

Review Article

Mechanisms of Resistance to EGFR TKIs and Development of a New Generation of Drugs in Non-Small-Cell Lung Cancer

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Gefitinib and erlotinib, which are epidermal growth factor receptor- (EGFR-) specific tyrosine kinase inhibitors (TKIs), are widely used as molecularly targeted drugs for non-small-cell lung cancer (NSCLC). Currently, the search for *EGFR* gene mutations is becoming essential for the treatment of NSCLC since these have been identified as predictive factors for drug sensitivity. On the other hand, in almost all patients responsive to EGFR-TKIs, acquired resistance is a major clinical problem. Mechanisms of acquired resistance reported in the past few years include secondary mutation of the *EGFR* gene, amplification of the *MET* gene, and overexpression of HGF; novel pharmaceutical agents are currently being developed to overcome resistance. This review focuses on these mechanisms of acquired resistance to EGFR-TKIs and discusses how they can be overcome.

1. Introduction

The epidermal growth factor receptor- (EGFR-) specific tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib are molecularly targeted drugs used for the treatment of non-small-cell lung cancer (NSCLC). In clinical trials, although response rates were approximately 10%–19%, in some cases dramatic responses have been observed soon after initiation of treatment, with this trend being particularly strong in Japanese patients, women, nonsmokers, and adenocarcinoma cases [1, 2]. In 2004, three research groups reported that the existence of the activating mutations of *EGFR* gene was a predictive factor for sensitivity to EGFR-TKIs [3–5]. Deletion mutations, mainly occurring around codons 746–750 in exon 19, and the substitution of leucine with arginine at codon 858 in exon 21 (L858R) comprise approximately 90% of these mutations [6]. These mutations are more prevalent in Asians, women, non smokers, and patients with adenocarcinoma, groups that match the highly gefitinib-sensitive clinical subset [6]. Many investigators have reported results from retrospective analyses of associations between

EGFR gene mutations and EGFR-TKI sensitivity. These analyses indicate that approximately 70%–80% of mutation-positive cases are EGFR-TKI sensitive whereas in wild-type patients the response rate is 10%–20% [6].

In recent years, three important findings have been reported regarding *EGFR* gene mutations and gefitinib treatment by Asian groups. First, in the IPASS trial, gefitinib treatment was compared with carboplatin and paclitaxel combination therapy in untreated East Asian patients with advanced pulmonary adenocarcinoma who were non-smokers or former light smokers [7]. The gefitinib group had a longer progression-free survival (PFS) than the carboplatin–paclitaxel group among all patient groups (hazard ratio for progression or death, 0.74). In the subgroup of patients who were positive for *EGFR* gene mutations, PFS was significantly longer among those who received gefitinib than among those who received carboplatin–paclitaxel therapy (9.5 months versus 6.6 months). Additionally, two Japanese groups reported the results of Phase 3 comparative clinical trials of gefitinib treatment and combined platinum-based treatment for *EGFR* gene mutation-positive patients. Both the WJTOG3405 [8]

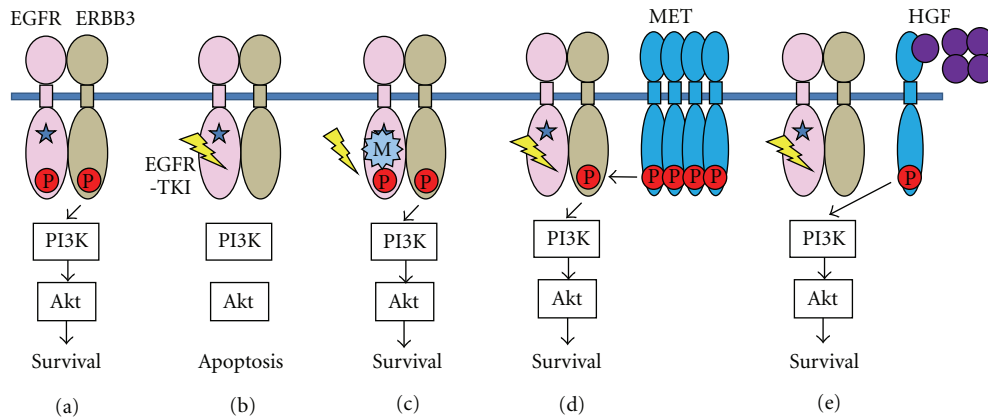


FIGURE 1: Mechanism of acquired resistance to EGFR-TKI (modified from reviews of Mitsudomi and Yataba [6] and Yano [13]). (a) Survival signal through the PI3K/Akt pathway in NSCLC cells with an EGFR activating mutation (star). (b) EGFR-TKI inhibits phosphorylation of EGFR and the survival signal is shut down, leading to apoptosis of cells. (c) Secondary T790M mutation prevents binding of EGFR-TKI to EGFR, resulting in cell survival. (d) Amplified MET causes phosphorylation of ERBB3. Even when phosphorylation of EGFR is inhibited by EGFR-TKI, activation of the PI3K/Akt pathway is maintained through ERBB3. (e) HGF induces activation of the PI3K/Akt pathway through MET; this activation is independent of ERBB3 or EGFR.

and NE J002 trials [9] showed better PFS for the gefitinib group (9.2 months versus 6.3 months and 10.4 months versus 5.5 months, resp.).

Although EGFR-TKI treatment shows good response rates and PFS in NSCLC patients with *EGFR* gene mutations as mentioned above, acquired resistance to EGFR-TKI treatment almost always develops after a median of approximately 10 months from the initiation of treatment. To date, several major mechanisms of acquired resistance, such as secondary mutation of the *EGFR* gene, amplification of the *MET* gene, and overexpression of HGF, have been reported and advances in the development of effective pharmaceutical agents against these mechanisms are being made. *EGFR* gene mutations such as exon 20 insertions [10, 11] and *KRAS* gene mutations [12] are believed to contribute to primary resistance to EGFR-TKI treatment. This review focuses on recent findings regarding the mechanisms of acquired resistance after initial response to EGFR-TKI therapy and discusses how they can be overcome.

2. Acquired Resistance

2.1. Secondary T790M Mutation of the EGFR Gene

2.1.1. About the Secondary T790M Mutation. A secondary mutation of the *EGFR* gene reported in 2005 was the first mechanism of acquired resistance to EGFR-TKIs to be identified [14–16]. When threonine-to-methionine mutations in codon 790 (T790M) in exon 20 of the *EGFR* gene occur additively as a secondary mutation, drug resistance is observed despite the occurrence of drug-sensitive activating mutations (Figure 1(c)). Crystal structure modeling has revealed that T790 is located in the ATP-binding pocket of the catalytic region and appears to be critical for the binding of erlotinib and gefitinib. T790 is often referred to

as the “gatekeeper residue.” Substitution of the threonine at this codon with a bulkier residue, such as methionine, is believed to sterically hinder the binding of these drugs. This amino acid change is not expected to interfere with ATP binding and, therefore, is not expected to alter the activity of the kinase on ligand stimulation [14]. A recent analysis showed that T790M mutations do not considerably affect the binding affinity between EGFR and EGFR-TKIs but instead increase the binding affinity between EGFR and ATP, causing a relative decrease in binding with EGFR-TKIs [17]. The authors reported that increased ATP affinity is the primary mechanism by which the T790M mutation confers drug resistance. An experiment using cell lines transfected concurrently with activating mutations and a T790M mutation also proved that resistance to gefitinib and erlotinib is evident when this mutation is present [14–16].

Analogous secondary mutations of the *BCR-ABL* gene in case of chronic myelogenous leukemia (CML) [18] and the *KIT* gene in case of gastrointestinal stromal tumor [19] have previously been reported as mechanisms of imatinib resistance, which is also a TKI. The structural similarity between ABL and EGFR tyrosine kinases is considerably high, and T315I in *ABL* corresponds to T790M in *EGFR* [20]. Using BaF3 cells, Azam et al. revealed that a gatekeeper threonine mutation stabilizes an active conformation of these tyrosine kinases through a network of hydrophobic interactions known as the hydrophobic spine and has a transforming function [21]. The authors anticipated that this regulatory mechanism would be conserved across the kinase family.

In summary, from the above three reports and subsequent reports on T790 mutations using clinical specimens from patients with acquired resistance to EGFR-TKIs [22–24], a secondary T790M mutation has been detected in approximately 50% of all patients.

2.1.2. Occurrence of T790M Mutations. Interestingly, T790M mutations exist as minor clones in almost all reported cases of acquired resistance. In every case, the results of sequence analysis or subcloning analysis indicate that the T790M mutant alleles or clones were lesser in number than wild-type alleles or clones. To address this issue, Inukai et al. proposed a clonal selection model of T790M mutant cells [25]. They used a highly sensitive method, that is, a mutant-enriched PCR assay to analyze clinical specimens from 280 sample cases who were not treated with gefitinib, and they detected 9 cases (3.6%) with a T790M mutation. Mutation was detected by direct sequencing in only one of the 9 cases; therefore the fraction of T790M mutant cells may have been considerably small. Inukai et al. hypothesized that gefitinib treatment may lead to the selection of T790M mutant cells and that even a small fraction of T790M-positive tumor cells at the beginning of treatment could lead to clinical gefitinib resistance because of selective proliferation of T790M mutant cells. Maheswaran et al. proved this hypothesis by analyzing circulating tumor cells using a highly sensitive method [26]. They analyzed pretreatment tumor samples from 26 NSCLC cases with activating *EGFR* gene mutations and found low levels of T790M in 10 of these cases (38%). Because detection of T790M required a relatively high number of amplification cycles, the T790M mutation has been inferred to be present in only few cells. When PFS was analyzed among these 26 cases after administering EGFR-TKI, the PFS for cases with T790M mutations during pretreatment was significantly shorter compared to that for cases without T790M mutations (median 7.7 months versus 16.5 months, $P < .01$). Given these results, the researchers inferred that the T790 mutant cells that existed as minor clones might be selected during treatment and become dominant after treatment.

This is analogous to the mechanism used by certain secondary mutations of the *ABL* genes in CML [27, 28]. Pre-existing small fractions of mutations become major clones when the tumor becomes resistant to imatinib, probably because of the selection pressure due to imatinib treatment [28].

Although rare, T790M mutations exist as major clones irrespective of gefitinib administration in certain patients [29, 30]. T790M mutations have also been reported to exist as germline mutations in families with a history of high incidence of familial lung cancer [31]. Vikis et al. reported that standalone T790M mutations exhibit increased kinase activity. Furthermore, the human bronchial epithelial cell line that transfects T790M mutations has a higher growth advantage than wild-type cells, although not as high as that of deletion mutations [32]. Mulloy et al. showed that the T790M mutant exhibits tyrosine phosphorylation levels comparable to those of wild-type EGFR, whereas the T790M/L858R double mutant exhibits a substantial increase in phosphorylation compared with the L858R mutant alone [33]. Godin-Heymann et al. also reported similar phenomena for both L858R mutations and a deletion mutation of exon 19, and discussed combination of activating mutations and T790M mutations is important to oncogenic performance [34]. Maheswaran et al. proposed that T790M may initially arise because of these oncogenic characteristics and

may rapidly emerge as a dominant allele after treatment [26].

2.2. *MET* Gene Amplification. In 2007, a research group at the Dana-Farber Cancer Institute reported that *MET* gene amplification is involved in acquired resistance to gefitinib [24]. The *MET* gene encodes a transmembrane tyrosine kinase receptor that acts as an HGF receptor and is involved with invasion, metastasis, and angiogenesis in tumors. The authors established gefitinib-resistant clones from a sensitive cell line with *EGFR* exon 19 deletion mutations (HCC827) by exposing the cells to increasing concentrations of gefitinib. In these resistant cells, T790M mutations were not observed but *MET* gene amplification occurred. *MET* causes phosphorylation of ERBB3, which in turn sustains the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signal downstream. Because of these phenomena, even with gefitinib inhibiting the phosphorylation of ERBB3 by EGFR, the proliferation signal is not inhibited because of the maintenance of the phosphorylation of ERBB3 by *MET*, which causes the cells to become resistant to gefitinib (Figure 1(d)). Conversely, when the *MET* signal was suppressed in resistant cells that were dependent on *MET* amplification, sensitivity to gefitinib was clearly restored. In the analysis of clinical specimens from 18 cases of acquired resistance, *MET* gene amplification was observed in 4 cases (22%), and T790M mutations were present in 10 of 18 cases, more than half as many as were expected. The coexistence of *MET* gene amplification and a T790M mutation was reported in one of these cases.

Bean et al. also focused on *MET* gene amplification by comparing genomic profiles of *EGFR* mutant tumors from untreated patients with those from patients with acquired resistance using array-based comparative genomic hybridization [35]. In an analysis of 43 patients with acquired resistance, *MET* gene amplification was observed in 9 cases (21%). In addition, 4 of the 10 tumors with *MET* gene amplification had a T790M mutation. These results indicate that *MET* gene amplification and T790M mutation may possibly occur independently. Combining both reports, *MET* gene amplification is shown to be present in approximately 20% cases of acquired resistance.

MET gene amplification in cases not treated with EGFR-TKI is said to occur with a frequency in the range of a few percent in all reports [35–38] but one [39], where it was reported to be approximately 20%. This leads one to think of the possibility that the few cells that have undergone *MET* gene amplification before treatment become major clones after EGFR-TKI treatment, similar to the previously described occurrence of T790M mutations. Turke et al. performed an analysis of lung cancer patients and cell lines using the high-throughput FISH method and identified a subpopulation of cells with *MET* gene amplification before drug exposure [40]. Particularly in the HCC827 cell line, they discovered that the subpopulation with pre-existing *MET* gene amplification was rapidly selected when hepatocyte growth factor (HGF) was administered along with EGFR-TKI. In analysis of clinical specimens having acquired resistance, *MET* gene amplification was observed in 4 out of 27 cases (15%) (T790M mutations were observed in 15 cases, 55%). Analysis

of the pretreatment specimens from those 4 cases indicated that the subpopulation with *MET* gene amplification was very small, less than 1% of the entire population. This proves that clonal selection occurs in the mechanism of *MET* gene amplification, as with the T790M mutation.

2.3. Overexpression of HGF. In 2008, a Japanese research group reported that overexpression of HGF, a specific ligand of MET, is involved in EGFR-TKI resistance [41]. They administered HGF to human adenocarcinoma cell lines that harbored *EGFR* exon 19 deletion mutations and showed that HGF induces resistance to gefitinib in a dose-dependent manner. Furthermore, by analyzing intracellular signal transduction pathways, HGF was shown to induce restoration of the PI3K/Akt signaling pathway through phosphorylation of MET. Interestingly, although *MET* gene amplification activates downstream signals by associating with ERBB3, HGF induces downstream signal activation through MET; this activation is independent of ERBB3 or EGFR (Figure 1(e)). Analysis using clinical specimens also showed cases of high HGF expression among acquired resistance cases that did not have a T790M mutation or *MET* amplification as well as among cases that exhibited primary resistance despite having EGFR-TKI sensitive activating *EGFR* gene mutations.

Turke et al. reported similar results [40]. They analyzed 27 acquired resistance cases and found that in the 16 cases for which pre- and posttreatment specimen pairs were available, HGF expression was significantly higher in the drug-resistant specimens than in the pretreatment specimens. They also found that in the 11 cases where only the specimens postresistance acquisition were available, HGF expression values were high and similar in value to those of the postresistance acquisition specimens in the paired cases. In addition to inducing EGFR-TKI standalone resistance, Yano et al. [41] found that HGF induced drug resistance by selective proliferation of clones with *MET* gene amplification. Although the frequency of HGF expression among cases of acquired resistance is unclear because of scarcity of reports, the frequency of coexistence of HGF expression with T790M mutations could possibly be high [42].

2.4. Other Mechanisms of Acquired Resistance

2.4.1. Other Secondary EGFR Gene Mutations. Other secondary *EGFR* gene mutations such as D761Y [22], L747S [43], and T854A [44] mutations have been reported. However, the frequency of all such mutations appears to be low in comparison with the T790M mutation and further analysis of such cases is required in the future.

2.4.2. IGF-1R. Guix et al. established gefitinib-resistant clones from a gefitinib-sensitive cell line of squamous cell lung carcinoma that amplified the wild-type *EGFR* gene (A431) by exposing the cells to increasing concentrations of gefitinib [45]. In the gefitinib-resistant cell line, hyperphosphorylation of the insulin-like growth factor-1 (IGF-1) receptor (IGF-1R) was observed instead of suppressed EGFR, and the PI3K/Akt signal was activated through IGF-1R. Gene expression analysis showed a considerably reduced

expression of IGF-binding protein 3 and IGF-binding protein 4 RNA, which are known to inhibit IGF-induced activation of IGF-1R. As these analyses were performed using cell lines, results from studies conducted using clinical specimens are now required for further understanding.

3. Strategies to Overcome Acquired Resistance

3.1. Irreversible EGFR Inhibitor. Gefitinib and erlotinib are reversible EGFR-TKIs, also known as first generation EGFR-TKIs. In contrast, the drugs currently being developed to overcome resistance are second generation EGFR-TKIs, typified by irreversible EGFR-TKIs. Unlike reversible EGFR-TKIs, irreversible EGFR-TKIs covalently and irreversibly bind a cysteine residue in EGFR to the amino acid position 797 [16]. This enables them to inhibit EGFR kinase activity even in the presence of an *EGFR* T790M mutation. Many of these irreversible inhibitors have demonstrated activity in preclinical studies against T790M mutations. HKI-272 [46, 47] and BIBW 2992 [48, 49], which are dual inhibitors against EGFR and HER2, and PF-00299804 [50], which is a multi-inhibitor against EGFR, HER2, and HER4, are representative agents currently undergoing clinical trial.

On the other hand, these irreversible EGFR-TKIs have yet to meet expectations in terms of clinical effectiveness. All current EGFR inhibitors possess a structurally related quinazoline-based core scaffold and are identified as ATP-competitive inhibitors of wild-type EGFR. One efficacy limiting factor is believed to be the dosage limitation imposed by the toxicity because of concurrent inhibition of wild-type EGFR [46]. Zhou et al. screened an irreversible kinase inhibitor library specifically against EGFR T790M and identified a covalent pyrimidine EGFR inhibitor [51]. These agents are 30- to 100-fold more potent against EGFR T790M, and up to 100-fold less potent against wild-type EGFR, than quinazoline-based EGFR inhibitors in vitro. These mutant-selective irreversible EGFR-TKIs could be clinically more effective and better tolerated than quinazoline-based inhibitors. The results of clinical application of these agents are expected.

Cell lines resistant to irreversible EGFR-TKIs have been established using methods similar to those used for establishing reversible EGFR-TKI-resistant cell lines. Using these cell lines, the mechanisms of acquired resistance have been revealed. Ercan et al. established a resistant cell line for an irreversible EGFR-TKI by subjecting a reversible EGFR-TKI resistant cell line with a T790M mutation (established by long-term exposure of exon 19 deletion mutant PC9 cells to gefitinib) to long-term exposure to PF-00299804 [52]. In the gefitinib-resistant cell line, clones with amplified T790M mutant alleles existed in small quantities. Selective proliferation of these clones occurred on long-term exposure to PF-00299804. The researchers suggested that although irreversible EGFR-TKI may be transiently effective against cancers harboring T790M mutations, clones harboring amplified *EGFR* T790M will rapidly emerge in vitro and in vivo through selection of pre-existing *EGFR* T790M or high-expressing clones, leading to clinical drug resistance. This is similar to the mechanism of clonal selection reported

for *MET* gene amplification [40]. In another study, Yamada et al. reported that HGF overexpression is involved not only with acquired resistance against reversible EGFR-TKIs but also with acquired resistance against irreversible EGFR-TKIs [53]. In the future, reports of further studies using clinical specimens can be expected, possibly leading to the development of pharmaceutical agents to overcome these mechanisms.

3.2. *MET* Inhibitors and HGF Inhibitors. The efficacy of inhibitors of *MET* gene amplification and HGF overexpression was revealed in a preclinical study. For *MET* gene amplification, the *MET* inhibitor PHA-665752 [24] and, for HGF overexpression, HGF-*MET* inhibitors, such as anti-HGF antibodies and the HGF-antagonist NK4, have been reported to be effective [54].

Establishment of resistant cell lines and the mechanism of acquired resistance against *MET* inhibitors have also been reported. McDermott et al. established such a cell line by continuously exposing EBC-1, an NSCLC cell line with *MET* amplification, to a *MET* inhibitor, PF2341066 [55]. In the PF2341066-resistant cell line, the EGFR signal pathways were activated. Resistance was suppressed by the combined application of PF2341066 and erlotinib.

MET gene amplification and HGF overexpression often overlap with T790M mutations. To prevent acquired resistance, combined use of an irreversible EGFR-TKI and *MET* inhibitors is being studied. Although tolerability issues may arise, the outlook appears promising.

4. Conclusion

Today, EGFR-TKIs are known to contribute considerably to the extension of PFS in prognosis for NSCLC. We predict that understanding and overcoming EGFR-TKI resistance mechanisms will lead to further extensions and we look forward to reviewing future analyses.

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