

Molecular Mechanisms of Resistance to Therapies Targeting the Epidermal Growth Factor Receptor

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ABSTRACT

Targeted therapies that inhibit the activity of tyrosine kinase receptors such as the epidermal growth factor receptor (EGFR) have shown activity against solid malignancies when used as single agents or in combination with chemotherapy. Although anti-EGFR therapies are active in some patients, eventually disease in nearly all patients will become refractory to therapy. Therefore, a better understanding of the mechanisms of resistance to anti-EGFR therapies is critical to further improve the efficacy of this class of agents. Mechanisms that mediate resistance to anti-EGFR therapies include the presence of redundant tyrosine kinase receptors, increased angiogenesis, and the constitutive activation of downstream mediators. Two recent landmark publications have also shown that specific mutations in the kinase domain of EGFR in some lung carcinomas are associated with markedly improved response rates to an EGFR tyrosine kinase inhibitor. Mutations in the EGFR receptor seem to play a significant role in determining the sensitivity of tumor cells to EGFR inhibitor therapy by altering the conformation and activity of the receptor. As the field of molecular therapeutics continues to evolve, a comprehensive understanding of resistance mechanisms will ultimately lead to refinements in our regimens to provide better care for patients with cancer.

INTRODUCTION

Despite advances in chemotherapy, most patients with cancer that has metastasized will succumb to the disease within 2 years of diagnosis. In an effort to improve survival, new therapeutic approaches focusing on the molecular mechanisms that mediate tumor cell growth or survival have gained much

attention. In particular, the epidermal growth factor receptor (EGFR) has been extensively investigated as a target for antineoplastic therapy. EGFR is overexpressed in a large number of tumors, including head and neck, breast, colorectal, lung, prostate, kidney, ovary, brain, pancreas, and bladder carcinomas (reviewed in ref. 1). The overexpression of EGFR in these carcinomas correlates with poor prognosis and decreased survival (2–4). In addition, ligands for EGFR, principally transforming growth factor α (TGF- α), are often produced by tumors that overexpress EGFR, leading to activation of survival pathways via autocrine loops. Signaling through the EGFR axis has been implicated in mediating multiple processes involved in tumor progression and metastasis, including invasion, angiogenesis, proliferation, and inhibition of apoptosis (reviewed in ref. 5).

Anti-EGFR-targeted therapies have improved the efficacy of conventional chemotherapy in both preclinical and clinical studies (reviewed in ref. 2). Although such therapies may lead to partial response or disease stabilization in some patients, many patients do not benefit from anti-EGFR therapy, and those who do eventually develop resistance to that therapy. Expanding our knowledge of the molecular alterations that occur with tumor progression and metastasis will provide insight into methods to optimize targeted therapies.

Biology and Function of EGFR. EGFR is a member of the structurally related erbB family of receptor tyrosine kinases, so named for its first identification as an oncogene encoded by the avian erythroblastosis virus. Four erbB members have been identified: EGFR (ErbB1), HER2/Neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) (ref. 6). All four erbB receptors are composed of an extracellular ligand-binding region consisting of glycosylated domains; a transmembrane domain containing a single hydrophobic anchor sequence; an intracellular region containing the catalytic tyrosine kinase domain, and a carboxyl-terminal region containing several tyrosine residues that become phosphorylated after receptor activation. Although structural similarity exists between the family members, important differences are also present. Unlike the rest of the erbB family, ErbB3 lacks tyrosine kinase activity. ErbB2 (HER2/neu) has no known ligand but is constitutively activated and amplified in many tumor types (5, 7).

EGFR was the first erbB family member to be described and remains the best characterized to date (8, 9). Epithelial cells and malignant tumors of epithelial origin express EGFR, but EGFR is not expressed on mature hematopoietic cells (9, 10). EGFR has six known ligands: EGF, TGF- α , amphiregulin, betacellulin, heparin-binding EGF, and epiregulin (11).

EGFR and other members of the erbB family form either homodimers or heterodimers upon ligand binding, resulting in conformational changes that allow activation of protein kinases and transphosphorylation of key tyrosine residues within the carboxyl-terminal domain. Phosphorylated tyrosine moieties serve as docking sites for proteins SH2 or phosphotyrosine binding domains, including the adaptor proteins Grb2 and Shc and enzymes such as phospholipase C, phosphatidylinositol-3 kinase (PI-3K), and the Src family kinases. These proteins go on

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to initiate multiple intracellular signal transduction cascades. After activation, the receptor is internalized, by which its degradation or recycling can transiently down-regulate signaling mediated from the receptor. Multiple signaling pathways related to cellular proliferation and survival are activated downstream of EGFR, including the ras/mitogen-activated protein kinase (MAPK), PI-3K/Akt, and signal transducer and activator of transcription (STAT) pathways. EGFR activation can also induce cell cycle progression via various mechanisms, including the up-regulation of cyclin D1 (12). EGFR stimulation can significantly increase the activity of c-Src, a signaling intermediate involved in cell cycle progression, motility, angiogenesis, and survival (reviewed in ref. 13).

Overview of Anti-EGFR Therapies. Among the several approaches used to inhibit EGFR function are neutralizing monoclonal antibodies (mAb) to the EGFR, receptor tyrosine kinase inhibitors (TKI), and toxins conjugated to mAbs. Both mAbs and TKIs have shown clinical benefit and continue to be evaluated in multiple clinical trials in a variety of tumors (reviewed in refs. 6, 14). Some of the anti-EGFR mAbs currently undergoing clinical investigation include cetuximab [IMC-C225 (Erbix)], panitumumab (ABX-EGF), EMD 72000, hR3 (the humanized version of ior-EGF/r3), and ICR62. These antibodies are similar in that they all bind to the extracellular ligand-binding domain of EGFR and competitively inhibit ligand binding, which in turn prevents dimerization and activation of the receptor tyrosine kinase. Binding of the mAb to the receptor can also lead to internalization of the receptor and transient decreases in receptor expression. Of these antibodies, cetuximab has achieved the most attention with its recent approval by the U.S. Food and Drug Administration (FDA) for use in refractory colorectal cancer.

TKIs are small molecules (usually quinazoline derivatives) that act by inhibiting EGFR phosphorylation and therefore receptor activation and signal transduction. In contrast to the anti-EGFR antibodies, TKIs do not lead to receptor internalization. EGFR TKIs fall into two broad classes: reversible inhibitors such as ZD1839 [gefitinib (Iressa)] and OSI-774 [erlotinib (Tarceva); formerly CP-358, 774] and irreversible inhibitors such as CI-1033 (a pan-HER TKI), EKB-569, and GW572016 (Lapatinib, a dual EGFR/erbB2 inhibitor). Unlike the reversible inhibitors, irreversible inhibitors covalently bind specific cysteine residues in the ATP binding site of EGFR. The clinical significance of reversible versus irreversible inhibition is uncertain at this point. In theory, the ability to irreversibly bind the tyrosine kinase domain could produce more sustained receptor inhibition.

EGFR Inhibitors for Patients with Epithelial Malignancies. Many EGFR inhibitors are now being investigated for use in treating various epithelial malignancies. The FDA recently approved two EGFR inhibitors, cetuximab and gefitinib, for clinical use; clinical trials involving these two agents highlight the successes and challenges of this genre of molecular therapy and are the focus of the following discussion.

Cetuximab is a human: murine chimeric anti-EGFR IgG1 antibody that has showed antitumor activity in EGFR-expressing tumors *in vivo* and tumor cell lines *in vitro* (15–17). Numerous preclinical studies provide evidence that cetuximab participates in inhibition of tumor cell cycle progression, promotion of apoptosis, and enhancement of antibody-dependent cellular

toxicity (reviewed in refs. 11, 18). Cetuximab has shown clinical benefit against several solid tumors, including colorectal cancer and head and neck squamous cell cancer (19–21). Cetuximab was recently approved by the FDA for recurrent metastatic colorectal cancer in patients who cannot tolerate irinotecan-based therapy. These recommendations were based mostly on a 9% response rate observed in a phase II trial of cetuximab for irinotecan-refractory EGFR-expressing colorectal cancer (21).

Although cetuximab has shown benefit as a single agent in colorectal cancer, it was primarily developed for use in combination with chemotherapy. In two trials involving patients with irinotecan-refractory metastatic colorectal cancer, consistent response rates of about 23% were shown in patients given a combination of cetuximab and irinotecan (22, 23). In effect, cetuximab was able to reverse the resistance of these tumors to irinotecan. After these results were reported, cetuximab was approved by the FDA for use in combination with irinotecan for treatment of irinotecan-refractory metastatic colorectal cancer that expresses EGFR. Similarly, in a phase II study of head and neck squamous cell cancer, the combination of cetuximab and cisplatin-based chemotherapy significantly improved the response of tumors that had been refractory to cisplatin (24). In 23% of cases in which tumors progressed on cisplatin-based therapy alone, clinical benefit was shown from the addition of cetuximab to cisplatin.

Interestingly, the effectiveness of EGFR inhibitors does not seem to correlate with the degree of overexpression of the EGFR. In patients with irinotecan-refractory colorectal cancer, the level of EGFR expression in the tumors did not correlate with the response rate to cetuximab, either as a single agent or in combination with irinotecan (14, 21). This observation stands in contrast to the use of the anti-HER2/neu receptor mAb trastuzumab for patients with metastatic breast cancer, in which the degree of HER2 expression by the tumor correlates with response to therapy (25, 26). The effectiveness of EGFR inhibitors may be directly related to dependence of an individual tumor on EGFR signaling for survival. On the other hand, a lack of response to EGFR inhibitors might suggest that the tumor can maintain critical survival pathways through either constitutively activated downstream mediators or alternative receptor signaling.

The effectiveness of combining EGFR inhibitors and chemotherapy may be related to the capacity of EGFR inhibitors to sensitize tumor cells to chemotherapy, possibly by inhibiting the mediators of antiapoptotic pathways such as BCL-2 and NF- κ B. In addition to down-regulating tumor survival mechanisms, EGFR inhibitors have also shown direct proapoptotic effects such as induction of Bax and activation of caspase-8 (27, 28).

The FDA approved the TKI gefitinib for locally advanced or metastatic non-small cell lung cancer refractory to both platinum-based and docetaxel chemotherapy. This approval followed two large phase II randomized trials of patients whose tumors were refractory to these chemotherapies to whom gefitinib was administered at doses of 250 or 500 mg/d. Symptomatic improvement was reported in ~40% of these patients. The response rate for gefitinib used as second- or third-line therapy ranged from 9% to 18% depending on the site of the study (29, 30).

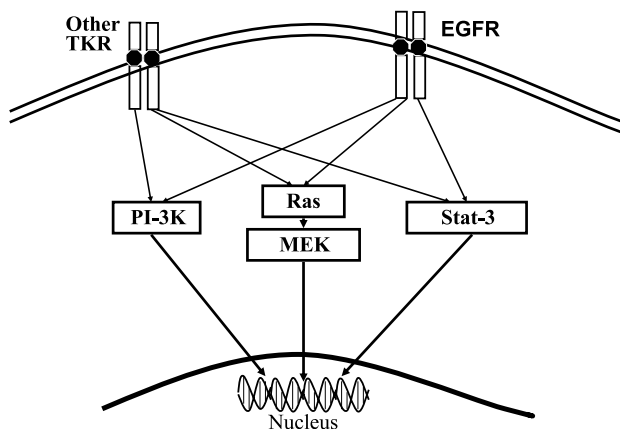


Fig. 1 Resistance caused by activation of other tyrosine kinase receptors (TKR) that bypass the EGFR pathway. Multiple TKRs share similar downstream pathways to EGFR. Tumor cells may be able to resist an inhibitor of a single receptor because of the presence of other activated TKRs that can also stimulate survival mechanisms.

After its successful use as single-agent therapy, gefitinib was tested in combination with traditional chemotherapy. Two large, randomized, placebo-controlled, double-blind phase III trials (INTACT 1 and 2) investigated the efficacy of gefitinib in combination with chemotherapy as first-line therapy for non-small cell lung cancer. In contrast to its success as a second- or third-line single agent, the addition of gefitinib to chemotherapy failed to improve survival, time to progression, or response rate compared with chemotherapy alone (31, 32). However, as discussed in the following section, a subset of patients with non-small cell lung cancer with activating mutations in EGFR was sensitive to gefitinib therapy. These trial results, along with others, have generated interest in identifying the mechanisms underlying resistance to EGFR inhibitors.

MECHANISMS OF RESISTANCE TO ANTI-EGFR THERAPY

Great interest exists in elucidating resistance mechanisms for anti-EGFR therapies as well as those for chemotherapy agents. The molecular mechanisms of resistance can be attributed to several general processes: (a) the presence of redundant tyrosine kinase receptors, (b) increased angiogenesis, (c) the constitutive activation of downstream mediators, and (d) the existence of specific EGFR mutations. Understanding the molecular mechanisms of resistance and sensitivity may lead to improvements in therapies that target EGFR.

Resistance due to Activation of Alternative Tyrosine Kinase Receptors that Bypass the EGFR Pathway. EGFR can enhance tumor cell survival and proliferation through multiple downstream signaling pathways. Other tyrosine kinase receptors such as c-MET (hepatocyte growth factor receptor), Ron (a protein tyrosine kinase related to c-MET), platelet-derived growth factor receptor, and insulin-like growth factor receptor-1 (IGF-1R) can also influence similar signaling pathways (Fig. 1). Activation of distinct growth factor receptors leads to initiation of pathways that impact multiple cell functions. For example, c-Met activation strongly promotes migration but can also increase proliferation,

survival, and angiogenesis. Thus, activation of several growth factor receptors may promote tumor progression by activating overlapping signal transduction pathways (33–35). The redundancy in these pathways likely exists because of the fundamental importance of the biological processes they regulate—cell survival and growth.

Activation of the tyrosine kinase receptor IGF-1R has been associated with resistance to EGFR inhibitors. IGF-1R activates many of the same downstream pathways as EGFR and can lead to tumorigenesis, increased proliferation, angiogenesis, and metastasis (36). PI-3K/Akt signaling is a critical component of the downstream mediation of EGFR and also plays a functional role in IGF-1R signaling (36). This redundancy may explain how the receptors can mimic the function of one another. Chakravarti et al. identified two glioblastoma cell lines that each overexpressed EGFR but exhibited very different responses to EGFR inhibitors (37). The resistant cell line significantly overexpressed IGF-1R and showed further increases in IGF-1R expression in response to EGFR inhibition by AG1478, an EGFR TKI (37). PI-3K/Akt signaling persisted in these resistant cell lines in response to AG1478 treatment, and these cells also maintained their invasive and antiapoptotic characteristics (37). These findings support the concept of redundant signaling through IGF-1R that maintains activation of critical pathways for survival in the presence of EGFR inhibition. Inhibiting both IGF-1R and EGFR significantly reduced the growth and invasiveness of cells that were resistant to EGFR inhibitors alone (37).

Further evidence that IGF-1R activation may bypass inhibition of other tyrosine kinase receptors comes from a study by Lu et al., who showed that the degree of overexpression of IGF-1R was inversely related to the response of breast cancer cells to trastuzumab, an antibody directed against ErbB2 (38). SKBR3 human breast cancer cells, which normally overexpress HER2/neu and minimally express IGF-1R, showed a 42% decrease in proliferation in response to trastuzumab (38). Unlike the parental cell line, SKBR3 cells that were engineered to overexpress IGF-1R showed no response to trastuzumab (38). When the IGF-1R was inhibited by IGF-binding protein-3 in the engineered cell lines, the response to trastuzumab returned to normal (38).

These studies clearly indicate that activation of alternative tyrosine kinase receptors in tumor cells may override the effect of EGFR family inhibitors. These examples suggest that a combination of growth factor receptor inhibitors may be able to overcome resistance to a single receptor inhibitor and thus more effectively inhibit pathways leading to cancer growth and survival. Recently, an enhanced response was shown in breast cancer cell lines treated with either recombinant bispecific antibodies to both EGFR and IGF-1R or a combination of single receptor antibodies compared with either antibody alone (39). Signaling pathways downstream of the receptors were also more effectively inhibited by the combination therapy (39). By combining therapies that attack multiple cell surface and intracellular signaling pathways, redundant receptor signaling might be blocked and greater clinical benefit achieved. Thus, identifying key downstream signaling molecules in which growth factor receptor signals converge may be important in the development of therapeutic agents that block signals from multiple activated growth factor receptors.

Angiogenesis, Vascular Endothelial Growth Factor, and Resistance to EGFR Inhibition. An increasing body of evidence suggests that EGFR-mediated pathways are intimately involved in tumor angiogenesis through up-regulation of vascular endothelial growth factor (VEGF) and other mediators of angiogenesis (40–44). Activation of EGFR by EGF or TGF- α has been shown to up-regulate VEGF expression in colorectal cancer, human glioma, prostate cancer, and head and neck squamous cell cancer cells *in vitro* (41–44).

In preclinical models, EGFR inhibitors have displayed antiangiogenic properties. Treatment of a variety of EGFR-expressing tumor cells with cetuximab resulted in down-regulation of various angiogenic mediators (45–47). The efficacy of cetuximab against tumor cells is more pronounced in xenografts than in cell culture, an effect that could be explained in part by the antiangiogenic consequences of EGFR blockade. Such antiangiogenic effects are an indirect result of EGFR inhibition on tumor cells, as cetuximab does not recognize murine EGFR and thus would not affect EGFR activation in tumor-associated murine endothelial cells.

Up-regulation of tumor angiogenesis promoting growth factors is a potential mechanism by which tumor cells may overcome the deleterious effects of EGFR inhibition. Through chronic administration of EGFR mAbs (cetuximab, mR3, and hR3) *in vivo*, Vilorio-Petit et al. generated EGFR inhibitor–refractory human squamous cell tumors that were analyzed for mechanisms of resistance (48). Resistant cell lines retained the same *in vitro* response as did the parental (sensitive) cells to EGFR mAbs (48). In contrast, resistant tumors had a significantly decreased *in vivo* response to anti-EGFR therapy compared with parental tumors (48). Unlike *in vitro* models of tumor growth, tumor growth *in vivo* relies on angiogenesis for development. Therefore, resistant tumors may overcome the inhibitory effects of EGFR blockade *in vivo* by increasing angiogenesis. As Vilorio-Petit et al. showed, the ability of resistant tumors to increase angiogenesis would not give an advantage to cell lines grown *in vitro*. In support of this model of resistance, five of six resistant cell lines expressed 2- to 4-fold more VEGF protein, which paralleled a corresponding increase in tumor angiogenesis (48). In squamous carcinoma cells engineered to overexpress VEGF and then treated with EGFR mAbs, the response to the EGFR mAbs was diminished relative to that of the parental tumors (48).

Ciardiello et al. confirmed that VEGF expression was elevated in EGFR inhibitor–resistant colon cancer (49). In that study, chronic administration of either EGFR mAbs or TKIs to athymic mice bearing human GEO colon cancer xenografts led to the development of resistant colon cancer cell lines that showed 5- to 10-fold increases in VEGF expression (49). In addition to the increase in VEGF expression, resistant tumors also exhibited increased expression of both cyclooxygenase-2 and activated MAPK, two upstream mediators of VEGF induction (49). These authors suggested that up-regulation of these mediators could contribute to increased angiogenesis and lead to resistance to EGFR inhibitors (49). In contrast to chronic therapy with EGFR inhibitors, chronic therapy with ZD6474, a TKI effective against both EGFR and VEGFR-2, did not result in the development of resistant tumors (49). Thus, targeting both EGFR and VEGFR-2 may represent a potential mechanism for overcoming the EGFR inhibitor–resistant phenotype mediated by increased VEGF expression.

A consistent pattern of resistance has been observed in the development of refractory tumors through chronic administration of EGFR inhibitors. Most tumors initially respond to EGFR inhibition with tumor shrinkage, and after a latent period (sometimes as soon as 90 days) new growth typically appears (48, 49). This pattern suggests that EGFR inhibition may select clones of preexisting cancer cells that rely on redundant signaling pathways or can overactivate survival pathways in response to the stress of treatment.

The development of resistant tumors with altered survival pathways leading to the latent growth observed during prolonged EGFR inhibitor therapy highlights the need for novel approaches to cancer therapy. Combining molecular therapies targeting several survival pathways, such as antiangiogenic therapy with EGFR inhibitor therapy, may minimize this method of resistance. In a test of this approach, Jung et al. gave a combination of mAbs to EGFR and mAbs to VEGFR-2 to treat gastric cancer grown in nude mice (50). Both mAbs were modestly effective at inhibiting tumor growth, but the combination achieved significantly greater tumor growth inhibition that was also associated with decreased tumor vascularity and increased tumor cell apoptosis (50).

Resistance Based on Constitutive Activation of Signaling Pathways Downstream of EGFR. Although EGFR activation can stimulate numerous downstream signaling pathways, other potential mechanisms can achieve similar signaling effects. Alterations in signaling mediators through genetic or epigenetic mechanisms may lead to constitutive activation and signaling of these downstream molecules. Thus, constitutive activation of signaling intermediates downstream of the EGFR would bypass the need for EGFR activation and thus decrease the efficacy of EGFR inhibition (Fig. 2). One of the most common examples of this potential resistance mechanism is the loss of PTEN/MMAC/TEP (PTEN) phosphatase function through genetic mutation.

PTEN is a lipid phosphatase and tumor suppressor protein that regulates the PI-3K/Akt signaling pathway (12, 51). The major substrate for PTEN is phosphatidylinositol 3,4,5-trisphosphate, a second messenger of PI-3K. With the loss of PTEN function, phosphatidylinositol 3,4,5-trisphosphate accumulates in the cell membrane, when it binds and activates Akt. Thus, loss of PTEN function results in overactivation of the Akt pathway, increasing its cellular antiapoptotic functions (Fig. 2; ref. 52). By regulating the activation of Akt, PTEN can also mediate the antiapoptotic downstream effects of EGFR signaling. Through Akt activation, EGFR induces Bad phosphorylation, thereby inhibiting its proapoptotic interaction with Bcl-2 and Bcl-x (12). Furthermore, the antiapoptotic genes *bcl-2* and *bcl-x* undergo transcription in response to EGFR activation (12). In addition to promoting cell survival, constitutive activation of the Akt pathway may also contribute to cell proliferation through multiple mechanisms such as activation of Raf kinase and also promote cell cycle progression through the cyclin-dependent kinase inhibitor p212.

Functional inactivation of PTEN (often loss of one allele followed by mutation in the other) have been observed in several human tumor types (53, 54). Bianco et al. observed increased resistance to gefitinib (relative to that of wild-type breast cancer cell lines) in MDA-468 breast cancer cells, which

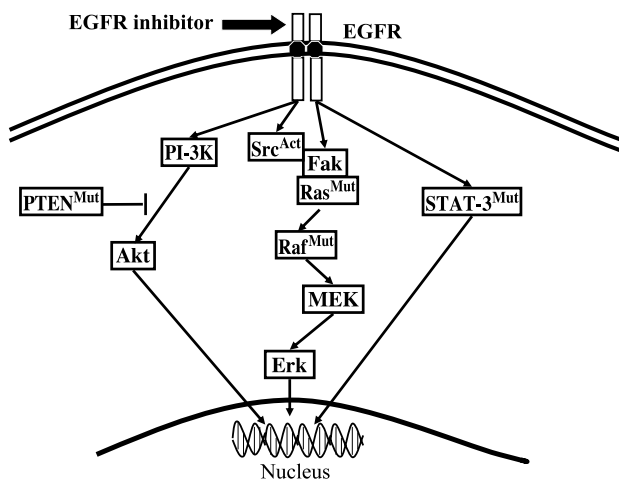


Fig. 2 Resistance based on constitutive activation of signaling pathways downstream of EGFR. Pathways potentially blocked by EGFR inhibitors can remain activated by mutations of downstream mediators. *Mut*, common mutations leading to up-regulation of the survival pathways; *Act*, increases in constitutive activation that are not usually due to mutations.

possess a mutant PTEN phosphatase (55). Up-regulation of PI-3K and Akt was shown in these cells after treatment with gefitinib (55). Furthermore, adenoviral infection of wild-type PTEN into MDA-468 cells led to a decrease in activation of the PI-3K/Akt pathway and an improved response to gefitinib (55). These investigators concluded that PTEN dysfunction leaves the PI-3K/Akt pathway unopposed and can thereby bypass EGFR inhibition (55). This genetic mutation can effectively circumvent the ability of EGFR inhibition to down-regulate antiapoptotic signaling.

The Akt pathway seems to have a major role in resistance to EGFR therapy. In one study, erlotinib-resistant clones of human squamous cell cancer cell lines (A431 and HN5) were generated and analyzed (56). Relative to the parental cell lines, the resistant clones had significantly higher levels of total and phosphorylated Akt, suggesting persistent downstream signaling even in the presence of EGFR inhibitors (56). Whether this effect resulted from selection or induction is unclear, but the findings implicate Akt as a major resistance factor for anti-EGFR therapy.

An important clinical trial examining potential markers of resistance in patients receiving anti-EGFR therapy used tissue sampling to assess levels of various surrogate markers of response. Rojo et al. conducted a phase II study evaluating gefitinib therapy for 75 previously treated patients with advanced gastric cancer (57). Patients were randomly assigned to receive either high-dose (500 mg/d) or low-dose (250 mg/d) gefitinib until the appearance of disease progression or unacceptable toxicity. EGFR inhibition was analyzed in endoscopically obtained biopsy samples of the tumor before therapy and on day 28 of treatment (57). Specimens were evaluated for EGFR, phosphorylated EGFR, MAPK, Akt, phosphorylated Akt, and Ki-67 (a marker of cellular proliferation; ref. 57). This novel approach of tumor assessment enabled the investigators to relate the response to therapy with signaling inhibition. In 17 paired tumor specimens, gefitinib significantly inhibited phosphorylation of EGFR and slightly decreased phosphorylated MAPK levels. The extent to

which proliferation was inhibited (indicated by Ki-67) did not depend on the dose of gefitinib. Disease control was achieved in 13 of 75 patients (partial response in 1 patient and stable disease in 12 patients; ref. 57). Tumors that showed low levels of activated Akt before therapy (defined by activity in <50% of tumor cells staining positive) exhibited significantly decreased tumor cell proliferation, represented by reduced levels of Ki-67 (57, 58).

Although EGFR inhibitors have been shown to inhibit the PI-3/Akt pathway, the consistent observation of elevated levels of activated Akt in resistant tumors implicates constitutive activation of Akt as an important mediator of EGFR resistance. As shown by Rojo et al., gastric tumors with high levels of Akt did not demonstrate decreased tumor proliferation in response to gefitinib (57). Future phase II trials with EGFR inhibitors could be directed at patients with EGFR-positive tumors with low levels of Akt (58) because such patients may be most likely to benefit from anti-EGFR therapy. Inhibition of EGFR alone may be inadequate in the subset of patients with tumors that demonstrate constitutively activated Akt. The challenge lies in detecting those tumors in which Akt is activated by EGFR versus those in which Akt is activated by alternative receptor systems or downstream pathways such as loss of function of PTEN. Alternatively, a trial of inhibitors to both EGFR and Akt or PI-3K for such patients could also be justified. Furthermore, the downstream targets of the PI-3K/Akt pathways, such as mTOR, are now being targeted in early-phase clinical trials, and combination of these agents with EGFR inhibitors may provide for rational selection of upstream and downstream mediators of tumor progression and metastasis. However, it is important to recognize that such combinations are speculative in nature and preclinical modeling is imperative to learn more about toxicity as well as potential efficacy.

Dysregulation of other downstream signaling mediators can also lead to constitutive activation of multiple survival pathways that may bypass EGFR inhibition (Fig. 2). Src family kinases (e.g., c-Src) are nonreceptor tyrosine kinases that mediate cell growth and survival. Constitutive activation of Src has been observed in several solid tumors, including colon, breast, ovarian, and pancreatic cancers (13, 59). Elevated Src activation directly correlates with a poor prognosis in colorectal cancer (13, 60). Increased tyrosine kinase activity of Src has been observed in 80% of colorectal cancer specimens relative to normal colonic epithelium (61). How c-Src and other Src family kinases are rendered constitutively active in cancer cells is not clear and even within an individual type of cancer. One potential mechanism is direct interaction with a receptor tyrosine kinase such as EGFR or other members of the erbB family. However, this is not the only mechanism by which Src, or other Src family kinases, can be activated in human cancer cells. Activation of Src by means other than EGFR signaling may bypass the effects of EGFR inhibition. Src may be activated downstream of other receptor tyrosine kinases such as platelet-derived growth factor receptor and cMET or by β_1 integrins (62–64). Activation of Src may also be achieved through elevated tyrosine phosphatase activity, which could remove the phosphate group from the regulatory tyrosine in the carboxyl-terminal tail. Ras mutations have also been identified in numerous tumor types, including lung, colon, and pancreatic cancers (reviewed in ref. 65). Ras mutations can lead to up-regulation of the Raf/MAPK pathway and increased activity of

multiple survival pathways (65). Although it is possible that tumors with high endogenous Ras activity due to mutations (such as pancreatic cancer) are more likely to be resistant to EGFR-directed therapies, this does not seem to hold true as there are promising data with anti-EGFR therapy (in combination with chemotherapy) in patients with pancreatic cancer (66, 67). This again highlights the complexity of cross talk between receptors and signaling intermediates.

Several members of the STAT family are downstream mediators of EGFR. However, STAT-3 can be mutated and constitutively activated in various human cancers such as breast and prostate cancers (68, 69; Fig. 2). In a study examining the contribution of STAT-3 to the progression of head and neck squamous cell cancer, a constitutively activated STAT-3 mutation was stably transfected into head and neck squamous cell cancer lines (70). The transfected cell lines showed increased Bcl-X and cyclin D1 expression (70). Tumors generated from the activated STAT-3 cell lines grew more rapidly *in vivo* than did control tumors. In both *in vivo* and *in vitro* models, the transfected cell lines did not exhibit any growth inhibition in response to EGFR TKI treatment (PD153035) as compared with that of the parental cells (70). This study showed that STAT-3 activation may be another potential resistance mechanism for EGFR antagonists.

Ligand-Independent Activation of EGFR and Resistance to Therapy. EGFR and multiple signaling mediators, including Src and Fak, can be activated through interactions with integrins and their ligands in the extracellular matrix (71, 72). Interactions between either β_1 or β_3 integrin and the receptor tyrosine kinases lead to clustering and activation of receptors (72). Src and p130Cas, a nonenzymatic focal adhesion protein, are required for integrin-associated EGFR phosphorylation. In this model, it is conceivable that the tyrosine kinase activity of EGFR could be activated via a novel pathway involving an interaction with integrins that could bypass the inhibiting effects of an EGFR antibody.

Urokinase plasminogen activator (uPA) receptor, a regulator of extracellular matrix degradation and tissue remodeling, has been implicated in ligand-independent activation of EGFR. Specifically, uPA receptor is a glycosylphosphatidylinositol-anchored receptor on the cell surface that converts pro-uPA to the active protease. uPA receptor can also activate Erk through interacting with $\alpha_5\beta_1$ integrin. This signaling pathway is mediated by EGFR tyrosine kinase phosphorylation, although it is more pronounced with increased uPA receptor expression (71). In a study of squamous carcinoma (T-Hep3) cells that naturally overexpress uPA receptor, EGFR could be activated by uPA receptor in a ligand-independent fashion (71). The addition of the EGFR TKI AG1478 to this system resulted in inhibition of both EGFR phosphorylation and Erk phosphorylation in response to fibronectin. Similar results were obtained with the use of a dominant negative mutant EGFR. Cetuximab, on the other hand, was unable to inhibit this pathway. Furthermore, inhibition of either uPA receptor or integrins led to significantly decreased EGFR phosphorylation and Erk activation. Because overexpression of uPA receptor is common in many malignancies (reviewed in refs. 73–75), this ligand-independent pathway may play a prominent role in resistance to EGFR mAb therapy.

EGFR Mutations and the Sensitivity to EGFR Inhibitors. Mutations of EGFR have been found in various human malignancies, including gliomas, non-small cell lung cancer, and breast and ovarian carcinoma. Of these mutations, EGFR variant III (EGFRvIII) is the best characterized and has been observed in up to 40% of glioblastomas (76). Although EGFRvIII is relatively common in gliomas, it has been documented in other human cancers such as breast and ovarian carcinomas. The EGFRvIII mutation results from an in-frame deletion from exons 2 through 7 in the extracellular domain of EGFR (77). The mutated gene generates a truncated receptor that is constitutively active. Expression of this mutation in U87 glioma cells leads to aggressive tumors that are resistant to chemotherapy (78, 79) and show enhanced growth and metastasis (78, 79).

The response of EGFRvIII to EGFR TKIs seems to be diminished relative to that of the wild-type EGFR. An investigation of gefitinib therapy for EGFRvIII-expressing glioblastomas showed that *in vitro* growth inhibition in response to gefitinib was significantly diminished in EGFRvIII-expressing tumors relative to that of tumors with wild-type EGFR (76). In the EGFRvIII-expressing tumors, phosphorylated Akt also was not inhibited by gefitinib, suggesting that signaling persisted downstream of the mutated receptor (76). The constitutive signaling and resistance to gefitinib in cells with the EGFRvIII mutation likely relate to conformation changes in the mutated receptor that affect the intracellular domain involving the ATP binding sites. No clinical data have been published regarding treatment of EGFRvIII-expressing tumors with EGFR mAbs despite the recent development of antibodies specific for the mutated receptor (77, 80, 81).

Interestingly, activating mutations in the EGFR have been identified that correlate with increased responsiveness to gefitinib in non-small cell lung cancer (Fig. 3; refs. 82, 83). Tumors from patients with non-small cell lung cancer treated with gefitinib at Massachusetts General Hospital since 2000

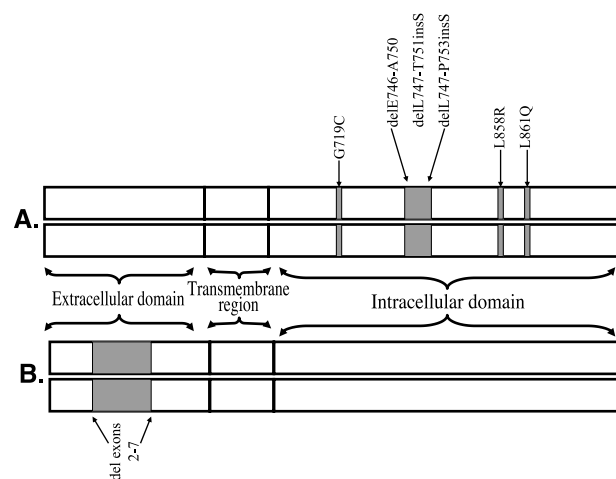


Fig. 3 EGFR mutations. *A*, representation of an activating mutation discovered in non-small cell lung cancer. Activating mutations such as these are all located in the tyrosine kinase domain and probably affect the conformation and activity of the receptor. *B*, EGFRvIII mutation commonly identified in gliomas, with the deleted sequence in the extracellular binding domain. *Shaded areas*, deleted sequences in EGFR.

were investigated for the presence of EGFR mutations (82). (Before being approved by the FDA in 2003, gefitinib was used as part of a compassionate-use expanded-access program.) In eight of nine patients with chemotherapy-refractory disease that responded to single-agent gefitinib therapy, similar heterozygous mutations were identified in the tyrosine kinase domain of EGFR (82). In contrast, no mutations were identified in the seven patients with unresponsive disease. In a separate study seeking EGFR mutations in pretreatment specimens, all five of five lung cancer specimens from patients who responded to gefitinib had similar mutations in the EGFR kinase domain. No mutations in EGFR were observed in specimens from patients whose disease progressed on gefitinib (83). Moreover, the patient characteristics that correlated with an improved clinical response also correlated with the presence of EGFR mutations (82). Specifically, mutations were more common in Japanese patients than in U.S. patients (29% to 2%) and more common in women than in men (20% to 9%). In clinical trials, Japanese and female patients showed higher response rates to gefitinib therapy (29, 30).

To further investigate the function of EGFR mutations, the two most common mutations (deletion L747-P753 and L858R missense) were expressed in SV40-transformed fibroblasts (82). Cell lines expressing either of the mutated EGFR constructs were two to three times more responsive to EGF than were cells with wild-type EGFR and showed prolonged activation after stimulation (82). Furthermore, cell lines expressing either of the mutated EGFR constructs showed a 10-fold greater growth inhibition at similar gefitinib doses than did wild-type EGFR-expressing cells (82). The kinase domain mutations may affect the corresponding protein conformation to increase the affinity for both ATP and gefitinib. The constitutive EGFR activity resulting from these mutations may overwhelm the contribution of other signaling pathways to cell survival, thus allowing the tumor cells to increase their dependence on the EGFR signaling pathway for survival. Thus, in this model, not only do the mutant receptors bind gefitinib with greater affinity but cells expressing these mutants are more sensitive to the inhibitor because of their increased dependence on the EGFR pathway for survival. Identifying EGFR mutations that correlate with other characteristics of non-small cell lung cancer that responded to gefitinib may highlight a possible means of targeting a subset of cancer patients for future trials of EGFR inhibitors. However, as experience with EGFR inhibitors increases in clinical trials and practice, it is likely that mutations will be found in tumors that result in *decreased* response to these agents. Furthermore, many patients without mutations in EGFR responded to therapy, and until further predictive factors are identified, these patients should not be excluded from therapies that target EGFR.

Two seminal publications on EGFR mutations and sensitivity to gefitinib (76, 77) were published just before the annual meeting of the American Society of Clinical Oncology in June 2004. Updates on this topic were presented at a "late-breaking" session at that meeting intended to put findings regarding EGFR mutations into perspective. The investigators cautioned the audience to recognize that data from relatively few patients were reported in the studies and that the mutations found in patients with lung carcinoma were *not* found in several other tumor systems, including colon carcinoma, head and neck cancer, pan-

creatic cancer, and other solid malignancies. Furthermore, retrospective analysis of tumor specimens from patients given gefitinib showed that some patients whose tumors did *not* harbor EGFR mutations also benefited from therapy, albeit at a lower frequency. However, further analysis of other potential genetic mutations within the EGFR gene outside of the kinase domains is currently being undertaken. Another important issue reported in these studies was that ligand binding to the EGFR accentuates the activity of the mutated kinase domain. Therefore, the possibility cannot be ruled out that antibodies that target EGFR would still be beneficial to patients who possess the mutated receptor. The current recommendation is that these agents be used according to the FDA guidelines or in clinical trials in which data can be collected prospectively in conjunction with tissue analyses. Although the oncology community would benefit from knowing whether EGFR mutations were present in all patients treated with EGFR inhibitors, development of high-throughput analyses for genetic mutations for large numbers of patients, in a prospective manner, would be a tremendous challenge. Most patients with lung carcinoma will not be able to have mutations in the EGFR kinase domain analyzed before therapy is begun, or even after therapy is initiated or completed.

These two landmark publications on EGFR mutations in lung cancer are of historic significance, as they clearly show the principle that understanding the molecular basis of malignant disease can improve outcome for patients. However, given the apparent lack of EGFR mutations in other tumor systems and that patients with lung cancer without the mutated EGFR can still benefit from therapy, we must continue to explore other mechanisms of EGFR sensitivity and resistance. One principle from the EGFR mutation studies is that EGFR is the driving force behind aberrant signaling in these tumors. However, as noted above, other growth factors and mutated signaling intermediates may also contribute to aberrant intracellular signaling that contributes to tumor progression and metastasis. Therefore, we must simultaneously investigate several molecular alterations that occur in epithelial malignancies to identify additional predictive factors to better identify patients who are likely to benefit from anti-EGFR therapy.

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