Molecular Mechanisms of Epidermal Growth Factor Receptor (EGFR) Activation and Response to Gefitinib and Other EGFR-Targeting Drugs
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Abstract The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, including EGFR, HER2/erbB2, and HER3/erbB3, is an attractive target for antitumor strategies. Aberrant EGFR signaling is correlated with progression of various malignancies, and somatic tyrosine kinase domain mutations in the EGFR gene have been discovered in patients with non–small cell lung cancer responding to EGFR-targeting small molecular agents, such as gefitinib and erlotinib. EGFR overexpression is thought to be the principal mechanism of activation in various malignant tumors. Moreover, an increased EGFR copy number is associated with improved survival in non–small cell lung cancer patients, suggesting that increased expression of mutant and/or wild-type EGFR molecules could be molecular determinants of responses to gefitinib. However, as EGFR mutations and/or gene gains are not observed in all patients who respond partially to treatment, alternative mechanisms might confer sensitivity to EGFR-targeting agents. Preclinical studies showed that sensitivity to EGFR tyrosine kinase inhibitors depends on how closely cell survival and growth signalings are coupled with EGFR, and also with HER2 and HER3, in each cancer. This review also describes a possible association between EGFR phosphorylation and drug sensitivity in cancer cells, as well as discussing the antiangiogenic effect of gefitinib in association with EGFR activation and phosphatidylinositol 3-kinase/Akt activation in vascular endothelial cells.

The epidermal growth factor receptor (EGFR) is a member of the erbB family of receptor tyrosine kinase proteins, which also includes HER2/ner (erbB2), HER3 (erbB3), and HER4 (erbB4). These receptors are composed of an extracellular ligand-binding domain, a transmembrane lipophilic domain, and an intracellular tyrosine kinase domain and, with the exception of HER2, all bind to receptor-specific ligands (Fig. 1A and B). Phosphorylation of the tyrosine kinase domain followed by homodimerization or heterodimerization between different receptors of the same family leads to protein activation (1). Receptor dimerization is promoted by ligand binding, high receptor density from overexpression, and mutations in the kinase domain. Protein activation on the cell surface of cancer cells is believed to promote signaling cascades, cell growth, differentiation, cell survival (apoptosis), drug and radiation sensitivity, cell cycle progression, and angiogenesis (Fig. 1A).

For cancer cells, various mechanisms of EGFR activation are now shown: overexpression of ligands and receptors, EGFR gene gain, and activating mutations. Under physiologic conditions, specific soluble ligands bind to the extracellular domains of EGFR, HER3, and HER4, but no ligand has been identified for HER2 (Fig. 1A). Of these ligands, EGF and transforming growth factor-α (TGF-α) selectively bind to EGFR, following dimerization as a homodimer or as a heterodimer with other members, and undergo autophosphorylation at specific tyrosine residues within the intracellular domain (Fig. 1B). This autophosphorylation activates downstream signaling pathways, including the Ras/Raf/mitogen-activated protein kinase pathway [extracellular signal-regulated kinase (ERK) 1/2], the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and the signal transduction and activator of transcription (STAT), and other pathways (Fig. 1A). ERK1 and ERK2 regulate cell survival and proliferation, whereas Akt as well as signal transduction and activator of transcription rather specifically regulate cell survival and apoptosis.

Recently, novel anticancer drugs targeting EGFR family members and other growth factor receptors have been developed. One of the EGFR tyrosine kinase inhibitors, gefitinib (Iressa), shows a highly specific affinity for EGFR and exerts its antitumor effects through inhibition of cell signaling(s) in cancer cells (2–4). EGFR and/or HER2 are highly expressed in many tumor types of epithelial origin, including breast, head and neck, bladder cancers, and non–small cell lung cancer (NSCLC; ref. 3). Expression of high levels of EGFR and/or HER2 has been associated with a poor prognosis, especially in NSCLC patients (5).

The discovery of EGFR mutations with or without gene gain has enabled an understanding of how to treat certain NSCLC...
patients with EGFR-targeting drugs from the standpoint of evidence-based therapeutic strategies. NSCLC patients responding to gefitinib or another EGFR-targeting drug, erlotinib, often carry various somatic mutations in the EGFR gene (4). Most of the identified mutations are located within exons 18 to 21, as activating EGFR mutations. A point mutation in exon 21 (L858R) and a deletion mutation in exon 19 (del E746-T751) offer a predictive marker for improved therapeutics with gefitinib or erlotinib. Moreover, the extent of EGFR gene gain also plays a critical role in the therapeutic efficacy of such drugs (6). However, it is also known that not all patients with EGFR mutations or gene gains are susceptible to EGFR-targeting drugs (6–10). Successful therapy by such EGFR-targeting drugs could be expected for patients whose EGFR family members are amplified, mutated, or overexpressed in cancer cells (11). In this article, we will discuss how the EGFR family of proteins could be specifically associated with drug sensitivity or the therapeutic efficacy of EGFR-targeting drugs.

**Activation of EGFR Downstream Signaling Molecules, K-ras Mutation, and EGFR Gene Gain**

EGFR mutation or EGFR gene gain is associated with a more favorable outcome following treatment with EGFR-targeting drugs, such as gefitinib or erlotinib (6, 12). In preclinical studies, we discovered that drug sensitivity to gefitinib is closely correlated with EGFR-dependent ERK1/2 and Akt activation (13). PC9, which harbors a deletion (del 746-750) in exon 19 of EGFR, was shown to be the most sensitive of nine lung cancer cell lines to growth inhibition by gefitinib and showed the closest coupling of growth arrest and Akt/ERK1/2 activation inhibition (Fig. 2A and C). Consistent with this finding, Paez et al. (8) reported that the lung cancer cell line H3255 harboring the L858R mutation in EGFR exon 21 was also highly sensitive to gefitinib (Fig. 2B and C) and also that both Akt and ERK1/2 pathways in H3255 were highly susceptible to the inhibitory effect of gefitinib. Increasing drug sensitivity to gefitinib of cancer cells harboring...
mutant EGFRs thus depends on how closely EGFR-driven signaling pathways (ERK1/2, Akt, and signal transduction and activator of transcription) are coupled with cell survival (apoptosis) and cell growth.

To understand in more detail which EGFR-driven signaling is specifically responsible for gefitinib-induced cytotoxicity and therapeutic efficacy, Sordella et al. (14) generated isogenic cell lines that expressed either wild-type (WT) EGFR or mutant EGFRs (L858R and del 746-750). Mutant EGFR selectively activated Akt and STAT 5 signaling, but not ERK1/2, to promote cell survival in lung cancer cells. Immunohistochemical analysis on advanced NSCLC showed

![Diagram](image-url)
Fig. 3. Formation of the HER2/HER3 or the EGFR/HER3 heterodimer enhances both PI3K/Akt activation and cellular sensitivity to EGFR-targeting drugs, including gefitinib. A, EGFR-targeting drugs preferentially inhibit Akt phosphorylation in a dose-dependent manner in LK2/HER2/HER3/EGFR cells but not in LK2/HER3/EGFR cells. B, gefitinib inhibits the formation of HER2/HER3 heterodimers, the association of HER3 with p85α, and the concomitant inhibition of HER3 tyrosine phosphorylation in LK2/HER2/HER3/EGFR cells. C, hypothetical model showing how overexpression of EGFR family proteins confers cellular sensitivity to EGFR-targeting drugs. In addition to experimental data of (A) and (B; ref. 30), coexpression of HER3 with HER2 and/or EGFR was shown to confer gefitinib sensitivity to cancer cells (13, 26, 34). HER3 also mediated PI3K/Akt activity through heterodimer formation with EGFR (WT and mutant type) in gefitinib-sensitive cancer cells but not in gefitinib-resistant cells (34). In this model, heterodimer formation of HER2/HER3 or EGFR/HER3 activates PI3K/Akt pathway that plays a pivotal role in drug sensitivity to EGFR-targeting drugs.
that patients with phosphorylated AKT–positive tumors have a better response rate, disease control rate, and time to progression than patients with phosphorylated AKT–negative tumors when treated with gefitinib (15). The Akt signaling pathway activated by EGFR harboring activating mutations or gene gain is rather more specifically involved in enhanced drug sensitivity and therapeutic efficacy than the ERK1/2 pathway, suggesting phosphorylated AKT as one of the molecular determinants of response to EGFR-targeting drugs. However, further study is required to determine how phosphorylated AKT expression can be applied to determination of the clinical therapeutic efficacy of gefitinib (16). Furthermore, recent studies showed that NSCLC patients with a EGFR mutation of del 746-750 had a longer median survival than patients with the L858R point mutation when treated with gefitinib or erlotinib (17, 18), suggesting some differences in activating EGFR signaling by each EGFR mutation.

K-ras is a downstream mediator of EGFR-induced cell signaling, and ras mutations confer constitutive activation of the signaling pathways without EGFR activation. Growing evidence indicates that K-ras mutations are important in the development of lung carcinomas (19). Pao et al. (20) examined 60 lung adenocarcinoma patients and showed that K-ras mutations are associated with a lack of sensitivity to gefitinib or erlotinib. K-ras mutations seem to be resistant to EGFR-targeting agents and are reported to be mutually exclusive to EGFR or HER2 gene mutations (21).

Increased EGFR gene gain is closely associated not only with gefitinib sensitivity but also with improved survival following gefitinib treatment in patients with advanced bronchoalveolar carcinoma (6, 22). EGFR gene gain is often observed in lung

Fig. 4. Correlation of the expression of four EGFR family members with drug sensitivity to gefitinib in human cancer cell lines. Western blot analysis was done using 50 μg total cellular protein, and growth inhibition assays were done using various concentrations of gefitinib in culture. Average IC_{50} values (μM) are presented from duplicate experiments. A, human cancer cell lines contain various lung cancer lines and two epidermoid carcinoma cell lines (A431 and KB3-1). Modified with permission (13). B, human cancer cell lines contain mainly breast cancer lines and other cell lines derived from various tumor types. Modified with permission (25).
cancers, and increased copy number is more frequently seen in patients harboring \textit{EGFR} mutations than those with the WT gene. Although the response rate to gefitinib for patients harboring mutations was 82%, it should be noted that amplified WT \textit{EGFR} was also associated with responses in some 11% of patients (23). Patients with an amplified mutant allele are thus expected to receive more benefit from gefitinib than those with an amplified WT allele (10, 23).

\begin{figure}
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\includegraphics[width=\textwidth]{fig5.png}
\caption{Inhibition of angiogenesis is also responsible for the antitumor effect of gefitinib. A, angiogenesis induced by EGF in mouse corneas is inhibited by gefitinib and partially by SU5416. a, hydron alone, control; b, EGF (200 ng); c, EGF with gefitinib (50 mg/kg/d, i.p.); d, EGF with SU5416 (25 mg/kg/d, i.p.). B, quantification of corneal neovascularization in mice after treatment with gefitinib or SU5416. a, drug administered i.p.; b, drug administered in pellet. *, \(P < 0.05\), significant difference from value for EGF alone. C, a model of EGF/TGF-\(\alpha\)-induced angiogenesis and gefitinib inhibition of the tumor angiogenesis process. Cancer cells often produce TGF-\(\alpha\), which induces the production of potent angiogenic factors, VEGFs, interleukin-8 (IL-8), matrix metalloproteinases (MMP), plasminogen activators (PA), platelet-derived growth factors (PDGF), and other factors. EGF/TGF-\(\alpha\) also directly activates EGFR in endothelial cells both in normal vessels and in tumor neovascularatures, which induce angiogenesis. Inhibition sites of gefitinib are indicated, suggesting that dual pathways are affected by gefitinib.}
\end{figure}
HER2 and Other EGFR Family Proteins

HER2 as well as EGFR is highly expressed in several solid tumors. For example, overexpression of the HER2 protein was reported in 20% to 30% of breast cancers, and EGFR is also overexpressed in ~40%. HER2 gene gain has been reported to be an independent predictive marker for overall survival and disease-free survival in node-positive patients (24). One representative molecular targeting drug is the human monoclonal antibody against HER2, trastuzumab (Herceptin), which improves the outcome of HER2-positive breast cancer. HER2 overexpression in various cancer cell lines or tumor xenografts also increases cytotoxicity and/or the antitumor effects of gefitinib (25, 26). In NSCLC patients, Cappuzzo et al. (27) reported that an increased HER2 gene gain is associated with gefitinib sensitivity in EGFR-positive patients. Those patients whose tumors had high HER2 copy number and EGFR mutation had the best objective response (53.8%) and disease control rate (76.9%), suggesting that HER2 fluorescence in situ hybridization analysis is a valuable method for selecting patients for tyrosine kinase inhibitor therapy. On the other hand, activating HER2 mutations, including an exon 20 point mutation (G776L), have been reported in lung adenocarcinomas (28). A NSCLC patient harboring mutations in both HER2 (G776L) and EGFR (A859T) experienced a response after treatment with trastuzumab, despite disease progression after prior tyrosine kinase inhibitor therapy (29). Both HER2 gene gain and mutation thus might be critical for cancer cell survival in NSCLC.

Although HER3 is unique among the EGFR family because it lacks tyrosine kinase activity, its six tyrosine phosphorylation sites effectively couple the protein to the PI3K/Akt pathway by providing excellent binding sites for PI3K/PI3K regulatory subunit of PI3K, p85α, through dimerization with HER2, which lacks the appropriate bindings site(s) for p85α (31, 32). HER3 exhibited a high level of basal tyrosine phosphorylated AKT in LK2/HER2/HER3/EGFR cells, whereas phosphorylated ERK1/2 was not inhibited in either LK2/HER2/EGFR or LK2/HER2/HER3/EGFR cells. This is suggestive of selective inhibition of Akt activation by gefitinib (Fig. 3A; ref. 30). HER3 efficiently recruits the regulatory subunit of PI3K, p85α, through dimerization with HER2, which lacks the appropriate bindings site(s) for p85α (31, 32). HER3 exhibited a high level of basal tyrosine phosphorylated AKT in LK2/HER2/HER3/EGFR cells, whereas phosphorylated ERK1/2 was not inhibited in either LK2/HER2/EGFR or LK2/HER2/HER3/EGFR cells. This is suggestive of selective inhibition of Akt activation by gefitinib (Fig. 3A; ref. 30). HER3 efficiently recruits the regulatory subunit of PI3K, p85α, through dimerization with HER2, which lacks the appropriate bindings site(s) for p85α (31, 32). HER3 exhibited a high level of basal tyrosine phosphorylated AKT in LK2/HER2/HER3/EGFR cells, whereas phosphorylated ERK1/2 was not inhibited in either LK2/HER2/EGFR or LK2/HER2/HER3/EGFR cells. This is suggestive of selective inhibition of Akt activation by gefitinib (Fig. 3A; ref. 30). Similar results have been reported by Moulder et al. (33) with human breast cancer cell lines. Engelman et al. (34) reported that HER3 was associated with PI3K exclusively in gefitinib-sensitive NSCLC cell lines harboring either WT or mutant (L858R and del 747-749) EGFR. Gefitinib dissociated this complex and released p85α in gefitinib-sensitive cell lines. HER3 thus activates PI3K/Akt signaling through dimerization with either EGFR or HER2 molecule only in gefitinib-sensitive cancer cells (Fig. 3C).

Moreover, concomitant overexpression of both HER2 and HER3 was detected in two of eight lung cancer cell lines in culture, and these two cell lines were found to be highly susceptible to gefitinib (13), again suggesting the close association of HER2 and HER3 coexpression with gefitinib sensitivity (Fig. 4A). Moasser et al. (25) also reported that four human breast cancer cell lines overexpressing HER2 together with HER3 expression were more sensitive to gefitinib than the other cell lines examined (Fig. 4B). This close correlation of HER2 overexpression with gefitinib sensitivity therefore occurs in both lung (Fig. 4A) and breast cancer cells (Fig. 4B). Gefitinib inhibition of cell growth is possibly mediated through blockage of HER2/EGFR and/or HER3/EGFR heterodimer formation (26). HER2 expression is thus expected to play a pivotal role in the therapeutic efficacy not only of HER2 monoclonal antibodies (35) but also of EGFR tyrosine kinase inhibitors.

EGFR-targeting drugs could overcome accumulating resistance to trastuzumab in human breast cancer cells, plausibly through modulation of PI3K/Akt signaling (36). Because EGFR and other family members are often overexpressed in various other tumor types, the notion of how EGFR-targeting drugs show their therapeutic efficacies against lung and breast cancer would be applicable to the further development of such therapeutic strategies against other tumor types.

Other Molecular Determinants of Responses to Erlotinib, Cetuximab, and Gefitinib

Even with the notable responses conferred by EGFR inhibitors where there are activating mutations, the responses are not always durable, and there remain patients who do not have responses at all, so that additional strategies are needed to increase the effectiveness of EGFR-targeted therapy (12). Gefitinib is the first EGFR-targeting drug to be registered for advanced NSCLC followed by erlotinib, which possesses slightly different pharmacologic characteristics. On the other hand, cetuximab, a chimeric monoclonal antibody targeting EGFR, has been registered for the treatment of metastatic colorectal cancer (37). Mukohara et al. (38) have reported differential effects of gefitinib and cetuximab against NSCLC cells harboring activating EGFR mutations. Whereas activating mutations were associated with sensitivity to gefitinib but not to cetuximab, one particular deletion mutant, del E746-A750, was associated with sensitivity to cetuximab. However, little clinical experience has been gained in the use of cetuximab in advanced NSCLC and other malignancies. Other EGFR-targeting treatments, including monoclonal antibodies, small molecules, and vaccines, are now in clinical trials (39).

Combining EGFR-targeting drugs with anticancer agents could modify the characteristics of drug sensitivity in ways that might be unique for each drug type. Cooperative growth inhibition is often observed following a combination of EGFR-targeting drugs against various cancer cell types (40–43). Huang et al. (40) showed that combining cetuximab with either gefitinib or erlotinib synergistically enhanced growth inhibition in head/neck cancer cells and other tumors.
types both in vitro and in vivo with a concomitant inhibition of EGFR phosphorylation. Furthermore, cetuximab resistance could be overcome by combination with erlotinib or gefitinib. The combination of cetuximab and erlotinib blocked erlotinib-induced EGFR up-regulation, resulting in apoptosis and growth inhibition of biliary tract cancer cells (42). Drug sensitivity and resistance could be regulated through common mechanisms among various EGFR-targeting drugs, such as protein expression levels, gene mutation, and gene gain of EGFR.

Van Schaeybroeck et al. (44) reported that EGFR activity contributes to colorectal cancer cell response to gefitinib alone and in combination with chemotherapeutic drugs. Colorectal cancer cell lines with high constitutive EGFR phosphorylation were found to be more sensitive to gefitinib than those with low EGFR phosphorylation. In addition, treatment with oxaliplatin or 5-fluorouracil increased EGFR phosphorylation; in those cell lines, a combination of treatment with gefitinib resulted in a synergistic effect on growth inhibition. This study strongly indicates EGFR phosphorylation levels in the absence or presence of chemotherapeutic agents as a plausible surrogate marker for therapeutic responses by EGFR-targeting drugs alone or in combination with chemotherapy.

### Antiangiogenic Effect of Gefitinib through PI3K/Akt Pathway

EGF and TGF-α are themselves known to be angiogenic factors (45, 46), and they also up-regulate expression of potent angiogenic factors, vascular endothelial growth factor (VEGF) and interleukin-8, in cancer cells (47, 48). EGFR activation is often linked to angiogenesis as well as to invasion and metastasis, all processes thus able to be affected by EGFR antagonists (see Fig. 1). We investigated whether the antitumor effect of gefitinib was partly attributable to antiangiogenic activity. EGF markedly induced angiogenesis in an avascular area of the mouse cornea at similar levels to VEGF, and this EGF-induced neovascularization was almost completely blocked by gefitinib (Fig. 5A; ref. 47). Moreover, EGF-induced production of the angiogenic factors interleukin-8 and VEGF was almost completely blocked by gefitinib in cancer cells and was partially inhibited by SU5416, a selective inhibitor of VEGF receptor tyrosine kinases (Fig. 5B). We recently reported that the EGF/TGF-α–dependent up-regulation of angiogenic factors, such as VEGF and interleukin-8, is specifically mediated through PI3K/Akt activation rather than through ERK1/2 in cancer cells (48). Expression of EGFR was also reported in tumor-associated endothelial cells (49, 50) and endothelial cells of neovasculatures (51), suggesting that endothelial cells could be one of the targets for anticancer therapy by EGFR-targeting drugs. The antiangiogenic effects of gefitinib could be mediated directly by blocking EGF-induced neovascularization and also indirectly by inhibition of VEGF or interleukin-8 production.

Treatment with anti-VEGF monoclonal antibody bevacicizumab (Avastin) in combination with anticancer agents provided the first clear demonstration of better survival outcomes over chemotherapy alone in patients with advanced colorectal cancers (52). VEGF and EGF exert their biological effects directly or indirectly on tumor growth and metastasis/invasion as well as on tumor angiogenesis. The biological effects of VEGF and EGF are mediated through activation of their specific downstream signalings, but both factors also share common downstream signaling pathways. This is thus the potential for improved therapeutic efficacy by combination of both EGF/EGFR–targeting and VEGF/VEGF receptor–targeting drugs. Clinical trials of combinations of these molecular targeting drugs have been applied to lung cancer and other tumor types (53, 54). Herbst et al. (55) have evaluated bevacizumab in combination with erlotinib for NSCLC in a phase I/II trial and observed encouraging antitumor activity and safety, supporting further development of this combination (55). Furthermore, several clinical trials with VEGF receptor tyrosine kinase inhibitors are also now in progress (53–55). These VEGF receptor tyrosine kinase inhibitors, such as vatalanib (PTK787/ZK222584), semaxanib (SU5419), sorafenib (BAV439006), and zactima (ZD6474), can also inhibit the tyrosine kinase activities of EGFR, platelet-derived growth factor receptor, c-Kit, Raf, and Fli-1; these drugs are thus considered multitargeted tyrosine kinase inhibitors. Of these multitargeted drugs, ZD6474, for example, has a potent inhibitory activity not only on VEGF receptor-2 tyrosine kinase of vascular endothelial cells but also on EGFR tyrosine kinase of cancer cells, resulting in the suppression of tumor angiogenesis, tumor growth, and invasion/metastasis. Whether the multitargeted therapeutic approach or the combination of selective targeting agents will have better therapeutic efficacy against each human tumor type is a matter of debate.

### Conclusion

In preclinical studies, HER2 and/or HER3 expression can sensitize cancer cells to gefitinib. Moreover, Akt activation following HER2/HER3 heterodimer formation seems to play a pivotal role in the sensitivity to EGFR-targeting drugs. EGFR-targeting drug sensitivity is largely dependent on the extent to which Akt or STAT activation as well as ERK1/2 activation is associated with EGFR-induced cell survival and cell growth in each cancer. Gene mutations, gene gains, and expression levels lead to EGFR activation without ligand binding, resulting in altered sensitivity to EGFR-targeting drugs. To predict the therapeutic efficacies of gefitinib and other EGFR-targeting drugs, standard assay systems, such as immunohistochemistry and fluorescence in situ hybridization, are required to evaluate EGFR mutations, gene gain, and protein expression levels. We expect that the combination of various tyrosine kinase inhibitors or multitargeted inhibitors might have better therapeutic benefits.

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