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Primary Double-Strike Therapy for Cancers to Overcome EGFR Kinase Inhibitor Resistance: Proposal from the Bench

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Abstract

Diverse molecular mechanisms that confer acquired resistance to EGFR tyrosine kinase inhibitors (TKIs) in lung cancers with sensitive *EGFR* mutations have been reported. However, it is not realistic to analyze for all these mechanisms at the time of resistance in clinical practice and establish adequate treatment targeting these numerous resistance mechanisms. Therefore, we believe that we should move our research focus from the exploration of "established" diverse resistance mechanisms to the elucidation of molecular mechanisms that enable cancer cells to remain alive at the early phase of the treatment. Here in this review, we summarize up-to-date molecular mechanisms that maintain residual tumor cells against EGFR TKI monotherapy in lung cancers with *EGFR* mutations. We classified these mechanisms into three categories. The first is a pre-existing minor subpopulation with a resistance mechanism such as a pretreatment T790M mutation that can be detected by highly sensitivity methods. The second is the reversible drug-tolerant state that is often observed in cell line models and accounts for the lack of complete response and continued survival of cells exposed to EGFR TKIs in patients. And the last is the role of the microenvironment, including survival signaling from fibroblasts or dying cancer cells and the role of poor vascularization. Primary double-strike cancer therapy, or even initial multiple-strike therapy, to cancer cells that cotarget EGFR and survival mechanism(s) simultaneously would be a promising strategy to improve the outcomes of patients with *EGFR* mutations.

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Keywords

EGFR mutation; Acquired resistance; Molecular mechanisms; Drug-tolerant state; Microenvironment; Tumor heterogeneity

On the basis of data from six phase III trials that compared gefitinib,[1,2] erlotinib,[3,4] or afatinib[5,6] with chemotherapy as initial treatment of patients with advanced NSCLC with sensitive *EGFR* mutations (exon 19 deletion or L858R mutation), EGFR tyrosine kinase inhibitor (TKI) monotherapy has become the standard frontline treatment for these patients.[7–9] However, acquisition of resistance to these EGFR TKIs at a median of 9 to 13 months is inevitable, thus restricting the improvement of patients' outcomes. Despite the fact that almost all cancer cells in these patients harbor "sensitive" *EGFR* mutations[10,11] and most patients have tumor shrinkage, complete responses are rare and all patients progress, indicating that a large number of cancer cells survive with the inevitable acquired resistance.

To understand and ultimately overcome the molecular mechanisms underlying the acquired resistance, a number of studies analyzed tissue specimens obtained from patients in whom acquired resistance developed.[12–17] Analyses of cell line models or xenograft models of development of acquired resistance against chronic exposure to these drugs have also shed light on mechanisms of acquired resistance.[18–23]

Resistance mechanism–based second-line treatment would be one of a number of reasonable treatment strategies to further improve patients' outcomes. However, our experience with the HCC827 lung adenocarcinoma cell line model[24] indicates that cancer cells are flexible enough to always find a way to survive. Therefore, we believe that we should move our research focus from the exploration of "established" diverse resistance mechanisms to the elucidation of molecular mechanisms that enable cancer cells to remain alive at the early phase of the treatment (mechanisms that allow survival of residual tumor cells[25]). Upfront polytherapy that cotargets residual tumor cells may improve treatment outcomes, as shown in highly active antiretroviral therapy, a combination of antiretroviral agents with different mechanisms of action against highly flexible human immunodeficiency virus.[26] Highly active antiretroviral therapy has changed a fatal disease, acquired immunodeficiency syndrome, into a chronic disorder in developed countries. Similar strategies involving a combination of agents with different mechanisms of action to prevent the emergence of resistance have also been applied in the treatment of tuberculosis[27] and hepatitis C virus.[28]

Here in this review, we summarize up-to-date molecular mechanisms that allow survival in the presence of EGFR TKI monotherapy in lung cancers with *EGFR* mutations. As shown in Figure 1, we classified these mechanisms into three categories, including a preexisting minor subpopulation with a resistance mechanism (Fig. 1*A*), a reversible drug-tolerant state (Fig. 1*B*), and the microenvironment (Fig. 1*C* and *D*).

Preexisting Minor Resistant Subpopulation

The evidence of a preexistent minor subpopulation with T790M mutation[12] has been reported since 2006,[29] with high-sensitivity methods used to detect this resistance

mutation.[30–34] Patients with the scant T790M mutation should be strictly distinguished from rare patients with double mutations (an activating *EGFR* mutation together with the abundant T790M mutation that is detectable in routine clinical molecular testing[35]), and some of them carry T790M mutation as germline mutations.[36–38] A recent ultrasensitive detection study in which droplet digital polymerase chain reaction was used to identify T790M mutation observed that 298 of 373 NSCLCs with activating *EGFR* mutation (79.9%) carried pretreatment T790M mutation. It is of note that the allele frequency of the *EGFR* T790M mutation was between 0.001% and 0.1% in most of the cases (95%),[39] and cases with abundant T790M allele (≥10%) are very rare (0.5%). It is unclear why cancer cells "prepare" this resistance mutation before EGFR TKI therapy. However, a recent study suggested that hypermethylation of the CpG dinucleotide in *EGFR* codon 790 easily leads to the C-to-T transition mutation, causing T790M mutation.[40] Therefore, it is possible that cancer cells may harbor several minor subpopulations with different a *EGFR* secondary mutation, including a TKI-resistant T790M mutation. Several studies have suggested that the pre-existing minor T790M mutation is related to decreased efficacy of gefitinib or erlotinib monotherapy,[30,34] whereas others have observed the opposite results.[32]

Preexistence of another resistance mechanism, MET proto-oncogene, receptor tyrosine kinase gene (*MET*) amplification, is also reported in clinical specimens by high-throughput fluorescence in situ hybridization analyses.[41] There are currently no data, as far as we know, regarding the preexistence of other resistance mechanisms, such as erb-b2 receptor tyrosine kinase 2 gene (*ERBB2*) amplification, AXL receptor tyrosine kinase activation, or SCLC transformation (although the presence of mixed small cell/adenocarcinoma at diagnosis has been recognized for many years).

In lung cancer cell lines with activating *EGFR* mutations, evidence supports the preexistence of minor resistant subpopulations, such as T790M mutation in PC9 cells[42] and *MET* gene amplification in HCC827 cells.[41] H1975 cells, with L858R mutation and T790M mutation, are the cell line model for aforementioned double mutations, but not for ones with a preexistent minor T790M subpopulation.

It is natural to consider that these preexistent minor subpopulation cells with a resistance mechanism are selected through the EGFR TKI therapy (see Fig. 1*A*) because most of the sensitive cells are killed and emerge as acquired resistance. This hypothesis is also supported by the fact that each lung cancer cell line with *EGFR* mutation has its "favorite" resistance mechanism: T790M mutation in PC9 cells, *MET* gene amplification in HCC827 and H4011 cells, and epithelial-mesenchymal transition (EMT) phenotype in HCC4006 and H1975 cells.[16,21,43–47]

Upfront polytherapy, targeting both EGFR and minor subpopulation cells with a resistance mechanism, may be a promising treatment strategy to overcome or delay the emergence of resistance. At the laboratory bench, studies using upfront polytherapies conferred different resistance mechanisms in PC9 cells,[48,49] HCC827 cells,[14] and HCC4006 cells[50,51] as compared with their favorite ones. In addition, in our experience, a longer exposure period was needed to establish cells resistant to a combination therapy of erlotinib plus a MET proto-oncogene, receptor tyrosine kinase (MET) TKI than to erlotinib alone in HCC827

cells.[14] In a clinical trial, osimertinib, a third-generation EGFR TKI that is able to inhibit the T790M mutation, has shown longer progression-free survival (PFS) in the frontline setting (19.3 months)[52] as compared with data from first- or second-generation EGFR TKIs (9.6–13.7 months[1–6]). This may due to the fact that most patients with *EGFR*-mutated lung cancer (79.9%) carry a tumor subpopulation with T790M mutation.[39] It is interesting to theorize whether a difference in PFS could be found between patients with a minor T790M subpopulation and those without T790M at frontline osimertinib treatment. Regarding *MET* gene amplification targeting, clinical trials with tivantinib, a small-molecule MET inhibitor, combined with erlotinib did not show a PFS benefit over erlotinib alone in a small *EGFR*-mutant subgroup with prior cytotoxic chemotherapy treatment.[53,54] Currently, most of the trials with a combination of EGFR TKI and MET TKIs (crizotinib, tivantinib, cabozantinib, volitinib, MSC2156119J, or INC280)[55] are focusing on *EGFR*-mutated patients after treatment failure with prior EGFR TKI (the phase II group 2 part of NCT02335944 uses patients without prior EGFR TKI therapy). Frontline combination of EGFR TKI and MET TKI in patients with *EGFR*-mutated lung cancer with a preexistent minor subpopulation with *MET*-amplified cells is an interesting strategy for another primary double-strike therapy candidate.

Reversible Drug-Tolerant State

In vitro observations demonstrated that the vast majority of *EGFR*-mutated lung cancer cells are killed within a few days upon exposure to a clinically relevant concentration of EGFR TKIs, whereas a small fraction of viable, largely quiescent cells (~0.3%) can still be detected 9 days later (see Fig. 1*B*).[56] In clinical practice, we can say that when a patient starts with 10^{11} cancer cells,[57] there are still 3×10^8 cells or more remaining even if an EGFR TKI eliminates cancer cells as effectively as in cell culture dishes. This drug-tolerant status is reversible, as cells propagated in drug-free media rapidly reacquire EGF TKI sensitivity and have also been shown to arise in cells established from a single-cell clone. Therefore, the mechanism(s) of cell survival is different from genetic changes such as acquisition of T790M mutation or *MET* gene amplification. However, recent studies have demonstrated that “irreversible” resistance mechanisms such as T790M mutation will occur in these drug-tolerant persisting cells after continuous exposure to EGFR TKIs.[42,58]

Survival signaling in drug-tolerant persisting cells is well studied for PC9 cells; it involves the insulin-like growth factor 1 receptor (IGF-1R) pathway or an altered chromatin state that requires the histone demethylase retinol binding protein 2/lysine demethylase 5A.[42,56] In PC9 cells, exposure to a combination therapy with an IGF-1R inhibitor, AEW541, and an EGFR TKI eliminated these persisting drug-tolerant cells.[42,56] However, it is unclear whether IGF-1R signaling or an altered chromatin state plays an important role in all *EGFR*-mutated lung cancer cell survival. Currently, there are no clinical data regarding the combination of IGF-1R inhibitor with EGFR TKI in patients with *EGFR*-mutated lung cancer, but discouraging results for this combination in a cohort of unselected patients with lung cancer have been reported.[59,60]

Another candidate pathway involved in drug-tolerant status against EGFR TKIs is nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) signaling. A pooled RNA

interference screen identified that down-regulation of several components of the NF-κB pathway specifically enhanced cell death induced by EGFR TKI in lung cancer cells with *EGFR* mutation.[61] The role of NF-κB activation in resistance to apoptosis with EGFR TKI treatment was confirmed by later research using patient-derived tumor xenograft models[62] and in three-dimensional cultured cell models.[63] The former study also identified signal transducer and activator of transcription 3 as the important downstream molecule in the NF-κB pathway that confers resistance to apoptosis,[62] confirming the importance of feedback activation of signal transducer and activator of transcription 3 in primary insensitivity to EGFR TKIs.[64,65] Recently, inhibition of casein kinase 1 alpha has been reported to prevent acquired resistance to EGFR TKI in vitro and in vivo through suppression of the NF-κB signaling pathway.[66]

Another RNA interference screen found that the canonical Wnt/beta-catenin pathway (in particular, the poly–adenosine diphosphate–ribosylating enzymes tankyrase 1 and 2 that positively regulate this signaling) contributes to the survival of cancer cells during EGFR inhibition.[67] In addition, tankyrase has recently been reported to allow cancer cells to survive against EGFR TKI through the Hippo/yes-associated protein pathway.[68] A tankyrase inhibitor, AZ1366, showed synergistic efficacy with gefitinib in H3255, H1650, HCC4006, and HCC4011 cells but not in HCC827 and PC9 cells.[69]

BCL2 like 11 (BIM) is another molecule that may be related to decreased apoptosis in response to EGFR TKIs. EGFR TKIs cause a rapid increase of BIM protein expression with proapoptotic BCL2 homology domain 3 (BH3) in lung cancer cells with *EGFR* mutation, and RNA interference–mediated knockdown of BIM eliminates EGFR TKI–inducible apoptosis, whereas addition of the BH3 mimetics (ABT-737) enhances EGFR TKI–mediated apoptosis.[70,71] Later studies found that pretreatment RNA levels of BIM predicted the capacity of EGFR TKIs to induce apoptosis,[72] and a common BCL2 like 11 gene (*BIM*) deletion polymorphism mediates intrinsic resistance to EGFR TKIs through the expression of BIM isoforms lacking BH3.[73] In addition, *EGFR*-mutant lung cancer cell lines that harbor the *BIM* deletion polymorphism are much less sensitive to EGFR TKI–inducible apoptosis, and the histone deacetylase inhibitor vorinostat could circumvent low EGFR TKI sensitivity in these cell lines.[74] However, the impact of *BIM* deletion polymorphism as a predictive biomarker for EGFR TKI is still controversial in clinical settings.[75–77]

Signals from the Microenvironment

It has also been shown that cancer cells receive survival signaling from the microenvironment that may modify drug efficacy (see Fig. 1*C*).[78] In 2008, Yano, et al. showed that addition of hepatocyte growth factor (HGF), but not epidermal growth factor, transforming growth factor-α, or insulin-like growth factor 1, in the conditioned media conferred resistance to EGFR TKIs in lung cancer cells with *EGFR* mutations.[79] HGF is often produced by fibroblasts, and lung cancer cells became resistant to EGFR TKIs when cocultured in vitro with HGF-producing fibroblasts or coinjected into immune-deficient mice.[80] Survival signaling may also be mediated by fibroblasts through hedgehog signaling.[81] Other growth factors or chemokines, such as fibroblast growth factor 2[44,82] or interleukin-8,[83,84] are also reported to cause established acquired resistance to EGFR TKIs

in vitro through overexpression by cancer cells themselves (autocrine). However, the role of these molecules produced from microenvironment noncancerous cells in the survival of residual tumor cells is unclear to date. Direct interaction of podoplanin-positive cancer-associated fibroblasts with cancer cells is also reported to cause resistance to EGFR TKIs in vitro, and *EGFR*-mutated lung cancer patients with podoplanin-positive cancer-associated fibroblasts demonstrated reduced PFS when treated with EGFR TKIs.[85] Fibroblast-mediated stimulation,[81] or many other factors including cigarette smoke extract,[86] may also cause a reduced EGFR TKI sensitivity at the early phase of the treatment through the induction of EMT.[18] Transforming growth factor-β receptor inhibition, together with EGFR TKI, is reported to inhibit EMT-mediated acquired resistance to EGFR TKI in vitro.[50] In addition, histone deacetylase inhibitors have been shown to overcome EGFR TKI resistance linked to epigenetic changes and EMT state in vitro[87–89]; however, the clinical data are limited so far.[90,91]

A recent study demonstrated that in addition to survival signaling from fibroblasts, secretomes produced by dying cells in response to EGFR TKI therapy may help residual cancer cells survive against the drug.[92] It is unclear whether this resistance conferred via the microenvironment can lead to development of bona fide resistance mechanism(s) in cancer cells. However, in one in vitro study, researchers observed that HGF exposure facilitated HCC827 cells' acquisition of *MET* gene–amplified resistance.[41]

Nonfunctional blood vessels in tumor tissues are also one of hallmarks of the tumor microenvironment (see Fig. 1*D*). This poor vascularization causes a hypoxic environment, and tumor hypoxia is associated with aggressive tumor phenotypes, treatment resistance, and poor clinical prognosis.[93] Several in vitro studies observed that *EGFR*-mutated lung cancer cells showed insensitivity to EGFR TKIs under hypoxic cell culture conditions compared with under normoxia conditions.[94,95] Poor vascularization is also considered to provide poor drug delivery to cancer cells, causing a lower EGFR TKI concentration, which is related to earlier development of resistance than with higher drug concentrations.[96,97] In clinical settings, the addition of bevacizumab (an antiangiogenic monoclonal antibody that targets vascular endothelial growth factor and may modify aberrant vessels around a tumor) to the EGFR TKI erlotinib has shown dramatic improvement of PFS (16.0 months versus 9.7 months).[98]

Conclusions and Future Perspectives

Here, we have summarized possible *scenarios* that lung cancer cells with *EGFR* mutation utilize to survive the early phase of treatment with EGFR TKIs. As shown in clinical trials with frontline osimertinib[52] or the combination of erlotinib plus bevacizumab,[98] primary double-strike therapies for cancer cells that cotarget EGFR and survival mechanism(s) simultaneously would be a promising strategy to improve the outcomes of patients with *EGFR* mutations. In addition, abundant data from the bench, as summarized here, may provide additional ideas for primary double-strike therapies for cancer cells. Outside the scope of this review, there are additional ideas for improved treatments for patients with lung cancer with *EGFR* mutations, such as combination of EGFR TKI with MEK inhibitors,[49,99] combination of EGFR TKI with immune checkpoint inhibitors, or combination of EGFR

TKI with cytotoxic agents[100], as summarized in a recent clinical review.[55] Although a dozen years have passed since discovery of the *EGFR* mutation,[101–103] this research area still holds substantial interest in the research community.

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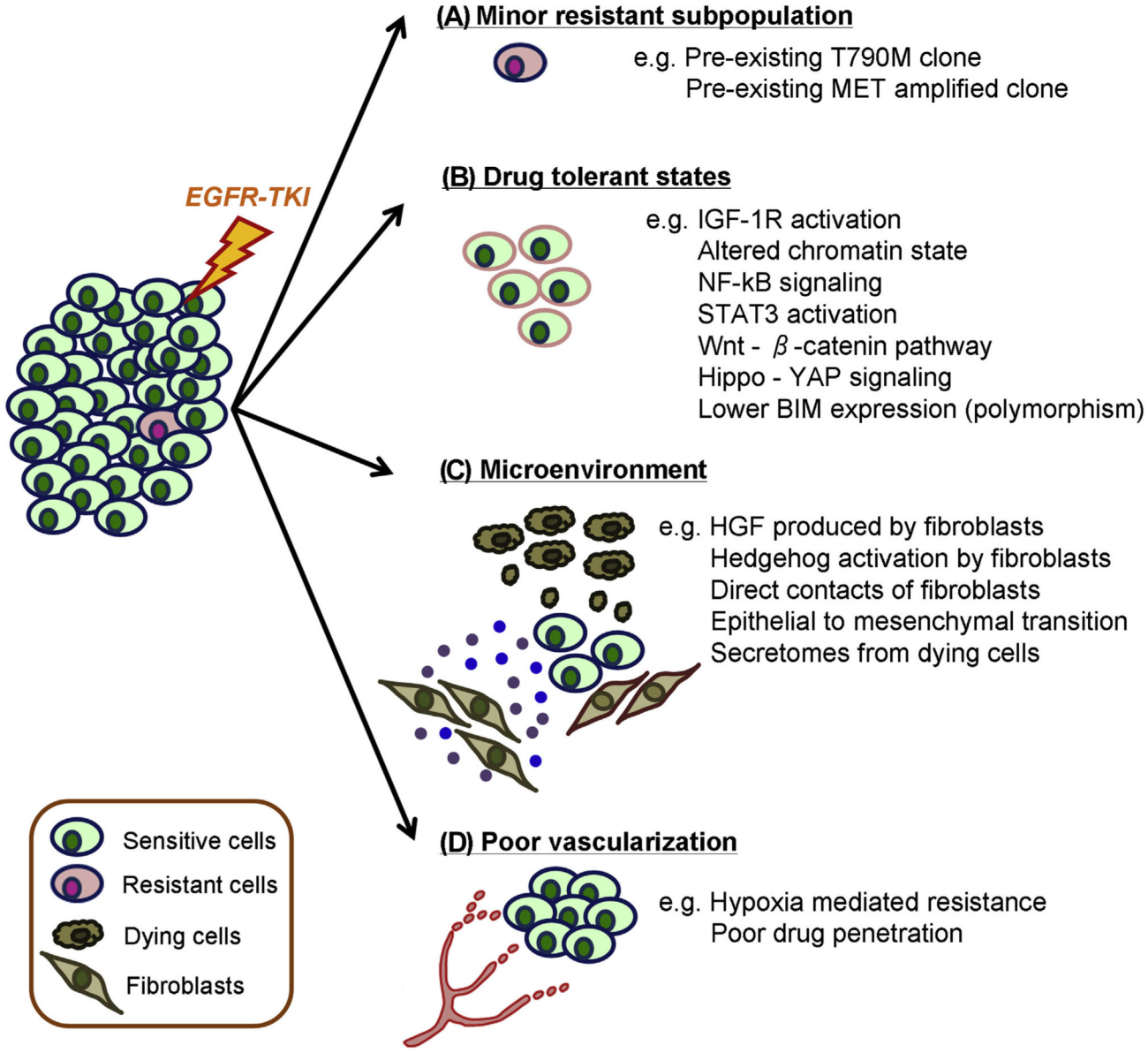

Figure 1.

Molecular mechanisms that support residual tumor cells in the early phase of EGFR tyrosine kinase inhibitor (TKI) monotherapy. Preexistence of a minor resistance subpopulation that will survive frontline EGFR TKI monotherapy (*A*), reversible drug-tolerant state mainly observed in cell line models (*B*), survival signaling from microenvironment (fibroblasts or dying cancer cells) (*C*), and role of poor vascularization (*D*) are shown. *MET,* MET proto-oncogene, receptor tyrosine kinase gene; IGF-1R, insulin-like growth factor 1 receptor; NF-κB, nuclear factor kappa light-chain enhancer of activated B cells; STAT3, signal transducer and activator of transcription 3; YAP, yes-associated protein; BIM, BCL2 like 11; HGF, human growth factor.