that nuclear EGFR was involved in ALK inhibitor sensitivity and thus ALK induction in 10004. Nuclear EGFR has been reported to have transcriptional factor activity that can promote transcription of cyclin D1 (S. Y. Lin et al. 2001a), iNOS, B-myb and Aurora kinase A (Cao et al. 1995; Hanada et al. 2006; Hung et al. 2008; S. Y. Lin et al. 2001b; Lo et al. 2005; Marti et al. 1991). Thus, transcription of ALK may be induced by nuclear EGFR.

However, in my ALK induction experiments, ALK was induced by gefitinib treatment, but was not observed by siEGFR after 6h, 24h, 48h or 72h of treatment **(**Figure 3-1), suggesting that EGFR was still needed for the induction of ALK but not necessary for its kinase activity. This is in accord with a previous finding that nuclear EGFR transcriptional function is through a transactivation domain in the c-terminus of EGFR while the tyrosine kinase domain is not needed for this function (S. Y. Lin et al. 2001a). Further, other studies have demonstrated that a kinase-dead EGFR can undergo endocytosis as well as translocate to the nucleus effectively (Sorkin and Goh 2009; Roepstorff et al. 2008; Brand et al. 2013), indicating that the translocation of EGFR into the nucleus may also be kinase activity independent. In addition, it has been reported that gefitinib, which blocks EGFR kinase activity, could further promote nuclear translocation of EGFR mediated by cetuximab, which competes with the ligand binding to the receptor (Tan et al. 2016), suggesting the induction of nuclear trafficking of EGFR was kinase activity independent. These sets of evidence suggest that both EGFR nuclear trafficking and transcription factor function are kinase activity independent. Therefore, it is plausible that gefitinib could promote EGFR nuclear trafficking and function as a transcription

factor in the nucleus, thus inducing ALK transcription (Figure 3-2), but siEGFR could not (Figure 3-1).

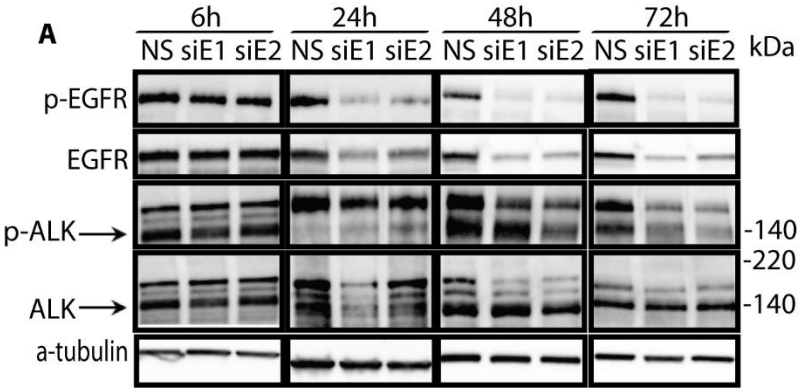


Figure 3-1. ALK protein and phosphorylation levels after siEGFR treatment in 10004 patient-derived tumor cells.

Patient-derived tumor cells from patient 10004 were treated with siEGFR or non-specific siRNA for 6h, 24h, 48h or 48 hours. Levels of total and phospho-EGFR and ALK as well as alpha-tubulin were assessed by immunoblot analysis. Total and phospho-ALK bands are indicated by arrows. The time points shown were blotted in separate gels.

Further studies are needed to test the hypothesis that gefitinib induced ALK was mediated by nuclear EGFR as a transcription factor. First, to test the hypothesis that ALK induction was at a transcriptional level, direct evidence on induction of transcription initiation rate of ALK RNA can be obtained using assays such as a nuclear run-on assay (Smale 2009). In a nuclear run-on assay, the isolated nuclei are incubated with labeled nucleotides. Attachment of new RNA polymerase to genes is prevented by inclusion of anionic detergent sarkosyl (Core et al. 2012). Therefore, only genes that already have an RNA polymerase will produce labeled transcripts. Hence, this assay can be used to identify the genes that are being transcribed at a certain time point.

Second, to test the hypothesis that nuclear EGFR as a transcription factor promotes ALK transcription, a chromatin immunoprecipitation (ChIP) could be performed to determine whether EGFR was associated with ALK gene promoter or other DNA biding sites.

Third, to test the hypothesis that induction of ALK transcription was due to an increase in EGFR nuclear translocation, inhibition of EGFR nuclear trafficking and induction of nuclear translocation are needed in combination with gefitinib treatment followed by ALK transcription rate and protein level assessment. EGFR nuclear trafficking can be inhibited by inhibiting Src family kinases and caveolin (Li et al. 2009), and can by induced by introducing nuclear localization sequence-tagged EGFR. ALK transcription initiation rate and ALK protein level can be measured by a nuclear run-on assay mentioned above and a western blot to determine the association between ALK induction and EGFR nuclear translocation.

If the experimental evidence supports the above hypothesis that induction of ALK was due to an increase in EGFR nuclear translocation, targeting EGFR nuclear trafficking

may be beneficial in combination with gefitinib treatment to prevent acquired resistance by upregulation of ALK.

If the experimental evidence disapproves the above hypothesis that induction of ALK was due to the transcription factor function of nuclear EGFR, an alternative possibility would be that ALK was regulated indirectly by EGFR as a transcription factor by upregulation of other genes. Other target genes regulated by nuclear EGFR as a transcription factor in EGFR inhibitor resistant HNSCC could be determined by ChIP with massively parallel DNA sequencing to identify the binding sites of nuclear EGFR after EGFR inhibitor gefitinib and cetuximab treatment. Other genes regulated by EGFR could be determined by RNAseq differential expression analysis comparing gene expression before and after gefitinib or cetuximab treatment. If additional genes regulated by nuclear EGFR are oncogenic, strategies targeting these genes in combination with gefitinib may be beneficial in preventing gefitinib resistance from happening.

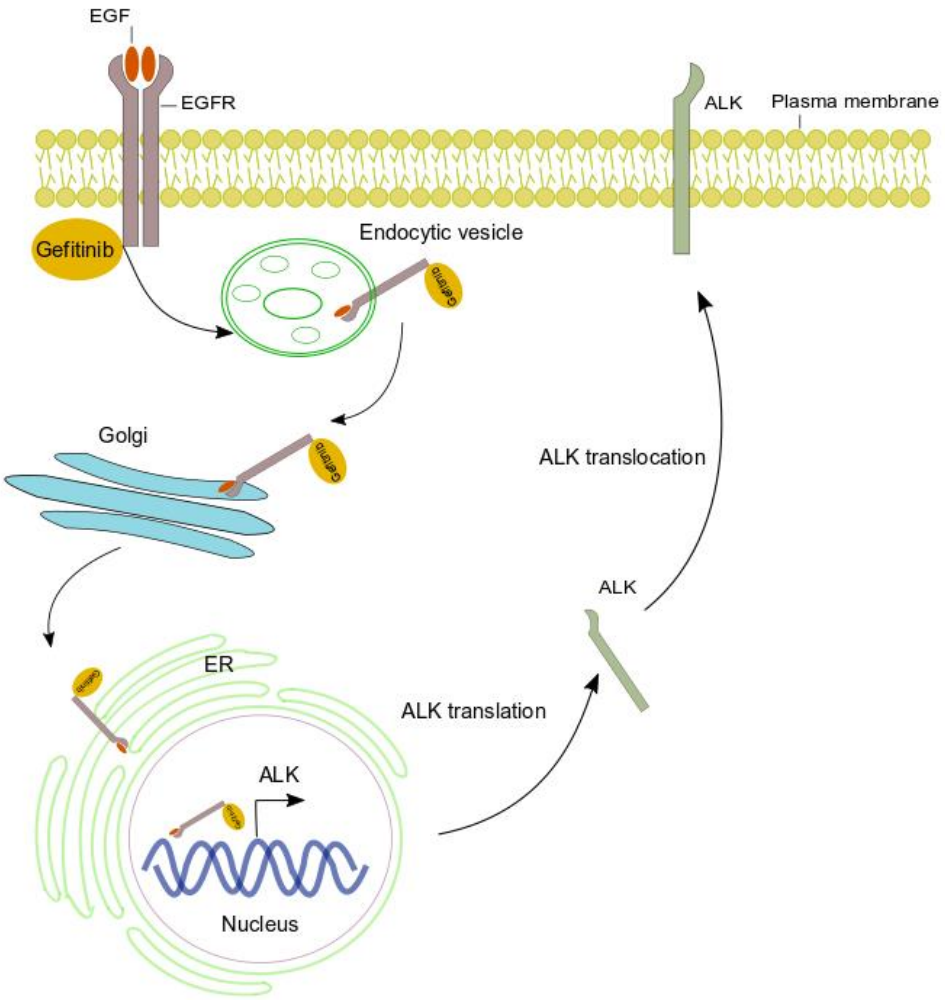


Figure 3-2. **Model of nuclear EGFR mediated ALK induction by gefitinib.**

Gefitinib induces internalization of EGFR to endocytic vesicles. EGFR then undergoes retrograde translocation through the Golgi apparatus to the endoplasmic reticulum (ER) (Y.-N. Wang, Wang, et al. 2010). At the ER, EGFR moves from the outer nuclear membrane to the inner nuclear membrane and is released into the nucleus (Y.-N. Wang,

Yamaguchi, et al. 2010). In the nucleus, EGFR functions as a transcription factor and facilitates ALK transcription and thus upregulation ALK protein level in the cell.

Preclinical studies for evaluating gefitinib and ceritinib combination for opening a clinical trail

In order to translate this study to clinic, *in vivo* efficacy of gefitinib and ceritinib combination in HNSCC patient -derived xenograft models would need to be evaluated. At the preclinical stage, the FDA will generally require, at a minimum, that sponsors: (1) develop a pharmacological profile of the drug; (2) determine the acute toxicity of the drug in at least two species of animals, and (3) conduct short-term toxicity studies ranging from 2 weeks to 3 months, depending on the proposed duration of use of the substance in the proposed clinical studies. For initial human effectiveness studies of the combination, FDA requires the combination starting dose, dosing escalation intervals, and doses to be used in dose-response studies be determined primarily from the phase 1 safety data for the individual new investigational drugs, if available. Since gefitinib and ceritinib have already been FDA approved as single agents for treatment of NSCLC, repurposing of this combination for HNSCC would primarily involve profiles of animal efficacy, pharmacokinetics and toxicology for combination of these two agents.

To meet FDA's requests, we would evaluate the gefitinib and ceritinib drug combination's toxic and pharmacologic effects through *in vitro* and *in vivo* laboratory animal testing. Acute and short-term toxicity tests would be performed in mice and rats. Investigations on drug absorption and metabolism, the toxicity of the drug's metabolites, and the speed with which the drugs and their metabolites are excreted from the body would be conducted. Depending upon the above results, an investigational new drug

(IND) application for a phase I trial including above information could be submitted to FDA for review before beginning clinical research.

The IND would include (1) above preclinical data on safety in animals and safety data in previous clinical trials as singe agents; (2) manufacturing information that can ensure that the company can adequately produce and supply consistent batches of the drug; (3) detailed protocols for the phase I study including patient enrollment criteria, dose escalation range, treatment time, number of patients based on results from above preclinical studies; (4) and information on the qualifications of clinical investigators-physicians- who oversee the administration of the experimental compound.

A design of a clinical trial to test the combination of gefitinib and ALK inhibitor could be a straightforward comparison to standard of care in patients, e.g. to those recommended for cetuximab, or would be incorporated in a "Serial Measurement of Molecular and Architectural Responses to Therapy" (SMMART) trial, currently under leadership of Joe Gray that seeks to individualize cancer treatment, initially of prostate cancer, pancreatic cancer and leukemia, but applicable to other cancers. Such a trial could enroll HNSCC patients for whom standard of care options have failed, and stratify patients to molecular targeted therapies or combination therapies determined by our functional analyses of vulnerabilities of their own tumor cells.

Further analysis of biomarkers, such as nuclear EGFR, to predict response, and potential drivers and effective targets in individual patients' cells in the functionally annotated OHSU cohorts and in TCGA promises a rich source of new options for HNSCC therapy.

Associations between mutation calling analysis and functional assay sensitivity

In a preliminary effort to identify potential molecular bases of drug sensitivity we have carried out mutational analysis of whole exome sequencing (WES) and from RNASeq data. On this basis, we developed the hypothesis of a conditional ALK dependence of the HNSCC tumor cells, and showed ALK induction *in vitro* and in tumors in mice in response to gefitinib, as a possible mechanism for the observed combination of EGFR and ALK inhibition as an effective combination for the most cases, given the drugs tested. This demonstrates the power of functional annotation of tumors, and provides an example of effectiveness of a drug combination that would not be discovered by genomics analysis alone. The future development of the OHSU functionally characterized cohort for expanding drug options for HNSCC patients will take advantage of pathways and targets in a more direct bioinformatics-driven manner. We highlight briefly a few initial findings for future following up.

NOTCH signaling has been reported to be highly altered in HNSCC, but its role in HNSCC is still being characterized. By WES, we found mutations in NOTCH pathway genes in 4 out of 6 patients we performed whole exome sequencing. In 10250, a patient that was resistant to most of the agents in the inhibitor assay, we found a deleterious mutation in NOTCH1 at 9,139403377 (C to A) with allele frequencies of 16%. In 10205, a patient that was resistant to all the inhibitors in the inhibitor assay, we found a very damaging mutation in FBXW7, a gatekeeper for NOTCH signaling, with an allele frequency of 37.5%. In 10004, there is also a novel damaging FBXW7 mutation at

153251924, A, G with an allele frequency of 36%. FBXW7 is ubiquitin ligase that targets Notch for degradation and is found mutated in 4.7% of cancers of HNSCC. FBXW7 may have a role outside of Notch signaling, as it targets many other known oncogenes including cyclin E, MYC, and JUN. In 10058, we detected damaging mutations in NOTCH1 at 9,139412690 (G to A) and in NOTCH2 at 1,120512298 (C to A) in the tumor, with allele frequencies of 36% and 23%. However, the roles of NOTCH pathway genes are still under debate in HNSCC. To determine the roles of these NOTCH pathway alterations in our patients' samples, further functional assays need to be performed in the NOTCH altered cases and compared to wild type cases.

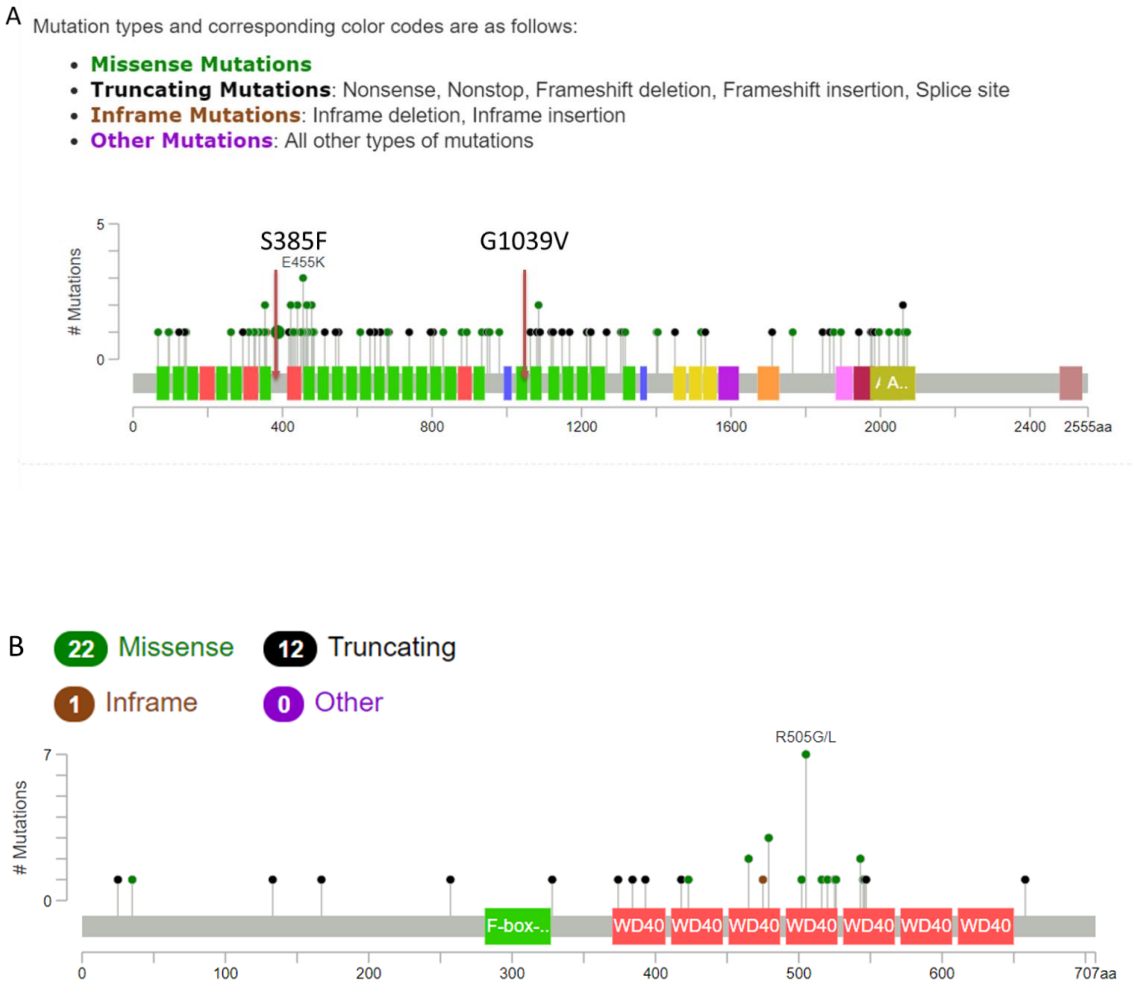


Figure 3-3. NOTCH1 (A) and FBXW7 (B) mutations and their locations in OHSU HNSCC cases.

Other than NOTCH pathway genes, which are of the most frequent alterations in HNSCC (Hayes, Grandis, and El-Naggar 2013), we discovered a novel somatic mutation (substitution G to A) in FGFR2 gene, at chromosome 10,123274645 (all the genomic coordinates for mutation calling are using the HG19 assembly of the human genome), resulting in an amino acid change in FGFR2 protein from arginine to tryptophan in patient 10004's tumor. We compared mutation calling analysis with functional data and noticed that 10004's tumor cells were also sensitive to siRNA pools targeting FGFR2, suggesting possible role of this mutation with FGFR2 inhibition sensitivity. As this mutation had an allele frequency of only 7% in this patient's tumor, and all mutations called will need to be validated by Sanger sequencing. From the functional predictions this is a damaging mutation. Further validation studies will be conducted to rule out sequencing error and to evaluate functional impact of this novel mutation.

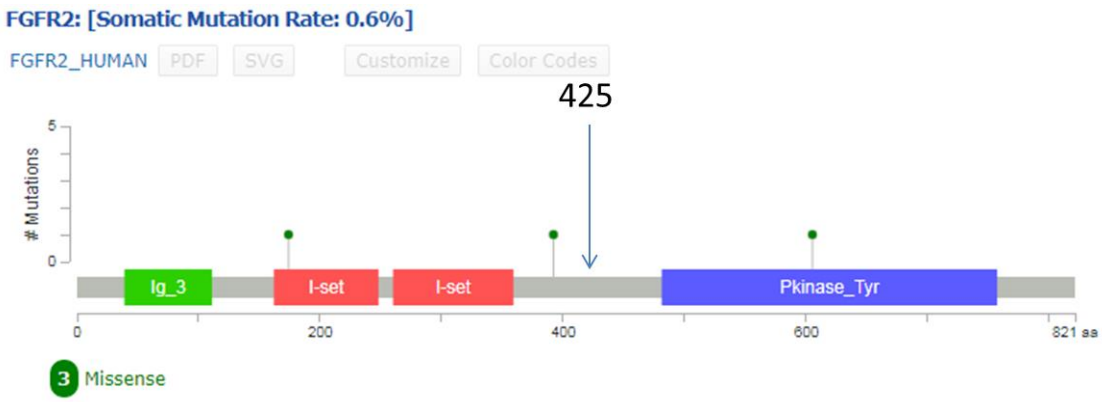


Figure 3-4. Novel somatic mutation in FGFR2 gene in 10004 patient's tumor, resulting in predicted damaging arginine to tryptophan mutation at residue 425.

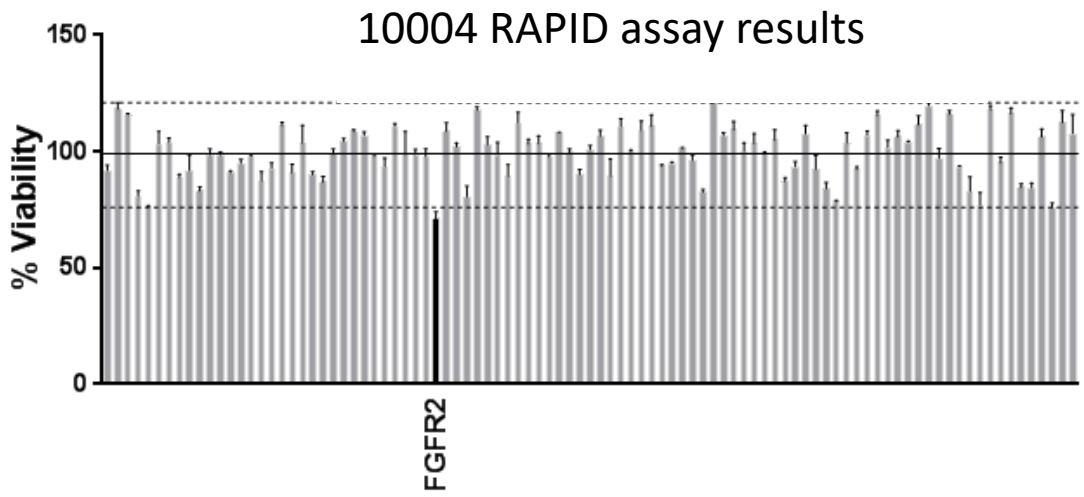


Figure 3-5. 10004 RAPID assay results.

siRNA targeting FGFR2 was an effective siRNA as a single agent in reducing cell viability in patient-derived tumor cells from 10004.

In addition, we detected an insertion at chromosome 7 position 55249012 (C to CGGT), resulting in a single amino acid insertion in the catalytic domain of EGFR (D725DG) in patient 10205's tumor with an allele frequency of 24%. This mutation is a previously reported somatic mutation and was predicated to be deleterious based on both COSMIC and our functional predictions. This patient is one of the anti-EGFR treatment resistant patients in inhibitor assay (Figure 2-9) and RAPID assay (Figure 2-14), suggesting further investigation of functions of this mutation and its relationship with EGFR inhibitor resistance. Notably, we detected an insertion mutation in EGFR in patient 10205's tumor, which represents a difficult to treat nasal HNSCC subtype, and this mutation was predicted to be deleterious with a significant score –7.55 by PROVEAN (Protein Variation Effect Analyzer), a software tool which predicts whether an amino acid substitution or indel has an impact on the biological function of a protein. This patient is one of the EGFR inhibitor resistant patients, suggesting further investigation of functions of this mutation and its association with EGFR inhibitor resistance, and possible new treatment options for this nasal HNSCC.

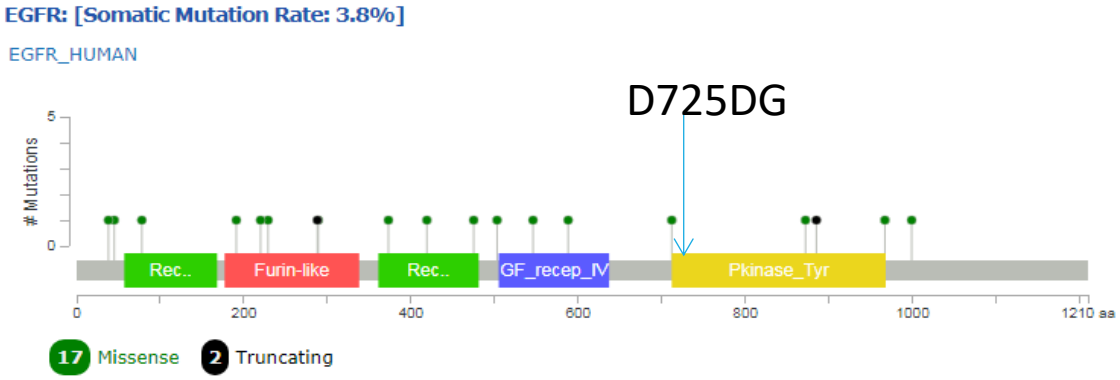


Figure 3-6. An insertion mutation in EGFR in 10205.

An insertion at chromosome 7 position 55249012 (C to CGGT), resulting in a single amino acid insertion in the catalytic domain of EGFR (D725DG) in patient 10205's tumor with an allele frequency of 24%.

Microenvironmental elements in EGFR and PI3K inhibitor resistance

Alterations in EGFR and PIK3CA are among the most frequent in the HNSCC TCGA cohort (Figure 3-8) (Cerami et al. 2012). Although numerous preclinical studies, including our data from inhibitor assays (Table 2-2), strongly suggest that targeting the PI3K pathways should be clinically beneficial in HNSCC (D'Amato et al. 2014), the dramatic response expected of PI3K inhibitors is not borne out in clinical studies(Jimeno et al. 2014). Major variances between preclinical studies and clinical trials may come from the lack of tumor microenvironment in preclinical models. It is likely that elements in the tumor microenvironment in patients may impede patients' response to anti-EGFR and PI3K treatments.

To test the microenvironmental factors that may affect drug response, a high throughput screening technology called microenvironment microarrays (MEMA) could be used to screen for stromal proteins or soluble factors that may be responsible for drug resistance. MEMAs are microarrays that have extracellular matrix (ECM, or substratum) molecules and cytokine/growth factors printed in pairs, providing over 1600 unique microenvironment conditions on the plates(C.-H. Lin, Lee, and LaBarge 2012). My preliminary data, has shown some differential responses to gefitinib under certain microenvironment conditions (Figure 3-8), although a robust statistical analysis of MEMAs was still under development at that time. In the future, this technology could be used to evaluate how growth, signaling, and response to EGFR and PIK3/mTOR inhibition, as well as other evidence based targets that are influenced by the tumor microenvironment, and can also be used to stratify patients by their tumor microenvironment based on whether it will promote or impair certain treatments.

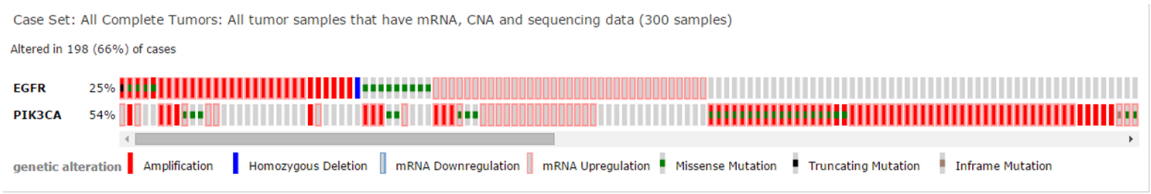


Figure 3-7. EGFR and PI3KCA alterations in HNSCC TCGA.

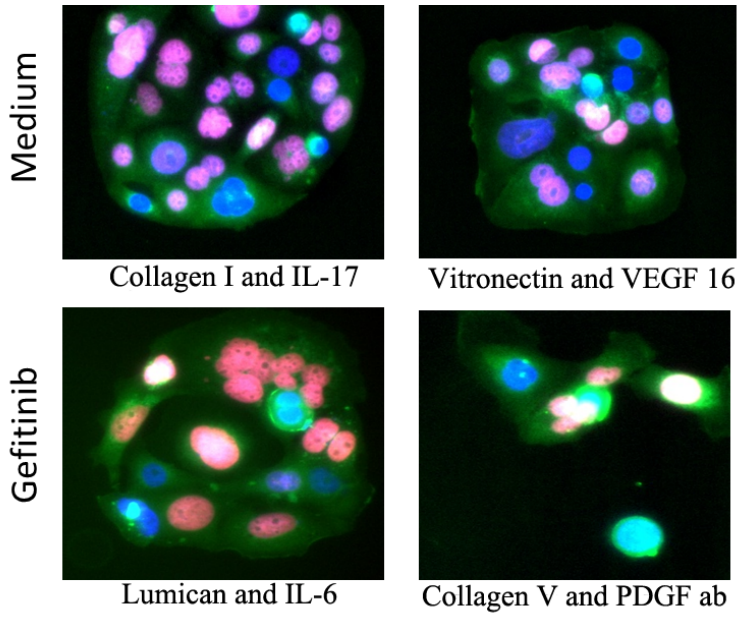


Figure 3-8. **Microenvironments affected 10205 patient-derived tumor cell response to EGFR inhibitor using microenvironment microarray.**

Patient 10205's tumor cells cultured in medium only and Gefitinib treatment under different conditions of microenvironmental elements. Red, EdU (proliferation); blue, DAPI (nucleus); green, b-tubulin (cytoskeleton). Under different microenvironment, number of cells that adhered (blue) and proliferated (red) differs.

Computational prioritization of targeted therapy

A bioinformatics tool, HitWalker (D. Bottomly et al. 2013), now updated to Hitwalker2 (Daniel Bottomly, McWeeney, and Wilmot 2016) was developed in our collaborator Dr. Shannon Mcweeney's lab to integrate –omics information into the complementary functional assays from patient samples to aid in the prioritization and visualization of these diverse data types. This method prioritizes patient variants relative to their weighted functional assay results in a protein-protein interaction network. The prioritization is performed using a random walk with restarts (RWR) algorithm. The RWR provides a measure of weighted proximity between a set of proteins associated with functional assay hits and a set of proteins containing variants. Variants are prioritized based on the resulting RWR association score attributed to the protein.

We will prioritize top effective drugs from inhibitor assays based on the results from each individual patient. Hitwalker analyses those significant responses to the inhibitors from the inhibitor assay for each patient and compare this information to the frequency of the variants in TCGA. We will select the top ranked variants across patients for further follow-up. Changes associated in the patient samples with target sensitivity in functional screens will be characterized by frequency, relationship to tumor TNM staging, tumor grade, site in the oral cavity, angio/lymphatic or perineural invasion, time of recurrence relative to diagnosis, and survival data, updated during the course of our study within our Molecular Profiling Resource tissue bank and database. Based upon these analyses, we will select several targets and corresponding drugs with highest potential as single agents or pairs with synergistic effect, for validation of whether mutation/aberration in the entire

OHSU dataset (weighted by frequency in TCGA HNSCC dataset) is responsible for drug effect. We will validate these by *in vitro* approaches, evaluation *in vivo* of target status in the original human tumor(s) and *in vivo* in the xenograft animal model.

Significance.

HNSCC is a serious global health problem, with estimated more than 550,000 new cases and 300,000 deaths annually (Papageorgiou and Avruch 2012). These cancers account for approximately 4% of all cancers in the United States (Siegel, Miller, and Jemal 2017). In the USA, it has been estimated that more than 65,000 men and women would be diagnosed with head and neck cancers in 2017 ("Cancer Facts & Figures 2017" n.d.), and an estimated 9,700 people will die of these cancers (The American Cancer Society, 2017).

Despite several efforts to identify biomarkers for early detection and develop new treatments, the overall survival rate and prognosis remain poor (Molinolo et al. 2009; Pisani, Bray, and Parkin 2002; Papillon-Cavanagh et al. 2017). The risk factors and carcinogens that participate in the development of HNSCC are smoking and HPV, which are well recognized (National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health 2014; Pullos, Castilho, and Squarize 2015). Local recurrence and metastasis are limiting factors for the success of the treatment (Chang and Wang 2016; Le, Squarize, and Castilho 2014). The five-year survival rate for regional and distant HNSCCs are 64.2% and 38.5%, respectively, and approximately 66% of the patients were diagnosed at these stages (Figure 3-9).

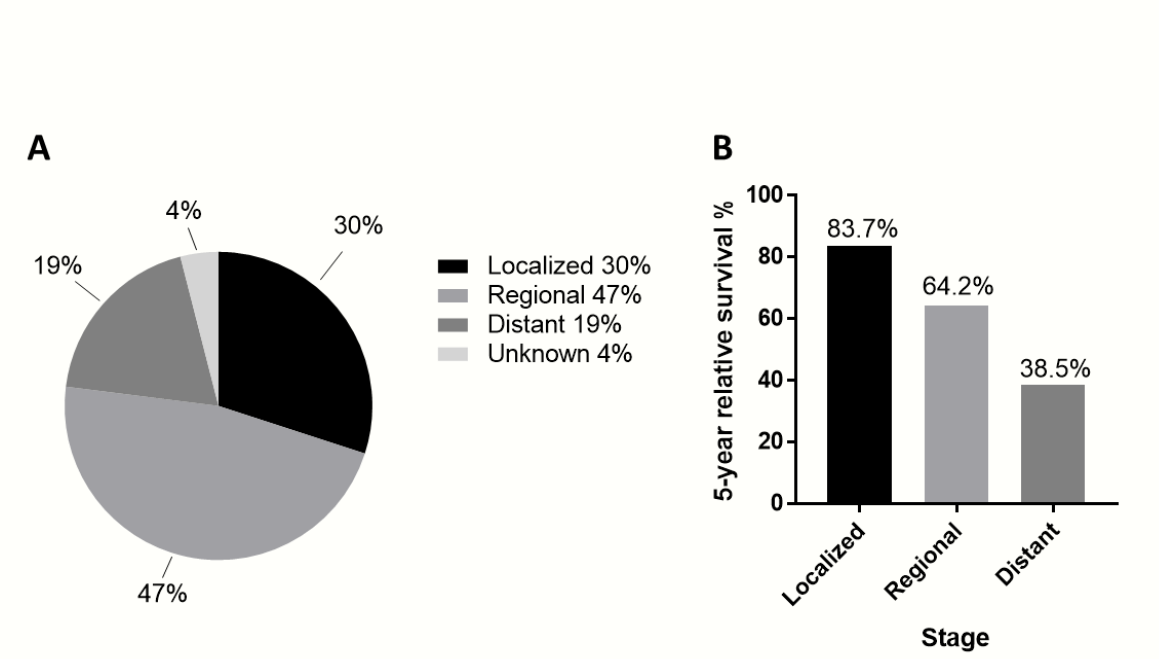


Figure 3-9. **HNSCC patient disease stages at diagnosis and 5-year survival by stages.**

Data plotted from Surveillance, Epidemiology, and End Results (SEER) database, 2007-2013, All Races, Both Sexes included.

Current diagnostic tests, including overexpression, mutation, or copy number variation of the EGFR gene, fail to predict HNSCC patient response to the only approved molecular targeted therapy, cetuximab, and this therapy fails eventually (Chong and Jänne 2013). Despite advances in surgery, chemotherapy, and radiation therapy, current treatments are disfiguring, result in nerve pain, impair eating and vocalizing, and diminish quality of life. Further, HPV-negative HNSCC patients exhibit worse outcomes to the current treatment options compared to the HPV-positive HNSCC (O'Sullivan et al. 2012b; Lassen et al. 2009b; Fakhry et al. 2008b; Ang et al. 2010b). Precision therapies for HNSCC could spare tissue destruction, improve quality of life, and extend life.

My graduate studies using functional screens in patient-derived models identified EGFR and ALK inhibitor combinations as effective combination therapies in HNSCC patient-derived tumor cells. Four different ALK inhibitors, including two that are FDA-approved, ceritinib and brigatinib, showed synergistic effects with gefitinib in patient-derived tumor cells, suggesting a potential benefit of using ALK inhibitors in combination with an EGFR inhibitor for treating HNSCC patients. Ceritinib, a next-generation ALK TKI with better selectivity to ALK than crizotinib (Cooper et al. 2015), has been FDA approved for NSCLC, therefore can be readily applicable in combination with EGFR inhibitor to HNSCC in clinical trials. Of note, brigatinib is a ALK and EGFR dual inhibitor, which can co-target EGFR and ALK in HNSCC even without EGFR inhibitor, reducing the chance of toxicity in patients with less dosage as being a single agent. These findings could provide more opportunities for late stage HNSCC patients with no options in the clinic.

My study provided evidence for ALK induction and activation after EGFR inhibitor treatment in HNSCC as a novel mechanism for and strategy to address EGFR inhibitor resistance in the clinic. These findings provide rationale to further investigate this phenomenon in other cancer types, such as NSCLC, colorectal carcinoma, pancreatic cancer, breast cancer and neuroblastoma, where EGFR inhibitors are relevant (Table 1-1). While whether this particular combination has sufficient additional clinical benefit to become a standard option for HNSCC patients remains to be seen, our approach, particularly for target sensitivities that may be observed in the achievable plasma concentration range, is providing promising leads for greatly expanding options for the treatment of HNSCC.

The inhibitor assay provides information on drug vulnerabilities even without prior genetic knowledge of the tumor. The use of patient-derived cells to functionally evaluate HNSCC cell sensitivity to drugs and siRNAs may provide critical guidance for precision medicine clinical trials in the future. The results of inhibitor and RAPID assays can be available in 4 to 8 weeks, well within the two year timeframe of recurrence of most HNSCC (C. R. Leemans et al. 1994b). Thus, providing functional information for stratification to match individual patients to effective drugs in clinical trials is feasible. Future development of HNSCC-specific inhibitor and siRNA panels is underway, creating HNSCC and skin SCC-specific panels based on data analysis using big data sets of HNSCC such as TCGA, as well as to expand annotation of drugs with more accurate target information and siRNAs targeting “dark” pathways that are not represented in the current inhibitor assay panels derived originally from studies of leukemia. This promises to be beneficial in identifying more effective agents and combination therapies for

HNSCC and has great potential for expandability to other cancers based upon pathway and target dependence.

The use of patient-derived cells to functionally evaluate HNSCC cell sensitivity to drugs and siRNAs may provide critical guidance for precision medicine clinical trials in the future. The inhibitor assay has been used in a clinical trial in refractory leukemia, with the primary endpoint of reduction of phosphorylated, activated gene targets, and has demonstrated remissions (NCT02779283). Because individual HNSCC patients' cells and tumors are evaluated in the timeframe of 4 to 8 weeks, well within the two year timeframe of recurrence of most HNSCC (C. R. Leemans et al. 1994b), it is feasible to translate new diagnostic and treatment tools arising from our study to precision treatments.

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