2009

THE PHARMACOGENOMICS OF EGFR-DEPENDENT NSCLC: PREDICTING AND ENHANCING RESPONSE TO TARGETED EGFR THERAPY

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ABSTRACT OF DISSERTATION

Justin M. Balko, Pharm.D.

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2009

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
Department of Pharmaceutical Sciences
at the University of Kentucky

By:

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Lexington, Kentucky

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The introduction of tyrosine kinase inhibitors (TKI) targeting the epidermal growth factor receptor (EGFR) inhibitors to the clinic has resulted in an improvement in the treatment of non small cell lung cancer (NSCLC). However, many patients treated with EGFR TKIs do not respond to therapy. The burden of failed treatment is largely placed on the healthcare field, limiting the effectiveness of EGFR TKIs. Furthermore, responses are hindered by the emergence of resistance. Thus, two questions must be addressed to achieve maximum benefit of EGFR inhibitors: How can patients who will benefit from EGFR TKIs be selected *a priori*? How can patients who respond achieve maximal benefit? To answer these questions, two hypotheses were formed. First, the EGFR-dependent phenotype, which is displayed by the tumors cells of those patients who respond clinically to EGFR TKIs, can be captured by genomic profiling of NSCLC cell lines stratified by sensitivity to EGFR TKIs. This gene signature may be used to predict the outcome of EGFR TKI therapy in unknown samples. Secondly, the predictive signature of response to EGFR TKI could provide insights into the underlying biology of the phenotype of EGFR-dependency. This information could be exploited to identify inhibitors which could be combined with EGFR inhibitors to elicit a greater effect, thereby minimizing resistance. The work herein describes the testing of these hypotheses.

Pharmacogenomics was utilized to define a signature of EGFR-dependency which effectively predicted response to EGFR TKI *in vitro* and *in vivo*. Furthermore, the signature was analyzed by bioinformatic approaches to identify the RAS/MAPK pathway as a candidate target in EGFR-dependent NSCLC. The RAS/MAPK pathway regulates expression and activation of EGF-like ligands. Furthermore, the RAS/MAPK pathway modulates EGFR stability in the EGFR-dependent phenotype. Further biochemical analyses demonstrated that the RAS/MAPK pathway mediates proliferation and survival of EGFR-dependent NSCLC cells. Finally, combinatorial treatment of EGFR-dependent NSCLC cell lines with small molecules targeting EGFR and the RAS/MAPK pathway yielded cytotoxic synergy. Thus, we have used pharmacogenomics methods to potentially improve NSCLC treatment by developing a method of predicting response and identifying an additional target to combine with EGFR TKIs to maximize responses.

KEYWORDS: Predictive pharmacogenomics, epidermal growth factor receptor, cancer, RAS/MAPK, EGFR ligands

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April 3rd, 2009

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DISSERTATION

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CHAPTER 1

A. LUNG CANCER OVERVIEW

Cancer overview

Cancer is the end-product of a series of genetic and epigenetic events within the cell and its environment that culminate in uncontrolled proliferation, survival, and eventually, malignant invasion into surrounding tissue. Commonalities among all cancers are the loss of expression or functionality of tumor suppressors (genes involved in negatively regulating proliferation and survival), as well as the gain of function of oncogenes. Tumor suppressors include genes which regulate apoptosis, such as p53, and those that control critical cell cycle checkpoints, such as retinoblastoma-1 (RB1), among others. Similarly, gain-of-function mutations in or increased expression of proto-oncogenes, such as the small GTPase RAS, also contribute to the formation of malignancy (1). Activated oncogenes such as RAS promote survival or drive cell proliferation by establishing self-sufficiency of growth signals. With few exceptions, multiple genetic and epigenetic events are required to fully transform normal into neoplastic tissue. However, it is likely that acquiring early lesions increases the probability of developing cancer by fostering an environment for additional cancer-promoting events. For instance, autosomal aberrations in BRCA1, a DNA repair enzyme, reduce the ability of the cell to correct errors in DNA replication, greatly increasing the likelihood of further mutations (2). Thus, those patients inheriting one or more of such genetic lesions are often diagnosed with cancer at a relatively early age.

As cancer progresses, tumor cells gain the ability to survive independently of the stroma. As a result, neoplastic cells enter the circulatory system and metastasize to distant tissues (1). Multiple organ involvement and ultimate failure is a hallmark of late-stage disease, typically resulting in death. Traditional treatments for cancer include resection of the malignant tissue, radiotherapy, and systemic chemotherapy. Importantly, resection is typically a useful approach in localized disease only. Thus, early detection is critical in cancer, as surgical resection is the only treatment modality which is considered potentially curative (3). Unfortunately, many aggressive cancers, including those of the lung, are characterized by a late stage of presentation resulting in high mortality rates (4).

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Lung cancer epidemiology and etiology

Cancers of the lung and bronchus pose a major health issue in the United States as well as in other developed nations. Since the early 20th century, a rise in industrialization and smoking has significantly increased the prevalence of lung cancer (5). Pulmonary cancer ranks first among all cancers in mortality, comprising over 28% of neoplastic-related deaths (4). In 2008, over 215,000 new cases of lung cancer are expected to have occurred (4). A close correlation exists between incidence and mortality of lung cancer, reflecting the reality that approximately 80% of lung cancer patients ultimately die of their disease. The Commonwealth of Kentucky reports one of the highest incidence rates of lung cancer of any state in the US, presumably due to high smoking rates and the local dependency on tobacco as an agricultural crop. The predominant contributing factor to the development of lung malignancy is exposure to tobacco smoke. Radon progeny, asbestos, arsenic, chloromethyl ethers and outdoor pollution are other exposure risks for the development of lung cancer (5).

Lung cancer typically presents as one of several different histological subtypes. The broad categorization of lung carcinoma is typically stratified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which includes adeno-, bronchioalveolar, squamous cell, and large cell carcinomas. Small cell and squamous cell carcinomas exhibit the strongest correlation to smoking. Adenocarcinoma, which arises from glandular tissue, is the most commonly occurring lung cancer subtype (6). The heterogeneity of lung cancer coupled with the diversity and multitude of underlying genetic alterations results in a highly complex disease state. Recent advances in our molecular and genetic knowledge of the disease have elucidated aspects of lung cancer biology that may be exploited through targeted biological and small molecule therapy.

Lung cancer genetics

Familial genetics plays a significant role in the development of lung cancer. The majority of people who smoke never develop lung cancer suggesting that genetic risk factors may predispose certain smokers to disease (6). In support of this, after adjusting for age, smoke exposure, occupation, and gender, first-degree relatives of lung cancer patients have a two-fold risk of developing lung cancer (7). On occasion, lung cancer can develop in nonsmokers at an early age, often fitting a Mendelian co-dominant inheritance model (8, 9). Further evidence for the role of genetic predisposition for

development of lung cancer is found in homozygous and heterozygous carriers of inactivating mutations in tumor suppressor genes such as RB1 or p53 (10, 11). However, these genes are involved in the development of a variety of cancers, and a lung cancer-specific gene has not yet been conclusively identified (12).

Aside from familial predisposition to the development of lung cancer, environmental factors such as tobacco smoke can facilitate the formation of a variety of genomic lesions directly resulting in pathogenesis (13). Although lung cancer is a highly heterogeneous disease, many of the common genetic and epigenetic events that comprise the general pathological course have been elucidated. Large scale genomic analyses demonstrate that a large number of somatic mutations are present in a typical lung tumor (14). Additional events such as chromosomal aberrations and epigenetic alterations frequently coincide with mutations (15). Constituents of tobacco smoke, such as polycyclic aromatic hydrocarbons, form DNA adducts which, when unrepaired, lead to apoptosis in normal cells. However, if repair or apoptotic mechanisms fail, the mutations are retained in the genome (13). Critical genetic lesions in p53, RB1, or RAS are present in a large number of lung cancers and correlate strongly with smoking (15). Their presence has been suggested to play a significant role in the initiation or progression of disease. A number of other genetic events have been shown to occur commonly in lung cancer and are reviewed elsewhere (15, 16). Among these are c-Kit mutations, amplification of MYC family members, and amplification of the pro-survival BCL2 protein. Although clearly important in the pathogenesis of lung cancer, the specific roles of these genes are less well-studied than p53, RB, RAS and EGFR.

The p53 transcription factor is one of the most frequently mutated genes in human cancers. Wildtype p53 is a DNA-binding protein that is regulated through transcriptional, translational, and post-translational events, including degradation targeted by the MDM family of ubiquitin ligases (17). Various stimuli, such as oncogenic signaling, hypoxic events, free radicals, and DNA damage activate wildtype p53 resulting in cell cycle inhibition at G1 and induction of apoptosis through both transcription-dependent and transcription-independent mechanisms (18, 19). Thus, p53 maintains a critical function in determining cell fate (i.e. repair or apoptosis)(19). Loss of p53 functionality promotes the progression of cancer by fostering an environment where additional lesions are more likely to occur. As many as 90% of SCLCs and 50% of

NSCLCs harbor mutationally-inactivated p53, correlating to tobacco use (18). Thus, loss of p53 activity is clearly an integral step in the development of lung cancer.

Lung cancer is also commonly associated with aberrations in RB1. Loss of RB1 was one of the first tumor suppressor genes linked to the formation of lung cancer. RB1 regulates an imperative checkpoint in cell cycle progression and its inactivation by hyperphosphorylation and subsequent dissociation from E2F is required for E2F-mediated S-phase entry (20, 21). Inactivating mutations in RB1 or in other components of the RB pathway, such as $P16^{INK4A}$, are present in almost all cases of lung malignancy (22). Interestingly, mutations in RB1 or loss of heterozygosity at the RB1 locus is present in almost all small cell lung cancer cases, while inactivation of the upstream pathway component $P16^{INK4A}$ is more common in NSCLC (22). Loss of RB pathway activity provides a means by which the tumor cell can avoid the regulatory constraints of cell cycle progression.

Another important genetic event in the pathogenesis of lung cancer is mutation of the RAS oncogene. The RAS family consists of three independently translated proteins: NRAS, KRAS, and HRAS. Under normal cell conditions, these proteins function as GTPases that are bound to GDP in their inactive state. Activation by upstream mitogenic stimuli results in the formation of GTP-bound RAS. Activated RAS binds downstream effector molecules and drives cell proliferation and survival. GTPase activating proteins facilitate the hydrolysis of GTP to GDP, restoring RAS to its inactive form (23). Of the three RAS family members, KRAS is most commonly studied in the context of lung cancer due to the frequency of observed aberrations. Mutations in codon 12 of exon 1 and codon 61 of exon 2 result in constitutive activation of KRAS and self-sufficiency of growth signals, culminating in uncontrolled cell proliferation and survival. KRAS lesions are highly oncogenic, as demonstrated by the observation that introduction of the mutant protein is sufficient to transform fibroblasts (24). Mutations in KRAS correlate with smoking status and occur in 40-50% of lung adenocarcinomas, but are relatively rare in other histological subtypes (25).

A final gene receiving increasing scrutiny that is involved in lung cancer is the epidermal growth factor receptor (EGFR) oncogene, and is the focus of this body of work. In the last decade, deregulation of EGFR through over expression, mutation, and various other mechanisms, has been shown to play a major role in a subset of NSCLC

tumors. This population of tumors comprises only 10-20% of all lung cancer, but tumors harboring activated EGFR represent an important and intriguing phenotype to study. Patients harboring EGFR-deregulated lung tumors are often highly responsive to EGFR tyrosine kinase inhibitors. Thus, our increasing understanding of both EGFR and the role of EGFR inhibitors in lung cancer represent a major breakthrough in therapy. Patients who respond to EGFR inhibitors tend to be non-smokers and have no overt etiological cause for disease. Thus, it is unclear what the pathologic causes and consequences are in tumors harboring activated EGFR. A thorough discussion of EGFR as it relates to lung cancer is presented below.

B. THE BIOLOGY OF EGFR

The epidermal growth factor receptor (EGFR/ErbB1/HER1) is a 170 kilodalton transmembrane receptor tyrosine kinase (RTK). EGFR is one of four structurally related RTKs known as the ErbB family and consists of three primary domains: an extracellular ligand-binding domain, a lipophilic transmembrane domain and a cytoplasmic tyrosine kinase domain (26, 27). EGFR exists as a monomer that homo- or hetero-dimerizes with other ErbB family members upon activation by extracellular ligands (FIG 1.1). Thus, heterodimerization presents a mechanism by which co-activation of different combinations of ErbB family receptors can modulate a diverse array of cell signals.

ErbB family members can be activated by many extracellular growth factor ligands. The ligands responsible for the activation of EGFR include epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin (AREG), betacellulin (BTC), epiregulin (EREG), and heparin-binding epidermal growth factor (HB-EGF) (28). Several of these ligands, including TGF-α, AREG, and HB-EGF, are synthesized as inactive pro-ligands tethered to the cell membrane. Processing of these ligands requires cleavage from the membrane by matrix metalloproteases (MMP) (29). Specifically, the ‘a disintegrin and metalloprotease’ (ADAM) family of MMPs is primarily responsible for this activity (30, 31). Following the release of ligand from the membrane, ligand binding to EGFR induces a conformational change in the receptor that promotes dimerization. Dimerization brings the cytoplasmic tails of the receptor monomers in proximity, resulting in autophosphorylation at tyrosine residues and activation of the kinase domain (26, 27). These phosphorylated residues serve as docking sites for a variety of second messengers, such as PI3K, STAT3, PLCγ, SRC,

SHC, and GRB2, which in turn can be activated or facilitate the activation of other molecules via the kinase activity of EGFR. The downstream effectors that can be activated by EGFR are diverse and are often cell type and/or context-specific. The resulting series of transduced signals largely culminate in the nucleus, modulating gene expression and driving cell cycle progression (32).

EGFR internalization and degradation

EGFR is a regulator of cell cycle progression and is involved in normal epithelial cell renewal and remodeling (33). Since control of cell cycle progression is a function that is frequently hijacked in the context of cancer, it is not surprising that EGFR signaling is a tightly regulated event. EGFR signaling can be negatively regulated by the activity of phosphatases, transcriptional and translational control, and post-translational processing. In addition to these regulatory events, degradation of the activated receptor is an important mechanism by which the cell can control the extent of signaling.

Once EGFR has been activated by ligand binding, the receptor is internalized in the endosome and either recycled to the membrane or ubiquitinated and degraded through the proteosomal pathway (33). Internalization is not required for receptor signaling, but is instead believed to be a mechanism by which the extent of signaling can be regulated (34). Phosphorylation at tyrosine 1045 (Y1045) activates the docking site for the Cbl protein, which along with E2 ligase, binds and facilitates the mono- and/or poly-ubiquitination of EGFR. Ubiquitinated EGFR is sorted to lysosomes where receptor degradation takes place (35). Interestingly, some ligand-EGFR complexes, such as EGF-EGFR are more likely to result in receptor degradation than other ligand-EGFR complexes (36). This has been suggested to be the result of a tighter binding efficiency of EGF in the acidic conditions of the endosome. In contrast, TGF-α dissociates from the receptor at the endosomal pH, deactivating the receptor and reducing ubiquitination before lysosomal sorting can occur. The dissociation and insufficient ubiquitination results in recycling of both the receptor and the ligand to the cell surface (36). Thus, differences in post-activation dynamics demonstrate one of the many diverse levels at which EGFR signaling and functionality can be modulated.

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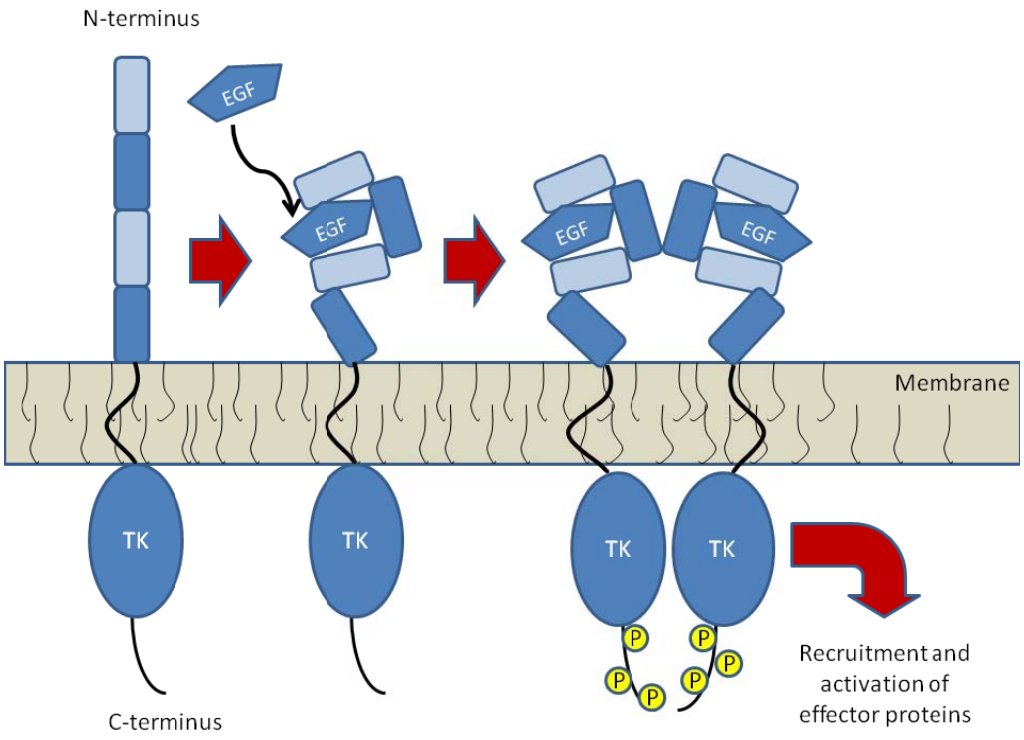
Figure 1.1: Activation of the epidermal growth factor receptor



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EGFR pathway activation

The activation of EGFR results in downstream signaling through a number of pathways such as RAS/MAPK, AKT, and STAT. Initial characterization of many of these pathways spawned a relatively naïve linear model for transduction of signals from the cell membrane to the nucleus (37). However, advancement in knowledge of the complexity of these pathways has produced a much more elaborate 'network' model where many downstream pathways participate in significant 'cross-talk' among themselves (Figure 1.2) (38-40). Additionally, many of these pathways can be activated by other receptor tyrosine kinases in response to a wide array of growth factors (38). Of the complex network of downstream signaling modules that emanate from EGFR, several pathways have been studied extensively.

The RAS/RAF/MEK/ERK (RAS/MAPK) pathway is a well-characterized signal transduction cascade activated by EGFR. EGFR can activate the small GTPase KRAS through several known mediators, including GRB2, SHC, and SOS (41). KRAS can also be activated through phosphorylation by other RTKs such as the insulin-like growth factor receptor (IGFR), MET, and the platelet-derived growth factor receptor (PDGFR) resulting in pathway cross-talk (38). GTP-bound KRAS transduces signals through a chain of phosphorylation events activating each successive kinase in the chain (RAF-MEK-ERK). These signals are transmitted from the cell membrane to the nucleus, ultimately modulating gene transcription (42). The ultimate kinase of the RAS/MAPK pathway, ERK, is translocated to the nucleus upon phosphorylation and activates transcription factors such as ETS and ELK (43). The genes regulated by RAS/MAPK activation, such as FOS, JUN, and MYC, drive cell proliferation and survival, two important oncogenic processes (44-46). Two components of this cascade, KRAS and BRAF, are oncogenes that are frequently mutated in a variety of cancers (47-57). Inhibitors of the RAS/MAPK pathway, specifically those targeting RAS, RAF, and MEK have been developed and tested clinically, but have yet to gain FDA approval. Only the multi-targeted kinase inhibitor sorafenib, which targets RAF, has entered into clinical use.

The second well-characterized pathway, phosphoinositol-3-kinase (PI3K)/AKT, also lies downstream of EGFR. The PI3K/AKT pathway is of significant interest in the context of cancer due to its role in protein translation and inhibition of apoptosis.

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Stimulation of RTKs such as EGFR can activate AKT via PI3K which in turn phosphorylates the BCL-2 family protein BAD, dissociating it from the BXL2/BCL2 complex and inhibiting intrinsic pro-apoptotic mechanisms such as the release of cytochrome C from mitochondria and caspase activation (58, 59). Importantly, AKT also feeds through the mammalian target of rapamycin (mTOR) pathway (60). The mTOR pathway plays an integral role in regulation of protein translation, and is a validated target of the FDA-approved anti-cancer drug, temsirolimus, in renal cell carcinoma (61, 62). Currently, no small molecule AKT inhibitors have obtained FDA approval although one is currently navigating clinical trials and shows promise (63). Several inhibitors are available for *in vitro* research, including wortmannin and LY294002, and these inhibitors demonstrate some activity in NSCLC cell lines (64).

A third effector pathway that may play a role in EGFR-mediated oncogenicity is the signal transducers and activators of transcription (STAT) module. STAT family members can be activated by direct EGFR-mediated phosphorylation or indirectly via SRC-mediated phosphorylation (65). Upon EGFR or SRC activation, STAT is phosphorylated at Y701, Y705, or Y694 (STAT1, STAT3, or STAT5 respectively)(65). Phosphorylation induces homodimerization, which results in transport of STAT3 to the nucleus where it binds DNA, promoting or repressing gene transcription activity. STATs, particularly STAT1, STAT3, and STAT5 have been extensively studied in cancer due to their role in regulating the expression of genes known to promote survival, cell proliferation, immune response, angiogenesis, and wound healing (66-68). STAT3/5 target genes include cyclin D, VEGF, and BCL2 among others (68). Interestingly, STAT1 has reduced expression in malignant tissue and transformed cells suggesting activity as a tumor suppressor, possibly through enhancement of p53 mediated apoptosis (69). In contrast, STAT3 and STAT5 frequently transmit oncogenic signals and are potential therapeutic targets in lung cancer (68, 70-74). However, no selective small molecule inhibitors of STAT3 or STAT5 have been extensively validated to date.

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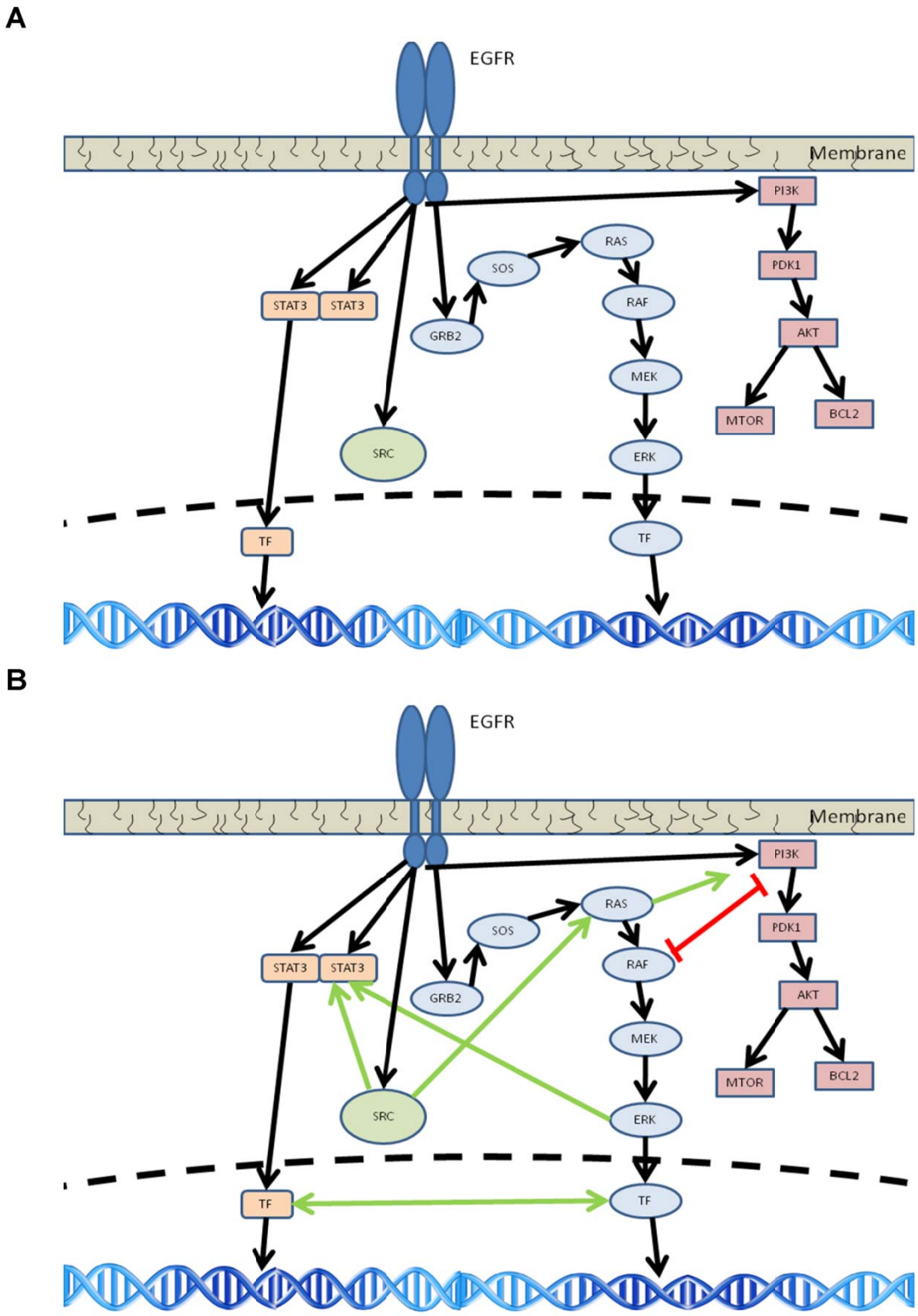
Figure 1.2: EGFR signaling pathways

(A) Linear model **(B)** Network model



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C. EGFR IN LUNG CANCER

EGFR can be oncogenic by a variety of mechanisms in cancer, including mutations, over expression of EGFR, over expression of ligands, defective down-regulatory mechanisms, and co-expression of other erbB family members (Figure 1.3). Somatic mutations in EGFR are frequently observed in solid tumors such as glioblastoma multiforme (GBM) and NSCLC. The location and nature of EGFR mutations are diverse, but primarily occur in one of three regions of the receptor. Mutations in the extracellular and intracellular domains are common in GBM and result in truncated receptors, many of which display ligand-independent activation (75, 76). The most frequent of these mutations, EGFR variant III (EGFRvIII) has also been reported to occur sporadically in NSCLC (77). EGFRvIII lacks extracellular amino acid residues 2-273, resulting from an in-frame deletion of exons 2-7 (78). Although the EGFRvIII mutant has lost the ability to bind ligand, the receptor displays ligand-independent activation of downstream pathways and impaired regulatory mechanisms, resulting in prolonged receptor activation (78, 79). EGFRvIII mutations often coincide with EGFR gene amplification and have been estimated to occur in approximately 3% of NSCLCs, usually in squamous cell carcinomas (77).

The most relevant EGFR mutations to NSCLC occur in the putative tyrosine kinase domain (exons 18-24) located in the cytoplasmic tail of the molecule (Figure 1.1). These mutations were only recently identified and occur in 10-20% of NSCLCs (80-88). Deletions in exon 19 (usually del746-750) and point mutations in exons 18 (G719A/C), 20 (T790M), and 21 (L585R) result in increased and prolonged activation of the receptor (89-93). Genes carrying these mutations, when ectopically expressed, are capable of transforming mouse fibroblasts. Additionally, lung-specific transgenic expression of EGFR del746-750 and L858R induce tumors in mice (90, 94). Clinically, these mutations have been found in higher prevalence in adenocarcinomas and bronchioalveolar carcinomas, women, patients of Asian descent, and nonsmokers (87, 95-97). Interestingly, many NSCLC tumors that harbor these mutations in EGFR are uniquely responsive to EGFR-targeted small molecule inhibitors (83, 95). In contrast, those tumors that contain the EGFR T790M mutation are resistant to EGFR inhibitor treatment (98-100).

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EGFR (chromosome 7p12) can also be over expressed, usually the result of genomic amplification. Increased EGFR copy number is found in the tumors of as many as 50% of lung cancer, GBM, and colorectal cancer patients (101-103). Over-expression of EGFR via genomic amplification leads to increased EGFR activation, likely due to spontaneous dimerization of the receptor at high density levels on the cell membrane (78, 104, 105). Aside from genomic amplification, EGFR over-expression may be the result of increased promoter activity or decreased regulation of transcription or translational mechanisms (78).

Activation of the EGFR receptor in the context of lung cancer can also result from increased expression and/or cleavage of ligands (106). Ligand-dependent EGFR activation can result in a feed-forward autocrine loop, further inducing the expression and/or activation of ligands (107). Increased ligand activity has been shown to promote oncogenesis in a number of models. One of the most well studied EGFR ligands in cancer is TGF-α. It has been known for more than a decade that transgenic mice (C57BL/6) engineered to over-express TGF-α develop a variety of hyper-, meta-, and neoplasias, demonstrating a possible role for TGF-α in the pathogenesis of cancer (108). The expression of EGFR ligands is required to achieve the oncogenic potential of wildtype-EGFR, as mouse fibroblasts over-expressing EGFR require TGF-α or EGF for transformation (109). Tumors that have been engineered to ectopically express EGFR ligands such as TGF-α demonstrate a significant growth advantage suggesting that TGF- α expression may drive the growth of more aggressive tumors. In one study, stable transfection of lung adenocarcinoma cells with TGF-α prior to orthotopic growth in nude mice produced lung tumors that were approximately 2-3 times the size of tumors grown from control lung adenocarcinoma cells (i.e. those not transfected with TGF-α (110). Other EGFR ligands, such as amphiregulin, promote additional oncogenic processes such as invasion and migration in normal and malignant mammary epithelial cells (111). As such, the combinatorial effects of over expression of many ErbB1 ligands could result in complex phenotypic interactions in cancer, promoting and maintaining oncogenic processes. Thus, over-expression of EGFR ligands can play an important role in some cancers.

Impairment in downregulatory mechanisms is yet another way that EGFR signaling can be deregulated in cancer. Defective downregulation of EGFR has been observed in tumors cells that harbor activating mutations in the tyrosine kinase domain

of EGFR (93). Cells harboring EGFR del746-750 or L858R demonstrate reduced ubiquitination in response to ligand binding, prolonging signaling (93). Although it is presently unclear what the precise mechanism is for this impairment, it is hypothesized that mutant EGFR undergoes enhanced heterodimerization with ErbB2, thus blocking the Y1045 site required for interaction with Cbl, the enzyme responsible for mediating ubiquitination of EGFR (93).

Finally, EGFR heterodimerization with other ErbB family members has been hypothesized to contribute to oncogenic properties of the tumor cell (78). Receptor heterodimerization is an important aspect of ErbB family signaling in normal cells. ErbB2 has no known ligand while ErbB3 lacks a putative kinase domain underscoring the necessity of heterodimerization of these proteins to effectively transduce extracellular signals. ErbB family members are over expressed in various cancer types resulting in aberrant oncogenic signaling (i.e. HER2/ErbB2 in breast cancer). Because ErbB2 lacks a putative activating ligand and ErbB3 lacks a kinase domain, the importance of ErbB heterodimerization and crosstalk to convey oncogenic signals appears to be paramount (112). Of the various ErbB combinations that can be expressed in tumor cells, EGFR-ErbB3 heterodimerization has been suggested to play a primary role in driving NSCLC tumor cell growth and mediating resistance to EGFR-targeted therapy (113, 114). Due to the potentially large number of combinatorial over-expression patterns of ErbB receptors in lung cancer, the role of receptor heterodimerization is not yet fully understood.

EGFR oncogene addiction

The term ‘oncogene-addiction’ originated from the finding that the tumor cells of greater than 90% of patients with chronic myelogenous leukemia (CML) harbor the t(9;22) translocation resulting in expression of the BCR-ABL fusion gene (115). BCR-ABL is a tyrosine kinase that is constitutively active in CML and drives cancer cell proliferation. Inhibition of BCR-ABL function by treatment with the BCR-ABL inhibitor imatinib in CML patients results in rapid and sustained remission in an overwhelming proportion of patients, suggesting that BCR-ABL is essential for tumor proliferation (115). This finding contradicted the hypothesis that all cancers are highly heterogeneous and are the end product of deregulated redundant and compensatory oncogenic signaling pathways.

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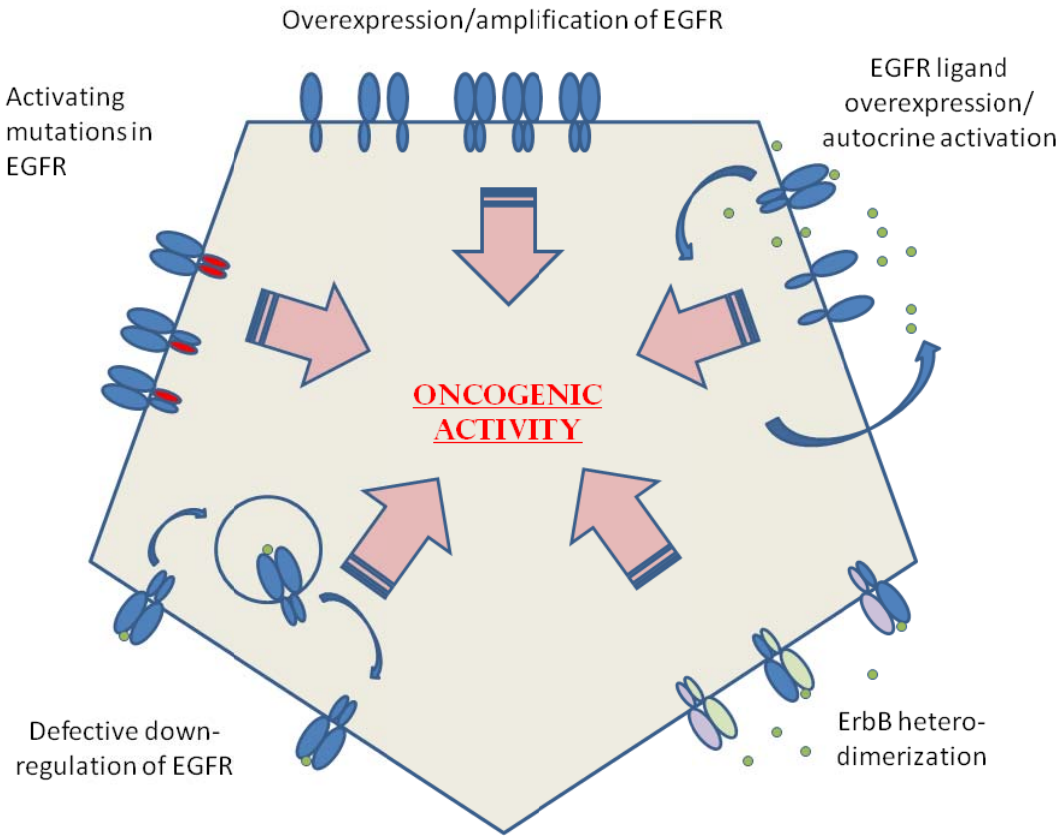
Figure 1.3: Mechanisms of oncogenic EGFR signaling



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Similar to CML, in NSCLC tumors displaying amplified or mutated EGFR, cell proliferation and survival appear to be dependent on the EGFR pathway. Cell culture models have demonstrated that cell lines harboring EGFR mutations undergo apoptosis when treated with EGFR-targeted agents or siRNA targeting the mutant receptors, much like that observed in CML patients treated with imatinib (83). Furthermore, animal models have demonstrated that EGFR activating mutations can transform lung epithelial cells, resulting in the formation of tumors when injected into immune-compromised mice, and inhibition of EGFR using small molecules results in dramatic regression of the tumor (90). Most importantly, large clinical studies have reported that a minority of patients (e.g. women, Asian, non-smoking, and/or adenocarcinoma histology) treated with small molecules targeting EGFR demonstrate massive tumor regression in cancers that were previously unresponsive to highly active chemotherapy (95). This population frequently displays some or all of the clinical or molecular characteristics described with respect to EGFR activation. Given that these neoplasms respond to inhibition of a single kinase in cases where broadly active chemotherapy was ineffective, it has been suggested that a subset of NSCLC are uniquely 'EGFR-dependent' in growth and survival characteristics (83, 95, 116, 117).

EGFR as a Target for Cancer Therapy

The finding that EGFR was activated through amplification or mutation in a variety of human cancers prompted the development of targeted EGFR therapeutics. Furthermore, preclinical models suggested that EGFR may mediate resistance to chemotherapeutic agents, thus targeting EGFR concomitantly with the administration of chemotherapeutic agents could improve benefit (118). The first anti-EGFR therapeutic to be approved was the EGFR tyrosine kinase inhibitor gefitinib (Iressa®, AstraZeneca) in May of 2003 (119). Gefitinib was approved by the FDA for the treatment of refractory NSCLC as an accelerated-track drug based on tumor response rates in phase III trials. However, when survival data became available for the trial, gefitinib failed to show benefit and was subsequently placed on restricted-use status. During this time, a second EGFR-TKI, erlotinib (Tarceva®, OSI Pharmaceuticals), completed Phase III trials. In contrast to gefitinib, erlotinib showed a 2-month improvement in median survival compared to placebo when used as monotherapy in previously-treated NSCLC (120). Based on these data, erlotinib was approved by the FDA in November of 2004 (121). Since its approval, erlotinib has also gained an indication for pancreatic cancer.

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Erlotinib is an orally available quinazolinamine compound with the formula $C_{22}H_{23}N_3O_4$ •HCl and a molecular weight of 429.9. Erlotinib is metabolized in the liver predominantly by CYP3A4, CYP3A5, and CYP2D6, but also by CYP1A2 (122). It has also been hypothesized that metabolism by CYP3A4 in the intestine, CYP1A1 in the lung, and CYP1B1 in tumor tissue could play a role in the metabolic clearance of erlotinib (122). Since smoking history appears to influence the efficacy of erlotinib in NSCLC, some reports have suggested that tobacco use may increase metabolic clearance of erlotinib via upregulation of CYP1A1 and CYP1B1, thus reducing drug exposure (123-126). The most common clinical adverse drug reactions to erlotinib include rash, diarrhea, and rarely, interstitial lung disease or liver toxicity. Interestingly, patients who develop a rash while on erlotinib have improved response rates, suggesting a pharmacokinetic component to the heterogeneous responses observed clinically (127, 128).

It is unclear why erlotinib appears to be more clinically efficacious than gefitinib in similar NSCLC populations. The two compounds are structurally related and metabolized by many of the same enzymes (122). Furthermore, both inhibitors appear to be particularly effective in subpopulations of NSCLC, such as those with EGFR mutations in exon 19 or 21 (87, 95, 129-132). Cross-resistance between the inhibitors has also been observed (133). One possible reason for the discrepancy in outcomes of the Phase III clinical trials is that erlotinib may achieve therapeutic concentrations for longer periods of time during administration due to its higher potency and less extensive metabolism by CYP3A than gefitinib (122).

EGFR-tyrosine kinase inhibitors in NSCLC

The EGFR TKI erlotinib has a unique role in the treatment of NSCLC. Typically advanced-stage patients who have been treated with multiple courses of chemotherapy are unable to tolerate additional toxic multi-drug regimens. Therefore, reduced-toxicity single agent treatments or supportive care are utilized in the second or third line setting. However, oral EGFR-TKI use, which demonstrated improved toxicity profiles in comparison with even single agent treatments, remains a useful option in the care of refractory patients. Erlotinib has been shown in a number of independent trials to demonstrate improved quality of life, observable responses and modest but significantly improved survival (120, 134-138).

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The treatment paradigm for use of erlotinib was affected by post-hoc analyses of the clinical characteristics of patients treated with EGFR TKIs which suggested that female gender, adenocarcinoma/bronchioalveolar carcinoma histology, and lack of smoking history were independent predictors of response to therapy (81, 132, 139). Furthermore, genetic analysis of the responding tumors identified the presence of novel EGFR tyrosine kinase domain mutations (L858R, del746-750 and other in-frame deletion mutations in this region) in the tumors of many of the responding patients (83, 87, 95). These mutations were subsequently found to result in constitutive activation of the receptor, and were proposed to be a positive predictor of EGFR-TKI response (83, 95). Both cell culture and mouse models of EGFR-mutated NSCLC reinforced this finding and suggested the presence of an oncogene-addicted phenotype in a subset of tumors (94, 140-142).

EGFR monoclonal antibodies

The second class of EGFR-targeted agents developed clinically was the IgG1 chimeric monoclonal antibody cetuximab. Monoclonal antibodies differ from small molecules in that they bind the extracellular domain of the target, and can elicit a wider array of cytotoxic effects, including inhibition of ligand binding, antibody-directed cell mediated cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC). Cetuximab was first approved by the FDA in February of 2004 for the treatment of colorectal cancer. Since its approval, cetuximab has also gained indications for head and neck malignancies and and sought approval for use in NSCLC. Although cetuximab has demonstrated efficacy in NSCLC, it has yet to enter routine clinical use, possibly because pivotal clinical studies were completed later than those of erlotinib, and cetuximab must be administered parentally versus erlotinib, which is administered orally. Cetuximab elicits anti-cancer effects through direct steric inhibition of the ligand binding domain, inhibiting downstream signaling. Additionally, ADCC is proposed to play a role in cetuximab activity, although the contribution of CDC is unclear (143-145). Thus, it is possible that cetuximab may have a different response profile than erlotinib in NSCLC.

Resistance to EGFR inhibitors

Resistance is a significant issue impairing the use of anti-EGFR therapies, particularly erlotinib, in NSCLC (99, 100, 146, 147). Resistance to EGFR-targeted agents can be primary or secondary in nature. Primary, or intrinsic, resistance to EGFR

inhibitors presents as rapidly progressing disease despite treatment and is seen in ~70% of patients treated with erlotinib. Secondary, or acquired, resistance to erlotinib typically appears in responding NSCLC patients within 12 months of initiation of therapy (99, 148). It is unclear whether secondary resistance to erlotinib arises from the selection of resistant tumor subclones that are present from treatment initiation, or whether it develops *de novo* in response to the offending agent. Multiple lesions have been shown to contribute to resistance to EGFR inhibitors in NSCLC, including somatic KRAS mutations, the EGFR T790M escape mutation, and amplification of MET (98, 146, 147, 149).

Primary resistance to EGFR targeted therapy is likely the result of lack of tumor cell dependency on the EGFR signaling pathway. Due to the genetically unstable nature of NSCLC, redundant pathways may be activated and are thus sufficient to maintain oncogenic signaling in the absence of EGFR activation (98, 113, 117, 150-154). One important mechanism of intrinsic resistance to EGFR inhibitors is the presence of mutations in the KRAS oncogene (147, 155). Mutations in KRAS, which occur primarily in exons 1 and 2 of the gene, are prevalent in a variety of cancers including lung, pancreatic, colorectal, lymphoma, and leukemia (48, 51, 52, 57). In particular, KRAS mutations are a regular event in the pathologic progression of colorectal cancers in familial adenomatous polyposis (156). Activating mutations in KRAS correlate with more aggressive tumors and poorer prognosis in both lung and colorectal carcinomas (157-161). More recently, the presence of KRAS mutations has been shown to be a strong negative predictor for response and benefit to anti-EGFR therapies in cancer patients (131, 147, 155). Although this finding was originally documented in NSCLC treated with erlotinib, more substantial data has supported this finding in colorectal cancers treated with the anti-EGFR monoclonals panitumumab or cetuximab (155, 162-170).

Acquired, or secondary resistance, is also a significant hurdle in the clinical use of EGFR inhibitors. Uncovered in clinical trials of erlotinib in NSCLC, a common mechanism of acquired tumor resistance is the development of the T790M lesion in exon 20 of EGFR. Substitution of threonine for methionine at this residue results in an increase in catalytic activity of EGFR through an increase in the ATP binding affinity at the tyrosine domain (91, 100). The presence of the T790M mutation was first noted to correlate with clinical resistance to erlotinib and gefitinib. However, the exact mechanism by which this mutation inhibits activity of EGFR-TKI is unclear. One

hypothesis suggests that steric inhibition of the drug binding site by the threonine/methionine substitution prohibits drug binding (171). An alternative hypothesis suggests that, due to the ATP-competitive nature of erlotinib binding, the increase in the ATP binding affinity of the mutant shifts the equilibrium in favor of ATP-induced activation, despite the presence of drug (100). Although T790M lesions are considered to be a secondary mechanism of resistance, detectable T790M mutations in the tumors of erlotinib-naive patients have been observed. The incidence of such findings is higher in studies that utilize detection methods with improved sensitivity, such as the SCORPION-ARMS method which incorporates real-time amplification of a mutation-specific allele with a fluorescence-labeled hairpin primer (172-177). These findings suggest that the lesion may be intrinsic to many EGFR-dependent tumors, although only in a small subpopulation of tumor cells (146, 178, 179). Thus, treatment results in selection of the resistant clones.

The distinct clinicopathologic and molecular characteristics of EGFR-dependent NSCLC suggest that there is a consistent underlying biology to the phenotype. However, single biomarkers such as mutation status and amplification (ie. either KRas or EGFR) and clinical characteristics such as gender, smoking status and histology are unable to adequately capture all patients harboring EGFR-dependent tumors. Therefore it is conceivable that using tools that integrate many pieces of biological information, such as pharmacogenomics, may offer better opportunities for defining the EGFR-dependent phenotype.

D. PHARMACOGENOMICS IN CANCER THERAPY

The advent of DNA microarray technology has enabled the high throughput analysis of gene expression data from nanogram quantities of RNA. A single tumor may harbor many genetic abnormalities that contribute pathologically to the manifestation of the disease (1). Single-gene or ‘candidate gene’ approaches, while useful scientifically, do not usually capture the true scope of clinical disease. Thus, our growing understanding of the heterogeneity of cancer has lead to the hypothesis that integration of many pieces of biological information will yield improved insight into tumor biology. This insight could facilitate the development of better mechanisms for making objective treatment decisions and determining which patients will benefit most from particular therapies. This global hypothesis is largely based on the seminal observation by Golub

and colleagues that hierarchical clustering of microarray data from the cancer cells of leukemia patients can be used in an unsupervised manner to identify coordinated patterns in the expression of genes. The individual clusters were shown to correspond to known clinical classifications of leukemias (i.e. acute lymphoblastic leukemia and acute myeloid leukemia) (180). The resulting gene signature was then used to accurately predict the status of unknown leukemias. This revelation opened the door to a number of scientific endeavors with the intention of using genomic data to diagnose, classify, prognose, and predict response to treatments and interventions.

In addition to providing multivariate data which can be used for classification and predictive purposes, gene expression data allow for the functional characterization of a biological system using annotation of gene sets associated with a phenotype. Routinely curated bioinformatics databases are widely available with which gene sets found to be altered or deregulated under specific experimental conditions can be explored. These databases support gene expression analyses by providing a framework for determining commonalities among perturbed genes. Specifically, gene ontology (GO) databases interweave known protein-protein interactions, biological functions, transcription factor binding sites, and biochemical pathway membership (181-183). Thus, new hypotheses and broader insights into affected processes and pathways can be formed (181, 184, 185).

High-throughput genomics can significantly advance clinical cancer therapy using both supervised and unsupervised means of analysis. The Vogelstein model for the progression of hereditary colorectal cancer suggests that progression from dysplastic to neoplastic disease is a molecularly-defined process (156). Therefore, each molecular step of pathologic progression may yield an identifiable genomic profile and this information could be used to differentially diagnose precancerous lesions from carcinomas or normal tissue, improving cancer detection. Alternatively, the metastatic and/or recurrence potential of a tumor can be identified from gene expression profiles (186-188). An excellent example of this is the OncotypeDx™ assay which is based on the expression levels of 21 genes (189, 190). The expression levels, along with the associated algorithm yields a ‘recurrence score’ that correlates with the probability of recurrence following resection of ER positive, node negative, HER2 negative breast cancers. This information can be used to determine whether a breast cancer patient undergoing surgical intervention should receive adjuvant cytotoxic chemotherapy. Since

the toxicity associated with therapy is high, the recurrence score provides valuable information on which to base clinical decisions. Thus, the OncotypeDx™ assay, which has entered routine clinical use, demonstrates that genomic data can be successfully incorporated into patient care.

Yet another way in which genomics can be used to improve clinical practice is in the prediction of drug sensitivity. Nevins and colleagues have shown that cell line and tumor sensitivity to chemotherapy can be extracted from gene expression data and developed into a predictive algorithm of response (191-193). Further, this group has demonstrated that gene expression profiles of ectopic signaling pathway activation can likewise be developed into a signature of sensitivity to targeted agents (194). When these predictive profiles are used for classification of unknown samples, a prediction of sample sensitivity can be made. The potential clinical utility of these predictive signatures is obvious. A number of clinical trials have been initiated to validate this predictive power of the signatures, although prospective clinical data has not yet been reported.

Despite the obvious need for discerning patients that will respond to certain therapies, developing tests for clinical practice using predictive genomics faces a number of hurdles. In particular, high dimensional data is generated when using microarray technology, creating a statistical dilemma. The resulting data are often described as 'tall data', referring to the large number of outcome variables compared with the number of individual experiments or replicates. Therefore, large samples sizes are necessary to minimize the inclusion of false-positive genes (i.e. those which are not truly influenced by or associated with the controlled variable (195-197). However, accumulating the number of samples needed to develop predictive signatures is frequently cost-prohibitive due to the high cost of the technology.

Also problematic is the identification of an appropriate model in which to determine the number of features, or 'signature' genes, which may be predictive (198). Certainly, *in vitro* models are the easiest and least costly to perform. Another advantage of *in vitro* models is the relative ease for manipulation of experimental conditions. However, the resulting data may not be useful *in vivo* due to the potential for substantial differences in expression patterns in two-dimensional cell culture versus three-dimensional tumor growth *in vivo*. Another complicating factor is one of tissue

procurement from clinical samples. While human tissue is perhaps most useful, it is subject to difficult regulatory requirements and is significantly more expensive. Further, gene expression data from tumors contain more noise (i.e. technical variability) as a result of procurement processes, net tumor content of the sample, and confounding biological and patient-specific variables (i.e. concomitant therapy, age, gender, etc). Therefore, the best method to develop a genomic profile of a disease or condition is unclear, with both *in vivo* and *in vitro* models demonstrating advantages and disadvantages.

In order to contend with the data- and cost-intensive nature of performing pharmacogenomic studies, a defined strategy for generation and validation of the predictive model must be decided upon and implemented *a priori*. When dealing with supervised methods of analysis (i.e. situations where the classification status of the samples are known and integrated into the analysis), the available starting data is typically stratified into 'training' and 'test' sets. The training data is used to generate the model based on the classification of a known response (e.g. sensitive or resistant). Thus, the features are selected and weighted based on the training data.

Internal validation is the next step in the development of a predictive model. One of the most commonly used internal validation methods is the leave-one-out cross validation (LOOCV) (199, 200). This method, which is a variation of bootstrapping or jack-knifing principles, requires iterative model rebuilding by removing one of the experiments from the training set, reselecting and weighting the features, and then applying the resulting model to the experiment that was excluded. The procedure is repeated with each of the training set experiments. A high LOOCV classification accuracy demonstrates robustness of the training data and model building method (i.e. discriminant analysis, partial least squares, principle component analysis, etc). Once a satisfactory model-building procedure is internally validated, the model is rebuilt using all of the training set data and applied to the test set. This initial form of external validation ensures that the final model can accurately predict group classification on external data that has not been used to build the model and provides an estimation of the expected predictive accuracy.

A more robust external validation, however, requires an extensive analysis of samples which are not part of the original dataset in order to obtain a better estimation of

the true predictive accuracy and to determine if a systematic bias (i.e. 'batch effect', or a non-biological difference) exists. Batch effects are frequently observed in microarray data due to within-set homogeneity relative to between-set heterogeneity; potential sources of these biases include tissue procurement methods, RNA isolation techniques, microarray hybridization protocols and equipment, and geographical and clinical population (201, 202). Batch effects can be corrected using a variety of methodologies when necessary, such as distance weighted discrimination (DWD) (202). External validation is particularly important if the original data was built using *in vitro* data, as the model must be validated on clinical samples, assuming this is the overall goal of the study.

While ascribing known biological importance to the genes comprising the predictive model is not required for utility of the predictive signature as a biomarker, a high accuracy rate of the predictive algorithm increases the confidence that the gene components of the model may be important to the phenotype (203). Therefore, it is of great interest to explore the genes comprising the model in order to mine information that may identify additional therapeutic targets or gain otherwise biologically useful knowledge. Working from a genomic signature backward, the 'candidate gene' approach may be useful in identifying a single relevant gene that biologically contributes to the phenotype (204). Further experimental testing of the importance of the gene in the biological system of interest could therefore yield a new target for cancer therapy or an as yet undefined signaling pathway component. As an alternative to the candidate gene approach, a systems biology approach can be applied to the gene signature in its entirety, gleaning commonalities in the genes with respect to signaling pathway membership, biological process, miRNA targeting, protein binding partners, common chromosomal aberrations and transcription factor binding sites (205). These methods of analysis could be used to infer an underlying deregulation within the system that can be exploited for new therapeutic endeavors.

E. PROJECT OVERVIEW

Pharmacogenomics offers both a mechanism to learn about a disease state from the analysis of high density genomic data and to utilize these data to directly influence clinical care. In this body of work, these approaches have been combined in order to achieve the overall goal of improving the utilization of EGFR-targeted therapy in NSCLC.

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Since the initial FDA approval of the EGFR TKI gefitinib for NSCLC in 2003, it has been relatively well-established that EGFR and KRAS genotyping can be of value in determining candidate patients for EGFR-targeted therapy (87, 88, 95, 99, 131, 147, 155, 163, 165-167, 206). However, single biomarkers are unlikely to capture the true heterogeneity of lung cancer. Multivariate biomarkers, while subject to increased statistical and logistical considerations, may offer improved predictive accuracy. Moreover, these data offer a means for exploring the underlying disease processes through systems biology approaches. Additional insights into the disease state can improve understanding of the disease and direct new therapeutic paradigms.

Although the introduction of EGFR inhibitors to the clinic has resulted in an improvement of in the treatment of NSCLC, the true potential of EGFR TKIs has yet to be realized. Many patients treated in clinical trials with erlotinib and gefitinib did not respond to therapy and therefore realized little benefit (134-137, 207-213). The costly burden of failed treatment is largely placed on the healthcare field, limiting the effectiveness of EGFR TKIs. Furthermore, clinical responses are frequently hindered by the rapid emergence of resistance (99, 100, 113, 146, 179, 214). Using knowledge gained from both responding and non-responding patients, as well as those who develop resistance, two questions must be addressed in order to achieve maximum clinical benefit of EGFR inhibitors: How can patients who will benefit from EGFR TKIs be selected *a priori*? How can patients who respond achieve maximal benefit and survival?

To answer these questions, two primary testable research hypotheses were formed. **First, I proposed that the EGFR-dependent phenotype, which is displayed by the tumors cells of those patients who respond clinically to EGFR TKIs, can be captured by genomic profiling of NSCLC cell lines stratified by sensitivity to EGFR TKIs and the resulting gene signature may be used to predict the outcome of EGFR TKI therapy in unknown samples. Secondly, I proposed that the predictive signature of response to EGFR TKI could aide in providing additional insights into the underlying biology of the phenotype of EGFR TKI sensitivity. This information could be exploited to identify inhibitors which could be combined with EGFR inhibitors to elicit a greater effect, thereby minimizing resistance.** This work documents the use of pharmacogenomics to improve the use of EGFR TKIs in lung cancer through patient selection and achieving combinatorial therapeutic benefit.

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CHAPTER 2

A. OVERVIEW

Small molecule tyrosine kinase inhibitors (TKI) of the epidermal growth factor receptor (EGFR) can induce both tumor regression and disease stabilization when used as second line therapy in patients with advanced non-small cell lung cancer (NSCLC) (86, 120, 215). Mutations in the tyrosine kinase domain of EGFR were observed in patients that responded to EGFR TKIs. Cell lines harboring mutated EGFR are dependent on EGFR for survival since inhibition of EGFR using TKIs, monoclonal antibody C225 or RNAi knockdown results in apoptosis (87, 95, 129, 216, 217).

While substantial data now exists that mutations in the tyrosine kinase domain of EGFR are associated with increased sensitivity to EGFR TKI, mutations in EGFR were not found to correlate with response to erlotinib in the BR.21 trial (218). More recent reports have suggested that increased EGFR gene copy number, co-expression of other ErbB receptors and ligands, and epithelial to mesenchymal markers are important in determining sensitivity to EGFR TKI (106, 219-221). There are conflicting reports about the role of RAS mutation and subsequent signaling in response to EGFR TKI (86, 106, 220). In addition, identifying patients who may clinically benefit from EGFR TKI other than through overt tumor response remains unclear. Importantly, tumor regression has been observed with these agents in patients that did not have identifiable EGFR mutations, suggesting other mechanisms, such as activation of parallel signaling pathways, underlie responsiveness to these agents (217, 222-224). Therefore, the clinical decision on how best to choose patients for EGFR TKI remains an important and ongoing dilemma.

Development of molecular profiles as predictive measures of outcome or response to therapy has increased significantly since the advent of large-scale genomic and proteomic approaches for classification of cancers (225). Microarray technology allows for interrogation of large numbers of genes that encompass variability found in biological conditions. However, methods of data analysis and modeling are hampered by the data itself in that it involves significantly more data points than experiments primarily due to the cost associated with performing many replicates (226, 227). Thus, building predictive profiles of clinical outcome or therapeutic response in non-small cell

lung cancers using large-scale genomic data is a daunting process, but may be necessary for improving patient-targeted therapy.

We developed a novel methodology using both bioinformatics approaches and supervised learning methods to model sensitivity to EGFR inhibitors with gene expression data from lung cancer cell lines. Cell lines were chosen as tumor surrogates for ease of handling, the ability to assay EGFR and downstream signaling events by biochemical methods, and the capacity to test inhibitors in a controlled environment. The predictive models were subjected to extensive leave-one (or a group)-out cross-validation. Out-of-sample validation using gene expression data from additional cell lines and human tumors were also employed. The predictive models described herein are both robust and accurate predictors of response that exceed the capacity of single parameters alone in NSCLC cell lines. Our data suggest that this finding may be translated to patient tumors with similar value, as sensitivity to erlotinib in a small cohort of clinical tumors was accurately predicted using our model.

B. METHODS

Cell Culture

A549 cells were grown in RPMI 1640 (Invitrogen) with 2 mM L-glutamine containing 10% fetal bovine serum (FBS) (BioWest), 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1 mM sodium pyruvate (Whittaker). H460 and UKY-29 cell lines (228) were generous gifts from Dr. Val Adams and Dr. John Yannelli, respectively, (University of Kentucky) and grown in DMEM (Invitrogen) + 10% FBS. H3255 cells were a gift from Dr. Frederick Kaye (NCI/Naval Medical Oncology, Betheseda, MD) and were grown in ACL4 media as described previously (87). K562 cells were a gift from Dr. Rina Plattner (University of Kentucky) and were cultured in suspension in RPMI 1640 and 10% FBS. Human cancer cell lines H1650, H1975, PC9, H358 and A431 were cultured as described (97, 229).

Cell Line RNA Isolation and Microarray Analysis

Cells were grown to subconfluence and passaged every three days. On the second day after passage, cell were harvested from a 150 mm plate and lysed in Trizol (Invitrogen). Total RNA was isolated and used for probe generation and hybridization to Affymetrix U133A DNA microarrays. Signal intensity values generated from Affymetrix

MAS v5.0 software was used for statistical analysis, described below. Independent replicates of A549 (n=8), UKY-29 (n=3), H3255 (n=3), PC-9 (n=5), and H1650 (n=6) were generated by using sequential passages of the cell populations. These replicates were treated as independent samples by the subsequent algorithms to identify differentially expressed genes and build the discriminatory training model. One replicate of each A431, H358, H460, H1975, and K562 was used for validation of the training model and a single replicate of each of the training lines omitted from the original models. Microarray analysis was carried out by the University of Kentucky Microarray Core Facility according to standard operating procedures. The microarray data are available on maduk.uky.edu.

External Cell Line Microarray Analysis

Two external datasets of NSCLC microarray data were extracted from Gene Expression Omnibus (GEO): GSE4824 and GSE8332. These datasets were comprised of panels of lung cancer cell lines which had previously been assessed for sensitivity to erlotinib (221, 230). The MAS5 normalized data were used to predict sensitivity using the 180-gene model developed in this study. IC50 values of ≤ 2 μM were considered sensitive in our analysis.

Tumor Acquisition and Microarray Analysis

Duke cohort: After appropriate informed consent and Duke IRB approval, the analysis used an initial cohort of 91 tumor samples obtained from patients with local and locally advanced (stage I-IIIa) NSCLC. From the resected lung specimens, percentage of tumor content and histological type of each tumor was ascertained before RNA extraction. Of the 91 RNA samples, 89 were of sufficient quality for gene expression analysis. Of the 89 samples, 40 were clearly identified as adenocarcinoma. Gene expression data was generated using an Affymetrix U133 2.0 plus array and processed as described previously (194). The Affymetrix data for these samples is deposited on GEO under accession number GSE3141. EGFR mutational status (for exon 19 deletion and L858R) was determined using previously described techniques (95).

Moffitt cohort: Patients undergoing surgical resection of adenocarcinoma of the lung were consented to have tumor tissue stored and banked through a University of South Florida IRB approved protocol. Processing of the samples was performed as

previously described (231). The data were obtained through personal communication with Dr. Eric Haura, M.D. from the H. Lee Moffitt Cancer Center, Tampa Fl.

University of Kentucky cohort: After appropriate informed consent and University of Kentucky IRB approval, samples were collected from patients in one of two manners: Retrospective collection of samples banked at the University of Kentucky Biospecimen Core was performed following identification of patients who were later treated with erlotinib. Patients were identified using an Honest Broker system which was supervised by the University of Kentucky IRB (see Appendix I). Chart review to identify RECIST response, patient demographics, and treatment history was completed by the treating oncologists and data was relayed to the investigators. Snap-frozen banked tumor and normal tissue was used for RNA isolation with RNEasy mini columns (Qiagen, Valencia, CA) according to the manufacturer's supplied protocol and downstream microarray analysis. DNA was also isolated for EGFR and KRAS mutational assessment (see below). Also, in a prospective manner, patients with recurrent NSCLC who were initiating erlotinib therapy were enrolled in a trial to assess the feasibility of tumor sample collection in a true clinical setting (see Appendices II and III). Briefly, CT-guided core needle biopsy or dissection of the primary tumor or metastatic site was performed. Cores or bulk tumor tissue were RNA-extracted for microarray analysis and DNA was sequenced for EGFR and KRAS mutations. Patient response and survival was recorded for 9 months following the initiation of erlotinib therapy. Five patients were evaluable (4 retrospective, 1 prospective).

DNA Content Analysis

Cell lines were plated to 60 mm dishes in 10% media. Cells were starved in 0.5% media for 24 hr before treatment with 1uM erlotinib (provided by Genentech, South San Francisco, CA) or DMSO for 72 hr. Floating and adherent cells were collected by trypsinization and centrifugation. Cell pellets were washed in 1X phosphate-buffered saline (PBS) and fixed in 70% ice-cold ethanol. Pellets were washed in 1X PBS,1% bovine serum albumin (BSA), and resuspended in 1X PBS, 1% BSA, 50 ug/ml propidium iodide (Roche), and 0.5 mg/ml RNase A (Sigma) at 4^{o}C. Cells were sorted by fluorescence activated cell sorting (FACS) (University of Kentucky core facility). Data was analyzed using ModFit LT (Verity Software, Topsham, ME). Apoptosis was recorded as the integrated sub-G_1 peak.

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K-Ras and EGFR Sequencing

Actively growing cells were scraped into 1X phosphate buffered saline (PBS) and pelleted by brief centrifugation. The cell pellets were lysed in 100 mM Tris HCl, pH 8.5; 5 mM EDTA; 0.2% SDS; 200 mM NaCl and 100 ug/mg proteinase K in a 500ul volume at 55^{o}C for several hours and the debris was pelleted by high speed centrifugation. Genomic DNA was precipitated from the supernatant, and the nucleic acid pellet was resuspended in 10 mM Tris-HCl, 1 mM EDTA. K-Ras exons 1 and 2 and EGFR exons 18-21 were independently sequenced as previously described (99, 232). Sequencing was carried out by the University of Kentucky Advanced Genetics Technology Center (AGTC) according to standard operating procedures.

Gene Selection

EGFR TKI-sensitive and resistant cell line expression data was filtered to remove probesets with less than 6 'present' calls (<1/2 smallest n) between groups. Probesets with no single unique sequence by BLAST alignment were removed from the list. The remaining genes were compared using Significance Analysis for Microarrays (SAM) (233). Those genes that were determined to be differentially expressed between sensitive and resistant cell lines were annotated using GATHER (205, 234); and only those genes that annotated to signal transduction at level 4 (GO:0007165) were included in the discriminant analysis. Duplicate genes (i.e. different probesets that annotate to the same gene) were filtered by removing the least significant probeset(s) as determined by a 2-sample, equal variance t-test.

Diagonal Linear Discriminant Analysis

The genes in the final dataset were ordered by p-value in a two sample equal variance t-test. Diagonal linear discriminant analysis (DLDA) was performed using the top 10, top 50, and the complete gene signature (180 genes) in order to assess the stability and robustness of the model. A leave-one-out cross validation and external validation was performed on additional cell lines and adenocarcinomas. Adenocarcinomas hybridized to U133 Plus 2.0 arrays were filtered to remove genes not present on the U133A chip and mean chip intensities were standardized to the complete training data set.

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C. RESULTS

Identification of sensitive and resistant cancer cell lines

Using lung cancer cell lines as tumor surrogates, we sought to find gene expression patterns that can predict the sensitivity to EGFR tyrosine kinase inhibitors. Published data, and our own, demonstrate that lung cancer cell lines are differentially sensitive to EGFR inhibitors, likely reflecting dependency upon EGFR or related signaling pathways (97). We identified lung adenocarcinoma cell lines sensitive to a representative EGFR TKI, erlotinib, by DNA content analysis using propidium iodide staining. Apoptosis was assayed by quantifying the sub-G1 peak following propidium iodide staining and FACS analysis in cells treated with 1 μM erlotinib for 72 hours or DMSO control (Figure 2.1). Several cell lines tested were sensitive to treatment with 1 μM erlotinib and these data are consistent with the findings of others (97, 221). We selected the A549 and UKY-29 cell lines for the drug-resistant training group, and the H1650, H3255, and PC-9 cell lines for the drug-sensitive training group.

Sequence analysis of EGFR and K-Ras genes

Since EGFR and K-Ras mutational status are thought to correlate with sensitivity and resistance to EGFR TKIs, respectively (147), we characterized the mutational status of EGFR and K-Ras in the cell lines. The status of K-Ras and EGFR has been previously determined in all of the cell lines used, except lung adenocarcinoma cell line UKY-29, isolated at the University of Kentucky. We performed direct DNA sequencing on PCR amplicons to identify mutations in EGFR exons 18-21 and K-Ras exons 1 and 2 in the UKY-29 cells as previously described (99, 131). The UKY-29 cells are wildtype for EGFR and harbor a mutation (G61H) in exon 2 of K-Ras, which has been observed in other NSCLC tumors and cell lines. A summary of the cell line data is shown in Table 2.1.

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Figure 2.1: Sensitivity to erlotinib in cell lines

Sensitivity to EGFR tyrosine kinase inhibitors was determined by treating cells with 1 μM erlotinib for 72 hr under serum-starved conditions. Apoptosis was assessed by integration of the sub-G_1 peak and compared to cells treated with equal volume of vehicle (DMSO). Experiments were repeated in triplicate with error bars representing SD. * denotes statistical significance ($p<0.05$, two sided t-test for unequal variances)

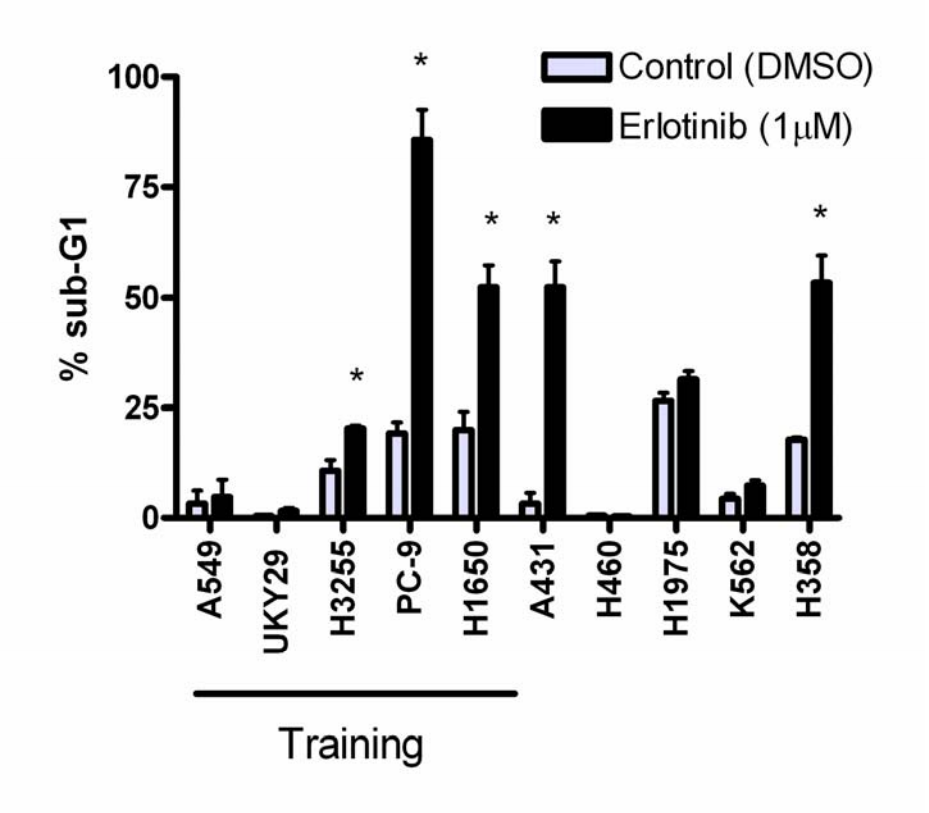

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Table 2.1: Characterization of cell lines used in training and validation

					Affymetrix U133A chips		
	Cell Line	Type	Sensitivity to EGFR TKI	K-Ras Status	(n) in training	(n) in validation	EGFR Status
Training	A549	AC	No	Mutant (Codon 12)	8	N/A	Wt [1]
	UKY-29	AC	No	Mutant (Codon 61)[1]	3	N/A	Wt [1]
	H1650	AC	Yes	Wt	6	N/A	Mutant (DelE746-A750)[1]
	PC-9	AC	Yes	Wt	5	N/A	Mutant (DelE746-A750)[1]
	H3255	AC	Yes	Wt	3	N/A	Mutant (L858R) [1]
Validation	H358	AC	Yes	Mutant (Codon 12)	0	1	Wt[1]
	H460	Large Cell	No	Mutant (Codon 61)	0	1	Wt[1]
	H1975	AC	No	Wt	0	1	Mutant (L858R, T790M) [1]
	K562	CML	No	Wt	0	1	Wt[1]
	A431	Epidermoid	Yes	Wt	0	1	Wt (Amplified) [1]

[1] Assayed in this study

AC: Adenocarcinoma

CML: Chronic Myelogenous Leukemia

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Microarray analysis and feature selection

Based on the observation that cancer cell lines and tumors are selectively susceptible to inhibition of the EGFR signaling pathway and that sensitivity may not be directly correlated to EGFR mutation or amplification in all cases, we sought to identify a gene expression signature that is predictive of EGFR TKI sensitivity. Using independent replicates of drug-resistant cell lines (n=11) and drug-sensitive cell lines (n=14), we generated gene expression data, and using both bioinformatics and statistical analyses identified a set of genes that predict sensitivity to EGFR TKI, outlined in Figure 2.2.

Specifically, gene expression data generated from Affymetrix U133A arrays was filtered based on present/absent calls and BLAST sequence alignment. The 12,019 remaining probe sets were analyzed by Significance Analysis for Microarrays (SAM), resulting in 1495 differentially expressed genes between the two groups, with a very low false discovery rate (0.025%) (233). We wished to focus on genes found primarily to function in signaling transduction in order to minimize noise from genes that are less likely to be responsible for differences in EGFR TKI sensitivity. To accomplish this, we annotated the list of 1495 differentially expressed genes using GATHER, a web-based gene ontology algorithm that detects enrichment of GO terms at all levels within a submitted list of genes (205). In the GATHER algorithm, p-values represent the probability of the term being similarly enriched in a randomly generated list of genes of identical size. A number of GO terms were significantly enriched within the 1,495 gene list, including signal transduction (GO:0007165, level 4, $p<0.0001$), G-protein coupled receptor protein signaling pathway (GO:0007186, level 6, $p<0.0001$) and cell surface receptor linked signal transduction (GO:0007166, level 5, $p<0.0001$), consistent with the hypothesis that altered signaling cascades may represent a significant proportion of the variability in EGFR TKI response. We selected only those genes that were annotated under signal transduction (GO:0007165, level 4) to constitute the signature of EGFR sensitivity.

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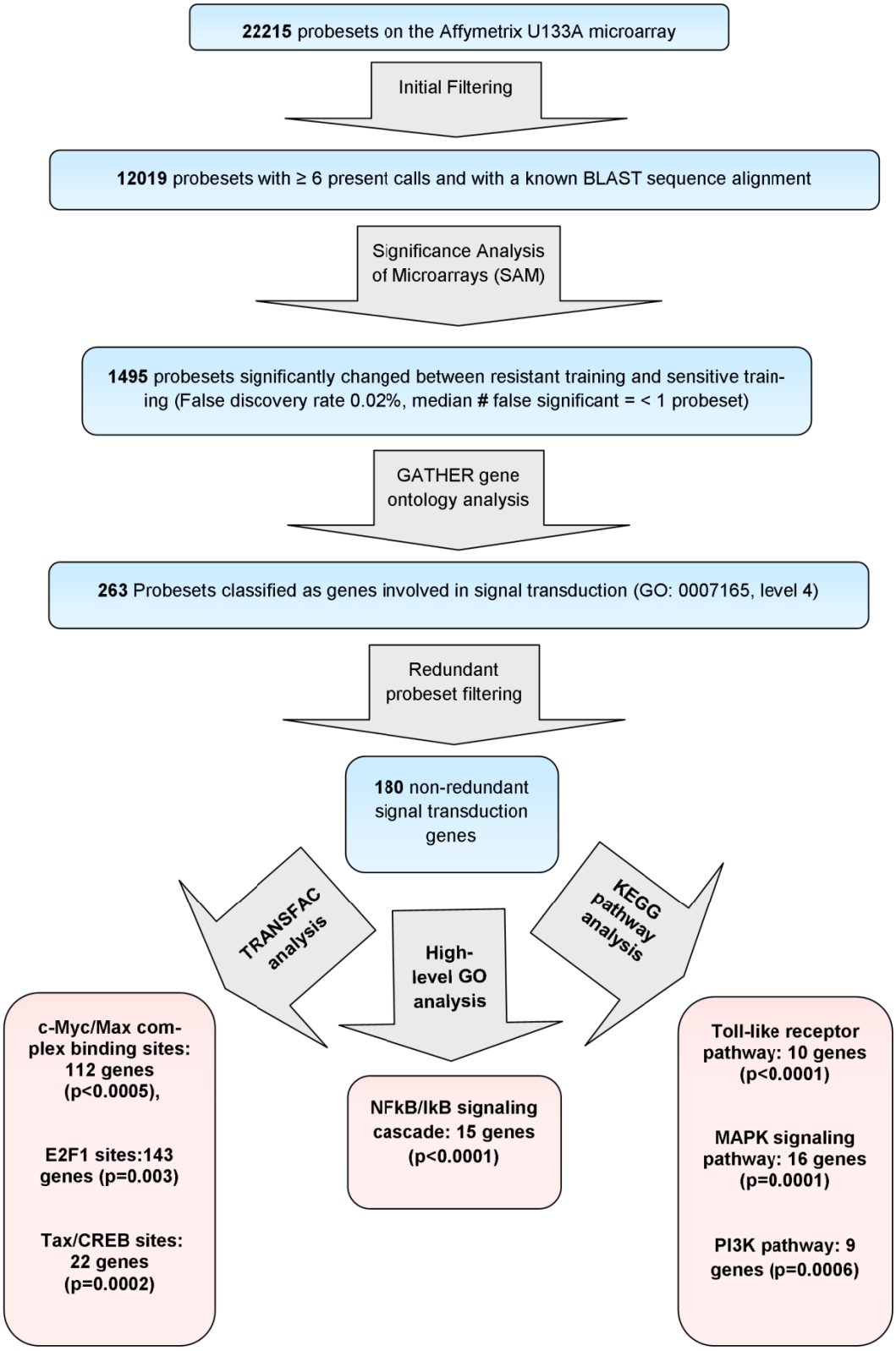
Figure 2.2: Feature selection and bioinformatics analysis for the 180 gene signature



After GATHER annotation, 223 probesets remained, and several of these probesets were redundant with respect to their target gene. To minimize bias in subsequent analyses, we kept only the most significant of the redundant probesets. When all filtering steps were complete, we identified a 180-gene signal transduction-oriented expression signature of EGFR sensitivity (see APPENDIX IV). The genes contained within the signature were re-annotated on higher levels of GO to more precisely characterize the biologic roles of these genes that are differentially expressed in EGFR TKI sensitive cells. Using GATHER's GO pathway analysis, we found significant deregulation of the NFkB/IkB signaling cascade (15 genes, GO:0007249, level 7, $p<0.0001$). Interestingly, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the 180-gene predictor revealed significant enrichment of pathways known to act downstream of EGFR, including the MAPK signaling pathway (16 genes, $p=0.0001$) and the phosphotidylinositol signaling pathway (9 genes, $p=0.0006$). We also queried for significant enrichment of transcription factor binding sites among the 180-gene signature using TRANSFAC via GATHER. The genes clustered into three interesting and significant classes of DNA-binding domains: c-Myc/Max complex binding sites (112 genes, $p<0.0005$), E2F1 sites (143 genes, $p=0.003$) and Tax/CREB sites (22 genes $p=0.0002$).

Internal and external validation using diagonal linear discriminant analysis

Diagonal linear discriminant analysis (DLDA) was performed on the 180-gene signature of EGFR sensitivity because this methodology performs well in classification problems concerning gene expression data (235). For each unknown subject, DLDA calculates the distance of the unknown to the average of the subjects in each group of the training set with respect to the common diagonal covariance matrix. The unknown is then classified into the closest group.

The model was trained using the H1650 (n=6), PC-9 (n=5), and H3255 (n=3) cell line samples as the sensitive group and the UKY-29 (n=3) and A549 (n=8) samples as the resistant group. The replicate measurements from each cell line were treated as independent samples by the subsequent algorithms to identify differentially expressed genes and build the discriminatory training model. We tested multiple predictive models,

including the 10 and 50 most significantly deregulated genes of the 180-gene signature to determine the robustness of the predictor (see APPENDIX IV).

We also performed a leave-one-out cross validation of the DLDA function. We assumed that one chip in the training set was an unknown, and then performed the complete analysis based on the remaining chips, beginning with the initial filtering steps. This was performed for each chip of the initial training set in turn. Specifically, each time a chip was removed from the training set the following steps were performed; presence/absence call filtering, SAM analysis on the newly filtered data set, with the same delta-threshold used in the complete analysis training set, gene ontology filtering, redundant probesets were removed, the diagonal linear discriminant function was fit from the remaining 24 chips, and then EGFR TKI sensitivity of the removed chip was predicted based on the newly fit diagonal linear discriminant function. This was performed using the top 10 and 50 genes in each iteration, as well as the full gene list (range: 171-208 genes). Leave-one-out cross validation yielded a 0% misclassification rate. Likewise, we also performed a leave-a-group-out cross-validation in which an entire cell line set was removed and the model was iteratively rebuilt. This approach resulted in correct predictions for PC-9, H3255, UKY29, and H1650 samples but incorrectly classified 3 of the 8 replicates of A549 (88% accuracy).

The models were then externally validated using a set of cell lines not used in training the model of EGFR TKI sensitivity as well as a single replicate for each of the training cell lines that were not incorporated into the training data (see Table 2.1). The K562 line was chosen as a negative control as it is a cancer cell line dependent on BCR-ABL expression to test if our predictor was recognizing non-specific dependence on any activated kinase. The 10-, 50-, and 180-gene models were used to classify all cell lines. The models classified all samples correctly, with the exception of the UKY-29 sample in the 10-gene model and the H1975 cell line in all 3 models. Additionally, we compared our genomic predictor (gene signatures and DLDA) to predictions based on mutational status alone, assuming sensitivity in the presence of exon 19 or 21 mutations, or resistance in the absence of EGFR mutations, or presence of an exon 20 mutation. Results are shown in Table 2.2.

To further assess the predictive accuracy of our model using external validation, we analyzed two independent NSCLC cell line datasets also assayed on Affymetrix

microarrays. The first dataset was from Girard and colleagues (GEO#GSE4824). These data include 14 lung cancer cell lines, of varied histologies, which were not included in our training model-Calu.3, H1299, H157, H1648, H2009, H2126, H820, HCC15, HCC2279, HCC4006, HCC44, HCC78, HCC827, and HCC95. Because NSCLC cell lines have a broad range of sensitivity to EGFR TKI, we chose an IC_{50} threshold of 2 μM to EGFR TKI as determined in Bunn et al for these 14 cell lines(230). Our genomic model of EGFR TKI sensitivity correctly classified 64% of the lines. Increasing the threshold to 3 μM added an additional correctly predicted sample (71%).

The final external NSCLC cell line dataset used was comprised of 42 NSCLC cell lines (GSE8332). These cell lines were assessed for sensitivity to erlotinib by Yauch et al (221). Again, using an erlotinib IC_{50} threshold of 2 μM (the same used by the authors in that study), we assessed the capacity of the 180-gene model to predict sensitivity. Using this threshold, the model correctly classified 34/42 cell lines, for a predictive accuracy of 81%.

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Table 2.2: Diagonal linear discriminant analysis of NSCLC cell lines

Predictions of EGFR TKI sensitivity are denoted for ten cell lines used in training/validation. Column 2 demonstrates experimental sensitivity to erlotinib. Column 3 demonstrates prediction of sensitivity using mutational status of EGFR. Columns 4-6 denote prediction of sensitivity of the cell lines using the 10, 50, and 180 gene signatures in DLDA. √: denotes correct prediction based on experimental sensitivity to EGFR TKI. *: Leave-a-group-out cross-validation incorrectly predicts 3 of 8 replicates of this cell line.

			Predicted sensitivity to EGFR TKI			
				Genomic signature / DLDA		
	Cell Line	Experimental Sensitivity to EGFR TKI (erlotinib)	Prediction based on analysis of mutational status alone (Exons 18-21)	10-genes	50-genes	180-genes
Training	A549	No	√	√*	√	√
	UKY-29	No	√		√	√
	H1650	Yes	√	√	√	√
	PC-9	Yes	√	√	√	√
	H3255	Yes	√	√	√	√
Validation	H358	Yes		√	√	√
	H460	No	√	√	√	√
	H1975	No	√			
	K562	No	√	√	√	√
	A431	Yes		√	√	√
		% Correct	80%	80%	90%	90%

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Independent external validation on resected lung adenocarcinomas

Given the accurate classification of the cell line data, we hypothesized that the signature of EGFR sensitivity should correctly classify resected tumors, and would result in appropriate predictions of response to EGFR TKIs *in vivo*. Two collections of resected adenocarcinomas, previously subjected to microarray analysis, were used to validate the predictive models of 10, 50, and 180 genes. The first set of tumor samples used for external validation of the model was obtained from H. Lee Moffitt Cancer Center and Research Institute (Tampa, FI). RNA was prepared from those tumors and was used for hybridization to Affymetrix U133A arrays. Tumors were also assayed by immunohistochemistry (IHC) methods, scoring for phosphorylated EGFR (pEGFR) as previously reported (229). Since persistently activated EGFR (pEGFR) may reflect underlying tumor reliance on EGFR and therefore sensitivity to EGFR TKI (229), we explored the relationship between classification by DLDA and pEGFR staining. Of the 19 tumors, 5 were either pEGFR-negative or exhibited very low pEGFR signal (<10 on a scale of 0-300) by IHC staining, while the remaining 14 stained with higher intensity of pEGFR. When the Moffitt tumors were predicted by DLDA, 4, 13, and 10 tumors classified as sensitive in the 10-, 50-, and 180-gene predictors respectively. Of the tumors that classified as sensitive, 100%, 92%, and 90%, respectively displayed higher degrees of pEGFR staining (>10). Of those tumors that were predicted to be resistant, 33%, 66% and 44% exhibited low levels or no pEGFR staining (<10), respectively. Tumor classification for the three models as well as IHC scoring is presented in Figure 2.3, panel A.

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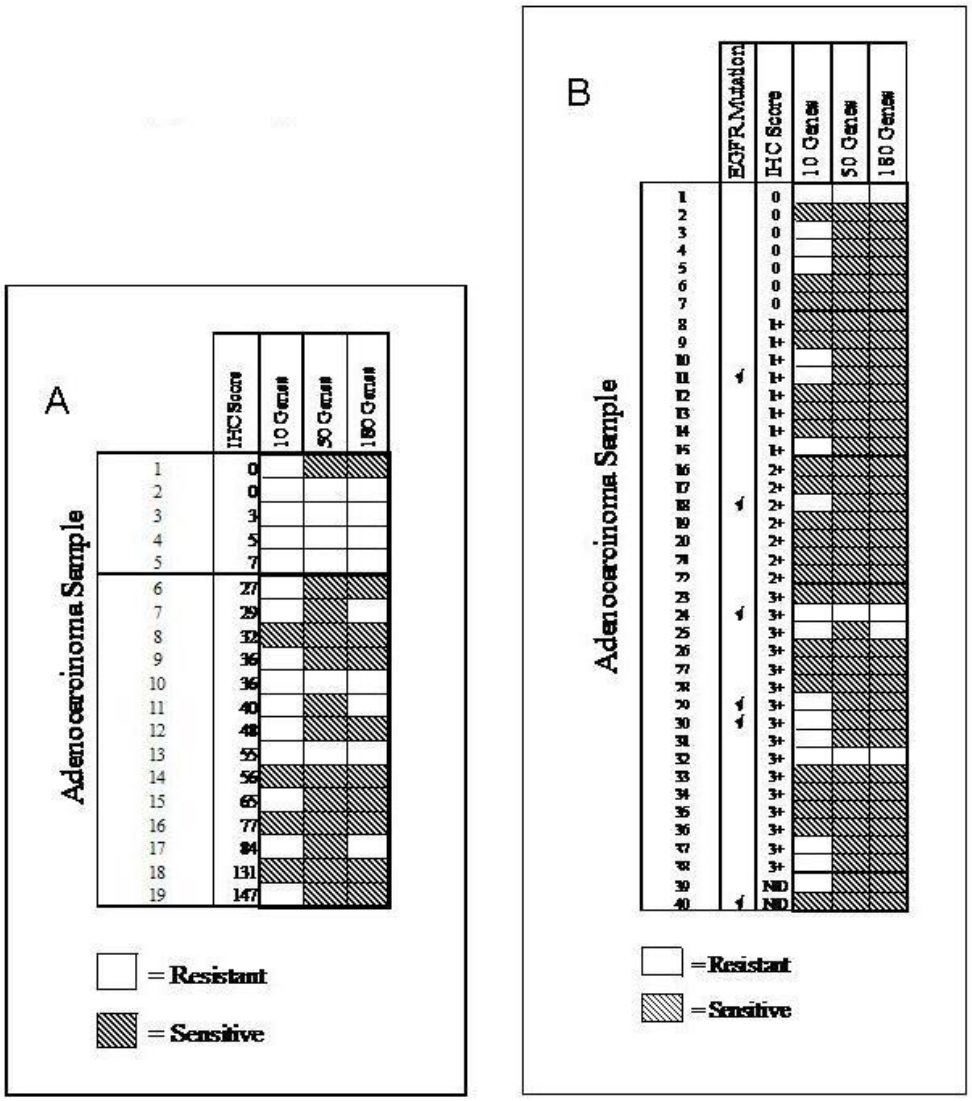
Figure 2.3: Classification of two independent collections of resected adenocarcinomas

(A) Tumors samples banked at H. Lee Moffitt Cancer Center and Research Institute were used for extraction of total RNA for probe preparation and hybridized to U133A arrays. IHC scoring was performed as previously described (229). Thatched boxes represent predictions of sensitivity. **(B)** Tumors samples banked at Duke University were used for extraction of total RNA for probe preparation and hybridized to U133 2.0 arrays. pEGFR scoring is reported on a 4 point scale (0-3+). The presence of activating mutations within EGFR is also reported. Sensitive predictions are represented by a thatched box.



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Because mutational status of EGFR has been shown in select studies to correlate with tumor response to erlotinib and gefitinib (223, 236), we chose to further validate our model on a series of resected adenocarcinomas for which mutational status, as well as pEGFR status was known. Adenocarcinomas from the Duke lung cancer cohort were used as a second test of our model. RNA from the samples was used for hybridization to Affymetrix U133 Plus 2.0 arrays, and these data were imported into our models (194). We were able to make predictions of EGFR TKI sensitivity of the Duke tumors using DLDA. We found that the 10-, 50- and 180-gene predictors identified 1/6 (17%), 5/6 (83%), and 5/6 (83%) of the tumors with EGFR mutations as sensitive, respectively. Of those tumors that classified as sensitive in the 10-, 50-, and 180-gene models, 82%, 78%, and 77% displayed positive staining for pEGFR, respectively. Of those tumors that were classified as resistant in the 10-, 50-, and 180- gene models, 24%, 33%, and 25% displayed no pEGFR staining, respectively. Classification of the data are shown for the Duke lung adenocarcinoma dataset in Figure 2.3, panel B.

Finally, to directly test the hypothesis that the 180-gene model could accurately predict response of lung tumors to erlotinib, we initiated a pilot clinical study and collected both retrospective and prospective data from patients at the University of Kentucky who were treated with erlotinib (see Appendices I, II, and III). Frozen tumor and matched normal resected tissue (where available) was utilized for DNA sequencing of KRAS and EGFR. RNA was isolated for microarray analysis. Radiographic response was recorded and compared with predictions using our 180-gene predictive model. In all cases, normal tissue predicted as 'resistant' to erlotinib. Three of five tumor samples predicted as sensitive, and of these, two of three demonstrated clinical benefit (stable disease or partial response). Two tumors predicted as resistant, and both of these patients demonstrated progression on erlotinib treatment. Also, 3 of the 5 patients in this cohort had cytotoxic therapy between the time of resection and the time of erlotinib treatment, suggesting that the predictive signature may be stable through other treatments, such as platinum agents that are commonly used in adjuvant or first-line regimens. We have assessed this hypothesis elsewhere, using ovarian carcinoma cell lines treated with platinum agents (237).

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Table 2.3: Results of predictive analysis of University of Kentucky Cohort

Sample	Sex	Eth	Smoking	Pack-year	Histology	Tissue	EGFR (ex19-21)	KRAS (ex1-2)	RECIST	Tumor prediction
L05-239	M	Ca	NS/FS	<1	BAC	Prim	L858R	WT	PR	sensitive
L06-312	F	Ca	FS	N/A	AC	Prim	WT	WT	SD	sensitive
L06-298	F	Ca	CS	N/A	Poor diff	Prim	WT	WT	PD	sensitive
L06-338	M	Ca	FS	25	AC	Prim	WT	WT	PD	resistant
BLA-06-ERL-02	M	AA	CS	47	Poor diff	Node	WT	N/A	PD	resistant

NS: non-smoker, FS: former smoker, CS: current smoker, BAC: bronchioalveolar carcinoma, AC: adenocarcinoma, WT: wildtype, PR: partial response, SD: stable disease, PD: progressive disease

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D. DISCUSSION

The EGFR TKI erlotinib was shown to increase survival in previous clinical trials when used as monotherapy in previously treated patients with advanced NSCLC (238). Toxicity to erlotinib is markedly lower than many alternative pharmacologic treatments, and would clearly be a preferred therapeutic option if survival were shown to be equivalent or better than treatment with other second line agents. Since only a fraction of patients respond to such therapy, *a priori* identification of responders could have a vast effect on healthcare costs and patient survival. Many clinical parameters that have been shown to correlate with response to EGFR TKIs, including smoking history, gender, ethnicity, and tumor histology. Additionally, EGFR expression levels, phosphorylation status of EGFR, and mutations within the kinase domain (81, 131, 236) also correlate with sensitivity to some degree. While each of these predictors of response result in some overlap, potential responders to EGFR targeted therapeutics may be overlooked. In the same vein, a significant number of patients selected for treatment with EGFR TKI will fail therapy. Therefore, we undertook this study with the hypothesis that a gene expression signature of response will capture more of the variability within the tumor and improve prediction of EGFR TKI sensitivity than currently preferred methods. Furthermore, closer examination of the genes within this signature will allow for greater understanding of the effects of aberrant EGFR signaling, as well as potential elucidation of new drug targets.

Using NSCLC cell lines as tumor surrogates and previous findings as guidance, we sought to train our model by stratifying cell lines by drug sensitivity. Three sensitive cell lines were chosen for training data: H3255, PC9, and H1650. A549 cell line and UKY-29 cell lines were resistant to treatment and used for training data. The cell lines resistant to EGFR TKI harbor K-Ras mutations while the sensitive cell lines used in the training set all harbor EGFR mutations, as previously reported, and this finding is consistent with the hypothesis that K-Ras mutations and EGFR mutations are mutually exclusive in NSCLC (147).

Our hypothesis is anchored in the concept that while many factors correlate with sensitivity to EGFR inhibition, distinct combinations of signaling pathway deregulation may underlie the observed phenotype. Therefore, a gene expression signature capturing this complexity should be a more accurate predictor of response to EGFR TKI,

and we defined a gene expression signature that utilizes our knowledge of signal transduction to model the phenotype of sensitivity.

Approximately 1500 genes were significantly different between our sensitive and resistant training cell lines, and while many of these genes may be important in our phenotype of response, we reasoned that a significant portion may be artifacts of two-dimensional growth and cell culture conditions. We filtered the 1500 differentially-expressed genes based on ontological annotation, allowing us to focus our signature on those genes that are important for cell signaling and are more likely to influence response to inhibition of the EGFR signaling cascade. To our knowledge, this is a novel approach to feature selection within a predictive gene signature study. A limitation of this approach is that genes that may contribute to pharmacokinetic variability such as transporters and metabolic enzymes would be omitted from the signature. Furthermore, markers of epithelial to mesenchymal transition (EMT), which have been shown to correlate with sensitivity to EGFR TKI (220, 221) are not present in our final predictive signature due to the filtering by gene ontology. It is of note that the SAM analysis identified several EMT genes as differentially expressed within the 1500-gene training data set, such as vimentin, E-cadherin, and β-catenin.

We defined a set of 180 features that represent differentially expressed genes that exhibit enrichment in signal transduction functions between EGFR-inhibition sensitive and EGFR inhibition-resistant cell lines, including a number of previously identified oncogenes such as SRC, BRAF, and PI3K that function downstream of EGFR activation. EGFR itself was identified as significantly deregulated and is consistent with the observation that EGFR expression may correlate with sensitivity (130).

GATHER allowed us to interrogate KEGG pathways in analysis of the genes included in the 180-gene signature and identified deregulation within the PI3K and MAPK pathways between sensitive and resistant cell lines. Interestingly, both of these pathways are downstream of EGFR, providing further evidence of their importance in NSCLC. Consistent with this finding, several subunits of PI3K were found highly expressed in the EGFR TKI sensitive cells, including both the catalytic and regulatory subunits.

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Analysis of transcription factor binding elements using GATHER also identified strong commonalities among the genes included in the signature. The high proportion of the genes are likely regulated by the E2F-family of transcription factors and/or c-MYC/MAX transcription factors suggesting common regulatory mechanisms may lead to the phenotypic difference of EGFR TKI-sensitive and -resistant cells. Importantly, both activating E2Fs and MYC are recognized as essential cell cycle regulators and bind to promoters of genes important for driving cell proliferation (239).

Our EGFR signature represent 180 genes that were observed to have large differences with low variability in our system (see Appendix IV). Since our leave-one-out cross-validation yielded a 0% misclassification error, there may be concern that over-fitting of the model has occurred. A full leave-one-out cross validation (i.e. features are reselected and model parameters are rebuilt at each iteration) is a stringent and relatively unbiased estimate of the model building algorithm error (240, 241). However, to ensure that the treatment of replicate cell line samples as independent samples in our model did not result in cross-validation bias, we performed additional internal validation experiments. Subsequent cross-validation was performed in which the entire data set from each cell line was removed (features were re-selected and weights were recalculated based on the data from only 4 cell lines, and the samples from the 5^{th} cell line were predicted using the new model). This method of cross-validation yielded a high degree of accuracy as well in that all cell lines predicted correctly, with the exception of 3 of 8 A549 samples (data not shown). We also constructed a second predictive model of EGFR TKI sensitivity using balanced numbers of replicates in both training classes (n=3 replicates/cell line). We found that although 111 genes of the resulting 169-gene model were common to the 180-gene signature the resulting model did not exactly replicate the classifications of the 10-, 50-, and 180-gene models. The differences could be due to a lack of statistical power in the second model.

We assessed the ability of this model to predict additional sets of gene expression data. To independently validate the signature, we used DLDA to classify cell lines that were not included in training the models. Additionally, we assessed the variability in predictive strength using multiple models. We found that predictions based on the most statistically significant 10 or 50 genes were similar to those made with the full data set. However, 10-gene model resulted in misclassification of both the UKY-29 and H1975 samples. This finding underscores the importance of including enough

features in the model to account for variability found in the biological system of interest, a lung adenocarcinoma. Interestingly, the H1975 sample is seemingly misclassified in the 50- and 180-gene models as well. This cell line harbors a second mutation in exon 20 that has been shown to confer resistance to the EGFR TKI gefitinib and erlotinib (99). Importantly, however, recent reports have shown that the irreversible inhibitors of EGFR such as CL-387, 785 overcome this resistance (242). Therefore, the double-mutant H1975 cell line, although insensitive to gefitinib and erlotinib, retains reliance on EGFR signaling pathways, providing an explanation for its classification using our models (89). Furthermore, when compared to predictions based on mutational status alone, the genomic predictors (50- and 180-gene models) perform better in determining *a priori* sensitivity (see Table 2.3).

We carefully selected the cell lines used as a validation set to ensure that our model was predictive of EGFR TKI sensitivity and not mutational status alone. The H358 adenocarcinoma cell line harbors a K-Ras mutant and no EGFR mutations, yet our predictor and data of others (221) identify this cell line as sensitive to EGFR inhibition. Furthermore, the A431 cell line was not derived from a lung adenocarcinoma, has both wildtype EGFR and K-Ras alleles, and is exquisitely sensitive to EGFR inhibition. However, K562 cell line is derived from a CML blast crisis patient, is wild-type for both EGFR and K-Ras, and is highly resistant to EGFR TKI. All three of these cell lines classify correctly and consistently among the 10-, 50-, and 180-gene predictors.

To strengthen confidence in our 180-gene model, we tested an unpublished, independently-derived set of NSCLC cell line microarray data (Girard, GEO # GSE4824). Our signature correctly classified 64-71% of the cell lines, depending on IC_{50} threshold selection of resistance to EGFR TKI as determined in Bunn et al (230). Of the four cell lines from the Girard set that were incorrectly predicted using our model, two were not of adenocarcinoma origin-H1299 (large cell carcinoma) and H157 (squamous cell carcinoma). Our predictor of sensitivity was trained using cell lines of adenocarcinoma origin and may then be more accurate when using similar data. Utilizing additional training data from cell lines of varied NSCLC histologies will likely improve the model for clinical use.

We next assessed the ability of the predictive models to classify lung adenocarcinoma tumors. Initially, in the absence of clinical outcome or survival data

from a prospective trial, we identified two datasets to which reasonable proxies for EGFR signaling and TKI sensitivity were available. These data included a set of 19 adenocarcinomas for which phosphorylated EGFR (pEGFR) was assessed using IHC and a set of 40 adenocarcinomas for which both pEGFR and EGFR mutational status was assessed. Classification based on 50 or 180 genes remained relatively constant, demonstrating robust predictive power. Furthermore, classification of the tumors using 50- and 180-genes models identify a majority of the pEGFR positive samples in both datasets, as well as capturing 5 of 6 EGFR mutants in the Duke tumor dataset.

We identified several tumors in both the Moffitt and Duke datasets that demonstrate no detectable expression of pEGFR but classify as EGFR TKI sensitive using the predictive gene expression model. It is possible that IHC analysis is less sensitive than classification using the gene expression profile and is also dependent on sections stained and phospho-specific antibody used. Alternatively, the tumors harboring low levels of pEGFR predicted to be sensitive to EGFR TKI might possess deregulation of parallel signaling pathways that result in a gene expression phenotype that closely resembles activation of EGFR, and accordingly, these patients classify as sensitive to EGFR TKI.

We classified 83% (5/6) of the Duke cohort that were EGFR mutants as sensitive to EGFR by gene expression signature. While the predictor seems to have misclassified one tumor that harbors mutant EGFR, we note that others have reported that cell lines with activating EGFR mutations are also insensitive to EGFR TKI, and our predictive models may have identified a tumor that will not respond to treatment (106). Further, the Duke cohort lacked analysis of the T790M mutation and MET amplification, two markers of clinical resistance to EGFR inhibitors that are not mutually exclusive with sensitizing EGFR mutations (98, 99). Thus, it could be that this EGFR-mutant sample contained one of the resistance-conferring lesions. Additionally, in non- Japanese populations screened by EGFR mutational status prior to treatment with gefitinib, the response rate among those patients with either deletion or point mutation of EGFR was found to be 75% suggesting that mutation of EGFR is not sufficient for EGFR TKI sensitivity (211). Thus, our tumor classifications accommodate the proportion of responders found in previous studies and while our approach may exceed those findings, future validation depends on comparing classification to response in a clinical study.

Because we did not have the EGFR TKI response data for the Moffitt and Duke tumor specimens, we used pEGFR staining and mutation status as surrogates for EGFR signaling, as described above. Combining both of the tumor data sets, our predictor of EGFR TKI sensitivity suggests that 80% of the tumors may be sensitive. Previous studies found that nearly 50% of patients with advanced stage IV NSCLC who had previously received cytotoxic chemotherapy had clinical benefit with EGFR TKI defined as either overt tumor response (shrinkage), stable disease, or symptomatic improvement (215). Since all the Moffitt and Duke tumors were of adenocarcinoma histology, a known clinical predictor of benefit to EGFR TKI, it is possible that the genomic predictor may accurately classify sensitivity in this group of tumors.

Finally, we designed a pilot clinical study to gather tumor samples from patients prior to treatment with erlotinib. Importantly, we were able to collect additional data in this study that was missing from the other clinical cohorts. Specifically, we tested for the presence of KRAS and EGFR T790M mutations to gain additional insight into the performance of our predictor in these genetic backgrounds. However, MET amplification was not tested in this study. Our hypotheses were supported by the results of the University of Kentucky clinical cohort, where accurate predictions of erlotinib sensitivity were made in 4 of 5 tumors. It is unclear whether there exists a difference in EGFR TKI sensitivity between early stage lung cancers and widely metastatic cancers that have previously received cytotoxic chemotherapy. However, our model appeared to perform well in predicting response in both early stage resected tumors and advanced disease. Studies are underway that address the sensitivity of early stage lung cancers to EGFR TKI. True assessment of the accuracy of our gene expression profiles to predict sensitivity of lung cancers to EGFR TKI will require more extensive prospective testing.

E. CONCLUSIONS

The gene expression signature of EGFR TKI sensitivity exhibits strong biological relevance as it encompasses many members of the EGFR signalling cascade. The prediction of sensitivity to EGFR inhibitors using DLDA models was accurate and robust within the cell line data. Furthermore, the DLDA predictive models suggest improved prediction of EGFR TKI sensitivity of human lung adenocarcinomas compared to single biomarkers alone. Clearly, the next step in assessing the ability of this signature to improve upon existing methods must be determined in a larger prospective clinical trial.

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We anticipate that use of gene expression predictors could advance patient-targeted therapy in this area.

CHAPTER 3

A. OVERVIEW

The epidermal growth factor receptor (EGFR) is activated by ligand binding to the extracellular domain resulting in either homo- or hetero-dimerization of the receptor and subsequent auto-phosphorylation of the intracellular domain. Activation of EGFR initiates a series of downstream signaling networks that integrate both proliferative and survival pathways (83, 97, 129, 232, 243, 244).

Mutations within the EGFR kinase domain (exons 18-21) result in increased pathway activity, and occur in 5-20% of NSCLC patients (81, 83, 87, 129, 131, 223). Of particular significance, NSCLC tumors harboring activating EGFR mutations rely upon constitutive pathway activation for survival, resulting in oncogene addiction (81, 83, 95, 129, 131, 140-142, 147, 223, 245). The inhibition of this pathway in NSCLC cell lines using EGFR tyrosine kinase inhibitors (TKI) or by siRNA-mediated knockdown of EGFR results in reduction in survival by colony-forming assays and reduction in proliferation by MTS assays (83, 97, 99, 232). Xenograft models of many of the same NSCLC cell lines respond to EGFR TKI *in vivo* (246). Tumors induced in the lungs of mice by tissue-specific expression of mutationally-activated EGFR also regress with EGFR TKI treatment (94). Furthermore, clinical treatment of carcinomas harboring mutations in the EGFR gene with the EGFR TKIs erlotinib or gefitinib has been reported to elicit higher response rates than those observed in wild type EGFR tumors or unselected patient populations (86, 87, 95, 96).

Interestingly, clinical response has been observed in NSCLC tumors with no identifiable EGFR mutation (218, 247). In concordance with these findings, various NSCLC tumor cell lines lacking known activating EGFR mutations have been found to be highly sensitive to EGFR inhibition (117, 220). As such, EGFR pathway addiction (reviewed in Chapter 1) may arise from other mechanisms in addition to activating mutations in EGFR (87, 93, 106, 114, 130, 207, 248). One potential mechanism was described by Fujimoto et al, who demonstrated that EGFR ligands are up regulated in adenocarcinomas that are sensitive to EGFR inhibition, suggesting the presence of an autocrine loop (106).

A number of ligands have been identified that are capable of binding EGFR, including epidermal growth factor (EGF), heparin-binding EGF-like ligand (HB-EGF), transforming growth factor–α (TGF-α), epiregulin (EREG) and amphiregulin (AREG). Deregulated expression and/or activation of these ligands may serve to potentiate an autocrine loop that contributes to EGFR pathway addiction either independently or in concert with EGFR-activating mutations. An autocrine loop involving TGF-α, EGFR, and the RAS/MAPK pathway has been suggested to exist previously, although its importance in EGFR-dependent lung cancer in unclear (107, 249, 250). Our own work has identified genes deregulated in the EGFR-dependent phenotype, which included genes involved in the MAPK pathway (see chapter 2) (251). Other epithelial cells, such as mammary and intestinal cells, also exhibit up-regulated EGFR ligands following RAS or RAF activation (252-254). From our data and the data of others, we hypothesized that signaling cascades downstream of RAS activation may play a role in modulating an autocrine loop controlling EGFR ligand activity in NSCLC.

In this study, we demonstrate that EGFR-dependent NSCLC cell lines display constitutive ERK1/2 activation. RAS/MAPK signaling contributes to regulation of EGFR ligand expression and activation in EGFR-dependent lung cancer cells, as well as in primary small airway epithelial cells and immortalized bronchial epithelial cells. Finally, we demonstrate that RAS/MAPK regulation of EGFR ligands is important for stabilization of EGFR protein levels. Antibody-mediated inhibition of ligand binding to EGFR results in degradation of the receptor, phenocopying the effects of small molecule-mediated RAS/MAPK inhibition. Our results demonstrate that EGFR-dependent NSCLC cell lines utilize RAS/MAPK activity to drive expression/activation of EGFR ligands which may serve to stabilize EGF receptor levels. These processes are potential contributors to oncogenic EGFR signaling in lung cancer.

B. MATERIALS AND METHODS

Cell culture

RPMI 1640 (with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5% glucose and 1.5% sodium bicarbonate) and DMEM were used as culture media and were supplemented with 10% fetal bovine serum (FBS) (Biowest, Miami, FL), 50 µg/ml penicillin and 50 µg/ml streptomycin. The human NSCLC cell lines A549, H460, UKY-29, NCI-H1975, NCI-H1650, NCI-H358, and PC9 were obtained and maintained as

previously described (251). Immortalized human bronchial epithelial cells (hBEAS2b) were obtained and cultured as recommended by the ATCC.

Adenoviral infections

The v-AKT and constitutively active STAT3 (STAT3ca) adenoviruses were provided by Dr. Eric Haura, M.D. of the H. Lee Moffitt Cancer institute (255). The constitutively active MEK-1 (MEK-1ca) adenovirus was generously provided by Dr. Janet Rubin of the University of North Carolina (256, 257). hBEAS2b were plated in full growth media approximately 24 hr prior to infection. Cell culture media was removed and cells were washed once in PBS prior to addition of adenovirus in infection media (basal growth media buffered with 25 mM HEPES). Cells were infected at a multiplicity of infection of 50 ffu/cell for one hour in a minimal volume and full growth media was then replaced overnight. Cultures were harvested 16 hr after infection for subsequent RNA or protein analysis.

Inhibitors and neutralizing antibodies

The MEK1/2 inhibitor U0126 was acquired from EMD Chemicals (Gibbstown, NJ). MG-132 was obtained from Dr. Hsin-Hsiung Tai, Ph.D., University of Kentucky. All inhibitors were solubilized in DMSO. Anti-EGFR LA1 and anti-HB-EGF neutralizing antibodies were acquired from Upstate (Lake Placid, NY) and R&D Systems (Minneapolis, MN) respectively, and were utilized at a final concentration of 10 μg/mL.

Microarray data

The NSCLC cell line dataset was extracted from the Microarray Database of University of Kentucky (251, 258). Small airway epithelial cell cultures infected with adenovirus, as described above, were used to purify RNA and generate probe for microarray hybridization as described in Chapter 2. All data were MASv5.0 normalized.

Quantitative real-time PCR

Total RNA was extracted from cells using RNEasy Mini kits (Qiagen, Valencia, CA) according to the manufacturer's supplied protocol. cDNA was synthesized by reverse-transcribing 1 μg of purified RNA using iScript (BioRad, Hercules, CA) according to the manufacturer's supplied protocol. Quantitative real-time PCR (qRT-PCR) was performed using Taqman Assays On-Demand primer/probes (Applied Biosystems,

Foster City, CA) for GAPDH, TGF-α, HB-EGF, and EGFR. PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Relative quantification was applied using a standard curve method and normalization to GAPDH expression.

Immunoblotting

Cells were plated in media containing 10% FBS and media was changed to 0.1% for a 24 hr incubation prior to collection. Cells were washed in PBS, harvested and lysed in RIPA buffer (50 mM Tris (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.25% sodium deoxycholate, 5 mM NaF, 5 mM Na_3VO_4, 10% glycerol, 1 mM phenylmethyl-sulphonylfluoride, and protease inhibitors) for 30 min on ice. Lysates were sonicated briefly on ice to disrupt membranes and then centrifuged at 13,200 rpm for 10 min and the protein concentrations of the supernatants were determined by BCA assay (Bio-Rad, Hercules, CA). Samples were separated by SDS-PAGE and transferred to PVDF membrane. Membranes were blocked with 5% nonfat dry milk or 5% BSA in TBS for 1 hr at room temperature and then incubated overnight at 4^{o}C with the appropriate antibody. Antibodies specific for phospho-MEK1, phospho-ERK1/2, total ERK1/2, phospho-EGFR (Tyr 1068), total EGFR, calnexin and PARP were all obtained from Cell Signaling Technology, Beverly, MA. Antibody specific for total MEK-1 (Santa Cruz Biotechnology, Santa Cruz, CA) was also utilized. Following incubation with appropriate horseradish peroxidase-conjugated secondary antibodies, proteins were visualized using an enhanced chemiluminescence detection system. Immunoblots were scanned at a resolution of 600 dpi and quantified by integrated density using ImageJ (National Institutes of Health) where applicable. Data were normalized to a loading control protein (actin or calnexin).

MEK inhibition assays

Cells were plated in 12-well plates (Costar, Corning, NY) at a density of $1x10^5$ cells per well. After 16 hr, the growth media was replaced with media containing 0.1% FBS and the cells were incubated for a further 24 hr. Subsequently, cells were treated with 10 μM UO126 for 24 and 48 hr by direct addition of the drug to the culture media. Control cells received an equivalent volume of DMSO. Following treatment, adherent and non-adherent cells were collected by centrifugation, washed in PBS, and lysed as previously described. For time-course analyses of ligand expression, cells were treated

as above, but were harvested at 0, 4, 8, 16, and 24 hr in RLT buffer (Qiagen, Valencia, CA). Total RNA was isolated using RNeasy mini kits according to manufacturer's instructions.

Determination of EGFR ligand concentration in conditioned media

Cells were plated at a concentration of $2x10^5$ cells/well of a 12 well dish and allowed to adhere overnight in full growth serum. The following morning, media was changed to media containing 0.1% FBS. Twenty-four hours later, cells were treated by adding U0126 to a final concentration of 10 μM to the existing media. Following this treatment, conditioned media was collected from wells at 0, 2, 6, and 24 hr post-treatment. Media was cleared by centrifugation at high speed for 10 minutes at 4°C and the supernatant was removed and transported to new tubes. Samples were snap-frozen and transported to Raybiotech® (Norcross, GA) for analysis by Quantibody Array. Concentrations of HB-EGF and TGF-α were determined and reported. The experiment was performed and analyzed in triplicate.

C. RESULTS

EGF-like ligands HB-EGF and TGF-α are constitutively expressed in EGFR-dependent NSCLC cell lines

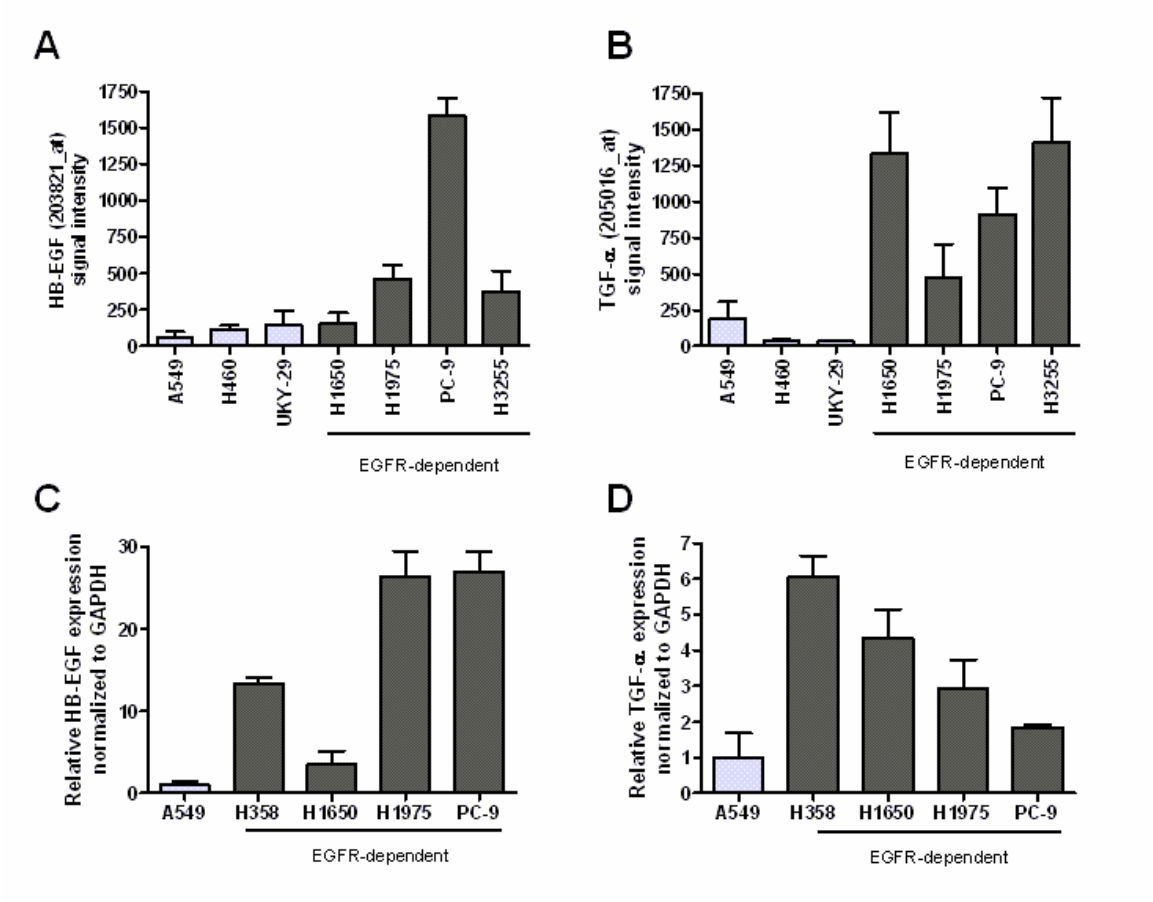
We explored our previously published gene expression data from a panel of NSCLC cell lines which was used to identify genes from the genomic signature of sensitivity to EGFR inhibitors that are important for mediating EGFR-dependency in NSCLC (251). The dataset included both lung cancer cell lines with and without EGFR-activating mutations (see Table 2.1). The data explored in this analysis were the pool of 1495 genes that were differentially expressed between EGFR-dependent and EGFR-independent cell lines, of which a subset of 180 signal transduction genes were found to be predictive of response to EGFR inhibition. Gene ontology trees are organized in a hierarchical manner, and we opted to utilize the entire 1495-gene dataset to include genes that are associated with cell signaling, although at a lower gene ontology level than that used to define the 180-gene subset. Therefore, using the entire 1495-gene dataset, we explored genes that appeared to be specifically deregulated in EGFR-dependent cell lines.

Interestingly, the EGFR ligands, heparin binding EGF-like ligand (HB-EGF) and transforming growth factor-alpha (TGF-α), were identified as significantly up regulated in the microarray experiments using EGFR-dependent NSCLC cells (Figure 3.1, panels A and B). These data were generated from cells actively proliferating in high-serum (10% FBS) containing media, which provides cells with exogenous survival factors.

To explore the effects of survival factors on ligand expression, HB-EGF and TGF-α mRNA levels were evaluated in three of these cell lines using qRT-PCR following 24 hours of serum-deprivation (0.1% FBS-containing media) (Figure 3.1, panels C and D). We chose these cell lines as they represent a variety of EGFR genotypes (see Table 2.1). We also included the H358 cell line as a representative EGFR-wt, EGFR-dependent cell line, and the EGFR-wt, EGFR-independent A549 cell line as a reference for a total of 5 cell lines. Relative expression levels of HB-EGF and TGF-α were consistent with the microarray data, with the exception of the EGFR-dependent PC-9 cell line. The PC-9 cell line demonstrated reduced expression of the ligands relative to the other tested cell lines by qRT-PCR in low serum conditions.

Figure 3.1: HB-EGF and TGF-α are constitutively expressed in EGFR-dependent NSCLC

Affymetrix MASv5.0 signal intensity values for **(A)** HB-EGF and **(B)** TGF-α, respectively, were extracted from the microarray data presented in Chapter 2. Only cell lines with data from at least 3 experiments were included. Bars represent average + SD. qRT-PCR analysis of expression of **(C)** HB-EGF and **(D)** TGF-α, respectively, in A549, H358, H1650, H1975, and PC-9 cells following 24 hours of serum-deprivation (0.1% FBS-containing media). Expression was determined by use of ABI TAQMAN probes and normalization of individual samples to GAPDH expression. Relative quantification was performed using a standard curve method. Bars represent mean + SD of three replicates.



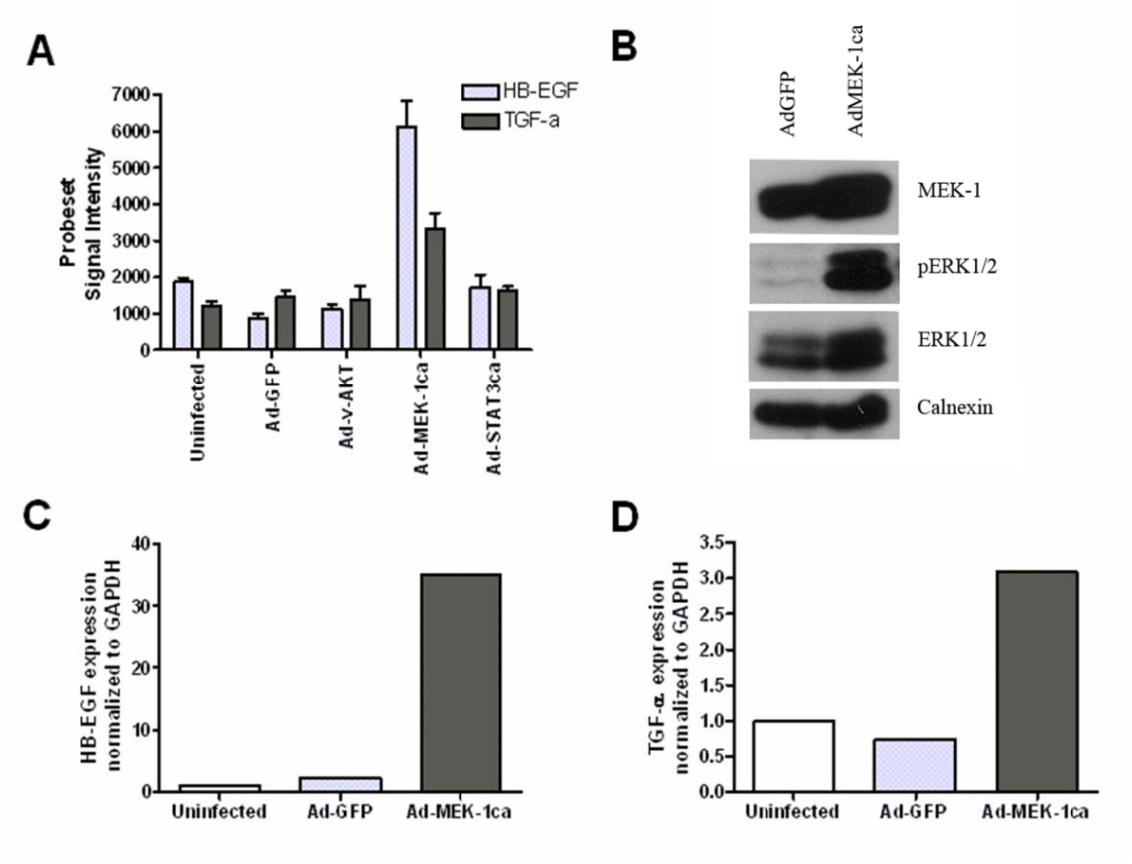
Ras/MAPK pathway activation induces expression of alternate EGF-like ligands

Analysis using TRANSFAC (234) and MAPPER (259) of the HB-EGF and TGF-α promoters identified the presence of ELK-1 and ETS-1 binding sites. These transcription factors are downstream of and controlled by ERK1/2, a member of the RAS/MAPK module (260-262). It is well established that EGFR can trans-activate the RAS/MAPK pathway (23). Additionally, both HB-EGF and TGF-α have previously been shown to be regulated via oncogenic RAS and/or RAF expression in intestinal and mammary epithelial cells (252, 253, 263). Finally, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the components of our 180-gene predictive signature identified enrichment of genes involved in the RAS/MAPK pathway (see Figure 2.2) (251). Therefore, both biochemical and bioinformatic analyses intersected at the RAS/MAPK pathway as a potential regulator of EGF-like ligand expression, and further exploration of this pathway in the context of EGFR-dependent NSCLC was warranted.

EGFR signaling is mediated through at least three downstream pathways in NSCLC: the RAS/MAPK pathway, the STAT3 pathway, and the PI3K/AKT pathway (78). Therefore, we next determined if the RAS/MAPK pathway could specifically regulate gene expression of HB-EGF and TGF-α in normal lung cells. We chose small airway epithelial cells (SAECs) as a model system because they are a primary cell line thought to be the precursor to adenocarcinoma and bronchioloalveolar carcinoma (264, 265). To isolate the effects of deregulation of downstream pathways of EGFR, we infected these cells with adenoviruses expressing either green fluorescent protein (GFP) as a control, v-AKT (Ad-v-AKT), constitutively active STAT3 (Ad-STAT3ca), or constitutively active MEK-1 (Ad-MEK-1ca), a penultimate kinase of the RAS/MAPK pathway. Uninfected cells were also analyzed as an additional control for the infection process. After 16 hours of virus infection, RNA was harvested for microarray analysis. We specifically analyzed the probesets corresponding to HB-EGF and TGF-α, and found that only Ad-MEK-1ca infection resulted in marked increase in expression of these EGFR ligands (Figure 3.2, panel A). To confirm these results, we repeated the ectopic expression of MEK-1ca in human bronchial epithelial cells (hBEAS-2B), a lung cell line which has been immortalized by SV40 large-T antigen. After 16 hours of infection, we confirmed ectopic expression (Figure 3.2, panel B) and isolated RNA to assess gene expression of both ligands by qRT-PCR. Again, gene expression of both ligands was significantly increased following infection of MEK-1ca (Figure 3.2, panels C and D).

Figure 3.2: Ectopic expression of MEK-1ca results in increased HB-EGF and TGF-α gene expression

A) Small airway epithelial cells were infected for 16 hr with recombinant adenovirus expressing GFP, v-AKT, MEK-1ca, or STAT3ca. RNA was isolated for downstream microarray analysis. Probesets for HB-EGF and TGF-α were extracted. Bars represent the average of at least 4 independent infections + SD. **B)** Human BEAS2b cells were infected with adenovirus expressing GFP or MEK-1ca for 16 hr. Immunoblots were performed for the indicated proteins and phospho-proteins. RNA from the experiment described in panel B was used to make cDNA and perform qRT-PCR for **(C)** HB-EGF and **(D)** TGF-α, respectively. Data were normalized to GAPDH expression level.



EGFR-dependent NSCLC cell lines exhibit constitutive ERK1/2 activation

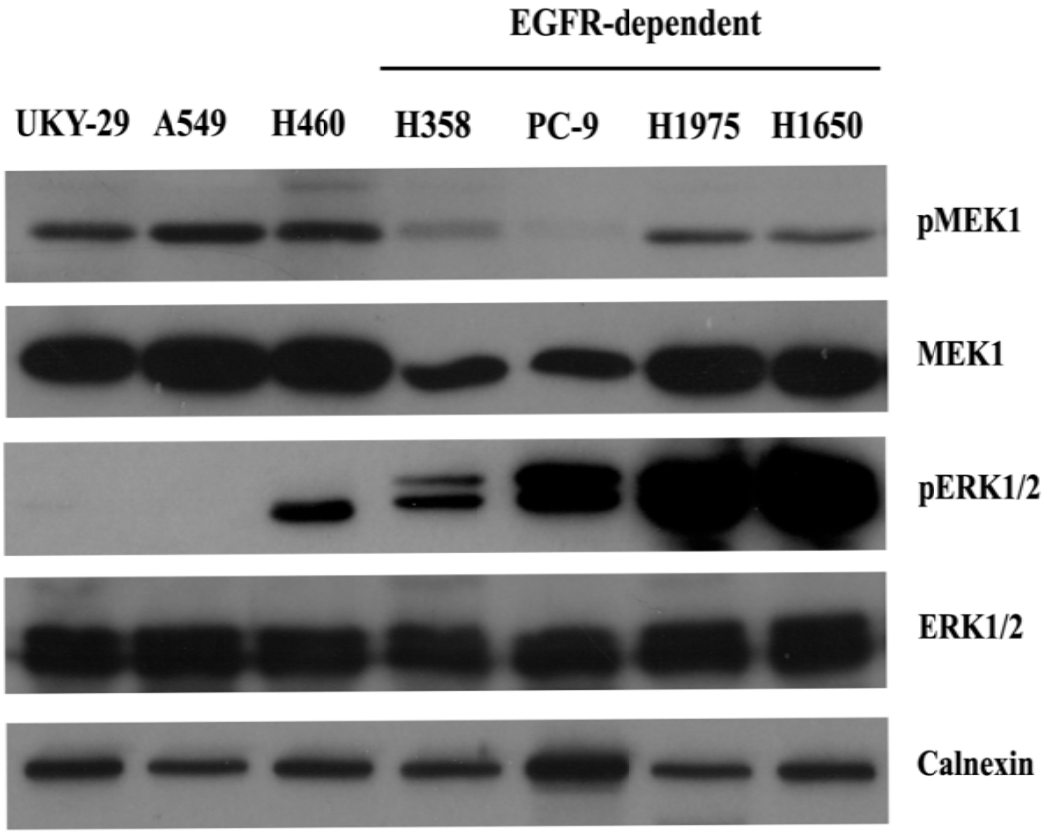
To determine whether HB-EGF and TGF-α expression correlates with activation of the Ras/MAPK pathway, we next assessed the level of activation of this pathway in the panel of NSCLC cell lines that we previously screened by microarray analysis. To determine baseline activity of this pathway, all cell lines were plated and cultured for 24 hr in low serum (0.1% FBS) containing media prior to lysis and western analysis for phospho-MEK-1 (pMEK-1) and phospho-ERK1/2 (pERK1/2) (Figure 3.3). The EGFR-dependent cell lines, which had the highest mRNA expression of HB-EGF and TGF- α, also demonstrated constitutive activation of ERK1/2.

MAPK inhibition disrupts expression and activation of HB-EGF and TGF- α in EGFR-dependent NSCLC cells

To confirm that the RAS/MAPK pathway drives expression of HB-EGF and TGF-α in EGFR-dependent NSCLC, we assayed mRNA expression by qRT-PCR of these genes for 24 hr following MEK1/2/5 inhibition with U0126 in three of the EGFR-dependent cell lines, as well as the EGFR-independent cell line, A549, as a reference (Figure 3.4, panel A and B). In the H358 and H1975 cell lines, U0126 significantly decreased expression of HB-EGF and TGF-α. For HB-EGF, this effect lasted the entire course of the experiment. However, TGF-α expression was decreased transiently, returning to baseline by 24 hours. In contrast, no decrease in the expression of either of the ligands was observed following MEK1/2/5 inhibition in the EGFR-independent A549 cell line or the EGFR-dependent H1650 cell line.

Figure 3.3: Constitutive ERK1/2 activity under serum starvation in EGFR-dependent NSCLC cell lines

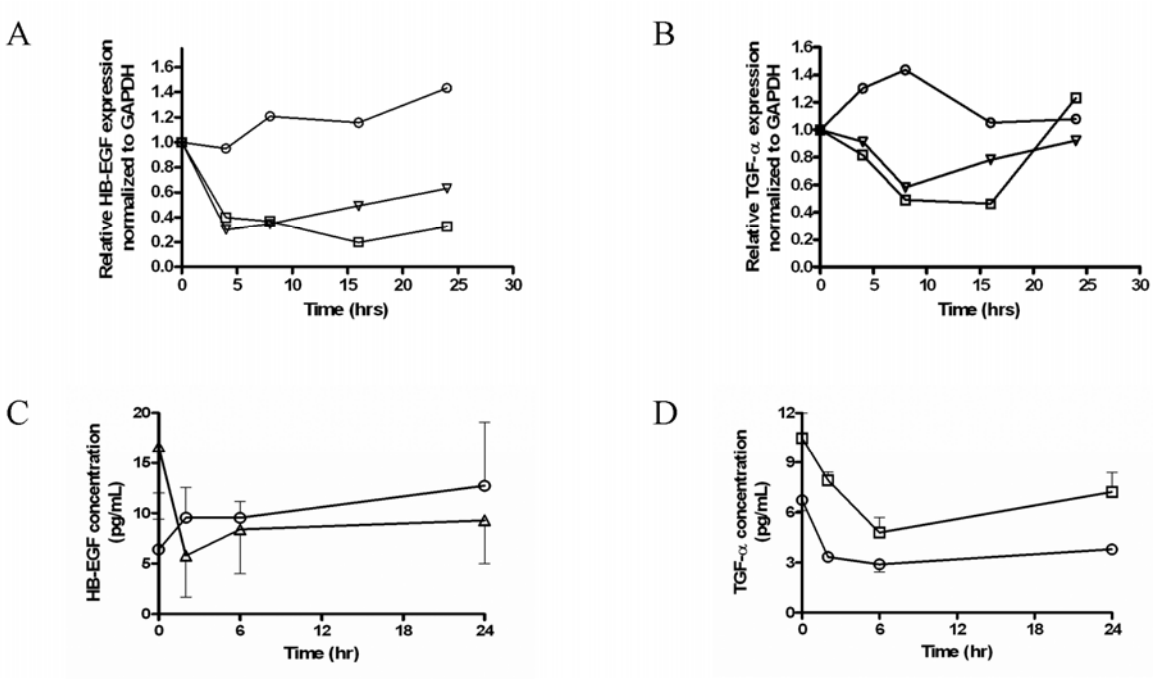
The indicated NSCLC cell lines were plated and allowed to adhere overnight in 10% serum-containing media. The following morning, media was replaced with 0.1% serum-containing media for 24 hr. Cells were harvested and analyzed by immunoblot analysis for the indicated proteins and phospho-proteins.



While inhibition of gene expression represents one mechanism by which ligand activity may be controlled by the RAS/MAPK pathway, it is also important to assess ligand activation and shedding from the plasma membrane. Alternate EGFR ligands, such as HB-EGF and TGF-α, are synthesized as pro-peptides and remain tethered to the cell membrane in an inactive form. Shedding and activation of these molecules requires the action of the ADAM family of matrix metalloproteinases (114). Vero-H cells (a monkey kidney epithelial line) have been previously utilized to demonstrate that shedding of HB-EGF is induced following constitutive RAS/MAPK activity (266). To evaluate the contribution of the Ras/MAPK pathway on ligand shedding and activation in NSCLC cells, we quantified both HB-EGF and TGF-α protein in conditioned media from each of these cell lines following MEK1/2 inhibition with U0126 (Figure 3.4, panel C and D, respectively). We found that in H1975 cells, the concentration of HB-EGF was rapidly depleted to approximately 30% of baseline levels within 2 hr, and was maintained at approximately 50% of baseline levels for the remainder of the 24 hr experiment. In H1650 cells, no change was observed in HB-EGF concentration in the supernatant. In H358 cells, HB-EGF levels were consistently below the level of quantification. The patterns observed in H1650 and H1975 cells were consistent with HB-EGF mRNA expression profile from the previous experiment (see Figure 3.4, panel A and B). In contrast, TGF-α concentrations decreased quickly in both H1650 and H358 cells and remained at approximately 50% baseline for the remainder of the 24 hr period. TGF-α was below the limit of quantification for all time points in the H1975 cell line. Overall, the patterns of TGF-α concentration observed for the cell lines did not reflect mRNA transcription, suggesting additional control of TGF-α shedding or activation through a MAPK-dependent mechanism.

Figure 3.4: Characterization of the effects of MEK inhibition on HB-EGF and TGF-α expression and shedding in EGFR-dependent NSCLC cell lines

Cells were plated and allowed to adhere overnight in 10% FBS-containing media. Media was then changed to 0.1% FBS-containing media for 24 hours before direct addition of U0126 to the culture supernatant. Cells were harvested at 0, 4, 8, 16, and 24 hours after addition of drug and RNA was used for downstream qRT-PCR analysis of **(A)** HB-EGF and **(B)** TGF-α expression. Values are reported as mean expression, normalized to the expression of the internal standard GAPDH and set to an arbitrary value of 1 for each cell line at the 0 hr. □ : H358 ○: H1650 Δ: H1975. Cells were treated as above, but supernatant media was collected and analyzed by the ELISA-based Quantibody ® array system to determine concentrations of **(C)** HB-EGF and **(D)** TGF-α, respectively. Values are reported as the mean of 3 experiments, with error bars representing the SEM. HB-EGF was below the limit of quantification for all time points in H358 and is not reported. TGF-α was below the limit of quantification for all time points in H1975 and is not reported. □: H358 ○: H1650 Δ: H1975.



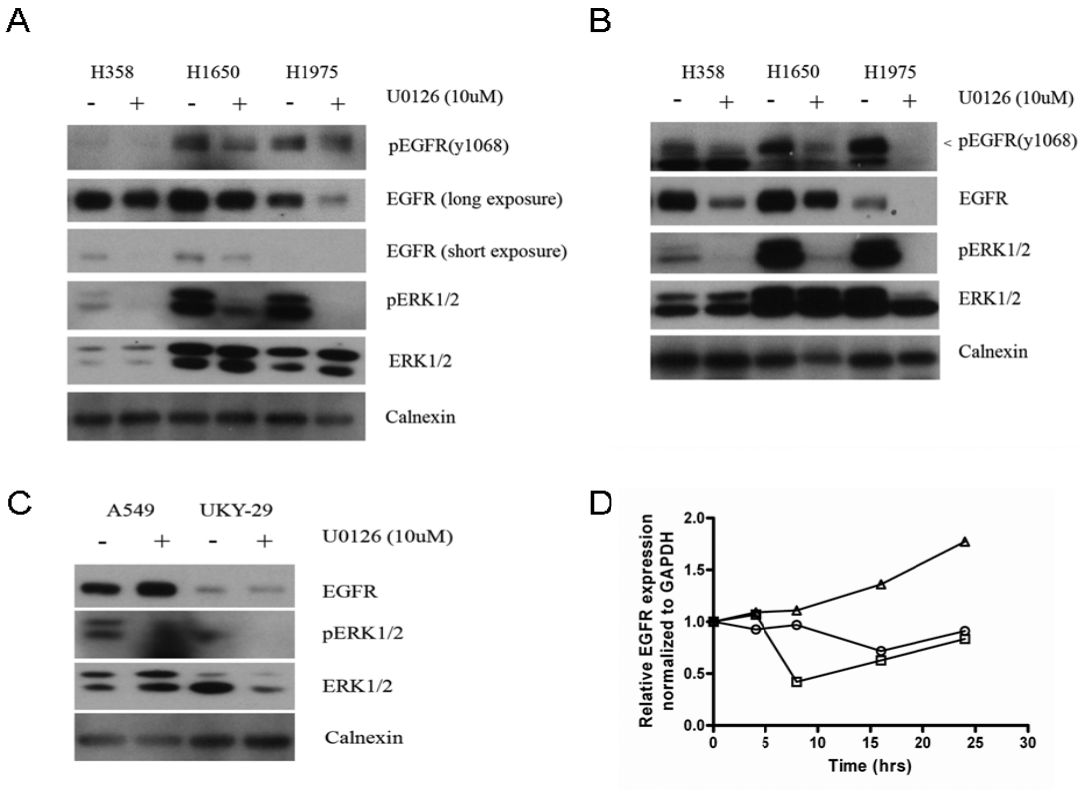
MAPK inhibition modulates EGFR stability through a transcription independent process

In order to determine the effects of decreased ligand expression and activity on EGFR signaling in each cell line following MEK inhibition, we performed western blot analysis to assess the phosphorylation of EGFR at tyrosine 1068 (one site which regulates RAS/MAPK pathway activity) following 24 and 48 hours of MEK1/2/5 inhibition with U0126 (Figure 3.5 panel A and B, respectively) (267). In each cell line, ERK1/2 phosphorylation was effectively inhibited by U0126 over the 48 hour period. A decrease in phospho-Y1068 was also observed in the U0126 treated cells compared to the DMSO-treated controls in each cell line tested. However, a decrease in total EGFR protein occurred concurrently in each of the cell lines, suggesting that the effects of MEK inhibition on EGFR-dependent survival may be due to a combination of decrease in ligand expression/activation and a down regulation of the EGF receptor itself. Down-regulation of EGFR was not observed in several EGFR-independent cell lines treated with U0126 (Figure 3.5, panel C), suggesting that this effect may be specific to the EGFR-dependent phenotype.

To further dissect these changes in EGFR stability, we assessed expression of EGFR by qRT-PCR over 24 hr following MEK1/2/5 inhibition, and did not observe a significant decrease in receptor gene expression in the H1975 or H1650 cell lines (Figure 3.5, panel D). The H358 cell line demonstrated a transient reduction in EGFR mRNA expression by ~ 70%. EGFR gene expression returned to baseline by 24 hr, failing to explain the prolonged reduction in EGFR protein.

Figure 3.5: MEK1/2/5 inhibition down regulates EGFR through a transcription-independent process in EGFR-dependent NSCLC cell lines

Cells were treated with 10 μM UO126 media containing 0.1% FBS for **(A)** 24 and **(B)** 48 hr. Expression of pEGFR (Y1068), total EGFR, pERK1/2, and total ERK1/2 was determined by immunoblot analysis. Blots were stripped prior to proceeding with the subsequent immunoblot. Blots were probed for calnexin as loading control. Panel A contains two exposures of the same immunoblot of total EGFR because saturation of the film occurred for the target band in the H1650 and H358 lanes before the band could be observed in the control lane for the H1975 cell line. **C)** EGFR-independent A549 and UKY-29 cells were treated and analyzed as described above (48 hr). **D)** Cells were plated and allowed to adhere overnight in 10% FBS-containing media. Media was then changed to 0.1% FBS-containing media for 24 hr before direct addition of U0126 to the culture supernatant. Cells were harvested 0, 4, 8, 16, and 24 hr later and RNA was used for downstream qRT-PCR analysis of EGFR expression. Values are reported as mean expression, normalized to the expression of GAPDH and set to an arbitrary value of 1 for each cell line at the 0 hr. □ : H358 ○: H1650 Δ: H1975.



Inhibition of ligand binding induces EGF receptor down regulation in the T790M, L858R EGFR mutant

Given these findings, we sought to identify a mechanism by which MEK inhibition could down regulate EGFR protein levels. EGFR can be ubiquitinated and degraded in the proteosome following ligand binding (268, 269). However, significant differences exist in the dynamics of endosomal EGFR sorting and subsequent proteosomal degradation following binding of the two most extensively studied EGFR ligands, TGF-α and EGF. TGF-α dissociates from EGFR at lower endosomal pHs more readily than EGF, and results in enhanced receptor and ligand recycling. EGF-EGFR interactions are more stable and result in degradation of the receptor-ligand complex (36, 268, 269). Thus, specificity of ligand binding could modulate EGFR turnover, depending on the ligands available in the tumor cell microenvironment.

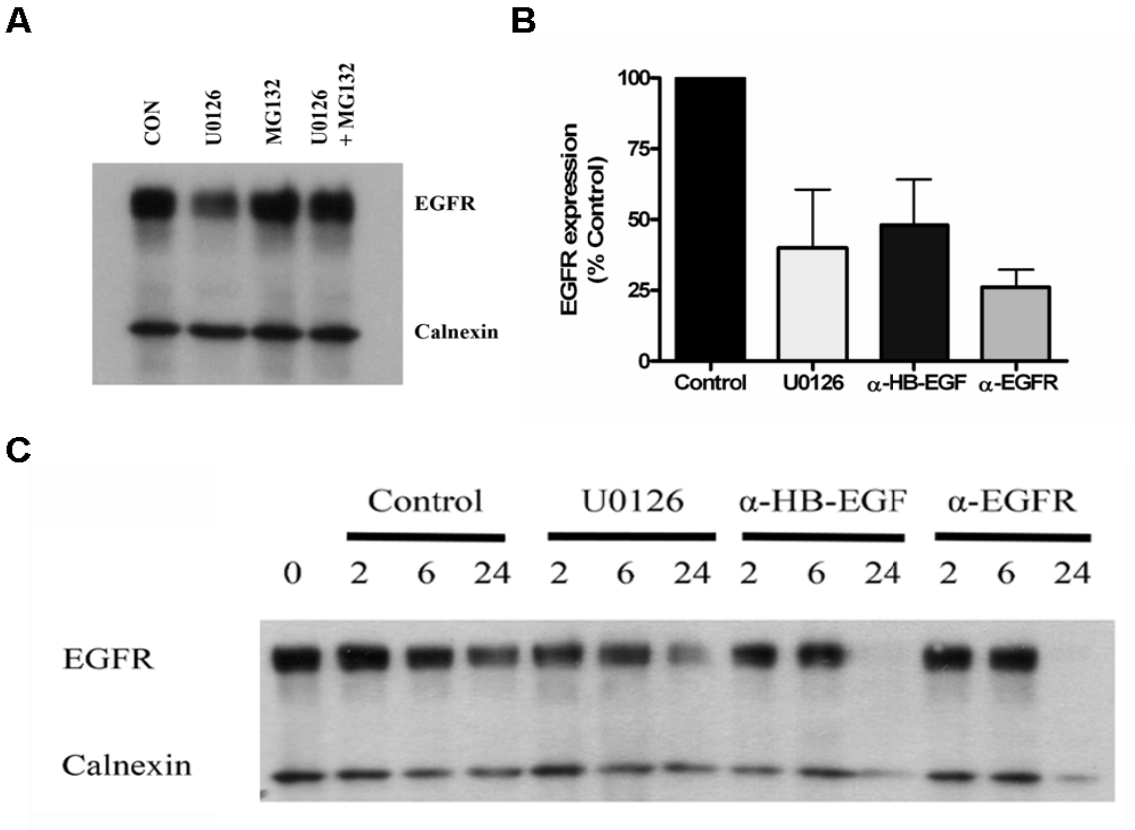
We therefore hypothesized that the persistent presence of alternate EGFR ligands, those other than EGF, such as HB-EGF or TGF-α could protect the receptor from degradation. To test this hypothesis, we utilized the EGFR double mutant (L858R/T790M) H1975 cell line. In previous experiments, the H1975 cell line responded the most dramatically to MEK inhibition in terms of EGFR down regulation. To determine if U0126-mediated down regulation of EGFR occurs via enhanced proteosomal degradation, we treated H1975 cells with either U0126 or the combination of U0126 and MG-132, a proteosomal inhibitor. U0126 treatment down regulated EGFR by approximately 30-40% at 24 hours, while the combination of U0126 and MG-132 treatment abrogated this effect. These results indicate that, in part, U0126 down regulates EGFR via enhanced proteosomal processes (Figure 3.6, panel A).

We have shown that H1975 cells express high levels of HB-EGF, but not TGF-α in cell culture supernatant and that U0126 treatment depletes HB-EGF and EGFR in these cells. Therefore, we treated H1975 cells with neutralizing HB-EGF targeted antibody or neutralizing EGFR ligand-binding domain antibody for 2, 6, or 24 hr to determine if interruption of HB-EGF binding to EGFR could produce a similar phenotype to MEK inhibition. We found that EGFR levels were significantly depleted after 24 hr in both neutralizing antibody treatment groups compared to control (Figure 3.6, panels B and C). Interestingly, this phenotype following EGFR-neutralizing antibody treatment was even more pronounced than U0126 treatment in this cell line. These data suggest

that persistent ligand binding of endogenous HB-EGF and/or other alternate EGFR ligands may protect the receptor from turnover, and could offer an additional explanation for the persistent EGFR levels observed in EGFR mutant NSCLC cell lines (93, 270).

Figure 3.6: MEK1/2/5 inhibition enhances proteosomal degradation of EGFR potentially through a EGFR ligand-dependent process

A) H1975 cells were serum starved for 24 hr in 0.1% serum-containing media before the direct addition of U0126, MG132, or the combination. Cells were harvested and lysates were prepared for immunoblot analysis of EGFR and calnexin. **B)** and **(C)** H1975 cells were serum starved in 12-well plates for 24 hr in serum-free media before treatment with 10 μM U0126, α-HB-EGF neutralizing antibody, or α-EGFR ligand binding domain neutralizing antibody for 2, 6, and 24 hr. DMSO + unconjugated goat anti-rabbit IgG was used as a negative control. Both adherent and non adherent populations were harvested by centrifugation, lysed in 1x RIPA, and used in immunoblot analysis for total EGFR and calnexin. The experiment was performed in triplicate and analyzed by densitometry. **B)** Bars represent mean signal + SD at 24hr. **C)** A representative immunoblot.



D. DISCUSSION

Understanding EGFR expression, activation, and turnover in the context of NSCLC is a subject of great significance and interest to both the basic science and clinical fields. Substantial research has been aimed at elucidating the relative contribution of downstream pathways to the phenotype of EGFR-oncogene addiction. Our results indicate that the Ras/MAPK pathway, through the expression and activation of EGF-like ligands, may serve to stabilize EGFR levels resulting in autocrine activity.

The work presented here demonstrate that EGFR-dependent cell lines express high levels of EGF-like ligand mRNA, regardless of culture conditions. Furthermore, constitutive activation of ERK1/2 correlates with expression of these ligands. Interestingly, phosphorylation of MEK-1 and its immediate downstream effectors ERK1/2 did not correlate in the NSCLC cell lines tested. Induction of the RAS/MAPK pathway by ectopic expression of constitutively active MEK-1 in non-malignant lung cells also induces ligand expression, mimicking the presence of an autocrine/paracrine loop in lung epithelial cells that is likely deregulated in EGFR-dependent NSCLC.

Loss of MAPK activity in EGFR-dependent lung cancer cell lines results in decreased expression and/or activation of EGF-like ligands, leading to reduced EGFR activation. Thus, in the absence of exogenous survival factors, the MAPK pathway is likely necessary for survival of EGFR-dependent lung cancer cells. This hypothesis will be tested in Chapter 4. The importance of the production and activation of EGF-like ligands in the EGFR–dependent phenotype is the subject of scrutiny. However, our work suggests that the mechanism of EGFR oncogene addiction may encompass either paracrine and/or autocrine signaling involving these ligands.

We have observed that activation of the EGFR pathway may also be modulated by inhibition of the RAS/MAPK cascade through a transcription-independent down regulation of the EGF receptor itself. Recent reports have suggested that EGFR mutations result in impaired degradation of the receptor following EGF activation (93, 271). This finding has been partially attributed to enhanced EGFR-HER2 interactions arising from constitutive phosphorylation of the mutant EGFR receptor. HER2-EGFR heterodimerization blocks the Cbl docking site on EGFR, thus interrupting Cbl-mediated

ubiquitination, and enhancing receptor recycling (93, 270). Similarly, EGFR-ligand interactions may also protect the receptor from degradation in EGFR-dependent cells.

A second mechanism that may be altered in EGFR-dependent NSCLC is EGFR turnover driven by inappropriate levels of EGF-like ligands. While EGF was utilized to stimulate EGFR in the aforementioned cell culture models, we were unable to detect endogenous EGF by ELISA in any of the NSCLC cell lines tested here (data not shown), suggesting that EGF may not be the most relevant stimuli in these cell lines. In contrast to EGF, TGF-α stimulation has been demonstrated to result in reduced degradation of the ligand-receptor complex via increased receptor recycling (36). Thus, TGF-α expression and activation may protect EGFR from degradation via shunting the receptor into recycling pathways. Although significantly less is known regarding the ligand-receptor dynamics of HB-EGF-EGFR, one could hypothesize that expression of alternative EGF-like ligands such as HB-EGF could similarly protect the receptor from degradation. Reduction in ligand activity by inhibition of the RAS/MAPK pathway might then restore normal receptor degradation rates, resulting in reduced EGFR protein levels. We have directly tested this hypothesis in H1975 cells by neutralizing HB-EGF as well as blocking the ligand binding domain of EGFR under conditions free of exogenous growth factors. Both neutralizing antibodies caused a substantial decrease in EGFR expression, similar to that observed with U0126. Interestingly, we were unable to achieve similar results in full-serum media, suggesting that growth factors contained in the media, such as EGF-like ligands, could supplement the loss of ligand activity induced with MEK1/2/5 inhibition or neutralizing antibody treatment. The results of this experiment suggest that RAS/MAPK-mediated expression of EGFR ligands, such as HB-EGF and TGF-α, may contribute to sustained EGFR expression and signaling in EGFR-dependent NSCLC.

Interestingly, an alternative and potentially contributing effect of ERK1/2 activation is phosphorylation of T669 of EGFR. Reconstitution experiments in CHO-GHR cells, which lack expression of EGFR, performed using stable expression of a T669A EGFR mutant, demonstrate that phosphorylation of this residue delays receptor trafficking, ubiquitination, and degradation (272). Furthermore, MEK inhibition phenocopied this effect in EGFR T669 expressing cells, but not in cells expressing the T669A mutant (272). Therefore, these results offer an additional explanation for how

RAS/MAPK activity in EGFR-dependent NSCLC cells may stabilize EGFR protein levels. The contribution of this mechanism remains to be studied in EGFR-dependent NSCLC.

Although each of the EGFR dependent cell lines demonstrated common trends when treated with MEK1/2/5 inhibitors, mRNA expression of HB-EGF and TGF-α was resistant to changes induced by MAPK inhibition in the H1650 cell line. Therefore, the H1650 cell line may utilize additional signaling pathways to maintain expression of these ligands. Interestingly, the H1650 cell line does not express the tumor suppressor PTEN, a primary mediator in PI3K/AKT pathway activity (273). PTEN deletion results in constitutive AKT activation and has been suggested to also play a role in mediating erlotinib resistance (154). In accordance with this observation, the H1650 cell line has been reported to be insensitive to EGFR inhibitors in some cell culture models (274). Therefore, it is possible that AKT pathway activation may compensate for loss of ERK activity in this cell line, although PI3K inhibition in H1650 cells did not reduce ligand expression (data not shown). Despite the heterogeneity observed among the cell lines tested, MAPK-dependent shedding of TGF-α was observed in H1650 cells, possibly accounting for the modest induction of apoptosis observed in this cell line. The ability of this cell line to evade some, but not all, of the effects of MEK inhibition on EGFR autocrine activity are intriguing and will be studied in the future.

E. CONCLUSIONS

The results of this study demonstrate that ERK activation is needed for EGFR-ligand activity and may protect the receptor from degradation in EGFR-dependent cells. These data offer mechanistic detail explaining the role of autocrine/paracrine signaling in maintenance of EGFR levels and subsequent downstream pathway activity. Further, these findings could indicate important therapeutic roles for HB-EGF neutralizing antibodies and/or MEK inhibitors in the treatment of EGFR-dependent NSCLC. It will be important to further these data by determining whether the effects of MEK inhibition in EGFR-dependent lung cancer cells translate to potential therapeutic benefit (i.e. decreased cell proliferation and/or survival). Also, future studies will determine if chemical inhibitors of MEK1/2, which are currently navigating clinical trials, may be useful in combination with EGFR inhibitors in the EGFR-dependent NSCLC phenotype.

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CHAPTER 4

A. OVERVIEW

The formation of resistance to EGFR inhibitors represents a hurdle in progressing toward a sustainable cure in EGFR-dependent NSCLC patients. Generation of the T790M mutation in exon 20 of the EGFR gene is the most common mechanism of acquired resistance documented to date, although intrinsic resistance via T790M has also been reported in clinical studies (91, 99, 178, 179). The T790M mutation confers enhanced EGFR activity and results in increased ATP binding affinity, dramatically raising the IC50 of the inhibitor (91, 100). Additionally, amplification of the c-MET gene has also been reported to facilitate resistance to EGFR inhibition, although it does not appear to occur as commonly as the T790M lesion (98, 113).

One strategy to combat acquired resistance to EGFR inhibitors is the clinical testing of second generation EGFR-TKI which irreversibly inhibit the ATP-binding domain of EGFR (214, 242, 275). Irreversible inhibitors could offer extended responses in patients with EGFR-dependent tumors when used front-line or may be used in salvage therapy for patients who initially respond to first-generation inhibitors and subsequently relapse. However, pre-clinical studies suggest that the T790M mutation can still be selected by exposure to clinically relevant concentrations of the irreversible EGFR inhibitor HKI-272 (146), perhaps as the result of the ATP-competitive nature of HKI-272 coupled with a comparably lower binding affinity of the drug for the mutated binding pocket than ATP. Therefore, the generation of T790M-mediated EGFR-TKI resistance could persist despite the irreversible mechanism of action of HKI-272, and the use of irreversible inhibitors of EGFR as a single-target treatment may not provide further benefit to patients harboring EGFR-dependent tumors.

Combinatorial therapy in cancer treatment results in improved response rates and reduced acquired resistance (276). Such strategies are widely implemented in antimicrobial and antiviral therapy, most notably in HIV treatment (277). When using traditional agents, combinatorial therapy in cancer often results in enhanced toxicity. However, combining EGFR inhibitors with traditional chemotherapeutic agents has not been successful in early clinical trials (135, 208-210). Little data is available regarding the combination of erlotinib with other kinase inhibitors, which may result in improved

response rates with lower toxicity than traditional chemotherapeutics. Identification of appropriate add-on therapeutics to EGFR inhibitors to improve therapy and combat acquired resistance requires an understanding of both the EGFR pathway and the effects of EGFR dependency on the lung tumor cell.

EGFR transduces signals from the extracellular space to the nucleus via several downstream pathways. The RAS/MAPK pathway lies downstream of EGFR, but can also be activated by other trans-membrane receptors, including c-MET (278). MEK1 and MEK2, the penultimate kinases of the classical Ras/MAPK pathway, activate ERK1 and ERK2, and are targeted by a number of kinase inhibitors currently navigating clinical trials (23, 279, 280). The more recently characterized MEK5 appears to activate ERK5 downstream of EGFR and RAS, independently of the classical RAF-MEK1/2-ERK1/2 pathway (281). Many of the commercially available MEK1/2 inhibitors such as U0126 and PD98059 have also been shown to affect MEK5, and therefore additional strategies, such as siRNA technology, are necessary to isolate the MEK1/2 pathway in pharmacological research.

Our observations, as well as those of others, demonstrate that ERK1/2 are constitutively active in EGFR-dependent NSCLC cells and this phenotype is associated with EGFR dependency (97). Furthermore, genomic profiling of EGFR-dependent NSCLC cells identified deregulation of the RAS/MAPK pathway (see Chapter 2). Inhibition of the RAS/MAPK pathway in EGFR-dependent NSCLC cells reduced the expression and/or activation of HB-EGF and TGF-α, two EGFR ligands (see Figure 3.4). These ligands were implicated in modulating the stability of EGFR protein levels, as MEK1/2/5 inhibition or inhibition of EGFR ligands with neutralizing antibodies resulted in decreased receptor levels in a transcription-independent process (see Figure 3.6).

In this study, we show that MEK1/2/5 inhibition reduces EGFR-dependent tumor cell proliferation, and induces apoptosis depending on trophic conditions. Importantly, MEK1/2/5 inhibitors are active in T790M mutant cell lines, supporting their use to reduce selective pressure for the T790M mutation in patients treated with EGFR inhibitors. Finally, combining MEK1/2/5 inhibitors with EGFR inhibitors resulted in synergistic activity, and the effect was the greatest in EGFR-mutant cells. Parallel experiments utilizing AZD6244 (ARRY-142886), a selective MEK1/2 inhibitor, as well as depletion of ERK1/2 using siRNA followed by EGFR inhibitor treatment further supported these data

and suggest that inhibition of MEK1/2 and not MEK5 drives the sensitive phenotype. Therefore, we conclude that combining EGFR and MEK1/2 inhibitors warrants clinical testing to enhance response and reduce acquired resistance by coordinately inhibiting at least two oncogenic signaling pathways. Overall these data support further research in evaluating the efficacy of combined MEK/EGFR inhibition in *in vivo* models of EGFR-dependent tumors.

B. METHODS

Cell culture

The human NSCLC cell lines A549, UKY-29, NCI-H1975, NCI-H1650, NCI-H358, and PC-9 were obtained and maintained as previously described (251). PC-9#37 was kindly provided by Dr. Jeffery Settleman, M.D. and is a randomly mutagenized subclone of the PC-9 cell line which contains the T790M mutation in the EGFR gene (146). This mutation is known to reduce the binding of EGFR inhibitors (99). All cells were maintained in RPMI 1640 (with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5% glucose, 1.5% sodium bicarbonate) containing 10% fetal bovine serum (FBS) (Biowest, Miami, FL), 50 µg/ml penicillin and 50 µg/ml streptomycin. Serum content of the media was reduced for particular experiments where indicated. Fraction V Bovine Serum Albumin (BSA, InVitrogen, Carlsbad, CA) was obtained at a stock concentration of 7.5% w/v. BSA was added to cell culture experiments at a 1:10 (0.75%) or 1:100 (0.075%) concentration where indicated.

Chemical compounds

The MEK1/2/5 inhibitors U0126 and PD098059, as well as the irreversible EGFR inhibitor CL-387,785 were obtained from EMD Chemicals (Gibbstown, NJ). The EGFR inhibitor erlotinib was provided by Genentech (San Francisco, CA). AZD6244 (ARRY-142866) was kindly provided by Astra Zeneca (London, UK). All inhibitors were solubilized in DMSO at a 1000X concentration such that the concentration of DMSO in treated cells did not exceed 0.1%.

Western blotting

Cells were washed in PBS, harvested and lysed in RIPA buffer (50 mM Tris (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.25% sodium deoxycholate, 5

mM NaF, 5 mM Na_3VO_4, 10% glycerol, 1 mM phenylmethyl-sulphonylfluoride, and protease inhibitors) for 30 min on ice. Lysates were sonicated for 2-3 seconds to shear DNA and cleared by centrifugation at 13,200 rpm for 15 min. Protein concentrations of the lysates were determined by BCA assay (Bio-Rad, Hercules, CA). Samples were separated by SDS-PAGE and transferred to PVDF membrane. Membranes were blocked with 5% nonfat dry milk or 5% BSA in TBS for 1 hr at room temperature and then incubated overnight at 4°C with the appropriate antibody as indicated. Antibodies specific for phospho-ERK1/2, total ERK1/2, p-AKT (Ser473), total AKT, activated CASP3 and total PARP were obtained from Cell Signaling Technology (Beverly, MA). MEK-1 and HA-tag antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Actin and calnexin antibodies were used as controls for equivalent protein loading and were obtained from Sigma-Aldrich (St. Louis, MO) and Cell Signaling Technology, respectively. Following incubation with appropriate horseradish peroxidase-conjugated secondary antibodies, proteins were visualized using an enhanced chemiluminescence detection system. Immunoblots were scanned at a resolution of 600dpi and quantified by integrated density using ImageJ (National Institutes of Health) where applicable.

Trypan blue exclusion assays

Cells were plated in 24-well plates at a density of 2x104 cells per well and allowed to adhere overnight in full growth media. The following morning, all cells were changed to media containing 0.1% FBS for 24 hours. Cells were then treated in one of the following conditions: 0.1% DMSO in media containing 10% FBS, 10 μM U0126 in media containing 10% FBS, 0.1% DMSO in media containing 0.1% FBS, or 10 μM U0126 in media containing 0.1% FBS. At 0, 24, 48, and 72 hr post-treatment, non-adherent (floating) and adherent cells were collected. Viable and non-viable cells were counted using trypan blue exclusion under light microscopy. Cell growth was determined at each time point by the percent of viable cells in the well compared to the viable cells counted immediately following treatment (0 hr). Cell viability was calculated as the number of viable cells counted at each time point as a percentage of the sum of non-viable and viable cells counted at that time point ([100% x viable] / [non-viable + viable]). Experiments were repeated at least 3 times to determine experimental variability. Data were analyzed by JMP (SAS, Cary, NC) using ANOVA for repeated measures to determine treatment effects.

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Clonogenic survival assays

Cells were plated to 6-well dishes at a density of 1000 cells/well. The following morning, media was changed to 5% or 0.1% serum-containing media, and cells were treated with 10 μM U0126 or DMSO control. After 48 hr, media containing drug was removed, cells were washed once in PBS, and then cells were replenished with 10% serum-containing media. After 7-14 days, cells were washed once in PBS and then fixed in ice cold methanol for 10 min. Cells were then stained with 0.5% crystal violet in 25% methanol for 10 minutes and excess dye was removed with multiple water washes. Colonies of greater than or equal to 10 cells were counted in 10 fields under light microscopy. The average number of colonies per visual field was then multiplied by 348.6 (number of fields calculated per well) to determine the number of surviving colonies. Colonies were counted by two investigators, one of whom was blinded to the purpose and design of the experiment. The average of the two counts was utilized for statistical analysis (two-tailed student's t-test). Each experiment was performed in triplicate.

Cell cycle/FACS analysis

Cells were plated on 60 mm dishes and allowed to attach overnight in full growth serum-containing media. The following morning, media was changed to 0.1% serum-containing media. Twenty-four hours later, sufficient serum was added to achieve a final concentration of 5% serum and cells were treated with 5 μM U0126 or 0.1% DMSO. After 24 hr, adherent and floating cells were collected and fixed in ethanol overnight. Cells were stained with propidium iodide and analyzed by flow cytometry for DNA content.

Adenovirus infection

Cells were plated at a density of $5x10^4$/well of 6-well plates and allowed to adhere for 6-8 hours. Once cells had attached, media was aspirated and the cells were infected with replication-deficient recombinant adenovirus expressing GFP or HA-tagged constitutively active MEK-1 at a multiplicity-of-infection of 500. The MEK-1ca construct was kindly provided by Dr. Janet Rubin, Ph.D. and the details regarding this construct are previously published (256). The infection was carried out in 1 mL of serum-free RPMI media supplemented with 25 mM HEPES buffer. The plates were returned to the

incubator and rocked every 15 minutes for 1 hr before the addition of 1 mL of full-serum media. Sixteen hours later, cells were treated with inhibitors or DMSO control in 0.1% FBS-containing media for a period of 48 hr. Floating and adherent cells were then harvested and analyzed by western blot as described.

siRNA transfection

Cells were plated and allowed to attach overnight in full growth serum-containing media. The following morning, cells were transfected with siRNA using siQuest (Mirus Bio, Madison WI) transfection reagent according to the manufacturer's protocol. Smartpool siRNA targeting ERK1 (MAPK3), ERK2 (MAPK1) or non-targeted siRNA was purchased from Dharmacon (Chicago, IL). Cells were collected at 48 hr and harvested for western analysis or re-plated for additional experiments.

Sulfarhodamine B assays

Sulfarhodamine B (SRB) was used to measure cell proliferation and viability using total protein content as a quantitative metric and is widely used by the NCI to screen compounds and quantify cytotoxicity. Cells were plated in 96 well plates at a density of 5x103 cells per well in 100 μL of full growth media and allowed to adhere overnight. The following morning, media was changed to 100 μL 5% FBS-containing media and cells were treated with DMSO control or serial dilutions of U0126 in an additional 100 μL of 5% FBS-containing media. After 24, 48, or 72 hr, media was aspirated and the cells were fixed for 30 minutes in 10% trichloroacetic acid at 4°C. Cells were then washed with tap water 5 times and allowed to air dry. Cells were stained with 0.4% sulfarhodamine B in 1% acetic acid for 10 minutes, washed 5 times with 1% acetic acid to remove unbound dye, and then allowed to air dry. Bound dye was then solubilized by the addition of 100 μL 10 mM Tris-HCl and shaking for 5 minutes. Absorbance was measured at 490 nM on a 96-well plate reader. At the time of treatment, additional cells were analyzed to determine baseline (time zero) signal. Blank wells and time zero signals were subtracted from the raw data, then normalized to the control (untreated) values. Fifty-percent growth inhibitory (GI50) and total growth inhibitory (TGI) values were determined by fitting a sigmoidal dose-response curve to the data and interpolating the concentrations at which drug inhibited 50% and 100% of the growth relative to time zero, respectively. Experiments were performed in quadruplicate and data was analyzed using Graphpad Prism (Graphpad Software, La Jolla, CA).

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Synergy studies

Cells were treated and analyzed by SRB as above. The MEK inhibitor U0126 and the EGFR inhibitor CL-387,785 were utilized at fixed (1:1) ratios of the IC50 as quantified in preliminary experiments. IC50 values were calculated similarly to GI50 values from SRB assays as described above, but without subtracting the time-zero signals. Synergy was assayed using Calcusyn (Biosoft, Ferguson MO). The combination index for the drug combination at the IC50 and IC75 was determined by the method reported by Chou and Talalay (282). Degree of synergy is based on the combination Index (CI) and is reported in the following manner: CI < 1, synergy; CI=1, additive; CI > 1, antagonism. The experiment was performed at least three times in all cell lines.

C. RESULTS

EGFR-dependent NSCLC cell lines display ERK-dependent growth

A panel of adenocarcinoma-derived NSCLC cell lines representing a variety of EGFR genotypes was assessed to determine activation of effector molecules downstream of EGFR. The characteristics of the cell lines used are presented in Table 4.1. Two signal transduction pathways downstream of EGFR that are known to contribute to the oncogenic phenotype include the RAS/MAPK pathway and the PI3K/AKT pathway. We first assessed the status of the RAS/MAPK pathway in EGFR-dependent cells. The PI3K/AKT pathway was also interrogated because it has been implicated in mediating many of the oncogenic effects of activated or mutated EGFR in NSCLC.

In order to evaluate constitutive activity of EGFR effector molecules, the cell lines were cultured for a period of 24 hr in low-serum (0.1% FBS) media prior to harvest and assessment by western blot of the activated effector proteins ERK1/2 and AKT (Figure 4.1, panel A). There was strong concordance between the EGFR-dependent phenotype and p-ERK expression relative to the reference EGFR-independent cell lines (UKY-29 and A549) under the same conditions, while the degree of p-AKT observed in the cell lines was variable.

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Table 4.1: NSCLC cell line characteristics

Cell line	EGFR-dependent	EGFR status	KRAS status	PTEN status
UKY-29	No	Wild-type	G61H	expressed
A549	No	Wild-type	G12V	expressed
H358	Yes	Wild-type	G12V	expressed
PC-9	Yes	Amplified, del746-750	Wild-type	expressed
PC-9#37	Yes	Amplified, del746-750, T790M	N/D	expressed
H1975	Yes	L858R, T790M	Wild-type	expressed
H1650	Yes	del746-750	Wild-type	deleted

N/D: No data

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To further explore the importance of ERK activity in these cell lines, the MEK1/2/5 inhibitor U0126 was used to determine the contribution of activated ERK on EGFR-dependent tumor cell growth. In parallel experiments, the pure MEK1/2 antagonist AZD6244 was utilized at a clinically achievable concentration (1 μM) to isolate the MEK1/2 pathway and confirm the findings (283). Using an SRB assay to monitor proliferation in the presence of drug, MEK1/2 activity was important in at least 4 of 5 cell lines tested (Figure 4.1, panel B). AZD6244, but not U0126, significantly reduced proliferation in the H1650 cell line. Additionally, we performed trypan blue exclusion assays on each of the cell lines under full serum conditions over a 72 hr period of 10 μM U0126 treatment and found that in 3 of the 4 EGFR-dependent cell lines tested (H358, PC-9, and H1975, but not H1650), a significant reduction in cell growth was observed by this method (see APPENDIX V, Figure V.1). Western analysis of p-ERK confirmed that U0126-mediated down regulation of ERK activity was sustained for at least 48 hours in all of the cell lines tested.

In light of our results using the specific MEK1/2 antagonist AZD6244, we wished to confirm specificity of these results to the MEK1/2 targets ERK1 and ERK2. To isolate this pathway, we performed siRNA-mediated depletion of ERK1, ERK2, and the combination (ERK1+ERK2). Forty-eight hours after transfection, a portion of the cells were harvested and ERK knockdown was confirmed by western blotting (Figure 4.1, panel C). Remaining cells were plated to 96-well dishes and cultured for an additional 48 hr to measure cell proliferation by SRB. ERK1 or ERK2 knockdown reduced cell proliferation considerably, although to varying degrees, depending on the cell line (Figure 4.1, panel D). Overall, ERK2 knockdown resulted in a more consistent reduction in cell proliferation across the cell lines. In 3 of 4 EGFR-dependent cell lines tested, combined ERK1/2 knockdown induced total growth inhibition (TGI).

To determine whether the effect of U0126 treatment on cell proliferation was due to growth arrest, DNA content analysis was performed in the cell lines following 24 hr of 5 μM U0126 treatment (Figure 4.1, panel E). Only the H358 and H1650 cell lines demonstrated a significant increase in cells in the G0 /G1 phase. Interestingly, although small molecule inhibition of MEK1/2 or siRNA-mediated knockdown of ERK1/2 resulted in reductions in cell growth in the H1975, PC-9, and PC-9#37 cell lines, we did not observe growth arrest by DNA content analysis in these cell lines. We hypothesized that the inhibition of cell growth due to loss of ERK1/2 activity observed in the EGFR-

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dependent cell lines could therefore be the result of contributing mechanisms such as cell death or apoptosis, as opposed to growth arrest.

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Figure 4.1: ERK1/2 is required for the proliferation of EGFR-dependent NSCLC cell lines

A) Cells were harvested following 24 hr of culture in low-serum media and lysates were assayed by western blot for the indicated proteins and phospho-proteins. **B)** Cells were treated with DMSO control, 10 μM U0126, or 1 μM AZD6244 in 5% media for 72 hr and assayed by SRB. Bars represent mean of four experiments +/- SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. **C)** Cell lines were transfected with non-targeted (NT) siRNA, or siRNA targeted for ERK2, ERK1, or the combination. After 48 hr, the cells were harvested and lysates were analyzed by SDS-PAGE and western blot to confirm successful knockdown. **D)** Cells from panel 'C' were trypsinized and split to 96-well plates. Cell proliferation was analyzed by the SRB assay at 0 and 48 hr. Data are expressed as percent of the signal achieved at day 0 for each transfection group, +/- SD (three replicates). **E)** Cells were treated with U0126 (5 μM) or DMSO control in 5% serum containing media. After 24 hr, cells were collected, fixed in ethanol, and stained with propidium iodide. DNA content analysis was performed by FACS analysis. Data are expressed as the percentage of cells in the G0/G1 phase. Bars represent mean of three experiments +/- SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

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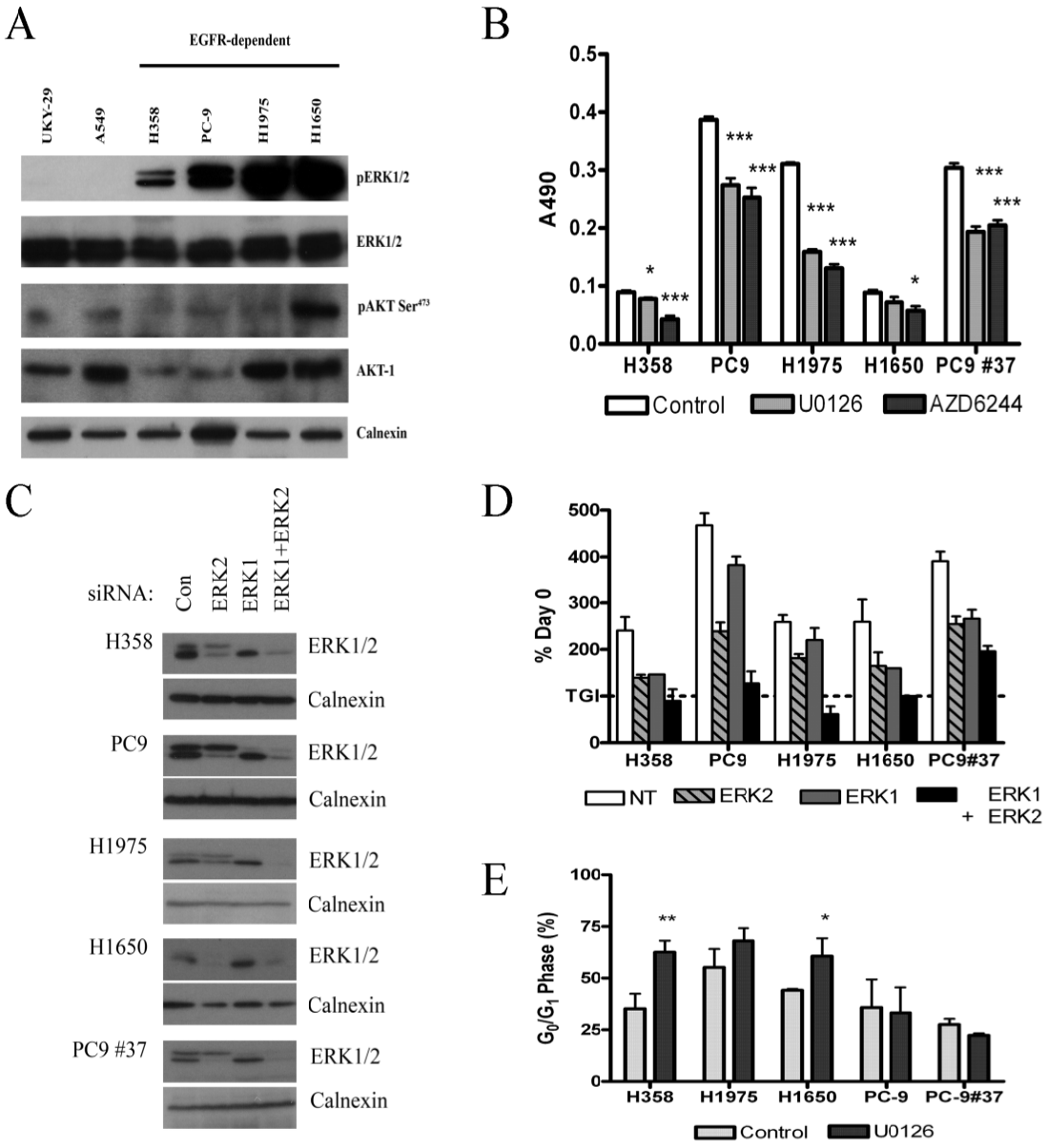
Figure 4.1 (continued): ERK1/2 is required for the proliferation of EGFR-dependent NSCLC cell lines



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Loss of ERK activity inhibits survival and induces apoptosis in EGFR-dependent NSCLC cell lines during trophic stress

We assessed whether EGFR-dependent NSCLC cell lines demonstrate reduced survival in response to MEK inhibition. In order to measure survival, we performed clonogenic survival assays in the EGFR-dependent cell lines following 48 hr of treatment with U0126 or DMSO in low serum media (0.1% FBS). A significant reduction in clonogenic potential was observed in each of the 4 cell lines tested (Figure 4.2, panel A, top). However, when the cells were treated in serum containing media (5% FBS) for 48 hr, this effect was largely abrogated (Figure 4.2, panel A, bottom). To further these results, we treated the cell lines with U0126 in low serum media (0.1% FBS) and assessed viability from 0-72 hours by trypan blue exclusion. The percent of viable cells was negatively affected by 10 μM U0126 treatment, particularly in the H1975 and H358 cell lines (see APPENDIX V, Figure V.2). The low serum conditions resulted in a considerable loss of viability in the PC-9 cell line regardless of U0126 treatment. However, as with the clonogenic assay, U0126 did not reduce viability when the experiment was repeated under full serum (10% FBS) conditions. These data suggest the presence of either a trophic effect, which alleviates the cytotoxicity of MEK inhibition under full serum conditions, and/or a stress-response effect, which facilitates an enhanced response to MEK inhibition in low serum.

We next characterized the cytotoxicity that was observed during MEK inhibitor treatment under low-serum conditions. Three of the EGFR-dependent cell lines were assayed for PARP cleavage as an indicator of apoptosis following U0126 treatment for 24 hr (Figure 4.2, panel B, top) and 48 hr (Figure 4.2, panel B, bottom) in low serum media. Treatment with U0126 resulted in a significant upregulation of the cleaved 85kD band, suggesting apoptotic activity due to MEK inhibition under trophic stress. These data were furthered by confirming the presence of a dose-response effect on PARP cleavage in H1975 cells following 24 hr of treatment with varying concentrations of U0126 (Figure 4.2, panel C). Furthermore, ectopic expression of a constitutively active MEK-1 mutant rescued H1975 cells from PD098059-induced PARP cleavage, but not U0126-induced PARP cleavage (see APPENDIX V, Figure V.3). In contrast to U0126, which inhibits the kinase activity of MEK1/2/5, PD098059 prevents the activation (phosphorylation) of MEK, thus genetically-modified MEK1 that is constitutively active would be expected to be refractory to inhibition by PD098059 (284). These experimental

results confirm the role of MEK1 and not MEK5 in mediating the survival of EGFR-dependent NSCLC cells.

We found that supplementing low serum media with bovine serum albumin abolished the ability of U0126 to induce apoptosis in the H1975 cell line (see APPENDIX V, Figure V.4). These findings suggest that drug binding to albumin may limit the effects of U0126, or alternatively, that albumin indirectly reduces low serum-mediated cell stress. Regardless, loss of ERK1/2 by siRNA treatment of cells grown in full serum media resulted in only moderate upregulation of cleaved PARP (Figure 4.2, panel D). Taken together, these data suggest that loss of ERK activity can induce apoptosis in EGFR-dependent cell lines depending on the serum content of the media.

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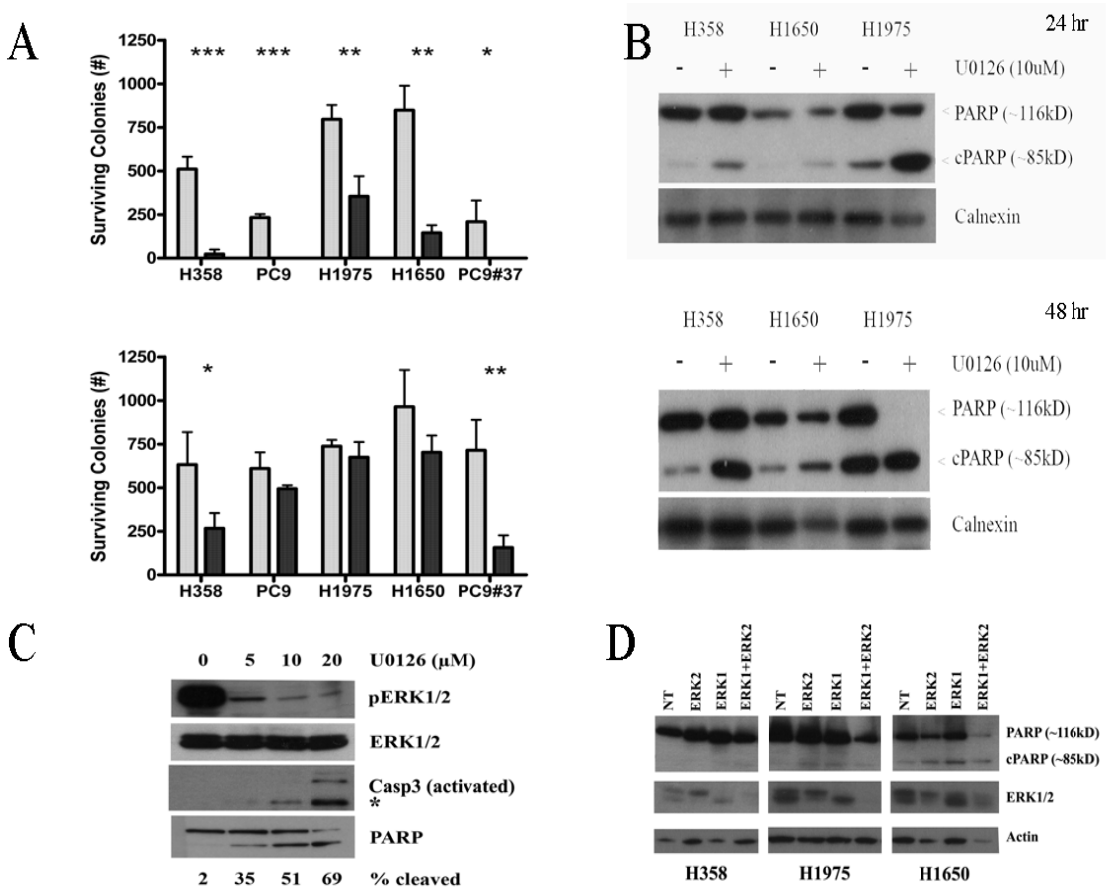
Figure 4.2: Loss of ERK activity inhibits survival and induces apoptosis in EGFR-dependent NSCLC cell lines during trophic stress

A) The indicated cell lines were assayed for their ability to form colonies following 48 hr of treatment with 10 μM U0126 (dark) or control (light) in either 0.1% (top) or 5% (bottom) serum containing media, followed by 7-14 days of culture in full-serum media. Colonies were counted as described in 'Methods'. Bars represent mean of three experiments +/- SD. * p<0.05, ** p<0.01, *** p<0.001. **B)** The indicated cell lines were treated in low-serum media with 10 μM U0126 and harvested at 24 hr (top) and 48 hr (bottom). Lysates were analyzed by SDS-PAGE and western blot for PARP. **C)** H1975 cells were treated in low serum media with 5, 10, or 20 μM U0126 for 24 hr. Cells were harvested and lysates were analyzed by western blot for the indicated proteins. Percent PARP cleavage was determined by densitometry (100 x cleaved PARP / cleaved PARP + full length PARP). **D)** The indicated cell lines were transfected with siRNA targeted for ERK2, ERK1, or the combination in full serum media (10% FBS). After 48 hr, floating and adherent cells were harvested and lysates were analyzed by western blot for PARP.



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Loss of ERK activity synergizes with EGFR inhibition in EGFR-dependent non-small cell lung cancer cell lines

We next tested whether inhibiting both MEK1/2/5 and EGFR could result in enhanced activity over either inhibitor alone. We first treated H358, PC-9, H1975 and H1650 cells with either erlotinib (500 nM), U0126 (5 μM), or the combination under low serum conditions to determine whether enhanced apoptotic activity could be achieved. After 24 hr, a statistically significant effect in PARP cleavage was observed in the combination treatment over either inhibitor alone, with two notable exceptions (Figure 4.3, panel A). First, the addition of U0126 to erlotinib in the PC-9 cell line did not result in a significant change in cleaved PARP, primarily because of the high sensitivity to erlotinib alone observed in this cell line. Secondly, the addition of erlotinib to U0126 in the H1975 and PC-9 #37 cell line did not result in a significant change in cleaved PARP, presumably due to the presence of the T790M mutation in these cell lines, which prohibits the binding of erlotinib to the target site on EGFR.

Given these results, we asked whether the combination of MEK1/2/5 and EGFR inhibitors could induce synergistic activity in EGFR-dependent NSCLC cells. Cells were treated with U0126, CL387,785, or the combination in fixed ratios of the IC50 for 48 hr in 5% serum containing media (Figure 4.3, panel B). The CL-387,785 compound was used since several of the cell lines tested (PC-9 #37 and H1975) harbor the T790M mutation, which strongly desensitizes the cell lines to erlotinib. A moderate level of serum was utilized to reduce trophic stress on the cells while limiting the presence of serum binding proteins such as albumin. In the EGFR-dependent cell lines, and in particular, the EGFR-mutant cell lines, the drugs displayed considerable synergy at a variety of dose levels. The drug effects in the EGFR-wt H358 cell line were additive at the IC75, but were synergistic at the IC50. In contrast, the effects in EGFR-independent, A549 cell line were moderate and appeared to be only additive (the 95% confidence interval includes '1') at both the IC50 and IC75.

To isolate the effects of ERK1/2 loss in combination with EGFR inhibition under full serum growth conditions, we performed siRNA-mediated knockdown of ERK1, ERK2, or the combination in four of the EGFR-dependent NSCLC cell lines. Forty-eight hours after transfection, cells were plated to 96-well dishes and treated with varying concentrations of erlotinib or the irreversible EGFR inhibitor CL-387,785 in 10% serum-

containing media. CL-387,785 was used only in those cell lines harboring the T790M mutation (PC-9#37 and H1975) as well as in the PC-9 cell line for direct comparison to the resistant (PC-9#37) clone. After 48 hr of treatment, cell proliferation was assayed by SRB (Figure 4.3, panel C). The concentration of EGFR inhibitor required for total growth inhibition (TGI) was interpolated from the dose-response curves, and was markedly reduced following either ERK1 or ERK2 knockdown (Figure 4.3, panel D). TGI for the ERK1 + ERK2 combination could only be calculated in the PC9#37 cell line, as ERK1/2 knockdown induced TGI without EGFR inhibitor treatment in the other cell lines tested (see Figure 4.1, panel D). These data support the hypothesis that MEK1/2 pathway inhibition, not MEK5 pathway inhibition, is likely responsible for the cytotoxic effects observed with U0126 either in the presence or absence of EGFR inhibitors.

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Figure 4.3: Loss of ERK activity synergizes with EGFR inhibition in EGFR-dependent non-small cell lung cancer cell lines

A) The indicated cell lines were cultured in low serum media (0.1% FBS) for 24 hr before addition of 5 μM U0126, 500 nM erlotinib, or the combination of both inhibitors. Following 24 hr of drug treatment, cells were harvested and lysates were assayed by western blot for PARP. A representative blot is pictured at left. Blots were scanned and analyzed by densitometry as described in Figure 4.2. Bar graphs (right) represent the mean +/- SD from three experiments. Means were compared using ANOVA with a Tukey's post-hoc test to contrast the individual groups. *p<0.05, **p<0.01, *** p<0.001 **B)** The indicated cell lines were treated for 48 hr in 5% FBS-containing media with fixed ratios of the IC50 of U0126, CL-387,785, or the combination before SRB analysis. Synergy was calculated using Calcusyn and the combination indices (CI) at the IC50 and IC75 for both drugs was graphed along with the 95% confidence intervals from three to four experiments. Interactions are reported as follows: CI <1: synergism, CI=1: additive, CI>1: antagonism. C) Cells were transfected with siRNA targeting ERK2, ERK1, or the combination, as described in Figure 4.1. After 48 hr, cells were trypsinized and re-plated to 96-well dishes in full serum media. After 16 hr, cells were treated with serial dilutions of erlotinib (H358 and H1650) or CL-387,785 (PC9, H1975, and PC9#37). Forty-eight hours later, inhibition of cell growth was assayed by SRB staining, and dose response curves were fit using Prism software. **D)** Using the data from panel C, the concentration of EGFR inhibitor required for total growth inhibition (TGI) was interpolated from the curves. *** TGI was achieved without the addition of EGFR inhibitors (i.e. combined siRNA transfection alone of ERK2 and ERK1 induced TGI).

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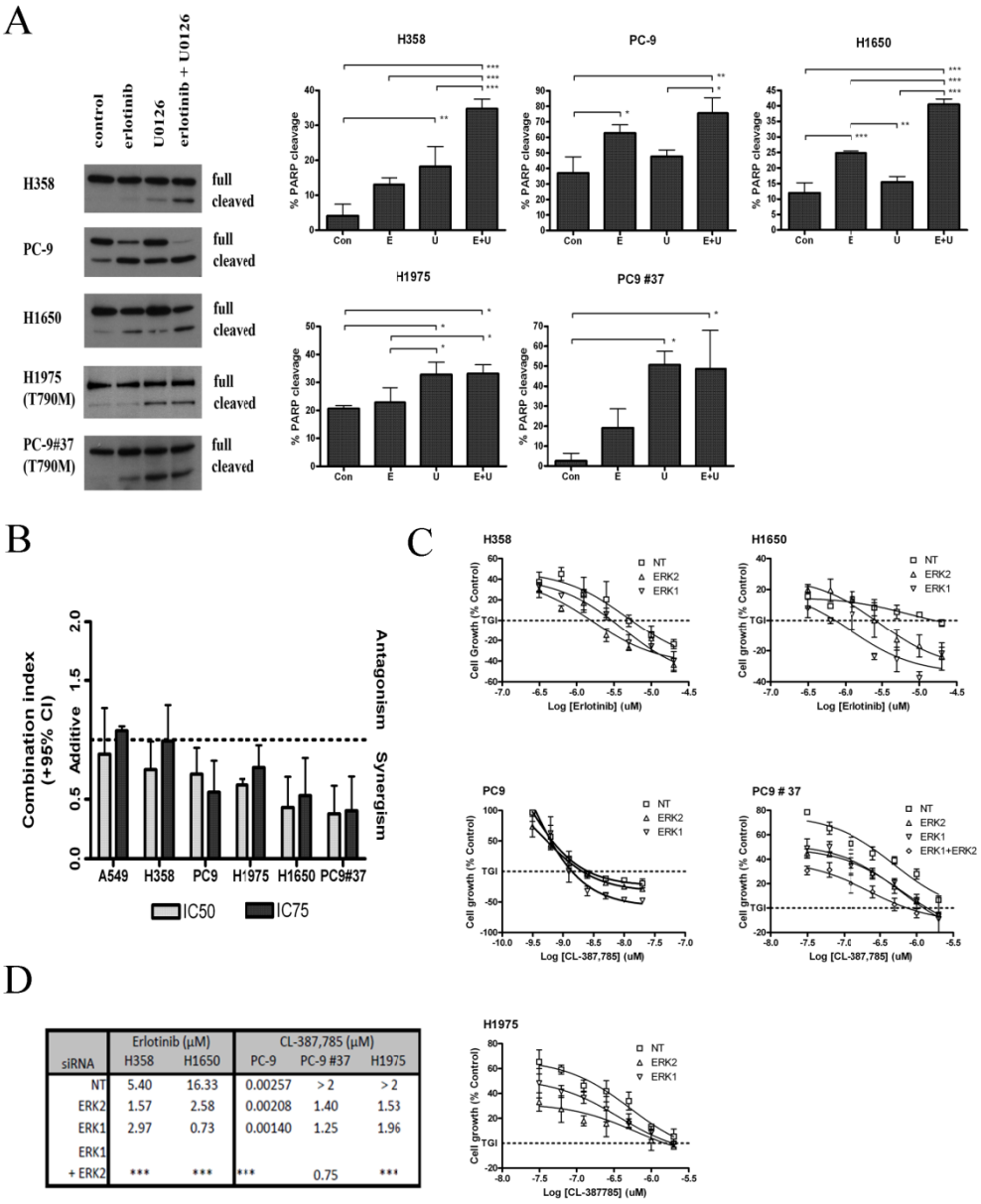
Figure 4.3: Loss of ERK activity synergizes with EGFR inhibition in EGFR-dependent non-small cell lung cancer cell lines (continued)



siRNA	Erlotinib (μM) H358	Erlotinib (μM) H1650	CL-387,785 (μM) PC-9	CL-387,785 (μM) PC-9 #37	CL-387,785 (μM) H1975
NT	5.40	16.33	0.00257	> 2	> 2
ERK2	1.57	2.58	0.00208	1.40	1.53
ERK1	2.97	0.73	0.00140	1.25	1.96
ERK1 + ERK2	***	***	***	0.75	***

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D. DISCUSSION

EGFR-dependent NSCLC represents a significant group of lung cancer cases, and is perhaps the most clinically treatable molecular phenotype in this devastating disease at this time. However, the outcomes of both preclinical and clinical studies suggest that improved strategies will be necessary to provide a sustainable systemic cure for patients harboring EGFR-dependent tumors. In this study, we sought to determine whether MEK1/2 inhibitors present promise for use as an add-on therapy for EGFR inhibitors in this patient subpopulation. The addition of a synergistic pathway inhibitor could enhance response rates, reduce acquired resistance, and prolong progression-free survival. Several MEK inhibitors are currently being tested in clinical trials, including AZD6244, although none have been tested in a clinically or molecularly defined population of EGFR-dependent NSCLC. Importantly, these inhibitors are specific for MEK1/2 and do not inhibit MEK5.

The MEK pathway is known to play a role in tumor cell survival and proliferation in a variety of cell culture and animal models (23). The compound utilized throughout the majority of this study (U0126) inhibits MEK1/2 as well as MEK5, and thus we have also utilized siRNA to isolate the MEK1/2-ERK1/2 pathway. Additionally, the selective MEK1/2 inhibitor AZD6244 phenocopied the growth inhibitory effects of U0126 and ERK1/2-directed siRNA, supporting our conclusions that the observed effects of U0126 are predominantly related to its inhibition of MEK1/2 and not MEK5.

We have demonstrated that EGFR-dependent NSCLC cells require the MEK1/2 pathway for sustained growth and survival signals. This dependence appears to correlate with the trophic conditions of the cellular environment. In low serum conditions, the free concentration of U0126 may be greater, resulting in stronger MEK inhibition and pronounced reductions in both proliferation and survival. However, in high serum conditions or low serum conditions with high concentrations of BSA, there may be reduced free drug due to serum-albumin binding.

EGFR mutations are thought to selectively activate the pro-survival PI3K/AKT pathway and may be primarily responsible for the transduction of constitutive oncogenic signaling emanating from activating EGFR mutations in NSCLC (83, 129, 285). Retrospective clinical data suggest that EGFR/PI3K/AKT axis activation may predict response to EGFR-targeted agents (117, 285). We have hypothesized that activation of

the PI3K/AKT pathway in EGFR-dependent tumors may override the importance of the MEK pathway in sustaining the anti-apoptotic phenotype and thus both pathways must be inhibited to most effectively induce apoptosis and elicit the strongest cytotoxic effects. In low serum conditions, only the H1650 cell line demonstrated pronounced AKT activation. This is likely due to the deletion of the PTEN gene in this cell line, which regulates PI3K activity (273). In concordance with this, we were not able to induce significant apoptosis in the H1650 cell line with a MEK inhibitor alone, suggesting that AKT activation could provide the necessary survival signals to sustain the anti-apoptotic phenotype. In other experiments, we found that U0126 treatment further activated AKT in this cell line, possibly through a feedback loop (see APPENDIX V, Figure V.5). Interestingly, combining a MEK inhibitor with an EGFR inhibitor in the H1650 cell line abrogated the expression of p-ERK1/2 as well as p-AKT, although total AKT expression was also reduced. Coordinate decreases in p-ERK1/2 and p-AKT corresponded to a significant induction of apoptosis, supporting the hypothesis that both MEK and AKT pathways must be inhibited to induce apoptosis.

Synergism between MEK and EGFR inhibitors was evaluated based on the following observations: 1) tumors harboring EGFR mutations are likely to respond to EGFR inhibitors; 2) EGFR mutations are known to selectively activate the PI3K/Akt pathway; and 3) coordinate deactivation of both the RAS/MAPK pathway and the PI3K pathway has been shown to result in improved anti-cancer activity (273). Our data show that synergistic cytotoxicity is achieved when combining MEK and EGFR inhibitors in EGFR-dependent lung cancer-derived cell lines. Our conclusions are strongly supported by the observation that siRNA-mediated knockdown of ERK1 and/or ERK2 sensitized the same cell lines to CL-387,785.

Finally, of particular importance, several of the cell lines displaying synergy between EGFR and MEK inhibitors harbored a T790M mutation. In clinical practice, a minority of EGFR-driven lung tumor patients present with detectable T790M prior to treatment, although most develop tumors harboring the T790M mutation during or following erlotinib treatment. This phenotype presumably arises due to selection of pre-existing subpopulations of cancer cells harboring T790M with erlotinib. The findings of others have suggested that even the newer generation irreversible inhibitors of EGFR such as HKI-272 may not be able to achieve concentrations sufficient to fully inhibit the T790M mutation *in vivo* (146). Thus, perhaps the observed synergistic interaction

between irreversible EGFR inhibitors and MEK inhibitors could reduce the selective pressure on the T790M mutation.

E. CONCLUSIONS

We have demonstrated that synergistic activity can be achieved with the combination of an irreversible EGFR inhibitor and a MEK inhibitor, suggesting that lower doses of both agents can be used to achieve an equivalent anti-cancer effect to either drug alone, reducing potential toxicity to these agents. Through enhanced activity, the combination of a MEK inhibitor and an irreversible EGFR inhibitor could remove the selective advantage on cells harboring the T790M mutation, thereby lengthening response time while minimizing resistance. Quantitative models of acquired resistance will be necessary to fully test this hypothesis. Additionally, *in vivo* animal model studies are warranted to determine if clinical trials should be targeted at the use of this combination in patients harboring EGFR-mutant NSCLC to enhance response and minimize acquired resistance.

CHAPTER 5

A. SUMMARY OF RESULTS

We hypothesized that gene expression profiling could enhance treatment of EGFR-dependent NSCLC by two methods: 1) to implement predictive classification of NSCLCs for the purposes of stratifying patients to EGFR-inhibitor therapy and 2) to provide new insights into the EGFR-dependent phenotype which could be chemically exploited. By coupling *in vitro* models with pharmacogenomic efforts, we have captured the phenotype of aberrant EGFR-dependent cell signaling in a gene expression profile. We conclude this work by demonstrating that these insights could be exploited using additional chemical inhibitors to enhance therapeutic outcome in EGFR-dependent NSCLC.

The first hypothesis, explored in Chapter 2, was tested by utilizing bioinformatics and systems biology approaches to define the180-gene profile of EGFR-dependency, both of which are novel methods of feature selection in this stratum of experimentation. The defined signature is capable of predicting the status of unknowns and exhibits biological relevance. The signature was validated in a panel of NSCLC cell lines with known sensitivity to EGFR inhibitors, and in NSCLC tumors stained for p-EGFR (a possible proxy of response to EGFR inhibitors) (251). Finally, we collected tumor tissue from NSCLC patients treated with erlotinib and found that the signature performed well in predicting RECIST response in this small cohort. Our results indicate that gene expression profiling could offer improved prediction over EGFR mutational status or immunohistochemical detection-based predictions alone, although additional validation will be required to robustly test this hypothesis. It is anticipated that through further modification and validation, the profile will yield a predictive diagnostic to identify response in patients with EGFR-targetable malignancies.

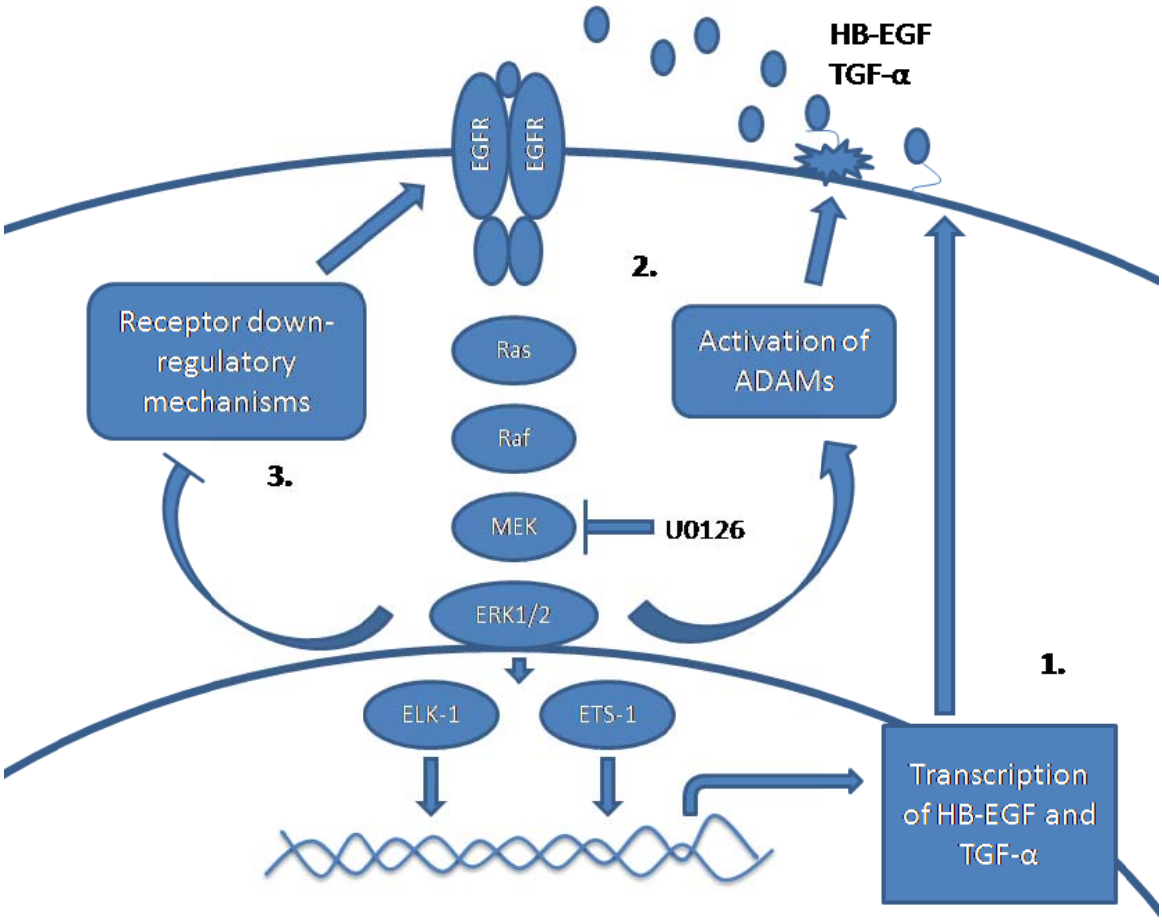
In Chapter 3, we explored the broader set of 1495 deregulated genes in EGFR-dependent NSCLC cell lines and found that high expression of EGFR ligands HB-EGF and TGF-α appears to be associated with EGFR-dependent phenotype. Further, we were able to identify at least three ways that RAS/MAPK activation could contribute to maintenance of the EGFR-dependent phenotype: 1) promoting expression of EGF-like ligands, 2) promoting shedding/activation of EGF-like ligands, and 3) modulating EGFR

protein stability and degradation (Figure 5.1). The RAS/MAPK pathway is constitutively active in EGFR-dependent NSCLC cell lines and directs expression of HB-EGF and TGF-α, as small molecule inhibition of MEK1/2/5 reduced EGF-like ligand gene expression. Importantly, redundant pathways may contribute to sustained expression of EGF-like ligands despite inhibition of the RAS/MAPK pathway, as was observed in the H1650 cell line. Thus, combining targeted agents may be necessary to achieve full abrogation of EGFR signaling in heterogeneous epithelial tumors (273, 286-290). We also discovered that proteolytic processing of TGF-α is mediated by the RAS/MAPK pathway, as MEK1/2/5 inhibition reduced the release of activated TGF-α from H1650 cells while sustaining gene expression levels. Finally, these results suggest that the availability of HB-EGF and/or TGF-α may be directly related to the stability of EGFR. Ligand activated EGFR is normally either recycled to the membrane or proteosomally degraded (78). In our experiments, MEK1/2/5 inhibition down regulated EGFR by enhancing proteosomal degradation and/or by reducing receptor-ligand recycling. To extend these results, the role of EGFR ligands in modulating EGFR stability was directly tested by neutralizing HB-EGF or the ligand binding domain of EGFR with antibodies. Both ligand-neutralizing antibodies phenocopied the effects of MEK inhibition in the H1975 cell line. Therefore, neutralizing antibodies directed at the ligand binding domain of EGFR (such as cetuximab), those directed at specific EGFR ligands, or small molecule inhibitors of the RAS/MAPK pathway may all offer potential therapeutic benefit in EGFR-dependent NSCLC because of the role of RAS/MAPK and EGF-like ligands in maintaining the EGFR-dependent phenotype.

Given our improved mechanistic understanding of the effects of RAS/MAPK pathway inhibition in EGFR-dependent NSCLC (see Figure 5.1), we tested the importance of this pathway in mediating tumor cell proliferation and survival (see Chapter 4). MEK1/2/5 inhibitors as well as the specific MEK1/2 inhibitor AZD6244 significantly inhibited the proliferation of EGFR-dependent NSCLC cell lines. We confirmed these results using siRNA-mediated knockdown of the MEK1/2 targets, ERK1/2. MEK inhibition also significantly impacted survival and induced apoptosis under low-serum conditions. Combining the MEK1/2/5 inhibitor U0126 with the irreversible EGFR inhibitor CL-387,785 resulted in cytotoxic synergy. Again, the role of the MEK1/2 pathway was isolated from the MEK5 pathway by combining EGFR inhibition with siRNA-mediated knockdown of ERK1/2. Thus, the combination of

targeting the RAS/MAPK pathway and EGFR appears to be highly effective in inducing cell cycle arrest/cell death in EGFR-dependent NSCLC.

Figure 5.1: Schematic for proposed contribution of RAS/MAPK pathway in modulating EGFR-dependent NSCLC signaling



B. EXPERIMENTAL CONSIDERATIONS

We combined pharmacogenomic and pharmacologic models to test our central hypothesis that gene expression profiles could be used to both select patients for EGFR inhibitor therapy and enhance therapy through the identification of additional molecular targets that could be exploited in combination with EGFR inhibitors. Using pharmacogenomic approaches, we were able to make broad inferences about the underlying deregulation occurring in EGFR-dependent NSCLC cell lines. Incorporating bioinformatics allowed us to then focus our results on candidate pathways and genes. Finally, the use of pharmacologic models allowed us to directly test the resulting specific hypothesis in a controlled experimental environment. Nonetheless, it is important to carefully consider the aspects of the experimental design for limitations and assumptions.

The most obvious caveat to the data presented herein lies in the extensive use of cell culture models. Cell culture models of cancer have the advantage of being malleable systems in which hypotheses requiring significant manipulation can be tested. However, cell culture models of cancer lack the heterogeneity and four-dimensional character of *in vivo* models, and therefore the conclusions that can be drawn from these models are limited. The impact of the model choice varies between the genomic and biologic/pharmacologic experiments.

Genomic models

We used gene expression data from NSCLC cell lines with known sensitivity to EGFR inhibition to populate a genomic dataset and identify a signature of EGFR-dependency. An alternative approach would be to utilize human tumors with known clinical outcomes to EGFR inhibitors. However, such data are not readily available and the cost of collecting and performing genomic analysis in a sufficient number of patients to confidently identify deregulated genes would be limiting, due to inter- and intra-sample heterogeneity (see Chapter 1 for a review). The relative homogeneity of cancer cell lines, both in replicates samples from the same cell line (inter-sample) and in cells constituting the clonal culture population (intra-sample), allowed us to achieve statistical confidence in the selected genes, and to reserve clinical data for validation. However, it is understood that this method may cause us to include features that may be artifacts of two-dimensional growth. Thus, continued validation in human tumors and possibly

modification of the profile will be important in further development as a useful clinical diagnostic.

Biologic and pharmacologic models

Cell culture models were also utilized for the biologic and pharmacologic experiments performed herein. Thus, there are important considerations associated with conclusions that were based on these models. As stated, a cell culture model of cancer does not likely capture the heterogeneity of the human condition. In order to reduce the impact of these limitations, we performed many of the experiments using a panel of cell lines representing different genetic backgrounds as opposed to a single representative cell line. Thus, our results may hold additional external validity over experiments performed in a single cell line, but the described models are still limited in their ability to capture three-dimensional growth characteristics and the contribution of the stromal cells surrounding a tumor. Additionally, pharmacokinetic and pharmacodynamic drug interactions between MEK inhibitors and EGFR inhibitors will be a crucial aspect of combinatorial therapy to explore before conducting clinical trials with this combination. As such, xenograft models will be important in validating and further exploring the findings of the biological experiments contained herein.

Another experimental consideration in the biologic and pharmacologic models is the use of serum starvation conditions to assess cell survival and apoptosis. We chose these conditions because we observed constitutive ERK activation that appeared to be uniquely associated with the EGFR-dependent phenotype under serum starvation. Importantly, serum starvation may not accurately reflect physiological conditions, since non-tumor cell derived growth and survival factors are likely to be present the tumor environment *in vivo*. However, the tumor environment *in vivo* is often hypoxic, creating regions of tumor necrosis and apoptosis (291). Therefore, the level of survival factors supplied to cells during culture in high serum medium, resulting in a low cell turnover rate *in vitro*, may conversely overestimate physiological conditions. We believe that it is reasonable to utilize low-serum media since one hallmark of cancer is the ability to survive independently of external growth signals. Growth and survival factors supplied under serum rich conditions may provide sufficient activation of alternative pathways to override the oncogenic self-sufficiency demonstrated by the EGFR-dependent cell lines. Furthermore, drug-binding plasma proteins may also play a role in reducing drug effects

on growth or survival at a given concentration, an effect that could be observed both *in vivo* and *in vitro*. Reducing the serum content of the media could have the effect of increasing free drug concentrations, and thus the observed pharmacologic activity. Importantly, the effects of MEK inhibition on cell survival and apoptosis were variable in the NSCLC cell lines used, depending on the serum content of the culture conditions. Thus, the model utilized is likely to have impacted our results. In the absence of *in vivo* data, it is unclear whether our low serum model is more or less representative of drug effects on cell survival than when a high serum model is used. Therefore, *in vivo* models will be important for further substantiating our hypotheses.

C. CONTRIBUTION TO THE FIELD

This work makes a substantial contribution to an improved understanding of the EGFR-dependent phenotype. The rising costs of health care require the identification of effective ways of minimizing non-beneficial medication use and maximizing outcomes. Predictive pharmacogenomics is a field that can meet these demands. Our work and the work of others have identified genomic signatures that could underlie responsiveness to EGFR inhibitors in the EGFR-dependent phenotype (230, 251, 292, 293) (see also Chapter 2). Coldren et al also published a genomic signature of response to erlotinib developed from NSCLC gene expression data (230). Their approach, while similar to our own utilized a larger number of cell lines in their model-training set, but did not use statistical replicates of each cell line. Importantly, they stopped short of validating their profile in human tumors. Fujimoto et al explored combinations of expression of ErbB family members and their ligands in EGFR-TKI sensitive NSCLC cell lines and mouse models of lung cancer (106). However, they did not attempt to translate their findings into a useful predictive model for classification of human tumors. Thus, the experiments contained in our work are the first to translate predictive signatures of EGFR-dependency successfully to human tumors. However, the cohort that has been used for validation is small, and thus significant work lies ahead.

Predictive gene expression signatures are being implemented in prospective clinical trials to guide the use of other cancer therapeutics (191-194). Other signatures have already been well established to be useful in this context, such as the Oncotype Dx and Mammaprint assays, which can predict the probability of metastatic recurrence

following resection in early stage breast cancer (190, 294-296). Thus, we provide an integral contribution to this movement. Further development of clinical diagnostics by others based on gene expression data will pave the scientific and regulatory avenues for the translation of our predictive signature into a clinically useful diagnostic. Likewise, successful clinical integration of our signature in EGFR-dependent NSCLC will further substantiate the approach of using genomic data to develop clinically predictive diagnostics. Thus, this outcome could facilitate a framework for the future development of gene expression predictors by others.

The signature we have identified also provides a wealth of data to mine for additional insight into the EGFR-dependent phenotype and these data could be further exploited to investigate new targets for therapy. These data are publicly available, and can be utilized by other researchers in the field to test their hypotheses or explore using candidate gene approaches. Our own mining of this dataset was successful in identifying the RAS/MAPK pathway as a significantly deregulated pathway in the EGFR-dependent phenotype. We further found that the expression and/or activation of EGFR ligands are modulated by RAS/MAPK activity. Regulation of ligand expression may be important for controlling EGFR receptor levels and this activity may be a factor in constitutive EGFR-mediated signaling. This concept remains to be rigorously tested, but provides a hypothesis to be further explored by those in the field.

Importantly, our work has restored focus on the RAS/MAPK pathway as an important target in EGFR-dependent NSCLC. Significant bodies of research have recently focused attention on the PI3K/AKT pathway in this phenotype. The PI3K/AKT pathway, downstream of EGFR and other growth factor receptors, has been shown to contribute to cell survival in EGFR-dependent cell lines and tumors (117, 129, 285, 297, 298). Furthermore, it has been suggested that inhibitors of the RAS/MAPK pathway may be more effective in tumors harboring mutant RAS or RAF that are mutually exclusive with tumors harboring EGFR mutations (23, 139, 147, 222, 299-303). Our work re-examines the RAS/MAPK pathway in the EGFR-dependent phenotype as a result of knowledge gained from our genomic studies. While inhibition of the RAS/MAPK pathway resulted in modest reduction of proliferation on EGFR-dependent NSCLC cells, significant synergy was observed when combined with EGFR inhibitors, enhancing apoptosis (see Chapter 4). Thus, based on the results of others in the field, our results obtained in combining EGFR and RAS/MAPK inhibitors were surprising. These data will

hopefully guide the effective combination of targeted agents to impact EGFR-dependent NSCLC therapy.

D. TRANSLATIONAL AND CLINICAL RELEVANCE

The findings of the work herein hold significant translational and clinical relevance. Each of the broad questions asked when formulating the hypotheses of this work were made with a particular concern for their impact on patient care. Furthermore, the approaches used, particularly in construction and validation of the genomic signature of EGFR-dependency, bridged both basic science and clinical fields, thus being largely translational in nature.

Improved methods of stratifying patients for targeted therapy in EGFR-dependent NSCLC are required to more appropriately utilize EGFR-targeted therapy in the clinic. The cost of erlotinib, estimated at over $3000 per month of therapy, underscores this need, as the impact of failed treatment on health care costs, quality of life, and outcome are driving forces in the focus on predicting response to cancer therapies. We have made a significant effort to validate the signature of response in clinical samples, but more validation is clearly necessary. Translating our findings in cell culture models to a clinically useful diagnostic is difficult, but addresses the broader clinical needs of the health care system. Successful implementation of the model could significantly impact health care costs and outcomes associated with erlotinib use.

Even those patients who achieve response or stable disease to EGFR inhibitors almost certainly progress through the development of resistance (98, 99, 113, 148). One well-established principle in anti-cancer therapy is the implementation of drug combinations to combat resistance. This principle is based on the assumption that the probability of a tumor cell becoming resistant to a combination therapy is the product of the probabilities of the development of resistance to each individual agent alone, provided there is not a common mechanism of resistance to both agents (276, 287). Thus, identifying effective drug combinations for treating EGFR-dependent NSCLC is paramount.

Combining MEK and EGFR inhibition could reduce resistance in EGFR-dependent NSCLC by several mechanisms. First, synergistic activity was observed between the agents in the cell culture models we utilized. Thus, one would expect more

significant initial clinical responses to the combination, reducing tumor burden and minimizing the probability of a drug-resistant tumor cell emerging (276). Second, common mechanisms of acquired resistance to EGFR inhibitors may result in reactivation of the RAS/MAPK pathway or reduction in the ability of the EGFR inhibitor to abrogate EGFR-RAS/MAPK signaling (98, 99, 147, 273). Therefore, treatment with both inhibitors could reduce the selective pressure for the development these mechanisms of tumor resistance. We anticipate that *in vivo* models and further clinical testing will define the clinical utility of this drug combination in EGFR-dependent NSCLC. Thus, the questions asked and approaches taken to answer those questions are translational and ultimately clinical in nature.

E. CONCLUSIONS

We conclude that the EGFR-dependent phenotype in NSCLC can be defined and captured in a gene expression profile. This profile could aide in identifying and stratifying NSCLC patients who will benefit from EGFR-targeted therapy. Enrichment of the treated population for responders will significantly impact the clinical utility of these agents. Furthermore, we conclude that coupling inhibitors of the RAS/MAPK pathway with EGFR inhibitors such as erlotinib could offer the potential to maximize benefit from targeted therapy in EGFR-dependent NSCLC through enhanced responses and minimized acquired resistance to therapy.

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APPENDICES

APPENDIX I: EXEMPT IRB SUBMISSION FOR RETROSPECTIVE CLINICAL STUDY

1. Background:

Gene expression signatures of objective response to anti-cancer agents are a novel approach to personalizing cancer medicine. New targeted agents such as erlotinib (Tarceva ™) and trastuzumab (Herceptin ™) among others are effective agents for the treatment of cancer, but activity varies depending on particular tumor and patient characteristics. Quantitative assessment of the expression of large numbers of genes may offer sufficient data to account for heterogeneity of disease in humans, and thus provide a way to predict response to targeted agents *a priori*.

We have previously published a signature of erlotinib sensitivity that appears to predict response in human cancer cell lines and possibly tumors. However, significant and vigorous validation of the ability of this signature to appropriately predict response is necessary. Erlotinib is not used extensively to treat non-small cell lung cancer at the University of Kentucky, although it is one of several recommended treatment options at particular points in care. The limited use of the agent precludes prospective collection of samples from lung cancer patients, as a large number would be required in order to obtain sufficient sample size to validate the ability of the signature to predict outcome. This is primarily because only 5-10% of all samples collected would likely be from patients who were later treated with erlotinib. Collection by such a process could be very time consuming and costly and thus highly inefficient.

Tissue banks nationwide represent a resource for acquiring retrospective samples in order to explore hypotheses in heterogeneous patient populations. The University Of Kentucky Biospecimen Core Program (BCP) has a large number of samples procured from lung cancer patients in the Kentucky area. Other publically available tissue banks include the Cooperative Human Tissue Network (CHTN). The CHTN is an NCI sponsored program that collects and banks samples at a number of institutional 'hubs' from myriad of disease states for distribution to academic investigators across the nation. All patient identifiers will be removed prior to distribution to us, as per the requirements of the program. More information on this program can be found at: http://chtn.nci.nih.gov/. We intend to utilize tissue banking resources,

specifically the BCP and CHTN, to validate and improve our predictive algorithm in hope of making a positive impact on cancer care.

2. **Objectives:**

The study will be a **retrospective** analysis of **existing** human lung cancer specimens for the purpose of understanding activated signal transduction pathways present in the tumor and how this correlates to response to small molecule inhibitors of those pathways. The goals of this work are to:

1. Determine the accuracy of a gene expression signature to predict response to the small molecule inhibitor erlotinib which targets the EGFR signal transduction pathway

2. Augment the predictive algorithm with additional gene expression data derived from human tumors

3. **Study Population:**

We will use samples that are available with no specific population of patients desired, other that those that had resected lung cancer and were treated with erlotinib.

4. **Subject Recruitment Methods**:

There will be no prospective recruitment. We will use the samples and associated clinical data provided by the BCP or the CHTN. In either case, to obtain pertinent clinical data, independent chart review will be performed by non-study associated personnel qualified to complete such review. For both of these tissue banks, patients signed a consent form stating the tumor tissue and clinical data could be used in a de-identified manner.

5. **Research Procedures:**

We plan to purify RNA from 5-10 milligrams of existing fresh frozen lung tumor tissue as provided by the Markey Cancer Center Biospecimen Core Program (BCP) or the Cooperative Human Tissue Network (CHTN). For samples from the CHTN, chart review will take place under the supervision of site-specific IRBs and only lung tumors which were treated adjuvantly with EGFR inhibitors (gefitinib, erlotinib) will be acquired.

For samples from the BCP at the University of Kentucky, the approach outlined below for identifying appropriate samples for collection will be used:

1) Dr. Susanne Arnold, M.D. and Dr. John Rinehart, M.D. of the Markey Cancer Center will act as moderators to ensure that no de-identified PHI will be made available to the PI. Since these are the two primary treating physicians at UK for non-small cell lung cancer, they will follow the procedures below to identify eligible samples for the study:

a) The physicians will compile a list of the patients they have treated with erlotinib (names, DOB, medical record numbers).

b) This list will be transferred to personnel at the UK BCP. The BCP personnel will then identify which, if any, of the patients from the list have specimens available.

c) The de-identified samples numbers will then be communicated to the PI and the samples will be delivered and analyzed by the PI laboratory. The RNA from the tumor samples will be used for hybridization to high-density DNA microarrays. Protein lysates may also be used to determine the activation patterns of particular pathways, including EGFR. For microarray analyses, the resulting genomic data will be subject to statistical analyses, both supervised and unsupervised learning methods, for the purpose of validating a genomic test to predict response to EGFR inhibitors. The information derived from this study will allow us to validate our previous *in vitro* work (Balko et al, BMC Genomics 2006).

d) The outcome of the analysis will be communicated to the physicians (Drs Arnold and Rinehart).

e) The physicians will request that the de-indentified sample numbers be matched with the patient list and this list will be communicated from the BCP to the physicians. The physicians will then match the analysis results with the patient data (see data collection), remove all identifiable patient information (i.e. MRN, DOB, and name) and forward the dataset back to the PI.

6. **Data Collection:**

Information to be collected by the oncologists, if available, will include:

a. Age, sex, race
b. Smoking history
c. Histology
d. Previous treatments (chemotherapy, radiotherapy)
e. Stage at erlotinib treatment
f. Dose of erlotinib
g. Length of treatment
h. Response (RECIST)
i. Progression free survival
j. Overall survival

7. **Potential Risks:**

There are no significant risks involved in this research protocol. No inheritable genetic information per se will be collected during the course of this research, and therefore the data collected will not be particularly sensitive data. The PI will not be given any identifiable information.

8. **Research Materials, Records and Confidentiality:**

The data acquired will be maintained only on the secure UK server. Specimens will not be linked to identifiable information and will be kept in locked facilities when not in use. No identifiable information will be acquired or utilized by the PI, and therefore there is no risk to confidentiality. The physicians functioning under the honest broker system will have access to identifiable information, but have access to this information in a regular basis since they are the treating physicians.

APPENDIX II: MEDICAL IRB SUBMISSION FOR PROSPECTIVE CLINICAL TRIAL

Medical IRB Research Description

1. Background

Lung cancers account for 30% of cancer-related deaths and are the most common cause of cancer-related mortality in the U.S. The Commonwealth of Kentucky leads the nation in incidence and mortality due to lung cancer [1]. Non-small cell lung cancer (NSCLC) is a disease characterized by late stage of presentation and intrinsic resistance to cytotoxic chemotherapy. Furthermore, a majority of NSCLC patients fail treatment and experience toxicity to standard treatments. Clinical and pre-clinical studies strongly suggest that a patient's tumor bears a molecular signature that can be predictive of response to targeted therapies [2]. We propose that the molecular signature of a patient's tumor can be captured and translated into a predictor of response of that individual to therapy. Using a predictive profile of response, we can avoid patient exposure to toxic and ineffective chemotherapeutic agents.

The majority of patients are diagnosed with NSCLC that is not surgically resectable, leaving systemic chemotherapy as their main treatment option. Platinum-containing doublets (using a taxane, gemcitabine or vinorelbine as the second agent) are utilized in front-line therapy with reasonable efficacy. Recently, bevacizumab has been added to platinum-containing doublets and has shown a survival improvement compared to chemotherapy alone in non-squamous histologies [3]. Unfortunately, many patients do not respond to initial treatment or relapse soon after cessation of chemotherapy. For patients with a performance status $\geq$ 2, second line therapy with a single agent is the standard of care. As single agents, docetaxel and erlotinib have all been shown to improve survival compared to best supportive care and pemetrexed has been proven to be equally effective as docetaxel. Gemcitabine, which is approved as a first line therapy, has significant activity as a single agent in second line therapy. As second line therapy, these agents have been shown to generate responses in approximately 10 percent of patients and prolong survival by approximately 3 months [4-7]. Patients who respond to therapy have a longer survival than those who do not respond, and the median duration of response ranges from 4-6 months while less than 30% have responses that last more than a year. While many patients experience

toxicity with commonly used chemotherapeutic agents, erlotinib, a small molecule therapeutic, results in the least amount of grade 3-4 toxicity.

The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), erlotinib represents a novel class of compounds shown in clinical studies to improve survival over best supportive care when used as single agent in second-line therapy for NSCLC. Erlotinib monotherapy in patients who received prior platinum therapy resulted in objective responses of approximately 13% and stable disease in nearly 30% of patients [8, 9].

No added benefit of EGFR agents was found when combined with chemotherapy despite preclinical evidence suggesting otherwise [10, 11]. A recent review of molecular events that predict responses to erlotinib in a large clinical trial show that response rates were more than double in patients with tumors expressing EGFR (versus non-EGFR expressers). Other factors which seem to predict response are EGFR amplification (versus no amplification), EGFR mutation (versus wild type), female gender (versus male), adenocarcinoma (versus all others), and non-smokers (versus current and former smokers) [12].

Table 1: Predictions of EGFR TKI sensitivity are denoted for ten cell lines used in training/validation. Checkmarks denote correct predictions.

	Cell Line	Sensitivity to erlotinib	Predicted sensitivity to EGFR TKI	
			Prediction based on EGFR exon 19-21 mutations	Genomic signature / DLDA 180- genes
Training	A549	No	√	√
	UKY-	No	√	√
	H165	Yes	√	√
	PC-9	Yes	√	√
	H325	Yes	√	√
Validation	H358	Yes		√
	H460	No	√	√
	H197	No	√	
	K562	No	√	√
	A431	Yes		√
		%Correct	80%	90%

It appears none are mutually exclusive. Likewise, gefitinib, an FDA-restricted EGFR tyrosine kinase inhibitor has the same mechanism of action as erlotinib. Studies evaluating gefitinib responders suggest that molecular analysis can identify patients with greater than a 90% chance of responding to treatment [13]. Patients that exhibit response to EGFR-targeted agents will frequently harbor a mutation in exon 19 or 21 of EGFR. The correlation of EGFR mutations with response and survival is controversial; the only method

demonstrated to predict response prospectively is to sequence exons 19 and 21 by standard methods from tumor tissue. However, efforts of our own using genomics approaches to select all responders provide an additional means for capturing the molecular signature of sensitivity in individual tumors [14].

Treatment decisions based on single parameters alone encompass only a modest percentage of responders. Post-hoc analyses of clinical trial data identified substrata of patients (female gender, never-smokers, harboring EGFR mutations or EGFR amplification) who exhibited higher response rates to erlotinib therapy (15). We hypothesized that a methodology that utilized a multi-gene molecular signature would be better suited than any single parameter in identifying responders to EGFR TKI. Our recent work identified a 180-gene expression signature of EGFR TKI response, which we have combined with predictive statistical analysis that can classify drug sensitivity in lung adenocarcinomas [14]. Data from our laboratory suggests that use of this classification tool can identify responding patients a priori more effectively than single parameters alone.

Using DNA content analysis, we determined erlotinib sensitivity in NSCLC cell lines. Using a genomic signature generated from a training set (5 of the 10 cell lines) and diagonal linear discriminant analysis, we correctly predicted sensitivity to first generation (reversible) EGFR TKIs in 90% of samples according to their sensitivity (Table 1). Importantly, predicting on mutational status alone yields only 80% accuracy. Furthermore, the predictor identified cell lines sensitive to newer-generation irreversible inhibitors (i.e. EKB-569) with 100% accuracy. These compounds are currently under clinical development.

We have also applied this algorithm to genomic data from NSCLC tumors, and have found that approximately 70% of adenocarcinomas predict as sensitive. In these datasets, however, the patients were not treated with erlotinib or gefitinib and therefore response cannot be ascertained.

Thus, the approach has the potential to identify patients that are sensitive to EGFR TKI resulting in marked gains in overall survival, reductions in toxicity, and improved cost-benefit ratios in second line therapy of NSCLC. Further, those who are not predicted to respond to erlotinib therapy could be treated with alternative regimens to

which they may be more likely to see benefit. A stepwise methodology that dissects the status of molecular targets of several commonly utilized and efficacious therapeutics provides a clinical tactic for prescribing the therapeutic most appropriate for the individual lung cancer patient. Therefore, we propose the following pilot study to develop preliminary data to examine this hypothesis.

2. Objectives

The objectives for this investigator-initiated pilot study are as follows: (1) to ascertain if a sufficient amount of RNA, DNA, and tissue for molecular and histological analyses can be obtained through CT-guided biopsy, (2) to determine the whether biopsy and molecular analyses can be completed in an appropriate time frame for second-line therapy decisions to be initiated (goal: 2-week window), and (3) to develop preliminary data exploring the hypothesis that NSCLC patients possessing intra-tumoral EGFR-activated signals exhibit improved clinical responses.

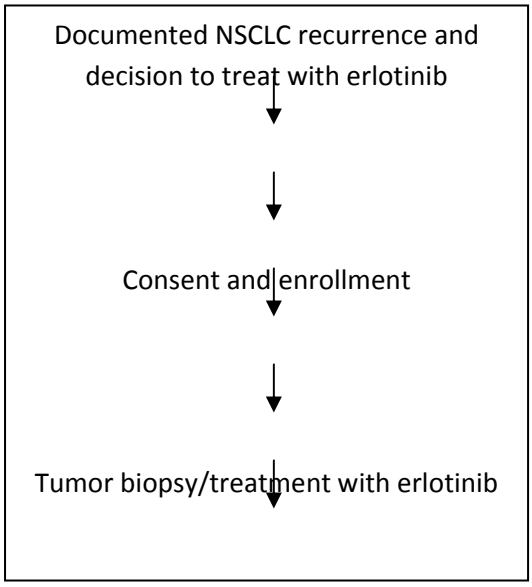

Figure 1: Proposed study design

Study Design

The purpose of this study is to generate preliminary feasibility data in preparation for a randomized prospective trial to prove the benefits of individualized 2nd line therapy in NSCLC. We propose this pilot study be undertaken to test the potential for improved response to a single, second-agent therapy based on the molecular characteristics of the patient. The study design is outlined in Figure 1.

Patient Consent and Tumor Biopsy

We will enroll twenty patients for this feasibility study. The patients will be referred to the study at the time of recurrence or relapse by John J. Rinehart, M.D. or Susanne Arnold, M.D. at the University of Kentucky, Markey Cancer Center. The decision to treat with erlotinib, as this is an acceptable second-line or third line therapy

for progressing or relapsed NSCLC, will be made by the physician and patient prior to consent and enrollment. Following consent, a CT-guided biopsy of the lung tumor will be scheduled within a two-day window and obtained by Majid Maybody, M.D. The biopsy will be analyzed by pathology to determine if tumor tissue is present. If so, genetic, genomic and histological analyses can proceed. Therefore, we expect completion of analytical studies within a 10-14 day window. The resulting outcome data can then be compared with response rates in the literature and greater statistical confidence will be achieved. In cases where the oncologist feels it inappropriate to continue treatment with erlotinib, alternative second-line therapies or best supportive care will be initiated. The patient response data will be removed from the analysis of *objective 3* if they do not complete at least 30 days of erlotinib treatment. The Consent form (C) is attached.

Tissue Handling and Assay Descriptions

The molecular analyses will use the biopsied specimen for both DNA and RNA preparation. Prior to microarray analysis, RNA will be extracted from tumor tissue (RNeasy, Qiagen), quantified by absorbance, and a determination will be made whether there is enough RNA to assay gene expression by DNA microarray (1-5ug required of total RNA).

The EGFR gene will be sequenced along exons 18-21, as described previously, using DNA extracted from tumor tissue [15]. Mutations, whether deletion in exon 19 or point mutants in exon 21, are considered 'activating' [16]. Point mutants in exon 20 (T790M) confer resistance to erlotinib [17]

Data from the microarray analysis will be imported into our predictive modeling software. In previous work (submitted for publication), we generated a gene expression predictor of EGFR-inhibitor sensitivity, and we will apply that predictive model to the gene expression data from each patient.

Study Evaluation

The feasibility study will be evaluated using the objectives stated earlier. ***Objective one*** assesses the amount of useful tumor tissue gathered from a CT-guided biopsy and will be gauged for each patient as DNA and RNA are prepared from the biopsied material. ***Objective two*** assesses time required for molecular analyses to be completed and provide information for therapeutic decisions and will be evaluated

following enrollment of the twenty patients. We hope that 18 of 20 patients can be evaluated within the 10-14 day window allowing assessment of molecular analyses prior to initiation of second-line therapy. To assess the effectiveness of using these molecular methods to determine second-line therapy, we will evaluate each patient following nine months of treatment by RECIST criteria or as the patient progresses or demonstrates toxicity to erlotinib. ***Objective three*** will assess response to therapy using Kaplan-Meier survival curves to compare patients who might be stratified by single molecular parameters alone (EGFR mutation/amplification) or by classification using the genomic predictor of response. We suspect that the genomic predictor will identify more responders than does the sequencing method for analyzing activation of EGFR. Thus, we believe many more responders to EGFR TKI can be treated with less toxic and more efficacious therapy than previously anticipated. The feasibility issues for molecular analysis can be resolved with this study and allow us to pursue a larger prospective study to utilize a decision tree algorithm testing two additional therapeutics.

4. Study Population

Inclusion Criteria

We will enroll the first 20 NSCLC patients who meet eligibility criteria without regard to race, gender, or age. All enrolled patients must meet each of the following inclusion criteria:

- Prior decision by both the physician and patient to initiate erlotinib therapy
- Previous treatment with front line systemic therapy (i.e. erlotinib will be used as 2nd line or higher therapy)
- Expected survival as determined by their oncologist of ≥ 3 months
- Eastern Cooperative Oncology Group (ECOG) Performance status of 0, 1, or 2
- Patient must have a tumor which is capable of being biopsied by CT-guided CNB without evidence of excess risk, as determined by the interventional radiologist (Dr. Majid Maybody, MD)
- Willing to undergo the CT-guided biopsy procedure, and assume risks associated with the procedure

Exclusion Criteria

Exclusion criteria include presence of any of the following:

- an ECOG performance status of ≥ 3
- Patients at high risk for bleeding episodes, including thrombocytopenia (PLT<50,000) or pathologic bleeding disorders during the initial evaluation
- Patients taking oral anticoagulants (i.e. warfarin), as these cannot be discontinued in a reasonable amount of time to allow for normalization of INR prior to undergoing the biopsy procedure

5. Subject Recruitment Methods and Privacy

Patients meeting recruitment criteria will be identified by John J. Rinehart, M.D. or Susanne Arnold M.D. at the time of their visit to the clinic. At that time, those patients will be referred to Esther P. Black, Ph.D., Assistant Professor in the College of Pharmacy, as potential participants in this study. Justin Balko, PharmD will be contacted to gain consent of the patient for participation in the study. We will not advertise this study to the general public.

6. Informed Consent Process

Informed consent will be carried out at time of evidence of recurrence or relapse by Justin Balko, PharmD, who will also serve as a witness. During administration of informed consent, the patient will be allowed to read or have read to them the document describing the study as a feasibility study to initiate the process for individualized care of recurrent lung cancer patients. Each patient will be informed that this is a genetic test that requires a biopsy sample of the tumor. The patient will be informed that they will be given a prescription for erlotinib and that their compliance will be monitored by routine calls to their designated pharmacy. If not used completely, the tumor sample will be banked for a defined period of time and then destroyed. The DNA and RNA samples will be stored in a locked sub-zero freezer and de-identified from the patient data. The patient will be asked to consent to secondary use of the genetic material for additional studies at this time. They may refuse to consent for secondary use, in which case excess sample will be destroyed.

The patient will also be informed that they will not be notified of the results of the study nor will they receive compensation for their participation. Each patient in responsible for the cost of the erlotinib and any necessary follow-up care, as these are not study-related but are costs of standard of care. The study will be responsible for the costs of the biopsy and genetic testing of the biopsied material.

7. Research Procedures

All procedures for this protocol are standard and have been previously completed by clinicians or scientific staff as outlined. If the patient has any medical issues during the study or does not comply with treatment parameters, they will be removed from study and standard of care will be initiated.

Day 0: Evidence of recurrence or relapse by Drs. Rinehart or Arnold. Referral to Dr. Black. Dr. Balko will witness, consent, and schedule core biopsy.

Day 1-3: CT-guided biopsy by Dr. Maybody. Prescription for erlotinib by Dr. Rinehart or Dr. Arnold. Biopsy in RNAice (-20C), sample for H&E to Pathology (Balko).

Day 4-7: Prepare RNA and DNA from biopsy and if remaining tissue, store at -20C (Balko). RNA to microarray core facility (Dr. Kuey-Chu Chen, Director) DNA to PCR amplification of EGFR exons for extraction and sequencing (Balko, AGTC core)

Day 8-14: Collection of microarray and sequencing data (Balko) Evaluation of molecular data ie. time to collection and analysis (Balko and Black)

Month 1-12: Evaluation of patient response by RECIST criteria (Rinehart or Arnold) Compliance follow-up every month (months 1-3), and every 3 months thereafter (Balko)

All procedures for this protocol are standard and have been previously completed by clinicians or scientific staff as outlined. If the patient has any medical issues during the study or does not comply with treatment parameters, they will be removed from study and standard of care will be initiated.

Study Timetable:

Procedure	PRE	Day 0	Day 1-3	Day 4	Day 5	Day 6	Day 7	Day 8-14	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8	Month 9	Month 10	Month 11	Month 12
PRE-STUDY ASSESSMENTS																			
Decision to Treat with Erlotinib	X																		
STUDY-INITIATION ASSESSMENTS																			
Eligibility Criteria		X																	
Consent		X																	
Concomitant Medication Record		X																	
Patient Compliance Record		X																	
Biopsy Scheduling		X																	
BIOPSY AND TREATMENT																			
CT-guided Core Needle Biopsy			X																
Patient Monitoring			X																
Begin Erlotinib Treatment			X																
LAB/STATISTICAL ASSESSMENTS																			
RNA and DNA purification				X															
EGFR Exon 18-21 Amplification					X														
Microarray hybridization (UK Core)					X														
DNA Sequencing (UK Core)						X													
Microarray Predictive Analysis								X											
DNA Mutational Assessment								X											
FOLLOW-UP ASSESSMENTS																			
Pharmacy Compliance Follow-up											X				X				X
Response, Survival Follow-up (RECIST)									X	X			X			X			X

8. Resources

Markey Cancer Center has solicited funds from the Lexington Foundation for Dr. Esther P. Black to complete this feasibility study from November 2006 to October 2007. The funding will only pay the costs of the CT-guided biopsy, genetic tests, and the salary of the College of Pharmacy graduate student responsible for the study. There are no

additional costs for storage of samples. No funding in provided for counseling or ancillary care for the patients enrolled in the study. There is no funding available outside for outsourcing communication specialists for the patients.

9. Potential Risks

Physical risks include those associated with CT-guided biopsy. The most common complications of percutaneous lung biopsy are pneumothorax (collapse of lung) and bleeding. Pneumothorax occurs in up to 30% of cases the majority of which are self limited and need no interventions. Of all biopsy induced pneumothraces about 5-10% will continue to enlarge some of which will require chest tube placement. Chest tubes are small bore (8 french) plastic catheters placed under CT guidance on the same biopsy session. Patients who receive chest tube are required to be admitted to hospital for an overnight observation. The vast majority of chest tubes can be removed the next day if the hole in pleura created by biopsy is sealed (what is called absence of “air leake”). In an extremely small number of patients the chest tube has to remain in place for a few days till “air leake” is resolved. These patients are required to stay in hospital for as long as they carry a chest tube.

It is recommended that patients who have undergone needle biopsy of lung not to fly for 48 hours post procedure. Pressurized cabin environment can potentially cause pneumothorax in these patients.

Bleeding occurs in 2-4% of cases the majority of which are self limited and need no interventions. In extremely rare cases angiography and embolization may be required.

Because the results of the study will not be shared with the patient, the physicians, or insurance providers there is likely no social or psychological risk to the patient. The genetic tests performed will not provide information about known hereditary disease and are therefore not a risk for the patient

10. Safety Precautions

The CT- guided biopsy provides only the necessary amount of tissue for the study with manageable risks for patient. The patient will be observed for several hours following the biopsy to monitor for adverse events, such as pneumothorax. Further

management of side effects or adverse events will be according to standard of care for each complication and may include hospitalization, chest tube insertion, monitoring in an intensive care unit, intubation, or other supportive care as deemed necessary by the treating physician.

Stopping rules for adverse events:

Anticipated complication rates for lung biopsy for this study include approximately 30% with pneumothorax and 2% with bleeding; the majority of these being self-limited and requiring no intervention. In the worst-case (conservative) scenario that these will occur independently, a stopping rule is designed using a composite complication rate of 32%. Patients will be closely monitored for these complication rates. The protocol will be stopped if sufficient evidence exists suggesting that among patients receiving lung biopsy, the true incidence of pneumothorax or bleeding exceeds 32%. Sufficient evidence will be taken to make an estimate whose lower limit to the corresponding one-sided 90% confidence interval exceeds 32%, and these estimates will be made after every 5th enrolled patient that is evaluable for this phase of the protocol. Operationally, any of the following observed ratios would lead to exceeding such a limit (ie. require stopping the study due to a safety concern): This will occur if 3 or more of the first 5; 6 or more of the first 10; 8 or more of the first 15; and 10 or more of the first 20 patients enrolled experience either pneumothorax or bleeding complications as defined above. If the true probability of complication development is 32%, the probability of stopping the study under these rules is roughly 6% after enrollment of 10 or more patients on the study. If the true probability is as high as 60%, the probability of stopping after 10 patients is approximately 65% and after 20 patients approximately 74%. Thus, this stopping rule has reasonable properties from a probabilistic viewpoint.

Stopping rules for serious adverse events:

If any patients experience Grade 2 pneumothorax or Grade 2 thoracic bleeding (symptomatic and/or requiring intervention) after lung biopsy, the study will be put on hold pending review by the safety committee.

Stopping rules for appropriate collection techniques:

In order to minimize undue patient risk associated with loss of study benefit, a stopping rule for assessment of appropriate tissue collection techniques will be used. It

is anticipated that at least 80% of enrolled subjects will contribute “viable” biospecimen material. Patients will be monitored and the protocol will be stopped and re-evaluated in regard to obtaining biospecimens if the true rate of obtaining a ‘viable’ specimen is smaller than 80%. Sufficient evidence will be taken to make an estimate whose upper limit of the corresponding one-sided 90% confidence interval is lower than 80%. Monitoring will occur after every 5th patient is enrolled in the study. Operationally, any of the following observed ratios would lead to stopping the study to re-evaluate the method for obtaining the biospecimens : The upper 90% CI will be lower than 80% when no more than 2 (2 or less) out of the first 5; no more than 6 (6 or less) of the first 10; no more than 9 (9 or less) of the first 15; or no more than 13 (13 or less) of the first 20 enrolled contribute a ‘viable’ amount of lung biospecimen. If the true rate of obtaining a ‘viable’ biospecimen is as poor as 50%, then this rule will result in a 50% probability of stopping the study after 5 patients have been enrolled and an 83% probability of stopping after 10 patients have been enrolled. If the true rate is 80%, then this rule will result in a 5.7% chance of stopping after 5 patients have been enrolled, and a 12% chance of stopping after 10 patients have been enrolled. Thus, this stopping rule has reasonable properties from a simple probabilistic viewpoint.

Protection of privacy:

All sample information will be de-identified and the matching patient information will be kept in a separate password-protected document to be accessed only by the principle investigator, Dr. E. Penni Black, PhD. No data will be released to the patient, the physician or insurance providers to protect confidentiality. No treatment decision will be based on the results of the molecular tests.

11. Benefit v. Risk

There is no direct benefit to patient of the results of this study, but the information achieved in the study may lead to an advancement of knowledge in individualizing treatment of non-small cell lung cancer patients.

The patient may risk injury due to the CT-guided biopsy and/or may have an adverse reaction to the erlotinib.

12. Available Alternative Treatments

The only alternative treatment is for the patient to not participate in the study. Since the study design is observational and does not include treatment intervention, the patient will continue to receive erlotinib as determined by the patient and their oncologist until progression.

13. Research Materials, Records, and Privacy

Initially, medical records will be reviewed to obtain patient information (i.e. age, sex, race, smoking history, histology or previous treatment). Samples will be de-identified such that only Dr. Black will retain a record of patient clinical information.

The samples (DNA and RNA) will be stored in a locked sub-zero freezer in locked room in the College of Pharmacy. If patients consent to a secondary study, the de-identified samples may be used for further testing but there will be no need for additional patient data.

For insuring privacy, neither patient, physician nor insurance provider will be informed of outcome of molecular testing. No treatment decision will be made with the molecular data and patients may withdraw from the study at any time.

If the study is published, no identifying patient information will be published.

14. Confidentiality

As described above, de-identified patient data will be kept only by Dr. Black; samples will only be available to Drs. Black and Balko. Biopsy samples that are unused will be destroyed after 6 months. Nucleic acid samples (RNA and DNA) will be retained in a sub-zero freezer in lab of Dr. Black with no patient identifier.

Data sharing may be arranged following publication of initial study as needed. No names or clinical data will be published.

15. Payment

CT- guided biopsy and genetic testing will be paid from the funds from the granting agency.

16. Costs to Subject

The patient or the patient's insurance provider will be responsible for the costs of the erlotinib and any necessary follow-up care.

17. Data and Safety Monitoring

The CT-guided biopsy is defined as greater-than-minimal risk. As such, each patient will be monitored by medical staff for several hours following the procedure. Each patient will be evaluated monthly by Dr. Rinehart or Arnold following prescription of erlotinib for assessment of compliance and safety.

18. Subject Complaints

The consent form contains phone numbers for Dr. Black and for the Office of Research Integrity for the purpose of monitoring complaints from the enrolled subjects

19. Research Involving Non-English Speaking Subjects or Subjects from a Foreign Culture

Given the small size of the study, we do not anticipate enrolling non-English speaking subjects. Furthermore, since this study does not provide patient-specific benefit, and therefore no loss of benefit will occur by excluding non-English speaking patients.

20. HIV/AIDs

N/A

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APPENDIX III: PROTOCOL FOR PROSPECTIVE CLINICAL TRIAL

A Phase I Feasibility Study of a Multivariate Genomic Predictor of Erlotinib Benefit in 2nd or 3rd line Non-Small Cell Lung Cancer

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1.0 INTRODUCTION

The introduction of molecular profiling into medical practice has yet to be realized on a clinically useful basis. The literature abounds with a number of basic science efforts to describe genome-wide expression patterns that characterize particular phenotypes. Oncology is an area of practice that could observe marked benefits in guiding therapies by such techniques. While microarray technology remains an expensive tool, the extensive costs of new cancer treatments demand effective methods to predict patients who will benefit from such resources and to triage those not likely to respond to alternative empiric regimens.

Erlotinib is a novel FDA-approved tyrosine kinase inhibitor which has been shown to offer survival advantages in 2nd and 3rd line non-small cell lung cancer therapy. However, there are substrata of the population which clearly exhibit improved responses and survival. Our lab has conducted extensive in-vitro experiments to characterize a genomic expression pattern distinctive of sensitivity to inhibitors of EGFR. We have combined this pattern with multivariate statistical methods with the ultimate goal of implementation in the clinical setting. We hypothesize that use of this methodology (biopsy of lung tumor tissue, molecular analysis, and predictive analysis) will be a feasible approach to guiding 2nd line therapy in NSCLC. In order for the approach to be useful, we will assay our ability to retrieve the appropriate amount of biological material (RNA and DNA) from CT-guided core needle biopsy. We will also attempt to complete the methodology in a 2-week window following recurrence. We have chosen a period of 2 weeks as a reasonable amount of time to delay initiation of second-line care after consultation with UK oncologists. Future studies will further confirm the usefulness of our genomic analysis in determining erlotinib candidates and improving survival in 2nd line NSCLC therapy.

2.0 SPECIFIC AIMS

To evaluate the feasibility of molecular profiling using genomic data in 2nd line NSCLC therapy, we will examine the following specific aims:

Determine the amount of tissue and biological material (RNA and DNA) acquired by core needle biopsy of primary non-small cell lung cancer tumors

Determine the length of time needed to perform computed tomography (CT) - guided core-needle biopsy, isolate biological material, and perform molecular analysis of erlotinib sensitivity in NSCLC patients

Generate preliminary data on the usefulness of a genomic classifier of erlotinib response in predicting erlotinib response and/or survival.

3.0 BACKGROUND AND PRELIMINARY DATA

3.1 Current therapy paradigm in NSCLC

Lung cancers account for 30% of cancer-related deaths and are the most common cause of cancer-related mortality in the United States. Unfortunately, the Commonwealth of Kentucky leads the nation in incidence and mortality due to lung cancer [1]. The majority of patients are diagnosed with NSCLC that is not surgically resectable, leaving systemic chemotherapy as their main treatment option. Platinum based doublets are utilized in front-line therapy with reasonable efficacy. Recently, bevacizumab has been added to platinum-containing doublets and has shown a survival improvement compared to chemotherapy alone in non-squamous histologies [2]. Unfortunately, many patients do not respond to initial treatment or relapse soon after cessation of chemotherapy. For patients with a performance status > 2, treatment with a single agent is the standard of care for 2nd line therapy. As single agents, docetaxel and erlotinib have all been shown to improve survival compared to best supportive care and pemetrexed has been proven to be equally effective as docetaxel. Gemcitabine, which is approved as a first line therapy, has significant activity as a single agent in second line therapy. As second line therapy, these agents have been shown to generate responses in approximately 10 percent of patients and prolong survival by approximately 3 months [3-6]. As such, patients who respond to therapy have a longer survival than those who do not respond, and the median duration of response ranges from 4-6 months while less than 30% have responses that last more than a year.

3.2 Erlotinib in 2nd or 3rd line therapy

While many patients experience toxicity with commonly used chemotherapeutic agents, erlotinib, an orally available epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), results in very low rates of grade 3-4 toxicity [7, 8]. Erlotinib represents a novel class of compounds shown in clinical studies to improve survival over best supportive care when used as single agent in second-line therapy for NSCLC.

Erlotinib monotherapy in patients who received prior platinum therapy resulted in objective responses in only 13% and stable disease in approximately 30% of patients [8, 9]. Importantly, the cost of erlotinib is exceedingly high. The University of Kentucky is contracted to obtain a single month's supply the typical daily dose (150mg daily) for approximately $1650 (~ $55/day). The actual cost to patient would well exceed this mark. This fact underscores the importance of developing efficacious strategies for identifying those who will benefit a priori.

No added benefit of EGFR inhibiting agents was found when combined with chemotherapy despite preclinical evidence suggesting otherwise [10, 11]. A recent review analyzed clinical and molecular characteristics that predict responses to erlotinib in a large clinical trial show that response rates were more than double in EGFR expressing patients (versus non-EGFR expressers), EGFR amplification (versus no amplification), EGFR mutation (versus wild type), female gender (versus male), adenocarcinoma (versus all others), and non-smokers (versus current and former smokers) [12]. It appears none are mutually exclusive. Furthermore, treatment decisions based on single parameters alone encompass only a modest percentage of responders.

Likewise, gefitinib, an FDA-restricted EGFR tyrosine kinase inhibitor has the same mechanism of action as erlotinib. Studies evaluating gefitinib responders suggest that molecular analysis can identify patients with greater than a 90% chance of responding to treatment [13]. Patients that exhibit response to EGFR-targeted agents will frequently harbour a mutation in exon 19 or 21 of EGFR [14, 15].

3.3 Genomic microarray data and multivariate statistical methods as a predictor of response to erlotinib therapy

Clinical and pre-clinical studies strongly suggest that a patient's tumor bears a molecular signature that can be predictive of response to targeted therapies [16]. We hypothesized that a methodology that utilized a multi-gene molecular signature would be better suited than any single parameter in identifying responders to EGFR TKI. Our recent work identified a 180-gene expression signature of EGFR TKI response, which we have combined with predictive statistical analysis that can classify drug sensitivity in lung adenocarcinomas (Balko et al, in Submission). Data from our laboratory suggests that use of this classification tool can identify responding patients a priori more effectively than single parameters alone.

Using DNA content analysis, we determined erlotinib sensitivity in NSCLC cell lines. Using a genomic signature generated from a training set (5 of the 10 cell lines) and diagonal linear discriminant analysis, we correctly predicted sensitivity to first generation (reversible) EGFR TKIs in 90% of samples according to their sensitivity (Table 1). Importantly, predicting on mutational status alone yields only 80% accuracy. Furthermore, the predictor identified cell lines sensitive to newer-generation irreversible inhibitors (i.e. EKB-569) with 100% accuracy. These compounds are currently under clinical development.

We have also applied this algorithm to genomic data from NSCLC tumors, and have found that approximately 70% of adenocarcinomas predict as sensitive. In these datasets, however, the patients were not treated with erlotinib or gefitinib and therefore response can not be ascertained.

4.0 STUDY SIGNIFICANCE AND RATIONALE

Using predictive measures of response, we can avoid patient exposure to toxic and ineffective chemotherapeutic agents. Thus, the approach has the potential to identify patients that are sensitive to EGFR TKI resulting in marked gains in overall survival, reductions in toxicity, and improved cost-benefit ratios in second line therapy of NSCLC. Further, those who are not predicted to respond to erlotinib therapy could be treated with alternative regimens to which they may be more likely to see benefit. We anticipate that the use of genomic approaches to select all erlotinib responders, not just those harbouring EGFR mutations, will provide the most effective means for predicting sensitivity in individual tumors.

4.1 Use of an invasive procedure for research purposes

The primary goal of this study is to determine if the biological analyses can be conducted and a test result can be achieved within 14 days of detection of recurrence. The secondary purpose of the study is to determine if the biological assay can predict sensitivity to erlotinib, and therefore further microarray and statistical analysis is only of benefit in cases where response data to erlotinib can be directly obtained, and will thus be conducted using tissue from recurrent lung cancer patients who have consented to erlotinib treatment. The Markey Cancer Center banks frozen resected tumor specimens which we have previously utilized to determine minimum amounts of lung tissue necessary to obtain sufficient amounts of RNA and DNA (IRB # 05-1025-X1G) and to conduct microarray analysis. The primary goal of this research, however, would preclude use of frozen specimens, since the test procedure will ultimately be conducted using biopsy core specimens following recurrence.

Due to the infrequency of erlotinib use in the past at University of Kentucky, we have not been successful in identifying banked and frozen specimens from patients who were later treated with erlotinib and to whom response data is directly available. Further, identification of patients who are to be treated with erlotinib and who have previously undergone surgical resection at UK with their tissues banked would result in a very low enrollment rate, and second, the molecular signature of the tumor would hypothetically be confounded by the effects of chemotherapy treatment between tissue extraction and erlotinib therapy. These reasons are presented in justification of using an invasive biopsy procedure for the research protocol.

4.2 Determination of RNA and DNA yield from core-needle biopsy of NSCLC tumors

In order for our predictive approach to be feasible and useful in clinical situations where surgical biopsy of tumor specimens is not indicated, we will need to determine if non-surgical biopsy (i.e. CT-guided core needle biopsy) will yield appropriate amounts of tumor RNA and DNA to perform molecular analyses.

We have determined that gross tumor specimens yield approximately 250ng RNA / mg tissue. In laboratory processed samples, the minimum yield was 150ng/mg tissue. Microarray analysis requires at least 1.5 ug of acceptable quality total RNA. Based on these parameters we estimate a requirement of approximately 10 mg of tumor tissue from a core needle biopsy for RNA extraction to produce sufficient quantity in the majority of samples.

Additionally, we will require 3-4 mg of tumor tissue for DNA extraction to perform sequencing of EGFR exons 18-21. This brings the total tissue required to approximately 14 mg tissue. It will be necessary to assess our ability to achieve this goal in at least 80% of cases to make the approach feasible. We do not have estimated weights of core needle specimens, although we anticipate that 1-3 specimens will meet our tissue weight requirements.

4.3 Determination of time required to complete analyses

As NSCLC patients are diagnosed with recurrence, time to initiate treatment is clearly an issue. Delay of treatment can presumably lead to progression of malignancy and worsening of disease and outcome. Thus, it is imperative that we assess our ability to perform the predictive analyses in as short of a period as possible. The biopsy procedure presents a particular obstacle since the patient must schedule time for the procedure promptly and return within 2-3 days. Based on communication with oncologists, we have determined that a 14 day window following recurrence detection is not deemed likely to affect patient outcome. For the purposes of this study, since all included patients will already be decidedly placed on erlotinib therapy and we have not yet proven the value of the test, we will not delay treatment to perform analysis. Instead we will assess our ability to schedule biopsy, isolate biological material, and perform predictive analyses within a simulated situation.

4.4 Generation of preliminary data for efficacy of the predictive analysis in classifying patients by survival

While this study is not statistically powered to detect improved survival between those patients predicted to respond and those patients predicted to be resistant, generation of preliminary data will allow us to evaluate trends in outcomes, and to determine whether it is useful to pursue our analysis in a larger interventional study.

5.0 PATIENT SELECTION

5.1 Inclusion Criteria

We will enroll the first 20 NSCLC patients who meet eligibility criteria without regard to race, gender, or age. All enrolled patients must meet each of the following inclusion criteria:

- Prior decision by both the physician and patient to initiate erlotinib therapy
- Previous treatment with front line systemic therapy (i.e. erlotinib will be used as 2nd line or higher therapy)
- Expected survival as determined by their oncologist of ≥ 3 months
- Eastern Cooperative Oncology Group (ECOG) Performance status of 0, 1, or 2
- Patient must have a tumor which is capable of being biopsied by CT-guided core-needle biopsy (CNB) without evidence of excess risk, as determined by the interventional radiologist (Dr. Majid Maybody, MD)
- Willing to undergo the CT-guided biopsy procedure, and assume risks associated with the procedure

5.2 Exclusion Criteria

Exclusion criteria include presence of any of the following:

- an ECOG performance status of ≥ 3

- Patients at high risk for bleeding episodes, including thrombocytopenia (PLT<50,000), prolonged prothrombin time, or known bleeding disorders

- Patients taking oral anticoagulants (i.e. warfarin), as these cannot be discontinued in a reasonable amount of time to allow for normalization of INR prior to undergoing the biopsy procedure.

6.0 OVERALL DESIGN AND ENDPOINTS

6.1 Study Design

This study will be performed as a pilot feasibility study evaluating the ability to achieve several criteria which are imperative to completion of a larger well-powered study evaluating the ability of predictive analyses to guide second line NSCLC therapy.

All enrolled subjects will be patients about to initiate 2nd or 3rd line therapy with erlotinib for NSCLC recurrence. We will assess our ability to perform a simulated intervention in an appropriate amount of time. This intervention would require scheduling and performing CT-guided core needle biopsy, tissue procurement and extraction of biological material, DNA sequencing and microarray analysis, and prediction of sensitivity based on analyses outcomes.

A secondary purpose of this study is to generate preliminary data on the accuracy of the molecular signature to predict response in a clinical situation.

All patients will be treated with erlotinib per standard of care. We will follow response and survival of patients and correlate these parameters with molecular predictions of sensitivity.

6.2 Endpoints

- The mass of RNA extracted from CT-guided core needle biopsy of primary or metastatic lung cancers

- The mass of DNA extracted from CT-guided core-needle biopsy of primary or metastatic lung cancers

- The time taken, in days, from enrollment of patient in the trial to completion of DNA sequence analysis as well as predictive genomic analysis

- Response rate to erlotinib therapy, as measured by RECIST criteria

- 1-year survival

7.0 METHODS

7.1 Patient Consent

Informed consent will be carried by Justin Balko, PharmD. During administration of informed consent, the patient will be allowed to read or have read to them the document describing the study as a feasibility study to initiate the process for individualized care of recurrent lung cancer patients. Each patient will be informed that this is a genetic test that requires a biopsy sample of the tumor. A concomitant medication form will be completed for the patient to ensure that the patient is not receiving any medications which may affect the efficacy of erlotinib (CYP450 3A4 substrates, inhibitors, inducers) and therefore represent a confounding variable. The patient will be informed that their compliance will be monitored by routine calls to their designated pharmacy by Justin Balko. Information regarding concomitant meds and pharmacy usage will be recorded and this information will be kept secured along with any other identifiable information by the PI as per the protocol. If not used completely, the tumor sample will be banked for a 6-month period of time and then destroyed. The DNA and RNA samples will be stored in a locked sub-zero freezer and de-identified from the patient data. The patient will be asked to consent to secondary use of the genetic material for additional studies at this time. They may refuse to consent for secondary use, in which case excess sample will be destroyed.

7.2 CT-guided core needle biopsy

At the time of consent, the patient will be referred to the radiology department in order to schedule a time for the core needle biopsy procedure. The procedure will be preferably scheduled within 3 days of the consent process.

All patients should have recent blood work for coagulation profile not older than two weeks including platelet count, INR and aPTT before the procedure. The minimal acceptable coagulation indices for core biopsy include a platelet count of at least 50,000, INR of less than 1.5 and aPTT of less than 34 seconds. Admission may be planned for those patients with laboratory values suggesting abnormal coagulation for corrective measures to be taken prior to procedure. The procedure is performed under conscious sedation with local anesthesia. Conscious sedation consists of intravenously administered fentanyl and midazolam. A sedation nurse constantly monitors patient's vital signs during procedure. For the purposes of sedation, all patients should be fasting for 8 hours before procedure. In each case, previous imaging studies are reviewed to determine the best approach for percutaneous biopsy. Patients are placed prone or supine in the CT scanner depending on the location of the lesion, and axial CT images are obtained through the thorax to localize the lesion. Intravenous contrast material is not routinely administered for this procedure. After an appropriate skin entry site is selected, a radiopaque marker is placed on the skin overlying the lesion with use of the axial laser light and confirmed by CT fluoroscopic imaging. The marker is removed and a mark is placed on the skin with an indelible pen. The depth of the lesion and the angle of the access trajectory are calculated on the scanner monitor in the plane of biopsy. The

skin site is prepared and draped in standard surgical fashion with povidone iodine. The skin access site is locally anesthetized with 1% lidocaine. A small dermatotomy is made with a no. 11 scalpel. A coaxial technique is used to reduce the number of pleural passes required thus minimizing the risk of pneumothorax. The lesion is accessed by a 17 gauge calibrated introducer needle. Proper positioning of the introducer needle is confirmed by CT fluoroscopy. The core biopsy samples are taken by an18-gauge calibrated automated cutting device through the introducer needle. Upon completion of procedure, CT fluoroscopic images of the biopsy area are taken to evaluate the presence of pneumothorax and hemorrhage. All patients will also be evaluated by CT scan of chest or chest x-ray two hours post procedure. In this two hour period all patients are monitored by the sedation nurse. In case of complications proper action will be taken immediately. These may include manual aspiration of pneumothorax, chest tube placement or angiography. Patients may be admitted if they undergo chest tube placement or angiography. All patients will be fully recovered from conscious sedation prior to discharge.

1-4 core specimens will be acquired. One adjacent sample will be taken to histopathology under the care of Kim Absher, MD to be assessed for tumor content. The remaining samples will be deposited into 15 mL snap-cap Falcon tubes containing 250uL RNA-later solution. These tubes will be pre-weighed on a Fischer Scientific digital AccuSeries balance (room 441, College of Pharmacy. The samples will be maintained at room temperature and taken to the laboratory of Esther Black, Ph.D in a sealed lockable container. Tubes will be reweighed to determine the weight of the tissue specimens. Total specimen weight and number of cores will be recorded. Only samples in which the adjacent core was found the histopathologist to have 50% or more malignant tissue will be processed further.

7.3 Patient safety measures

7.3.1 Diagnostic labs

All patients will have diagnostic labs drawn prior to undergoing core-needle biopsy. Prothrombin time, INR, and CBC will be determined to ensure minimal risk of bleeding during the procedure.

7.3.2 Selection of biopsy site

Biopsy site will be selected by the oncologist and/or diagnostic radiologist with the purpose of minimizing risk to the patient. Sites within the lung which are considered to be sub-optimal for biopsy due to risk of complications will not be biopsied. Pulmonary or extra-pulmonary sites will be used in such a scenario, provided the site is determined to carry less of a risk to the patient. Good medical judgement will be used by the clinicians in determining the biopsy site.

7.3.3 Core needle biopsy good medical practices

The most common complications of percutaneous lung biopsy are pneumothorax (collapse of lung) and bleeding. However, not all patients on this protocol will receive biopsy of lung nodules, and may undergo biopsy of tissues with lower associated risk. Pneumothorax occurs in up to 30% of cases the majority of which are self limited and need no interventions. Of all biopsy induced pneumothoraces about 5-10% will continue to enlarge some of which will require chest tube placement. Chest tubes are small bore (8 french) plastic catheters placed under CT guidance on the same biopsy session. Patients who receive chest tube are required to be admitted to hospital for an overnight observation. The vast majority of chest tubes can be removed the next day if the hole in pleura created by biopsy is sealed (what is called absence of “air leake”). In an extremely small number of patients the chest tube has to remain in place for a few days till “air leake” is resolved. These patients are required to stay in hospital for as long as they carry a chest tube.

It will be recommended that patients who have undergone needle biopsy of lung not to fly for 48 hours post procedure. Pressurized cabin environment can potentially cause pneumothorax in these patients.

Bleeding occurs in 2-4% of cases the majority of which are self limited and need no interventions. In extremely rare cases angiography and embolization may be required.

Because the results of the study will not be shared with the patient, the physicians, or insurance providers there is likely no social or psychological risk to the patient. The genetic tests performed will not provide information about known hereditary disease and are therefore not a risk for the patient

The patient will be observed for several hours following the biopsy to monitor for adverse events, such as pneumothorax. Further management of side effects or adverse events will be according to standard of care for each complication and may include hospitalization, chest tube insertion, monitoring in an intensive care unit, intubation, or other supportive care as deemed necessary by the treating physician.

7.3.4 Stopping rules

7.3.4.1 Lung biopsy complications

Anticipated complication rates for lung biopsy for this study include approximately 30% with pneumothorax and 2% with bleeding; the majority of these being self-limited and requiring no intervention. In the worst-case (conservative) scenario that these will occur independently, a stopping rule is designed using a composite complication rate of 32%. Patients will be closely monitored for these complication rates. The protocol will be stopped if sufficient evidence exists suggesting that among patients receiving lung biopsy, the true incidence of pneumothorax or bleeding exceeds 32%. Sufficient evidence will be taken to make an estimate whose lower limit to the corresponding one-sided 90% confidence interval exceeds 32%, and these estimates will be made after

every 5th enrolled patient that is evaluable for this phase of the protocol. Operationally, any of the following observed ratios would lead to exceeding such a limit (ie. require stopping the study due to a safety concern): This will occur if 3 or more of the first 5; 6 or more of the first 10; 8 or more of the first 15; and 10 or more of the first 20 patients enrolled experience either pneumothorax or bleeding complications as defined above. If the true probability of complication development is 32%, the probability of stopping the study under these rules is roughly 6% after enrollment of 10 or more patients on the study. If the true probability is as high as 60%, the probability of stopping after 10 patients is approximately 65% and after 20 patients approximately 74%. Thus, this stopping rule has reasonable properties from a probabilistic viewpoint.

7.3.4.2 Serious lung tumor complications

If any patients experience Grade 2 pneumothorax or Grade 2 thoracic bleeding (symptomatic and/or requiring intervention) after lung biopsy, the study will be put on hold pending review by the safety committee.

7.3.4.3 Collection of viable tumor tissue

It is anticipated that at least 80% of enrolled subjects will contribute “viable” biospecimen material. Patients will be monitored and the protocol will be stopped and re-evaluated in regard to obtaining biospecimens if the true rate of obtaining a ‘viable’ specimen is smaller than 80%. Sufficient evidence will be taken to make an estimate whose upper limit of the corresponding one-sided 90% confidence interval is lower than 80%. Monitoring will occur after every 5th patient is enrolled in the study. Operationally, any of the following observed ratios would lead to stopping the study to re-evaluate the method for obtaining the biospecimens : The upper 90% CI will be lower than 80% when no more than 2 (2 or less) out of the first 5; no more than 6 (6 or less) of the first 10; no more than 9 (9 or less) of the first 15; or no more than 13 (13 or less) of the first 20 enrolled contribute a ‘viable’ amount of lung biospecimen. If the true rate of obtaining a ‘viable’ biospecimen is as poor as 50%, then this rule will result in a 50% probability of stopping the study after 5 patients have been enrolled and an 83% probability of stopping after 10 patients have been enrolled. If the true rate is 80%, then this rule will result in a 5.7% chance of stopping after 5 patients have been enrolled, and a 12% chance of stopping after 10 patients have been enrolled. Thus, this stopping rule has reasonable properties from a simple probabilistic viewpoint.

7.4 Extraction of biological material

7.4.1 RNA

RNA will be purified by incubating the tumor tissue in RNA-later overnight, at least 16 hours at 4 C. Tissue will be cut and weighed on ice (30mg max) and placed in a 1.5 mL Kontes tube. 100uL RLT buffer (+β –mercaptoethanol, Qiagen) will be added to the sample and the tissue will be disrupted with a Kontes tissue grinder. 500uL RLT buffer will then be added and the sample will be ground again. The sample will then be loaded

into a Qiashredder (Qiagen) column per directions and centrifuged to homogenize the sample. The sample will then be loaded onto a micro RNEasy prep column (Ambion) and RNA will be eluted per manufacturer directions.

7.4.2 DNA

DNA will be purified by incubating the tumor tissue in RNA-later overnight, at least 16 hours at 4 C. Tissue will be cut and weighed on ice (10 mg max) and then digested at least 5 hours at 50 C in digestion buffer (100mM Tris, 5mM EDTA,0.2% SDS, 200mM NaCl, 100mcg/mL). Tubes will be vortexed vigorously for 1 minute, then centrifuged for 15 minutes at 13K RPM. Supernatant will be transferred to a new tube and DNA will be precipitated with 500uL isopropanol. DNA will be pelleted with high speed centrifugation and the pellet will be washed in 70% ethanol. The washed pellet will be resuspended in EB buffer (Qiagen) and quantified by UV spectrophotometry. DNA will be used as template for amplification reactions of exons 18-21 DNA. PCR products will be resolved on a 1% agarose gel and the appropriate DNA fragment will be excised and purified used Qiaquick Gel Extraction Kit columns (Qiagen). Amplified DNA fragments will then be provided to the AGTC core facility along with sequence-specific primers for exons 18-21.

7.5 Core facility procedures

7.5.1 Microarray

Confirmation of RNA integrity, probe generation, and hybridization to U133A microarrays will be completed by the UKMC core array facility as per standard protocols.

7.5.2 Sequencing

DNA sequencing of exon 18-21 will be completed by the UK AGTC core facility per standard protocols.

7.6 Predictive analyses

Affymetrix MASv5.0 data will be imported into R and the diagonal linear discriminant analysis script will be run on the sample, as well as two control samples. The result will be recorded.

7.7 Patient follow-up

All patients will be monitored via monthly (Months 1-3) and 3 month (Months 6, 9, and 12) chart review by Justin Balko, PharmD, a study co-investigator. Data to be collected will include severe adverse effects (Grade 3/4), objective response, patient survival, and disease state measures (recurrence, progression, etc).

8.0 STUDY CALENDAR/TIME TABLE

Procedure	PRE	Day							Month										
		0	1-3	4	5	6	7	8 - 14	2	3	4	5	6	7	8	9	10	11	12
PRE-STUDY ASSESSMENTS																			
Decision to Treat with Erlotinib	X																		
STUDY-INITIATION ASSESSMENTS																			
Eligibility Criteria		X																	
Consent		X																	
Concomitant Medication Record		X																	
Patient Compliance Record		X																	
Biopsy Scheduling		X																	
BIOPSY AND TREATMENT																			
CT-guided Core Needle Biopsy			X																
Patient Monitoring			X																
Begin Erlotinib Treatment			X																
LAB/STATISTICAL ASSESSMENTS																			
RNA and DNA purification				X															
EGFR Exon 18-21 Amplification					X														
Microarray hybridization (UK Core)					X														
DNA Sequencing (UK Core)						X													
Microarray Predictive Analysis								X											
DNA Mutational Assessment								X											
FOLLOW-UP ASSESSMENTS																			
Pharmacy Compliance Follow-up											X				X				X
Response, Survival Follow-up (RECIST)									X	X			X			X			X

9.0 STATISTICAL CONSIDERATIONS AND DATA MANAGEMENT

Formal statistical analysis will not be undertaken in this pilot feasibility trial. However, all data collected will be recorded and evaluated, including variability and trends of data.

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APPENDIX IV: 180-GENE SIGNATURE OF EGFR SENSITIVITY

Probeset	Gene	Description	p-value
205891_at	ADORA2B	adenosine A2b receptor	1.65347E-12
213434_at	EPIM	epimorphin	2.03526E-12
211475_s_at	BAG1	BCL2-associated athanogene	1.2089E-11
201716_at	SNX1	sorting nexin 1	1.3942E-11
219933_at	GLRX2	glutaredoxin 2	2.82157E-11
204513_s_at	ELMO1	engulfment and cell motility 1	2.92588E-11
203011_at	IMPA1	inositol(myo)-1(or 4)-monophosphatase 1	4.20475E-11
202743_at	PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma)	4.51605E-11
204491_at	PDE4D	Phosphodiesterase 4D, cAMP-specific	8.05036E-11
204000_at	GNB5	guanine nucleotide binding protein (G protein), beta 5	8.7681E-11
204115_at	GNG11	guanine nucleotide binding protein (G protein), gamma 11	1.02678E-10
218913_s_at	GMIP	GEM interacting protein	2.64411E-10
200994_at	IPO7	importin 7	2.65447E-10
202286_s_at	TACSTD2	tumor-associated calcium signal transducer 2	2.75325E-10
209035_at	MDK	midkine (neurite growth-promoting factor 2)	7.31553E-10
218995_s_at	EDN1	endothelin 1	7.75626E-10
219855_at	NUDT11	nudix (nucleoside diphosphate linked moiety X)-type motif 11	8.77697E-10
209678_s_at	PRKCI	protein kinase C, iota	1.04253E-09
202501_at	MAPRE2	microtubule-associated protein, RP/EB family, member 2	2.31343E-09
212117_at	RHOQ	ras homolog gene family, member Q	3.22134E-09
206277_at	P2RY2	purinergic receptor P2Y, G-protein coupled, 2	3.92313E-09
209295_at	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	4.33798E-09
205376_at	INPP4B	inositol polyphosphate-4-phosphatase, type II, 105kDa	4.50987E-09
206722_s_at	EDG4	endothelial differentiation, lysophosphatidic acid GPCR,4	7.96715E-09
205673_s_at	ASB9	ankyrin repeat and SOCS box-containing 9	1.24878E-08
201471_s_at	SQSTM1	sequestosome 1	1.34231E-08
204352_at	TRAF5	TNF receptor-associated factor 5	1.46887E-08
206907_at	TNFSF9	tumor necrosis factor (ligand) superfamily, member 9	1.57771E-08

218150_at	ARL5	ADP-ribosylation factor-like 5	2.04888E-08
205459_s_at	NPAS2	neuronal PAS domain protein 2	2.22961E-08
205455_at	MST1R	macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	2.45512E-08
202641_at	ARL3	ADP-ribosylation factor-like 3	2.78193E-08
201667_at	GJA1	gap junction protein, alpha 1, 43kDa (connexin 43)	2.86113E-08
210512_s_at	VEGF	vascular endothelial growth factor	2.90316E-08
212104_s_at	RBM9	RNA binding motif protein 9	5.42805E-08
200762_at	DPYSL2	dihydropyrimidinase-like 2	5.43168E-08
221235_s_at	TGFBRAP1	transforming growth factor, beta receptor associated protein 1	5.51367E-08
211302_s_at	PDE4B	phosphodiesterase 4B, cAMP-specific	5.51731E-08
205080_at	RARB	retinoic acid receptor, beta	7.03586E-08
202266_at	TTRAP	TRAF and TNF receptor associated protein	7.2889E-08
205240_at	GPSM2	G-protein signalling modulator 2 (AGS3-like, C. elegans)	8.30858E-08
213798_s_at	CAP1	CAP, adenylate cyclase-associated protein 1 (yeast)	8.61121E-08
221819_at	RAB35	RAB35, member RAS oncogene family	8.9216E-08
207011_s_at	PTK7	PTK7 protein tyrosine kinase 7	9.78716E-08
204255_s_at	VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor	1.1087E-07
208864_s_at	TXN	thioredoxin	1.34274E-07
209885_at	RHOD	ras homolog gene family, member D	1.50021E-07
201923_at	PRDX4	peroxiredoxin 4	1.6148E-07
204392_at	CAMK1	calcium/calmodulin-dependent protein kinase I	2.24378E-07
203269_at	NSMAF	neutral sphingomyelinase (N-SMase) activation associated factor	2.59238E-07
205924_at	RAB3B	RAB3B, member RAS oncogene family	2.77173E-07
202853_s_at	RYK	RYK receptor-like tyrosine kinase	3.45502E-07
202530_at	MAPK14	mitogen-activated protein kinase 14	3.53556E-07
219936_s_at	GPR87	G protein-coupled receptor 87	4.06845E-07
203665_at	HMOX1	heme oxygenase (decycling) 1	4.37988E-07
205926_at	IL27RA	interleukin 27 receptor, alpha	5.33484E-07
202105_at	IGBP1	immunoglobulin (CD79A) binding protein 1	6.14358E-07
213324_at	SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	8.2585E-07
205709_s_at	CDS1	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 1	8.69007E-07
207303_at	PDE1C	phosphodiesterase 1C, calmodulin-dependent 70kDa	9.11398E-07

204484_at	PIK3C2B	phosphoinositide-3-kinase, class 2, beta polypeptide	9.23963E-07
38269_at	PRKD2	protein kinase D2	9.25184E-07
211171_s_at	PDE10A	phosphodiesterase 10A	1.0636E-06
212757_s_at	CAMK2G	calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	1.08937E-06
202167_s_at	MMS19L	MMS19-like (MET18 homolog, S. cerevisiae)	1.18954E-06
202932_at	YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	1.24119E-06
209110_s_at	RGL2	ral guanine nucleotide dissociation stimulator-like 2	1.343E-06
205055_at	ITGAE	integrin, alpha E	1.48098E-06
203910_at	PARG1	PTPL1-associated RhoGAP 1	1.52987E-06
203388_at	ARRB2	arrestin, beta 2	1.59381E-06
212099_at	RHOB	ras homolog gene family, member B	1.76917E-06
204497_at	ADCY9	adenylate cyclase 9	1.78932E-06
208091_s_at	DKFZP564 K0822	hypothetical protein DKFZp564K0822	1.81223E-06
213135_at	TIAM1	T-cell lymphoma invasion and metastasis 1	1.84133E-06
203837_at	MAP3K5	mitogen-activated protein kinase kinase kinase 5	1.89236E-06
206549_at	INSL4	insulin-like 4 (placenta)	2.17123E-06
205880_at	PRKD1	protein kinase D1	2.29581E-06
211471_s_at	RAB36	RAB36, member RAS oncogene family	2.29859E-06
210058_at	MAPK13	mitogen-activated protein kinase 13	2.44208E-06
40850_at	FKBP8	FK506 binding protein 8, 38kDa	2.88345E-06
208819_at	RAB8A	RAB8A, member RAS oncogene family	3.20502E-06
202203_s_at	AMFR	autocrine motility factor receptor	3.36339E-06
201431_s_at	DPYSL3	dihydropyrimidinase-like 3	3.54374E-06
217976_s_at	DNCLI1	dynein, cytoplasmic, light intermediate polypeptide 1	3.62385E-06
201097_s_at	ARF4	ADP-ribosylation factor 4	3.81095E-06
203077_s_at	SMAD2	SMAD, mothers against DPP homolog 2 (Drosophila)	3.81843E-06
203081_at	CTNNBIP1	catenin, beta interacting protein 1	3.83717E-06
219357_at	GTPBP1	GTP binding protein 1	3.95215E-06
212255_s_at	ATP2C1	ATPase, Ca++ transporting, type 2C, member 1	4.40768E-06
218360_at	RAB22A	RAB22A, member RAS oncogene family	4.52284E-06
202545_at	PRKCD	protein kinase C, delta	4.62498E-06
203679_at	TMED1	transmembrane emp24 protein transport domain containing 1	4.8425E-06
212070_at	GPR56	G protein-coupled receptor 56	5.80992E-06

204622_x_at	NR4A2	nuclear receptor subfamily 4, group A, member 2	5.98479E-06
202844_s_at	RALBP1	ralA binding protein 1	6.56314E-06
204547_at	RAB40B	RAB40B, member RAS oncogene family	6.59316E-06
219032_x_at	OPN3	opsin 3 (encephalopsin, panopsin)	7.0888E-06
212422_at	PDCD11	programmed cell death 11	7.19593E-06
203266_s_at	MAP2K4	mitogen-activated protein kinase kinase 4	7.43037E-06
205220_at	GPR109B	G protein-coupled receptor 109B /// G protein-coupled receptor 109B	7.59026E-06
212181_s_at	NUDT4	nudix (nucleoside diphosphate linked moiety X)-type motif 4	8.07236E-06
208072_s_at	DGKD	diacylglycerol kinase, delta 130kDa	8.08474E-06
218329_at	PRDM4	PR domain containing 4	8.32807E-06
210288_at	KLRG1	killer cell lectin-like receptor subfamily G, member 1	8.456E-06
206099_at	PRKCH	protein kinase C, eta	9.06238E-06
205481_at	ADORA1	adenosine A1 receptor	1.02958E-05
217839_at	TFG	TRK-fused gene	1.04379E-05
202340_x_at	NR4A1	nuclear receptor subfamily 4, group A, member 1	1.11601E-05
212873_at	HA-1	minor histocompatibility antigen HA-1	1.24958E-05
202020_s_at	LANCL1	LanC lantibiotic synthetase component C-like 1 (bacterial)	1.31667E-05
209666_s_at	CHUK	conserved helix-loop-helix ubiquitous kinase	1.3401E-05
200651_at	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	1.41049E-05
201401_s_at	ADRBK1	adrenergic, beta, receptor kinase 1	1.43031E-05
203185_at	RASSF2	Ras association (RalGDS/AF-6) domain family 2	1.51579E-05
202401_s_at	SRF	serum response factor	1.58311E-05
203726_s_at	LAMA3	laminin, alpha 3	1.62925E-05
217496_s_at	IDE	insulin-degrading enzyme	1.74448E-05
206118_at	STAT4	signal transducer and activator of transcription 4	1.80022E-05
208641_s_at	RAC1	ras-related C3 botulinum toxin substrate 1	1.92909E-05
206044_s_at	BRAF	v-raf murine sarcoma viral oncogene homolog B1	1.98244E-05
205349_at	GNA15	guanine nucleotide binding protein (G protein), alpha 15 (Gq class)	2.02216E-05
1007_s_at	DDR1	discoidin domain receptor family, member 1	2.03771E-05
58994_at	FLJ20241	putative NFkB activating protein	2.12741E-05
201895_at	ARAF	v-raf murine sarcoma 3611 viral oncogene homolog	2.13489E-05

211499_s_at	MAPK11	mitogen-activated protein kinase 11	2.17506E-05
203567_s_at	TRIM38	tripartite motif-containing 38	2.2332E-05
210621_s_at	RASA1	RAS p21 protein activator (GTPase activating protein) 1	2.24105E-05
219646_at	FLJ20186	hypothetical protein FLJ20186	2.24905E-05
218856_at	TNFRSF21	tumor necrosis factor receptor superfamily, member 21	2.28896E-05
205147_x_at	NCF4	neutrophil cytosolic factor 4, 40kDa	2.44098E-05
215177_s_at	ITGA6	integrin, alpha 6	2.57288E-05
202564_x_at	ARL2	ADP-ribosylation factor-like 2	2.63409E-05
207630_s_at	CREM	cAMP responsive element modulator	2.73227E-05
212629_s_at	PKN2	protein kinase N2	2.80355E-05
201181_at	GNAI3	G protein alpha inhibiting activity polypeptide 3	2.8689E-05
207375_s_at	IL15RA	interleukin 15 receptor, alpha	2.91388E-05
201983_s_at	EGFR	epidermal growth factor receptor	2.95162E-05
205263_at	BCL10	B-cell CLL/lymphoma 10	3.07237E-05
218186_at	RAB25	RAB25, member RAS oncogene family	3.17395E-05
207111_at	EMR1	egf-like module containing, mucin-like, hormone receptor-like 1	3.39972E-05
219290_x_at	DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides	3.51493E-05
206456_at	GABRA5	gamma-aminobutyric acid (GABA) A receptor, alpha 5	3.61634E-05
219537_x_at	DLL3	delta-like 3 (Drosophila)	4.0124E-05
200923_at	LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	4.18435E-05
201390_s_at	CSNK2B	casein kinase 2, beta polypeptide	4.2931E-05
211992_at	WNK1	WNK lysine deficient protein kinase 1	4.54378E-05
205992_s_at	IL15	interleukin 15	4.58957E-05
200991_s_at	SNX17	sorting nexin 17	4.73166E-05
221610_s_at	STAP2	signal-transducing adaptor protein-2	4.76896E-05
201508_at	IGFBP4	insulin-like growth factor binding protein 4	4.83857E-05
219327_s_at	GPRC5C	G protein-coupled receptor, family C, group 5, member C	6.06081E-05
200985_s_at	CD59	CD59 antigen p18-20	6.20756E-05
202315_s_at	BCR	breakpoint cluster region	6.45513E-05
200627_at	TEBP	unactive progesterone receptor	6.4762E-05
201288_at	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	6.49524E-05
205854_at	TULP3	tubby like protein 3	7.0941E-05
204369_at	PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide	7.12144E-05

202012_s_at	EXT2	exostoses (multiple) 2	7.25418E-05
206204_at	GRB14	growth factor receptor-bound protein 14	7.62668E-05
201980_s_at	RSU1	Ras suppressor protein 1	7.72429E-05
210105_s_at	FYN	FYN oncogene related to SRC, FGR, YES	7.73012E-05
218589_at	P2RY5	purinergic receptor P2Y, G-protein coupled, 5	8.07908E-05
202150_s_at	NEDD9	neural precursor cell expressed, developmentally down-regulated 9	8.37333E-05
212273_x_at	GNAS	GNAS complex locus	8.74661E-05
200833_s_at	RAP1B	RAP1B, member of RAS oncogene family	8.96348E-05
214724_at	DIXDC1	DIX domain containing 1	9.31294E-05
207643_s_at	TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A	9.56597E-05
219020_at	FLJ14249	HS1-binding protein 3	9.74361E-05
203895_at	PLCB4	phospholipase C, beta 4	0.000101184
204336_s_at	RGS19	regulator of G-protein signalling 19	0.000106767
217792_at	SNX5	sorting nexin 5	0.000113975
210056_at	RND1	Rho family GTPase 1	0.000121576
32137_at	JAG2	jagged 2	0.000123516
205596_s_at	SMURF2	SMAD specific E3 ubiquitin protein ligase 2	0.000126851
205698_s_at	MAP2K6	mitogen-activated protein kinase kinase 6	0.000143749
218931_at	RAB17	RAB17, member RAS oncogene family	0.000144453
217763_s_at	RAB31	RAB31, member RAS oncogene family	0.000179924
214875_x_at	APLP2	amyloid beta (A4) precursor-like protein 2	0.000185475
209184_s_at	IRS2	insulin receptor substrate 2	0.000193615
204602_at	DKK1	dickkopf homolog 1 (Xenopus laevis)	0.000260573

APPENDIX V: SUPPLEMENTARY FIGURES FOR CHAPTER 4

Figure V1: MEK inhibition reduces growth in EGFR-dependent NSCLC cell lines

EGFR-dependent NSCLC were cultured in low-serum media for 24 hr before replacement with-full serum media (10% FBS) plus 10μM U0126 (grey). An equal volume of DMSO was used as control (black). Cell proliferation was measured by trypan blue exclusion at 0, 24, 48, and 72 hr after treatment. Data were normalized to the 0 hr cell counts and is expressed as percent viable cells +/- standard deviation.

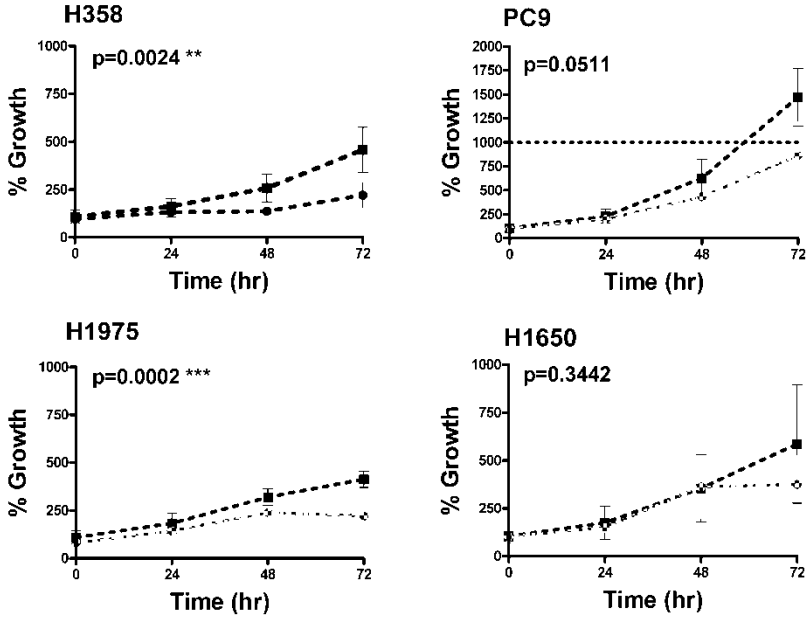


Figure V2: MEK inhibition reduces cell survival in EGFR-dependent NSCLC cell lines

EGFR-dependent non-small cell lung cancer cell lines were cultured for 24 hr in low serum media (0.1% FBS) and subsequently treated with 10 μM U0126 (grey) or DMSO control (black) in fresh low serum media or full serum media (10% FBS). Cell proliferation was measured by trypan blue exclusion at 0, 24, 48, and 72 hr after treatment. The percent viable cells in each group were determined as described in 'Methods'. Data represent mean +/- standard deviation. Data were analyzed by ANOVA for repeated measures, and p-values are reported in the inset.

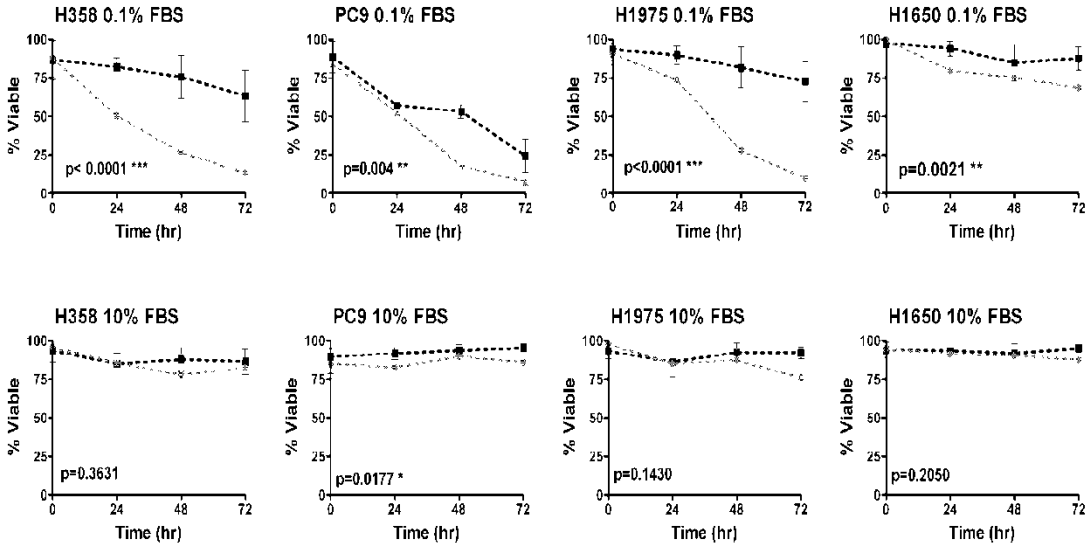


Figure V3: Ectopic MEK expression rescues EGFR-dependent NSCLC cells from PD098059-induced apoptosis

H1975 cells were infected with recombinant adenovirus expressing GFP or constitutively activated MEK-1. After 16 hr, the cells were treated in low serum media (0.1% FBS) with DMSO, U0126 (20μM), or PD098059 (25μM or 50μM). After 48 hr, floating and adherent cells were harvested and lysates were analyzed by western blot for PARP.

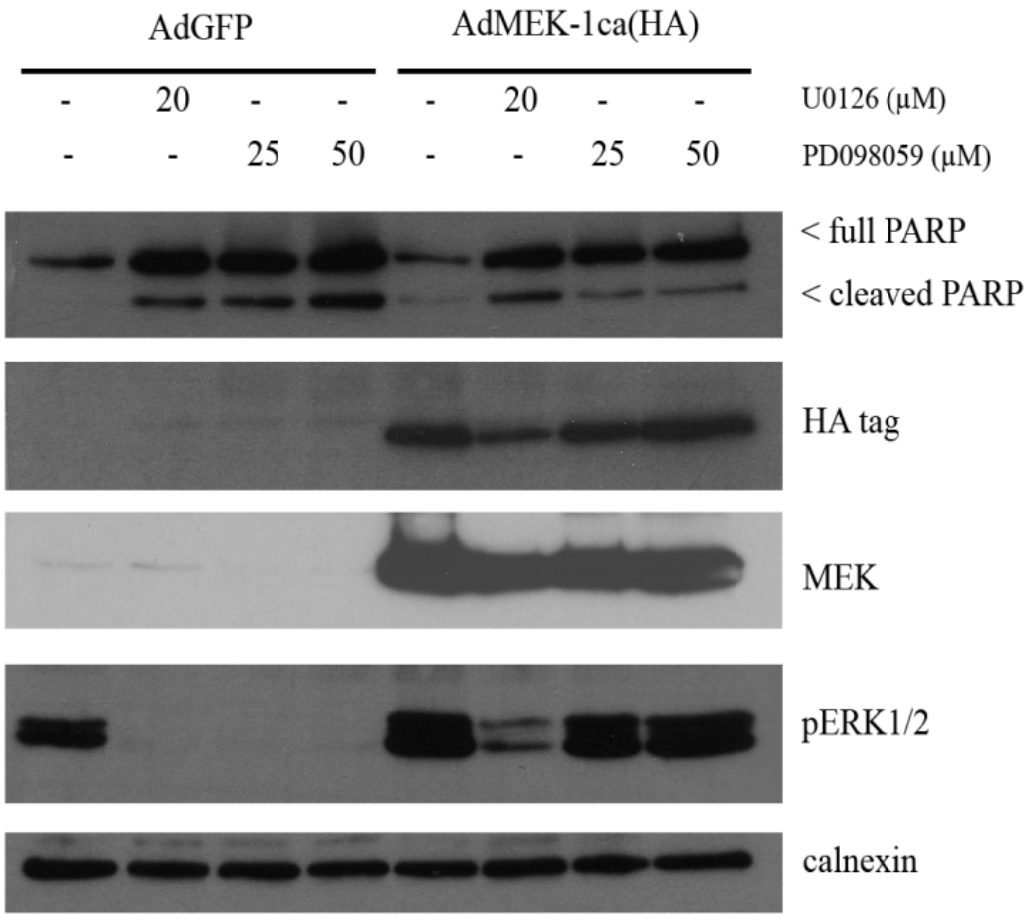


Figure V4: Bovine serum albumin protects EGFR dependent NSCLC cell lines from U0126-induced apoptosis in low serum media

H1975 cells were treated with U0126 for 48 hr in either low (0.1% FBS) or high (10% FBS) serum media. Bovine serum albumin was also added to the indicated wells at the time of treatment (+: 0.075% BSA, ++: 0.75% BSA). Floating and adherent cells were harvested and lysates were prepared and analyzed by SDS-PAGE and western blotting for the indicated proteins.

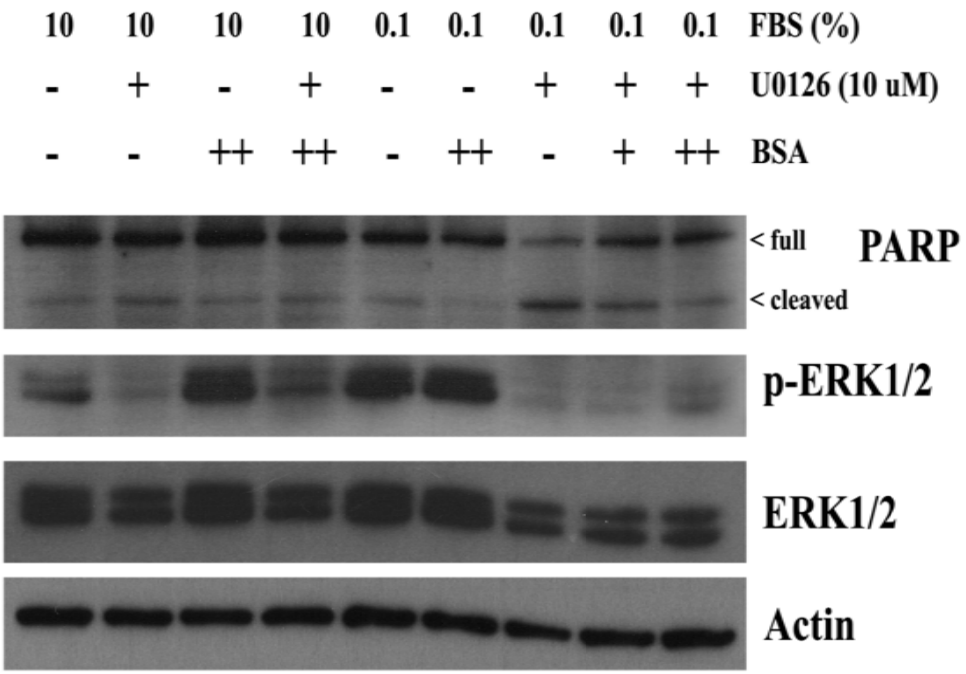
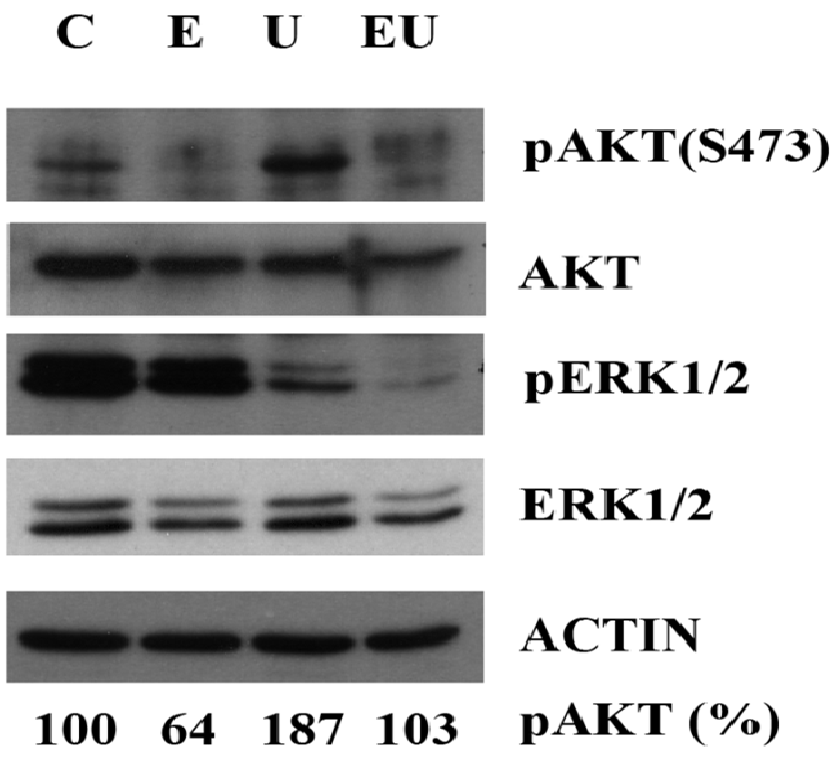


Figure V5: Combined inhibition of MEK1/2 and EGFR abrogates the U0126-mediated activation of AKT

H1650 cells were treated with DMSO (C, 0.1%), U0126 (U, 5μM), erlotinib (E, 500nM), or the combination (EU) for 24 hr in low (0.1% FBS) serum media. Floating and adherent cells were harvested and lysates were prepared and analyzed by SDS-PAGE and western blotting for the indicated proteins. pAKT was quantified by densitometry (100 x pAKT signal/total AKT signal).



VITA

Justin M. Balko, Pharm.D., Ph.D.

Born: April 16th, 1980, Binghamton NY

EDUCATION

University of Kentucky, Lexington KY, 2004-Present
Ph.D., Clinical and Experimental Therapeutics Track, Department of Pharmaceutical Sciences
Mentor: E. P. Black, Ph.D.

State University of New York at Buffalo, Buffalo, NY, 2000-2004
Pharm.D., School of Pharmacy and Pharmaceutical Sciences

University of the Sciences in Philadelphia, Philadelphia, PA, 1998-2000
Pre-pharmacy undergraduate coursework

RESEARCH

Basic science/laboratory

March 2005-April 2009

Graduate student in pharmacology
Mentor: Esther P. Black, Ph.D.

Dissertation project: Pharmacogenomics in EGFR-dependent NSCLC: predicting and enhancing response to targeted EGFR therapy

Summary:

My dissertational work has focused on dissecting the EGFR-dependent phenotype in non-small cell lung cancer, as defined by an apoptotic response of both tumors and cell lines to selective inhibitors of EGFR. I have utilized genomic methods, including DNA microarray and quantitative real-time PCR, to define patterns of gene deregulation in a cell culture model of EGFR-dependent NSCLC for the purposes of 1) predicting clinical

response to EGFR inhibitors (see 'Clinical Research' experience) and 2) identify potential mechanisms of EGFR activation and dependency in NSCLC.
During the course of my dissertational work, I have implemented genomic and systems biology approaches in identifying a gene expression signature of response (GEPR) to EGFR targeted therapies. This GEPR incorporates a diagonal linear discriminant function to predict the sensitivity of unknown samples to EGFR inhibitors and has performed well in both external *in vitro* and clinical datasets. A more extensive validation of this GEPR in predicting response to EGFR inhibitors and monoclonals in multiple tumor types is underway.
I have also utilized this GEPR to identify additional targetable pathways that are deregulated in the EGFR-dependent phenotype. My hypothesis is that these pathways could be exploited by combinatorial inhibition to improve EGFR-targeted lung cancer therapy. I have demonstrated that EGFR-dependent NSCLC cells rely upon the downstream RAS/MAPK pathway for both growth and survival, depending on the environment of the cell. Combining selective inhibitors of the MAPK pathway with EGFR inhibitors resulted in mathematically-verified cytotoxic synergy and enhanced apoptotic response compared to EGFR inhibitors alone. Combining agents targeting these pathways clinically could improve responses and reduce the development of resistance to targeted therapy in non-small cell lung cancers.

Clinical research:

October, 2006 – present:
A Phase I Feasibility Study of a Multivariate Genomic Predictor of Erlotinib Benefit in 2nd or 3rd line Non-Small Cell Lung Cancer
Markey Cancer Center/University of Kentucky College of Pharmacy
Sponsored by the Lexington Foundation

Contributions: Trial design, preparation of research justification, protocol and consent forms; Administration of informed consent to enrolled patients; handling, preparation, and processing of tissue samples; clinical data extraction; statistical analysis.

TEACHING

Spring 2009: Overview of Chemotherapeutic Agent Pharmacology – Advanced Pharmacotherapeutics – PY3 (1 hour)

Spring 2008: Monoclonal Antibody and Tyrosine Kinase Inhibitor Pharmacology – Advanced Pharmacotherapeutics – PY3 (1 hour)

Fall 2006: Lung Cancer – Advanced Therapeutics – PY3 (2 hours)

Fall 2005-Spring 2006: Teaching Assistant, Pharmacotherapeutics for professional pharmacy students. Assisted with lecture materials and grading of pharmacokinetics assignments.

Fall, 2004-Spring 2005: Teaching Assistant, CAPP III, IV, and V courses for professional pharmacy students. Assisted with pharmacy practice laboratory exercises and led small group discussions on issues in patient care.

CLINICAL EXPERIENCE

January, 2006 – present:

Markey Cancer Center, University of Kentucky
Cancer Clinic Pharmacist

Summary: Dosing adjustments and calculations, patient counseling and teaching, pharmacotherapy consults, dispensing of chemotherapeutics.

GRANTS AND AWARDS

Joan's Legacy Foundation (Co-wrote with PI) - Dual targeted therapy: can MEK inhibition improve response and reduce acquired resistance in EGFR-dependent NSCLC? – $100,000, 2009-2011

University of Kentucky K30 translational science seed grant, 2006 – $5,000

Sullivan University Nanotechnology Symposium Outstanding Research Paper Award (AM Al-Ghananeem, EP Black, JM Balko, and E Romond) 2008

Rho Chi Research Day Abstract Award – 1st place, 2007

Elizabeth S. Helton Travel Award, 2007

Keystone Symposia 'Cancer and Kinases, Lessons from the Clinic' Best Graduate Student Abstract Award, 2005 (NIH/NIAID #1R13CA117397-01)

Kentucky State Research Challenge Trust Fund Fellowship 2004-2007

NYSCHP Corporate Future of Pharmacy Award, 2004

MANUSCRIPTS

Balko JM, Black EP. A gene expression predictor of response and progression-free survival to cetuximab in KRAS wild-type metastatic colorectal cancer. BMC Cancer 2009, 9:145

Al-Ghananeem AM, **Balko JM**, Black EP, Malkawi AH, Romond E, Crooks PA, DeLuca P. Paclitaxel-Loaded Nanoparticles Based on Hyaluronic Acid Chemically Cross-Linked with Glutaraldehyde for Intratumor Cancer Therapy. AAPS PharmSciTech. 2009 Apr 21.

Balko JM, Jones BR, Coakley VL, Black EP. MEK and EGFR inhibition demonstrates synergistic activity in EGFR-dependent non-small cell lung cancer. Cancer Biology and Therapy, 2009, 8:6

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Balko JM, Potti A., Saunders C., Stromberg A., Haura EB, Black EP. Gene expression patterns that predict sensitivity to epidermal growth factor receptor tyrosine kinase inhibitors in lung cancer cell lines and human lung tumors. BMC Genomics 2006, 7:289

Gao, J, McConnell MJ, **Balko JM**, Li J, Black EP, Johnson JJ, Lloyd MC, Altiok S, Haura EB. MUC1 mediates cell survival and metastasis potential of NSCLC cell lines through interactions with tyrosine kinases and STAT3 signaling pathways. International Journal of Oncology, In Press.

BOOK CHAPTERS

Balko, JM, Adams, V. Lung Cancer. Pharmacotherapy: Principles and Practice, 1st Edition.

Balko, JM, Adams, V. Lung Cancer. Pharmacotherapy: Principles and Practice, 2nd Edition. In Press.

ABSTRACTS

Balko JM, Jones BR, Black EP. Toward dual therapy for EGFR-dependent non-small cell lung cancer: a role for MAPK inhibitors? AACR Clinical Trials and Personalized Medicine, Monterey, CA. July, 2008.

Balko JM, Jones BR, Coakley VL, Black EP. MAPK induction of EGF-like ligands contributes to EGFR stability in EGFR-dependent NSCLC cell lines. Molecular Targets: Drug Design and Diagnostics for the 21st Century, Lousiville KY, February, 2008.

Balko JM, Black EP. Inhibition of EGF-like ligand activity disrupts the MAPK-driven EGFR autocrine loop affecting survival and proliferation of lung cancer cell lines. AACR Molecular Diagnostics in Cancer, Atlanta, GA August, 2007.

Al-Ghananeem, AM, Black EP, **Balko JM**, Faraj J, Malkawi, Ahmad, DeLuca P, Crooks PA, Romond E. Paclitaxel-loaded cross-linked hyaluronan nanoparticles for breast cancer therapy. 16th International Symposium on Microencapsulation, Lexington KY September, 2007.

Balko JM, Jones BR, Black EP. Signaling through the MAPK pathway is necessary for maintenance of an autocrine loop in EGFR-inhibition sensitive non-small cell lung cancers. Molecular targets in cancer Keystone Symposia, Whistler, BC, March 2007.

Black EP, **Balko JM**, Saunders CS, Stromberg A, and Haura EB. Gene expression predictors of response to epidermal growth factor receptor-targeted therapeutics. Rho Chi Research Symposia, University of Kentucky, March 2006.

Black EP, **Balko JM**, Saunders CS, Stromberg A, and Haura EB. Computational approaches for modeling activated epidermal growth factor receptor as a predictor of response to targeted therapies. Cancer and Kinases Keystone Symposia, Santa Fe, NM February 2006

PRESENTATIONS

Predicting clinical response to EGFR-targeted agents: experience in NSCLC and metastatic CRC. Pharmacy Practice and Science Department Works-In-Progress Seminar, University of Kentucky, March 2009

Personalizing cancer care: matching drugs to patients. University of Kentucky Pharmacy Grand Rounds, September, 2008.

Exploring mechanisms of EGFR oncogene addiction in non-small cell lung cancer. Drug Discovery Seminar, University of Kentucky, November, 2007

Inhibition of EGF-like ligand activity disrupts the MAPK-driven EGFR autocrine loop affecting survival and proliferation of lung cancer cell lines. Invited presentation, Lucille Markey Cancer Center Research Day, University of Kentucky, August, 2007

Predicting clinical response to inhibitors of EGFR and exploring mechanisms of EGFR dependency in non-small cell lung cancer. Drug Discovery Seminar, University of Kentucky, May, 2007

Development of a Genomic Predictor of Response to EGFR Inhibitors in Non Small Cell Lung Cancer. Pharmacology Seminar, University of Kentucky, January, 2006

Modeling gene expression predictors of oncogenic signaling pathways in non-small cell lung cancer. Pharmacology Seminar. University of Kentucky, May, 2005.