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Bypass Mechanisms of Resistance to Receptor Tyrosine Kinase Inhibition in Lung Cancer

Matthew J. Niederst^{1,2} and Jeffrey A. Engelman^{1,2,*}

¹Massachusetts General Hospital Cancer Center, Charlestown, MA 02129, USA.

²Department of Medicine, Harvard Medical School, Boston, MA 02115, USA.

Abstract

Receptor tyrosine kinases (RTKs) are activated by somatic genetic alterations in a subset of cancers, and such cancers are often sensitive to specific inhibitors of the activated kinase. Two well-established examples of this paradigm include lung cancers with either *EGFR* mutations or *ALK* translocations. In these cancers, inhibition of the corresponding RTK leads to suppression of key downstream signaling pathways, such as the PI3K (phosphatidylinositol 3-kinase)/AKT and MEK (mitogen-activated protein kinase kinase)/ERK (extracellular signal-regulated kinase) pathways, resulting in cell growth arrest and death. Despite the initial clinical efficacy of *ALK* (anaplastic lymphoma kinase) and *EGFR* (epidermal growth factor receptor) inhibitors in these cancers, resistance invariably develops, typically within 1 to 2 years. Over the past several years, multiple molecular mechanisms of resistance have been identified, and some common themes have emerged. One is the development of resistance mutations in the drug target that prevent the drug from effectively inhibiting the respective RTK. A second is activation of alternative RTKs that maintain the signaling of key downstream pathways despite sustained inhibition of the original drug target. Indeed, several different RTKs have been implicated in promoting resistance to *EGFR* and *ALK* inhibitors in both laboratory studies and patient samples. In this mini-review, we summarize the concepts underlying RTK-mediated resistance, the specific examples known to date, and the challenges of applying this knowledge to develop improved therapeutic strategies to prevent or overcome resistance.

Introduction

Receptor tyrosine kinases (RTKs) function as key regulators of cell growth, proliferation, and survival by transducing signals initiated by growth factors to the MEK (mitogen-activated protein kinase kinase)/ERK (extracellular signal-regulated kinase), PI3K (phosphatidylinositol 3-kinase)/AKT, and STAT (signal transducer and activator of transcription) pathways, among others (1). In nontransformed cells, activation of RTKs and downstream signaling pathways is reversible and tightly regulated, rendering cells dependent on extracellular cues from the environment. However, in cancer cells, these pathways are often constitutively activated. Indeed, because a growing number of cancers have been systematically assessed for mutations and copy number changes, it has become increasingly evident that the majority harbor genetic alterations in either RTKs themselves or components of the downstream signaling pathways (2). These alterations often result in constitutive signaling output that is not susceptible to normal regulation, leading to

*Corresponding author. jengelman@partners.org.

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dysregulated cell growth, survival, and division, all of which are hallmarks of cancer (3). In some cancers with genetic activation of an RTK, that RTK is the predominant activator of downstream signaling pathways. In such instances, the cancer is “addicted” to that RTK, and inhibition of the mutant kinase leads to simultaneous suppression of multiple downstream pathways, often resulting in cell growth arrest and death (4, 5) (Fig. 1). Two of the most well-studied oncogene addiction paradigms include non–small cell lung cancers (NSCLCs) with either mutations in *EGFR* [which encodes epidermal growth factor receptor (EGFR)] or translocations in *ALK* [which encodes anaplastic lymphoma kinase (ALK)], both of which are sensitive to drugs that inhibit the mutant RTK (6, 7). As the genetic characterization of cancers continues to expand, the number of such examples will likely increase, as will the proportion of patients who benefit from tyrosine kinase inhibitors (TKIs).

A growing number of TKIs, such as erlotinib and gefitinib (EGFR inhibitors) in *EGFR*-mutant NSCLC and crizotinib [an ALK and ROS1 (c-ros oncogene 1) inhibitor] in *EML4-ALK*-translocated (ALK-positive) NSCLC, have been approved by the Food and Drug Administration, and many others are currently in clinical or preclinical development. When used in the appropriate genetically defined population, these targeted therapies often demonstrate clinical efficacy, leading to a significant reduction in tumor burden often associated with the abatement of symptoms (8–11). Despite these promising results, there are significant limitations. First, patients who initially respond almost universally relapse, and second, a substantial fraction of patients who have the appropriate genetic abnormality fail to respond altogether (for example, *EGFR*-mutant lung cancers that fail to respond to EGFR inhibitors). These two types of resistance are often referred to as “acquired resistance” and “intrinsic resistance,” respectively (12). Although this mini-review will focus on resistance mechanisms in *EGFR*-mutant and *ALK*-positive NSCLCs, similar concepts are likely shared by other cancers with addiction to a specific RTK.

RTK Activation in Acquired Resistance

Many mechanisms of acquired resistance have been identified over the last several years. A large proportion is associated with reactivation of the key intracellular signals that were originally suppressed by inhibition of the oncogenic RTK. These resistance mechanisms usually fall within one of two categories. The first is the development of secondary mutations in the oncogenic kinase that abrogates the inhibitory activity of the drug, and therefore, the original activated kinase continues to activate the downstream signaling pathways. For example, the development of a T790M resistance mutation is detected in more than 50% of *EGFR*-mutant lung cancers with acquired resistance to gefitinib or erlotinib (13–15). Similarly, in *ALK*-positive cancers, initial reports suggest that resistance mutations in *ALK* are observed in about 33% of cancers with acquired resistance to crizotinib (16). An analogous resistance mutation was recently observed in one case of lung cancer with a *ROS* translocation that had become resistant to crizotinib (17). The second category does not involve mutation of the target, but is associated with reactivation of the downstream signaling pathways through mechanisms independent of the original drug target. This may result from mutational activation of components of the downstream signaling pathways, such as *PIK3CA* or *BRAF* (18, 19), that directly activate the PI3K/AKT and MEK/ERK pathways, respectively, or activation of another RTK that sustains downstream signaling despite inhibition of the oncogenic RTK (20, 21). One could refer to this latter type of resistance as “bypass track” signaling, as the second RTK provides an alternate route around the inhibited target to activate downstream signaling, much like a replacement blood vessel diverts the blood around a blocked artery in heart bypass surgery (Fig. 1). The understanding of bypass track resistance is a focus of current research aiming to develop drug combinations that overcome therapeutic resistance in lung cancer.

One of the earliest suggestions that RTK bypass signaling could promote resistance to targeted therapies came in the setting of *EGFR*-mutant NSCLC. In these cancers, amplification of the *MET* gene, which encodes the MET RTK, was observed in cancers with acquired resistance to EGFR TKIs, but not in the pretreatment samples (20, 21). *MET* amplification had been initially discovered in an *EGFR*-mutant cell line that was cultured in the presence of gefitinib until resistance developed. In that model, MET was found to promote resistance by reactivating both PI3K/AKT and MEK/ERK signaling despite the inhibition of EGFR. Furthermore, the combination of a MET inhibitor and an EGFR inhibitor was both necessary and sufficient to block downstream signaling and induce marked tumor regressions in vitro and in vivo (20, 22). A subsequent study revealed that there were rare cells (less than 1%) with *MET* amplification in pretreatment samples from several patients whose resistant tumors ultimately developed *MET* amplification, raising the possibility that these resistant cells existed at low frequency before treatment (22). In addition to *MET* amplification, activation of MET by its ligand hepatic growth factor (HGF) is sufficient to promote resistance through activation of downstream signaling (22, 23). Thus, MET activation, by either gene amplification or ligand stimulation, can cause bypass resistance to EGFR inhibitors in *EGFR*-mutant lung cancer.

Since those initial observations, several other RTKs have been implicated in driving resistance to EGFR TKIs (Table 1). *ErbB2* amplification, as identified by FISH (fluorescence in situ hybridization), was observed in a subset of drug-resistant, *EGFR*-mutant cancers that did not harbor T790M-resistant mutations. Increased ErbB2 protein abundance was also detected in a cell line model of acquired resistance to EGFR TKI (24). In addition, activity of the insulin-like growth factor 1 receptor (IGF1R) can promote acquired resistance to gefitinib in *EGFR*-amplified and *EGFR*-mutant cancer cell line models (25). In gefitinib-resistant A431 epidermoid carcinoma cells, loss of expression of *IGFBP3* and *IGFBP4*, which encode insulin-like growth factor binding proteins 3 and 4, respectively, leads to increased IGF1R/PI3K/AKT pathway activity and maintenance of PI3K/AKT signaling despite EGFR inhibition (25). Likewise, PC9 NSCLC cells incubated in the presence of next-generation EGFR inhibitors PF299804 or WZ4002 (which have the capacity to suppress EGFR-T790M activation) had decreased IGFBP3 abundance and IGF1R-dependent maintenance of PI3K/AKT signaling in the presence of an EGFR inhibitor (26). In both cases, inhibition of IGF1R by either a targeted monoclonal antibody or a kinase inhibitor was sufficient to restore sensitivity to EGFR inhibition, and the combination was necessary to suppress PI3K/AKT signaling (25, 26). Notably, IGF1R expression is detected in a majority of NSCLC tumors by histological analysis, lending credibility that this mechanism could mediate resistance in some patients (27). The AXL RTK has also recently been implicated in acquired resistance to TKIs in *EGFR*-mutant NSCLC, in which the expression of AXL and its ligand GAS6 was increased in 20 and 25% of drug-resistant patient tumors, respectively (28). AXL abundance was similarly elevated in *EGFR*-mutant NSCLC cells with acquired resistance to erlotinib in vitro and in vivo. The increased expression of AXL in resistant cells was coincident with the induction of an epithelial-to-mesenchymal transition (EMT), and EMT has even been observed in *EGFR*-mutant lung cancer specimens with acquired resistance to EGFR inhibitors (18, 28). Other studies in large panels of genetically diverse cell lines have shown that both AXL and EMT are associated with intrinsic resistance to EGFR inhibitors (29, 30). In addition to the RTK-mediated mechanisms of resistance described above, activation of fibro-blast growth factor receptor 1 (FGFR1) through an FGF2-FGFR1 autocrine loop was also identified as a mechanism of resistance in a PC9 cell line model (31). The data suggest that the bypass RTK (MET, AXL, IGF1R, or FGFR1 in the examples presented) mediates resistance by activating one or more downstream pathways that are normally activated by the targeted RTK, and that resistance could be overcome by targeting the bypass RTK in combination with the oncogenic RTK.

Similar to *EGFR*-mutant cancers, RTK-mediated bypass resistance has also been described in *ALK*-positive lung cancers with acquired resistance to crizotinib. In resistant cell line models, *EGFR* has been identified as a bypass RTK, and activation of *EGFR* maintains ERK signaling despite *ALK* inhibition to cause resistance. In these models, combined inhibition of *EGFR* and *ALK* suppresses both AKT and ERK pathways and impairs cell viability (16, 32). Analysis of patient samples revealed increased phosphorylation of *EGFR* in 44% of crizotinib-resistant patient tumors compared with their respective preresistant samples, lending credence to the notion that *EGFR* activation may promote resistance in the clinic (16). In one crizotinib-resistant patient, amplification of *c-KIT*, which encodes the c-KIT kinase, was detected along with increased expression of *SCF*, which encodes stem cell factor, a ligand for KIT (16), neither of which was observed in the pretreatment sample. Laboratory studies demonstrated that both KIT and SCF were required to confer crizotinib resistance in NCI-H3122 cells in vitro. Resistance was overcome in these cells by combining imatinib (a BCR-Abl kinase inhibitor) with crizotinib. As an aside, it is also notable that the MET ligand HGF promoted resistance to the *ALK* inhibitor TAE684 but not crizotinib (33, 34). Because crizotinib is also a potent MET inhibitor, MET activation would not be expected to be a resistance mechanism to crizotinib. These results point out the possibility that MET activation has the potential to induce resistance to second-generation *ALK* inhibitors (such as LDK378) that do not target MET. In summary, *ALK*-positive lung cancers may use several other RTKs to promote resistance to *ALK* inhibitors, similar to that observed in *EGFR*-mutant lung cancers.

The findings described demonstrate that activation of RTKs can drive resistance to targeted therapies. Most of those findings have been developed from laboratory models of acquired resistance and from examination of patient samples. Recently, laboratory investigators have used larger-scale, unbiased approaches to identify which receptors have the potential to cause resistance. In one study, a panel of cancer cell lines driven by different RTKs (*EGFR*, *ALK*, *HER2*, etc.) was treated with a variety of growth factors to determine which were capable of promoting resistance (34). In another study, a similar panel of cell lines was co-incubated with a group of stromal cells to identify secreted factors that may promote resistance (35). These studies revealed that multiple growth factor–RTK pairs are capable of promoting resistance in several distinct oncogene-addicted paradigms but that variability exists within cell lines sharing the same oncogenic RTK and between cell lines with different oncogenic RTKs, potentially because of the specific spectrum of RTKs expressed in each cancer cell line. A recent study by MacBeath and colleagues provides additional insight by functionally grouping different RTKs together into distinct classes based on network modeling derived from shared and unique signaling (36). They hypothesize that RTKs with similar signaling networks would readily compensate for one another. For example, *EGFR*, *FGFR*, and *MET* are all found in the same class, suggesting that *MET* and *FGFR* would readily drive bypass resistance to *EGFR* inhibitors. These findings are consistent with those by Settleman and colleagues, who reported that FGF and HGF can confer resistance to an *EGFR* TKI in *EGFR*-mutant NSCLCs (34). As discussed further below, additional studies are needed to verify which RTKs are actually promoting resistance in patients. Still, it is clear that these types of investigations yield key insights into predicting which RTKs have the capacity to substitute for the driver oncogene, and they serve as powerful starting points when searching for the specific RTKs responsible for promoting resistance in individual patients.

RTK Activation in Intrinsic Resistance and Drug-Tolerant Persisters

Although many of the studies mentioned examined RTK activation as a mechanism of acquired resistance, the lines between intrinsic and acquired resistance are blurred. Specifically, in some drug-sensitive cancers, the minor proportion of cancer cells that

survive the initial therapy may do so because of RTK bypass tracks preexisting within a subpopulation of cells. These cells may then proliferate in the presence of an inhibitor and ultimately lead to clinical resistance. This is highlighted by the finding that *MET* amplification was observed in a rare subset of pretreatment cells in some *EGFR*-mutant NSCLC patients who subsequently developed *MET* amplification in their resistant specimen (22). Other studies have also demonstrated that the subset of cells that survives after initial treatment with targeted therapies may be maintained by bypass track signaling emanating from RTKs. Settleman and colleagues demonstrated that these “drug-tolerant persister” cells have marked changes in gene expression because of an altered chromatin state (37). These cells exhibited high IGF1R activity and were sensitive to a combination of anti-EGFR and anti-IGF1R therapies. Cortot *et al.* likewise found that resistance to an EGFR TKI involves a multistep process that is mediated early by signaling from IGF1R. After continuous culture in the drug (for 4 to 6 months), the resistant cells no longer had increased IGF1R activation, but rather exhibited increased ERK signaling (26). These results suggest that as resistance evolves, bypass signaling by alternative RTKs may lead to the initial survival of cancer cells treated with TKIs, and that these cells may ultimately give rise to the fully resistant phenotype. These findings indicate that the use of multiple RTK inhibitors may block the survival of intrinsically resistant sub-populations in the tumor and may lead to greater and more durable remission in patients.

The relief of negative feedback loops is another means of resistance, particularly to inhibitors of the PI3K/AKT and MEK/ERK signaling pathways. Normally, these pathways induce negative feedback loops that suppress RTK signaling. In other words, when these pathways are directly inhibited with small molecules, RTK signaling is increased, thereby diminishing the impact of such inhibitors. For example, in ovarian and breast cancer cells, treatment with PI3K pathway inhibitors releases negative feedback mechanisms, resulting in increased signaling by multiple ErbB RTK family members (38, 39). Similarly, inhibition of MEK increases activation of ErbB and/or IGF1R signaling in several cancer types (40, 41). Of particular interest are the recent findings that *BRAF*-mutant colorectal cancers fail to respond to vemurafenib, a BRAF kinase inhibitor, because initial inhibition of ERK leads to increased signaling output from RTKs (particularly EGFR) that restore ERK signaling despite the presence of vemurafenib (42, 43). In these cancers, a combination of vemurafenib and EGFR inhibitors blocks ERK reactivation and induces tumor regressions in mouse xenografts. Combinations of EGFR and BRAF inhibitors are now being pursued in the clinic for *BRAF*-mutant colorectal cancer (clinical study numbers NCT01750918, NCT01719380, and NCT01787500).

Targeting RTK-Driven Resistance in the Clinic

A major challenge in overcoming bypass track resistance is to accurately identify the specific RTK that mediates resistance in an individual tumor. In some instances, bypass RTKs can be detected by genetic activation of the RTK (such as *MET* amplification). However, in most cases, activation of the RTK (be it IGF1R, AXL, EGFR, or others) is not readily identifiable by genetic studies, and their role in resistance is only inferred by immunohistochemical or other protein detection methods using resistant biopsies. However, the increased abundance of an RTK does not mean that it is necessarily mediating resistance. For example, the abundance of AXL is increased in a substantial subset of *EGFR*-mutant lung cancers with acquired resistance to EGFR inhibitors, but AXL expression is also associated with EMT (28, 30, 44). Thus, one cannot be certain whether AXL is primarily a biomarker of EMT or if AXL is truly mediating resistance in all of the tumors where increased protein abundance is detected.

Of course, these concerns extend to most conclusions based simply on genetic expression or protein abundance. Furthermore, IHC (immunohistochemistry) is often not highly quantitative, and it has significant limitations in determining the levels of RTK activation. One could easily envision that a technological advance, such as an unbiased mass spectroscopy method, may improve quantitative assessment of RTK activation. It is also possible that assessment of RTK ligands and other quantitative assays, such as reverse-phase protein arrays, may improve the predictive power by accurately identifying bypass RTKs. However, the gold standard for determining if a specific RTK is truly mediating resistance will be the observation of clinical remissions when an inhibitor of the suspected RTK is added to the original TKI in resistant tumors. Short of these clinical observations, efforts to develop cell lines and patient-derived xenografts directly from resistant biopsies may facilitate the functional studies necessary to directly assess RTK dependence and to complement biomarker analyses. Indeed, patient-derived resistant models have already been used to elucidate important mechanisms of resistance to crizotinib in *ALK*-positive NSCLC (16, 32).

Multiple clinical trials are examining the efficacy of combining an RTK-directed therapy along with an inhibitor of the primary TKI. For *EGFR*-mutant NSCLC, combinations targeting *EGFR* and *MET*, *IGF1R*, or *FGFR* are all currently under way (study numbers NCT01186861, NCT01515969, and NCT00965731). Studies pairing *EGFR* inhibitors with antibodies targeting *ErbB3* have also been initiated (NCT00994123). Additionally, crizotinib is being tested in combination with pan-*ErbB* family inhibitors, which could potentially serve as an option for *ALK*-positive cancers that are resistant to crizotinib because of *EGFR* activation (NCT01121575). However, even when such combinations demonstrate signs of overcoming resistance, there are several challenges that must be addressed for this approach to have a major impact in the clinic. First, one would need to identify which additional RTK to target in a specific patient. In general, these early-stage trials do not include assessment of the activation of the secondary RTK as a biomarker for inclusion into the study. Once the safety of these combinations has been tested, a greater emphasis can be placed on matching the patient with the appropriate therapy to ensure maximum efficacy. Second, there may be several different resistant subpopulations of cells within a tumor mass that are mediated by different RTKs, and these may differ among patients with the same tumor type (16). Thus, the potential for heterogeneity of resistance mechanisms within a single patient may limit the efficacy of a simple two-drug combination.

Although treating cancers after the development of resistance with specific combinations of RTK inhibitors may be informative in proof-of-concept studies and could provide meaningful benefit to patients, it seems unlikely to us that treating cancers with such combinations after resistance emerges will have a truly transformative impact on patients. It is tempting to speculate that the best strategy to produce a lasting effect on cancers is to treat with combinations up front to prevent potentially resistant clones from emerging. However, because many RTKs can cause resistance in a given cancer (Table 1), innovative treatment approaches that use alternating and intermittent dosing of different RTK-based combinations in a proactive manner (that is, before overt clinical resistance develops) are needed. Because RTKs can often be targeted with antibodies, and these often do not share the same gastrointestinal toxicities that are observed with many small-molecule inhibitors, it may prove less toxic to use multiple combinations of individual RTK-targeted antibodies in combination with the original TKI. One could envision these combinations being administered using alternating and intermittent regimens to thwart the emergence of resistance. Although such an innovative approach may substantially delay the development of resistance and provide increased clinical benefit, it may not be sufficient to transform

these paradigms into curative ones for a large proportion of patients. However, it may serve as a good start.

Conclusions

Inhibitors of RTKs can be highly effective therapies in cancers with genetic activation of the target receptor. However, this efficacy is limited by the likely development of acquired resistance. The study of *EGFR*-mutant and *ALK*-positive NSCLCs with acquired resistance to TKIs has led to the identification of several resistance mechanisms. One common paradigm is that other RTKs can restore the activation of key intracellular signaling pathways despite inhibition of the oncogenic kinase, leading to resistance. Although laboratory experiments have implicated several different RTKs in resistant cell line models, it is necessary to continue to validate specific RTKs in resistant patient samples and further demonstrate that they are directly responsible for resistance to TKIs in the clinic. New therapeutic combinations that couple an inhibitor of a potential bypass RTK along with an inhibitor of the original target RTK are entering the clinic. To maximize the potential patient benefit, identifying the appropriate patients for each specific combination, determining when combination strategies should be implemented, and testing the potential of alternating different treatment regimens will all be critical to prevent or delay resistance.

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Fig. 1. Activation of a secondary RTK can create a bypass track that promotes resistance to TKIs.

In drug-sensitive cancers (left), the mutant oncogene exerts unilateral control over RAS, MEK/ERK, and PI3K/AKT pathway signaling. When inhibited by the appropriate TKI, these pathways are suppressed, resulting in cell cycle arrest and apoptosis. In drug-resistant cancers with a bypass track (right), the secondary RTK reactivates the signaling of at least one of the key downstream pathways, whereas the primary oncogene remains inhibited. Sustained activation of these pathways leads to continued cell proliferation and survival in the presence of TKI.

Table 1

Previously published examples of RTK-mediated resistance in *EGFR*-mutant and *ALK*-positive cancers.

Oncogene	Bypass RTK	Drug	References
EGFR	MET amp	Gefitinib/erlotinib	(18-23)
EGFR	MET [*] (HGF)	Gefitinib/erlotinib	(22, 23)
EGFR	HER2 amp	Gefitinib/erlotinib	(19, 24)
EGFR	IGF1R	Gefitinib	(25)
EGFR	IGF1R	Erlotinib, afatinib, WZ4002	(26, 37)
EGFR	FGFR	Gefitinib	(31)
EGFR	AXL [*] (GAS6)	Gefitinib/erlotinib	(28)
EML4-ALK	EGFR [*] (AREG/NRG1)	Crizotinib, TAE684	(16, 33)
EML4-ALK	c-KIT amp	Crizotinib	(16)

* Examples in which ligand-induced activation of bypass RTK activation led to resistance; amp, amplification.