Tumor heterogeneity and resistance to EGFR-targeted therapy in advanced nonsmall cell lung cancer: challenges and perspectives

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Abstract: Lung cancer, mostly nonsmall cell lung cancer, continues to be the leading cause of cancer-related death worldwide. With the development of tyrosine kinase inhibitors that selectively target lung cancer-related epidermal growth factor receptor mutations, management of advanced nonsmall cell lung cancer has been greatly transformed. Improvements in progression-free survival and life quality of the patients were observed in numerous clinical studies. However, overall survival is not prolonged because of later-acquired drug resistance. Recent studies reveal a heterogeneous subclonal architecture of lung cancer, so it is speculated that the tumor may rapidly adapt to environmental changes via a Darwinian selection mechanism. In this review, we aim to provide an overview of both spatial and temporal tumor heterogeneity as potential mechanisms underlying epidermal growth factor receptor tyrosine kinase inhibitor resistance in nonsmall cell lung cancer and summarize the possible origins of tumor heterogeneity covering theories of cancer stem cells and clonal evolution, as well as genomic instability and epigenetic aberrations in lung cancer. Moreover, investigational measures that overcome heterogeneity-associated drug resistance and new assays to improve tumor assessment are also discussed.

Keywords: NSCLC, EGFR, TKIs, drug resistance, tumor heterogeneity

Introduction
Despite many novel cancer treatments developed in the past decades, advanced lung cancer, mostly nonsmall cell lung cancer (NSCLC), is still the leading cause of cancer death and poses a great threat to public health. So far, early resection is still the only way to cure the disease. However, more than two-thirds of the patients are beyond the curable stage at diagnosis. The estimated 5-year survival rate of advanced NSCLC is less than 10%, and the median life expectancy is only 4 months if left untreated. Chemotherapy may slightly prolong survival, but the adverse effects are sometimes unbearable, and the tumor eventually becomes resistant to the drug.

Based on the “oncogene addiction” theory, the development of new compounds targeting tumor-driving pathways (eg, the epidermal growth factor receptor [EGFR] signaling pathway) brings new hope to cancer patients. Identification of mutations in these pathways, followed by targeted therapy, gives rise to personalized therapy in several types of cancer. In advanced NSCLC, tyrosine kinase inhibitors (TKIs; eg, gefitinib and erlotinib) competitively block EGFR and suppress tumor growth, conferring survival benefits with acceptable adverse effects on patients who have failed chemotherapy. In patients with NSCLC and sensitive EGFR mutations, TKIs are recommended as first-line treatments because of their significantly increased response rates and prolonged progression-free survival in this group of patients.
Although early results are encouraging, EGFR TKIs only show effects in a small number of patients (−10%). Moreover, even tumors that initially respond to the TKIs eventually become refractory to the therapy, and the tumors rapidly regrow. Mechanisms underlying this later-acquired drug resistance are still unknown. However, recent reports reveal a critical role of tumor heterogeneity in the development of drug resistance. It is now believed that cancer is actually a process of clonal evolution and that every single tumor is a complex hierarchy of tumor subclones resulting from distinctive microenvironmental adaptation. These heterogeneous tumor cells provide the material that Darwinian selection can work on; so the fittest subclones can survive through the therapeutic intervention and then dominate regrowth of the tumor. In this review, we provide an overview of currently recognized causes of tumor heterogeneity, including cancer stem cells (CSCs) and genomic instability, as well as epigenetic aberrations, and discuss their roles in NSCLC. Spatial and temporal tumor heterogeneity as a mechanism of the primary and acquired EGFR TKI resistance in advanced NSCLC is also elucidated. Finally, new approaches to tackling the challenge of tumor heterogeneity are summarized, which may bring new hope to future targeted therapy.

Origins of tumor heterogeneity in NSCLC

CSC and acquired drug resistance in NSCLC

According to the CSC theory, individual tumors are organized into a hierarchy composed of subsets of tumorigenic stem cells and their nontumorigenic progeny cells. Heterogeneous subclonal lineages in solid tumor are branched from distinctive CSCs and are dynamically maintained by these regenerating cells. Thus, identification of the CSCs followed by specific treatment targeting developmental signaling pathways (eg, Notch, hedgehog, and transforming growth factor-beta pathways) may be more effective in suppressing tumor growth and preventing drug resistance.

The existence of CSCs in lung cancer is supported by the fact that only a small population of tumor cells (<1.5%) from adenocarcinoma samples possess clonal forming and tumorigenic ability. These lung CSCs are thought to be derived from the self-renewing epithelial and bronchioalveolar cells as a result of oncogenic KRAS and EGFR activation and may exhibit increased aldehyde dehydrogenase activity. It has been demonstrated that CSC-like cell population, characterized by elevated expression of cell cycle genes and increased aldehyde dehydrogenase activity, is increased in the EGFR mutant lung cancer cell line with acquired resistance to erlotinib, indicating a potential role of CSCs in EGFR TKI resistance. However, some nontumorigenic progeny subclones may regain tumorigenic capacity and transform back to stem-like cells. Without specific biomarkers, it is very difficult to distinguish CSCs from nontumorigenic cells. Therefore, CSCs targeting therapy are still quite premature, and research in this field is complicated.

Genomic and chromosomal instability of NSCLC

In addition to CSCs, genomic and chromosomal instability may also contribute to heterogeneity in solid tumors. In normal lung cells, the genome is replicated with high fidelity, and mutations are poorly tolerated, which is attributed to stringent intrinsic checking mechanisms such as base and nucleotide excision repair, mismatch repair, telomere maintenance, and double-strand break repair. Malfunctioning checking mechanisms greatly increase the mutation rate and significantly accelerate the process of clonal evolution, leading to carcinogenesis and tumor heterogeneity. Chromosomal instability has been shown to correlate with shorter survival in patients with NSCLC, possibly because these unstable cancer cells display a higher multidrug-resistant capacity compared with stable cells. In mice, lung adenocarcinoma with chromosome instability induced by overexpression of a mitotic checkpoint gene Mad2 is highly aneuploid, correlating with a higher tumor recurrent rate after anticancer treatment. Moreover, next-generation sequencing techniques have identified a number of hallmark genomic mutations that are involved in DNA maintenance and mitotic progression, which may predict the prognosis of NSCLC. Some of these genomic mutations may be used for targets able to be drugged, highlighting the importance of maintaining genomic stability for tumor control.

Epigenetic aberrations of NSCLC

Apart from genomic mutations, gene expression and phenotypic changes of tumor cells can be affected by epigenetic aberrations including abnormal DNA methylation at CpG islands, dysregulated histone modification, and changes in pathways regulating these epigenetic mechanisms. Compared with genomic mutations, epigenetic changes are reversible and more plastic under environmental pressures, adding further complexity to tumor heterogeneity and contributing to the development of drug resistance. In NSCLC, global DNA hypomethylation is considered a main
cause of genomic instability, as well as abnormal oncogene expression. A recent analysis of cancer-specific differentially DNA-methylated regions by whole-genome bisulfite sequencing reveals drastic stochastic differences in the differentially methylated regions (DMRs) and a significant loss of sharply delimited methylation boundaries at CpG islands of the DNA in samples from several malignant tumors, including lung cancer. Loss of the epigenetic marks in these cancer cells perhaps indicates the epigenome has been “reset”; thus, these cells have greater potential to reshape themselves under selective environmental pressures. Consistently, status of global histone modification is strongly associated with prognosis of patients with NSCLC, and it can predict tumor recurrence in early-stage patients. Notably, epigenetic mechanisms may function in a different dimension from genomic heterogeneity during tumor evolution, through which the tumor cells are not only passively selected by the environments but also may actively change themselves to adapt external stress.

Microenvironmental adaptation and tumor heterogeneity

The extrinsic compartments of tumor cells are composed of disorganized stromal cells including fibroblasts and vasculature and immune cells. Distinct microenvironments characterized by varied degrees of selective pressures such as oxygen, acidity, and tumor growth factors may select for mutations that engender subclonal survival and expansion, thus exerting great influence on tumor heterogeneity. Moreover, the extrinsic tumor microenvironment may also participate in the development of drug resistance by forming an adaptive, reciprocal signaling loop with tumor cells, thus providing a protective compartment in response to anticancer treatments. Hepatocyte growth factor (HGF), for instance, is secreted by stromal fibroblasts under the stimulation of tumor-derived factors and strongly propagates tumor expansion by activating MET signaling pathway in NSCLC. High levels of tumor HGF are associated with EGFR TKI resistance because the activation of MET induces the common downstream prosurvival signaling, bypassing EGFR inhibition. Moreover, the tumor vascular network featured by dysregulated angiogenesis and reorganization of existing vessels also contributes to tumor heterogeneity. Poorly developed tumor vasculature results in variations in oxygen and nutrient supply within the tumor, as well as affecting drug delivery. Microvessel density as an index of angiogenesis may predict survival of the patients with lung cancer, and elevated expression proangiogenic ligand vascular endothelial growth factor (VEGF) is associated with poorer prognosis in NSCLC. It is therefore conceived that coinhibition of VEGF signaling may confer a benefit on tumor suppression. However, coadministration of VEGF targeted therapy has been shown to reduce the delivery of radiolabeled chemotherapy in patients with NSCLC, as determined by positron emission tomography. Nevertheless, a combinational strategy targeting tumor stromal cells remains one of the most important research areas in personalized therapy and will be further discussed later.

Spatial tumor heterogeneity and TKI resistance in NSCLC

Intertumor heterogeneity and TKI resistance

As previously mentioned, individual tumors are driven by distinct prosurvival signaling pathways as a result of heterogeneous genomic mutations. Optimal outcomes of personalized therapy can be achieved by matching the specific lesions with the corresponding treatments, whereas tumors without sensitive mutations are unsusceptible to treatment. So far, a number of mutations with the tumor driving capacity have been identified (Table 1). Frequencies of these driver mutations in NSCLC are associated with histologic subtypes, sex, ethics, age, and past smoking history. The patients with wild-type EGFR are insensitive to gefitinib or erlotinib treatment. KRAS and BRAF mutants, being mutually exclusively expressed with EGFR in ~25% and 3% of patients with NSCLC, respectively, predict poorer prognosis in patients after EGFR-targeted treatment. Similarly, NSCLC patients harboring EML4-ALK fusion oncogene (~7%) are sensitive to TKI crizotinib but resistant to EGFR TKIs. Hence, intertumor heterogeneity of different mutations may confer primary resistance to any targeted therapy.

The sensitivity of different EGFR mutations to TKIs also varied between individuals. The EGFR gene is located on chromosome 7p12–7p13, whereas the NSCLC-relevant mutations occur in exons 18–21, encoding the kinase domain of the receptor. Although more than 188 EGFR mutations have been identified, 85% of TKI-sensitive clinical cases harbor only two major mutations: in-frame deletions of exon 19 (45%–50%) and a point mutation L858R in exon 21 (40%–45%; Figure 1). Other uncommon TKI-sensitive mutations include amino acid substitution mutations of G719X in exon 18 and L861X in exon 21. These mutations cause profound activation of both prosurvival and antiapoptotic signaling cascades but also enhance the affinity of the receptor to gefitinib and erlotinib. In contrast, an insertion
Table 1 Distribution of known tumor-driving mutations and chromosomal fusions in advanced NSCLC

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**Note:** Data obtained from previous reports.54,55,60,75,76,77

**Abbreviations:** NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; TP53, tumor protein p53; KRAS, Kirsten rat sarcoma viral oncogene homolog; BRAF, v-raf murine sarcoma viral oncogene homolog B; ALK, anaplastic lymphoma kinase; PIK3CA, phosphatidylinositol-4,5-bisphosphate-3-kinase, catalytic subunit alpha; AKT1, v-akt murine thymoma viral oncogene homolog 1; MAP2K1, mitogen-activated protein kinase 1; HER2, human epidermal growth factor receptor 2.

In lung cancer, whole-genome analysis of samples from different regions of the tumor is still lacking. By conducting deep digital sequencing analysis of 17 treatment-naïve lung adenocarcinomas, Govindan et al.15 found a multiclonal signature indicated by distinct variant allele frequency features in more than half of the samples. Some of these subclones were believed to be involved in progression and migration of the tumor.15 It has been reported that some TKIs desensitizing mutations such as EGFR T790M mutation97,98 and amplification of MET99,99 (both discussed later) may concomitantly present with TKIs sensitizing EGFR mutations in a minor group of patients before the TKI treatment. Considering heterogeneity within the tumor, it is not hard to imagine some

**Intratumor heterogeneity and TKI resistance**

Apart from differences between tumors, intratumor heterogeneity also presents at histologic, cellular, molecular, genetic, and epigenetic levels.94 Early histological analysis of dissected NSCLC samples showed varied levels of extracellular receptors and stress-responsive genes in different regions of the tumor, indicating distinct subclonal interplay with external stress.95,96 Using a next-generation whole-genome sequencing technique, Gerlinger et al reveal that heterogeneous somatic mutations, divergent allelic profiles and ploidy heterogeneity vastly present in different regions of microdissected samples from the same renal tumor.94 About 63%–69% of all somatic mutations are not detectable across every tumor region, and genes associated with good or poor prognosis can be detected at different regions of the same tumor, both of which reflect drastic subclonal diversity within the tumor.94 The presence of intratumor heterogeneity largely diminishes the reliability of single-tumor biopsy prevalently used in hospital and poses a challenge to the current protocol of personalized therapy.94

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less-prevalent subclones may be missed by the single-region biopsy, so that the actual coexistence rate of TKI-sensitive and TKI-resistant subclones may even be higher, which may be further examined by genotyping microdissected samples from surgically resected lung tumors.

Notably, some researchers claim coexistence of both wild-type and mutant EGFR within the same tumor, as well as discordant EGFR mutational status between the primary tumor and the metastatic sites. These results are challenged by the reports showing highly homogeneous distribution of EGFR mutations across the primary tumor and the metastatic lesions. The high wild-type EGFR detection rate in the former studies was thought to be explained by normal tissue contamination, as well as technical variations (e.g., the minor presence of EGFR mutations that may be neglected by less-sensitive methods). Moreover, although some groups reported a conversion from EGFR mutant to wild-type after TKI treatment in occasional cases, most of the studies show highly consistent EGFR mutations in tumors before and after the treatment. It is hard to imagine why the conversion rate of mutant to wild-type after TKI treatment is so low if intratumor heterogeneity of EGFR mutation is not rare. In addition, Govindan et al applied computational analysis to the deep sequencing data of NSCLC samples and found that EGFR mutation exists in all subclones as a founder mutation of the tumor, suggesting this mutation may be acquired at the very initial phase of tumorigenesis.

**Temporal tumor heterogeneity and acquired TKI resistance in NSCLC**

Temporal heterogeneity reflects the dynamic tumor evolution in response to environmental changes including therapeutic interventions. Adaptive tumor subclones after this natural selection may then dominate the tumor clonal architecture, leading to disease progression. Therefore, temporal tumor heterogeneity is perhaps more related to acquired resistance to chemotherapy or EGFR-targeted therapy in NSCLC.

It has been noticed that the predictive value of pretreatment EGFR mutation status seen in the first-line TKI regimen is diminished in cohorts of TKI treatment after chemotherapy, and the tumor seems to be less sensitive to EGFR-targeted therapy after chemotherapy. Bai et al compared EGFR mutation status before and after neoadjuvant chemotherapy in both lung tumor tissue and blood samples and found that EGFR mutant-positive NSCLC significantly decreased after chemotherapy. However, these results may be misinterpreted because the reduction of EGFR mutant in the blood can also be affected by the amount of tumor cells being exposed to circulation, and chemotherapy may increase fibrotic content in the tumor, thus affecting the detection of mutant EGFR in the tumor. De Pas et al compared EGFR expression on mediastinal lymph nodes at the primary biopsy with its expression on both the primary tumor and the residual mediastinal nodes after three cycles of chemotherapy in 47 patients with NSCLC and found reduced EGFR immunoreaction in 12% of the patients. Platinum-based agents may reduce the sensitivity of lung cancer cells to EGFR TKIs as a result of PTEN loss and EGFR-independent AKT activation. Although the precise mechanism of this chemotherapy-induced resistance to TKIs is still unclear, this issue merits further investigation because it may necessitate rebiopsy after chemotherapy for better guidance in targeted therapy.

**Secondary mutations and acquired TKIs resistance in NSCLC**

In relapsed NSCLC after TKI treatment, secondary mutations that desensitize EGFR to its inhibitors are frequently detected. T790M, which represents threonine-to-methionine substitution at position 790 in the gatekeeper residue of EGFR kinase domain because of a point mutation within exon 20, presents in about 50% of EGFR mutant NSCLC’s that become resistant to TKIs after the treatment. EGFR of lung cancer cells with both TKI-sensitive mutations and T790M mutation regain affinity to ATP at the hydrophobic pocket, hence abrogating competitive inhibition of the receptor by gefitinib and erlotinib. Other secondary mutations in the EGFR kinase domain that are associated with acquired resistance include L747S, D761Y, and T854A. L747S resulting from deletion in exon 19 is located at the head of the loop between strand β3 and helix αC of the receptor and is believed to attenuate BIM upregulation and mitochondrial apoptosis induced by TKIs. D761Y from exon 19 mutation occurs in the middle of the helix αC and forms a salt bridge that interacts with the α- and β-phosphates when ATP is present, reducing sensitivity of the EGFR to TKIs. T854A mutation in exon 21 interferes the contact of erlotinib to the ATP pocket of the receptor, hence abrogating the inhibition of tyrosine phosphorylation by erlotinib. These secondary mutations are rarer and cause less-potent resistance to TKIs than T790M.

Notably, it remains debatable whether T790M mutation truly developed after TKI exposure or whether the mutation preexists before the treatment and become more prevalent in the tumor architecture under selective pressure of the TKIs. There have been some reports showing T790M coexists with sensitive
EGFR mutations before TKI treatments in a minor group of patients.\textsuperscript{97,125} Moreover, T790M mutation is significantly less prevalent in brain metastases, possibly because routine doses of TKIs hardly penetrate the blood–brain barrier and thus exert less selective pressure on the brain lesion.\textsuperscript{54,126,127} In fact, the presence of secondary mutations in resistant NSCLC may not be an “all or none” phenomenon but, rather, a dynamic process in association with environmental pressure. Discontinuation of EGFR targeted therapy in resistant patients often leads to accelerated tumor growth and symptom progression,\textsuperscript{128} suggesting at least some tumor cells are still under suppression by the EGFR inhibitors.\textsuperscript{54,128} Notably, in the absence of EGFR inhibition, the expression of mutations associated with acquired resistance may reversibly disappear, as revealed by serial biopsies.\textsuperscript{112,128} Consistently, reinitiation of EGFR-targeted therapy after a short period of TKI suspension may slow disease progression again in some cases.\textsuperscript{112,128,129} Hence, larger cohorts are needed to evaluate whether dynamic mutation monitoring may benefit the patients who acquired resistance to targeted therapy.

Bypass signaling of EGFR inhibition
Compensatory activations of the “oncogene addiction” pathways via bypass signaling after EGFR inhibition represent another mechanism of acquired TKI resistance (Table 2). Focal amplification of MET oncogene, for instance, is observed in about 5%–20% of NSCLCs after treatment with EGFR inhibitors.\textsuperscript{109,112,136} MET encodes a transmembrane tyrosine kinase receptor for HGF. Activation of MET phosphorylates the downstream ERBB3, activating Akt signaling. Amplification of MET oncogene causes hyperactivation of PI3K and Akt, bypassing the blockade of this pathway by EGFR inhibitors and promoting tumor survival.\textsuperscript{136} In addition, amplification of HER2 and PI3KCA mutations, both of which activate the common downstream pathways, are detected in about 12% and 5% of EGFR mutant NSCLC patients, respectively.\textsuperscript{57,112,131} Similar to T790M mutation, MET amplification may also exist before the treatment.\textsuperscript{99} Turke et al reported MET amplification in rare tumor cells (<1%) from treatment-naïve NSCLC samples (5 of 27 patients).\textsuperscript{99} Four of these five patients later developed TKI resistance as a result of MET amplification, suggesting acquired resistance may emerge as a result of the survival advantage of pre-existing resistant tumor subclones.\textsuperscript{12,99,112}

Phenotypic changes and epigenetic alterations in acquired TKI resistance
Apart from genetic aberrations, phenotypic changes of the tumor cells such as epithelial to mesenchymal transition\textsuperscript{132–134} and transformation to a small-cell-like carcinoma\textsuperscript{77,81} are frequently observed in NSCLC after TKI treatments, possibly as a result of altered epigenetic modifications.\textsuperscript{135} For instance, gefitinib-induced DNA hypermethylation is associated with decreased expression of micro-RNA (miRNA)-200c, resulting in overexpression of aldehyde dehydrogenase isoform 1, which contributes to the epithelial-to-mesenchymal transition and the presence of stem cell-like properties.\textsuperscript{26} Moreover, DNA hypermethylation at the promoter region of death-associated protein kinase may silence this gene in the NSCLC cell line,\textsuperscript{136} leading to erlotinib resistance, whereas transient induction of death-associated protein kinase by gene transfection resensitizes the cells to erlotinib.\textsuperscript{136} Other hypermethylated tumor DNA such as Wnt antagonist SFRP\textsuperscript{57,137} and mitotic stress checkpoint gene CHFR\textsuperscript{138} have also been reported to affect TKI sensitivity. Although it is still unclear to what extent epigenetic mechanisms are involved in acquired TKI resistance, considering its importance in clonal evolution and tumor heterogeneity, more efforts should be invested on research and new drug development in this area.\textsuperscript{38}

Advances in molecular diagnosis and tumor monitoring in NSCLC
Next-generation sequencing for the search of new molecular targets
Next-generation high-throughput sequencing techniques now allow us to map the genomic landscape of lung cancer at lower cost, but at orders of magnitude higher speed, compared with the traditional Sanger technique.\textsuperscript{146,147} These new

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These new
platforms exponentially enhance our capacity to search for new oncogenes that can be made into drugs. Imieliński et al conducted whole-exome and whole-genome sequencing on 183 paired samples of lung adenocarcinoma and normal lung tissues from treatment-naive patients, from which 25 hallmark carcinogenic genes including 19 previously reported genes and 6 novel mutants (NKP2-1, TERT, PTEN, MDM2, CCND1, and MYC) were identified. They also detected frequent somatic mutations in epigenetic and splicing factor genes including U2AF1, ARID1A, RBM10, SETD2, and BRD3, suggesting epigenetic regulation as a new hallmark of lung carcinogenesis. In 2012, a comprehensive genomic analysis of squamous cell lung cancer was reported by the Cancer Genome Atlas Research Network, showing statistically recurrent mutations in 11 genes, including TP53 mutation in almost all specimens and significantly altered signaling pathways such as NRF2/KEAP1, PI3K/AKT, and CKN2A/RB1. By matching these genomic aberrations with the currently available US Food and Drug Administration-approved targeted therapeutic agent library, the researchers found 64% of patients in this study possess at least one gene that can potentially be a drug. Moreover, the emerging single-cell profiling technique based on whole-genome amplification enables us to process sparse clinical biopsy samples from fine-needle aspirates or core biopsy specimens in which only hundreds of tumor cells are available. With the development of these advanced techniques, we should be able to obtain a better view of tumor pathology, and new drug development may be greatly accelerated in the foreseeable future.

Dynamic tumor monitoring by circulating biomarkers

Because cancer cells continue to evolve, dynamically monitoring molecular changes in the tumor should be involved in the personalized therapy. Rebiopsy of the tumor is a straightforward measure but usually confronts ethical issues. Instead, dynamic monitoring circulating tumor biomarkers, also called liquid biopsy, may become a more realistic option.

Circulating tumor cells and DNA

Malignant tumor cells are known to present in the blood of patients. However, isolation of these cancer cells from blood samples is rather difficult. Maheswaran and coworkers developed a microfluidic-based device in which blood flows through highly condensed micropores coated with epithelial-cell adhesion molecule antibody. The device is able to capture and quantify circulating tumor cells (Ct-cells) at relatively higher efficiency. By combining this isolating technique with the ultrasensitive Scorpion Amplification Refractory Mutation System, the researchers claimed 100% specificity and 92% sensitivity in detecting EGFR mutations and secondary T790M mutation from Ct-cells compared with direct tumor biopsy. Using this technique, they also found an increase of T790M mutant allele in Ct-cells after TKI treatment as a sign of drug resistance. Moreover, using the whole-genome amplification technique, Ni et al recently reported whole-exome sequencing profiles of single Ct-cells obtained from patients with lung adenocarcinoma. These cells exhibit distinct patterns of single-nucleotide variation, but highly reproducible copy number variations. Genes relevant to drug resistance and phenotypic transitions were found to be enriched after chemotherapy. In addition, the detection of mutated tumor DNA in cell-free plasma has also been reported, and the cost is relatively lower, as cell isolation is not required. However, the sensitivity of direct genotyping for DNA mutation in the plasma is markedly lower (43%–73% compared with for direct tumor biopsy), possibly because circulating tumor DNA (Ct-DNA) is more fragmented and it is difficult to extract fragmented tumor DNA from the blood for subsequent amplification. Nevertheless, it has been reported that sensitive EGFR mutants are reduced in the Ct-DNA from the patients with NSCLC after TKI treatment, accompanied by the emergence of resistant DNA mutants. Although larger cohort studies are required before the spread of plasma genotyping, preliminary results have shown a promising future of both Ct-Cells and Ct-DNA in tumor monitoring.

Other circulating nucleotide biomarkers

In addition to Ct-Cells and Ct-DNA, specific miRNAs and methylated tumor DNA in the plasma may also be used as biomarkers of tumor evolution in patients with NSCLC. Compared with Ct-DNA and Ct-Cells, detection of methylated DNA in the plasma is less expensive and more time-saving. It also has been reported that methylated RARB2 and RASSF1A are increased in the plasma of patients with NSCLC. Plasma levels of these methylated genes decrease after tumor resection and chemotherapy, whereas a rebound rise of these plasma biomarkers manifests tumor relapse. So far, very few methylated biomarkers have been identified in the plasma with a predictive value of TKI resistance. Salazar et al showed that unmethylated CHFR may predict prolonged survival in patients receiving EGFR TKIs. Other hypermethylated tumor DNA associated with TKI resistance,
such as death-associated protein kinase,\textsuperscript{136} SFRP5,\textsuperscript{137} may also be worth further validation as plasma biomarkers for tumor monitoring. Moreover, by introducing microelectronic technology, multiple methylated tumor DNAs can be detected and quantified simultaneously, which may further improve efficiency of the detection.\textsuperscript{167}

miRNA is a class of short (18–25 nucleotides), noncoding RNA that binds to complementary mRNA for selective degradation, thus causing a cascade of effects.\textsuperscript{168} Circulating miRNAs, being more stable than other plasma nucleotides, may emerge as another group of biomarkers for cancer monitoring.\textsuperscript{168–170} In female nonsmokers with lung adenocarcinoma, levels of plasma miR-195 and miR-122 can differentiate the patients who may benefit from EGFR TKI treatment.\textsuperscript{171} Circulating miR-21, an important regulator of cell apoptosis and proliferation,\textsuperscript{172,173} was found to be elevated in the patients with NSCLC after TKI treatment, indicating drug resistance.\textsuperscript{172} With the development of nanotechnology, highly sensitive probes in plasma are able to directly detect miRNA without labeling or amplification, which may significantly reduce the time and cost of cancer monitoring.\textsuperscript{174}

Proteomic biomarkers and VeriStrat

In addition to nucleotide biomarkers, proteins in the blood may also provide valuable information for tumor evaluation before and after EGFR-targeted therapy. VeriStrat, a mass spectrometry-based high-throughput platform, can analyze proteomic profiles using only 5 µL serum from the patients and subsequently classify the patients for “good” or “poor” response to TKIs.\textsuperscript{175} To our knowledge, VeriStrat is the only liquid biopsy tool for NSCLC patient stratification before TKI treatment that has been validated in a Phase III clinical trial,\textsuperscript{176,177} which revealed its prognostic value in predicting overall survival and time to progression.\textsuperscript{177–180} However, the VeriStrat test seems to be more powerful in selecting patients who may have worse outcomes on TKIs than on chemotherapy, rather than finding patients who may benefit more from TKIs because the overall survival of “good” patients on either treatment is similar.\textsuperscript{177} In addition, results of VeriStrat may also indicate temporal changes of the tumor. Lazzari et al processed sequentially collected serum samples from 111 NSCLC patients receiving gefitinib and found that one third of these patients converted from good to poor after the treatment, in relevance to drug resistance.\textsuperscript{181} Further proteomic analysis identified overexpressed serum amyloid A protein 1 (SAA1) in plasma from patients with poor VeriStrat results, which is responsible for the generation of four mass signals in the test.\textsuperscript{182} Dynamic change of this protein in the plasma during the course of TKI treatment may merit further investigations.\textsuperscript{182}

Advances in the next generation of targeted therapy

Next-generation irreversible TKIs

Apart from the advances in tumor diagnostics, new inhibitors of EGFR have also been developed and examined in clinical trials (Table 3). First-generation TKIs such as erlotinib and gefitinib inhibit EGFR receptors by reversibly competing with ATP, but the inhibition can be abrogated by secondary EGFR mutations (eg, T790M) because of the increased affinity of EGFR to ATP.\textsuperscript{183} Second-generation TKIs (eg, afatinib [BIBW2992], dacomitinib [PF00299804], and neratinib [HKI-272]) form irreversible covalent bonds with the EGFR receptor and also inhibit other EGFR family members (ERBB2 and ERBB4), and thus may be more potent in tumor suppression.\textsuperscript{183–185} Afatinib, an irreversible EGFR/ERBB2 dual inhibitor, has been extensively studied in a serial of clinical trials of NSCLC.\textsuperscript{186–190} In patients who had failed both chemotherapy and TKI treatment, afatinib significantly prolonged progression-free survival and increased tumor response rate compared with placebo, although no benefit was seen in overall survival.\textsuperscript{187} Dacomitinib, another pan-ERB inhibitor, also exhibits superiority over erlotinib as the second-line therapy after chemotherapy in unselected patients with NSCLC.\textsuperscript{191} The main adverse events of the second-generation TKIs include diarrhea, dysphagia, and mouth sores,\textsuperscript{192} possibly as a result of pan-ERB inhibition.\textsuperscript{193} Severe gastrointestinal reactions of the second-generation TKIs may reduce bioavailability of the drugs.\textsuperscript{194} To specifically target T790M mutation, several third-generation TKIs have been developed and are currently undergoing clinical or preclinical investigations. Of note, these highly specific inhibitors may cause much less damage to EGFR in the gastrointestinal tract and skin hair bulbs compared with their predecessors,\textsuperscript{194,195} and hence intestinal absorption of these drugs may be increased. It is hoped these new drugs may resolve the current clinical dilemma and improve outcomes of targeted therapy.\textsuperscript{12}

Combinational therapy

As previously discussed, activation of pathways bypassing EGFR is another adaptive mechanism against TKIs. In murine lung tumors cotransfected with mutant EGFR and inducible MET oncogenes, treatment with EGFR inhibitor alone failed...
### Table 3 Clinical trials of next-generation TKIs in advanced NSCLC

<table>
<thead>
<tr>
<th>Targets and trial code</th>
<th>Trial Phase</th>
<th>Lines of treatment</th>
<th>EGFR mutations</th>
<th>Trial design</th>
<th>Outcomes</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><strong>Second-generation irreversible TKIs</strong></td>
<td></td>
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<tr>
<td>Afatinib (BIBW2992) EGFR/HER2</td>
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<td>LUX-Lung 1 IIb/III</td>
<td>Third/fourth</td>
<td>Unselected</td>
<td>Afatinib versus placebo in Asian patients</td>
<td>Improved PFS (3.3 versus 1.1 months) and ORR (7% versus 0.5%), no benefit in term of OS</td>
<td>187</td>
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<tr>
<td>LUX-Lung 2 II</td>
<td>First/second</td>
<td>Positive</td>
<td>Afatinib after chemotherapy</td>
<td>ORR, 61%</td>
<td>190</td>
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<tr>
<td>LUX-Lung 3 III</td>
<td>First</td>
<td>Positive</td>
<td>Afatinib versus pemetrexed + cisplatin</td>
<td>Improved PFS (11.1 versus 6.9 months)</td>
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<tr>
<td>LUX-Lung 4 II</td>
<td>Second</td>
<td>Positive in 72.6% of patients</td>
<td>Afatinib after TKIs</td>
<td>ORR, 8.2%; median PFS: 4.4 months; median OS, 19 months</td>
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<tr>
<td>LUX-Lung 5 III</td>
<td>Second</td>
<td>Unselected</td>
<td>Afatinib + chemotherapy versus chemotherapy after afatinib</td>
<td>Ongoing</td>
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<td>LUX-Lung 6 III</td>
<td>First</td>
<td>Positive</td>
<td>Afatinib versus gemcitabine + cisplatin in Asian patients</td>
<td>Improved PFS (11.0 versus 5.6 months)</td>
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<tr>
<td><strong>Dacomitinib (PF00299804) EGFR/HER2/HER4</strong></td>
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<td>NCT00769067 II</td>
<td>Second/third</td>
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<td>Dacomitinib versus erlotinib after chemotherapy</td>
<td>Improved median PFS (2.86 versus 1.91 months) and median OS (9.53 versus 7.44 months) ORR, 8%; median PFS, 18 weeks</td>
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<tr>
<td>NCT00548093 II</td>
<td>Second/third</td>
<td>Positive</td>
<td>Dacomitinib</td>
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<td><strong>Neratinib (HKi-272) EGFR, HER2</strong></td>
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<td>Canertinib (CI-1033) EGFR/HER2/HER4</td>
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<td>NCT00266877 II</td>
<td>Second</td>
<td>Positive</td>
<td>Neratinib after TKIs</td>
<td>ORR, 3% (all in patients with G719X mutation)</td>
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<td>NCT00050830 II</td>
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<td>Unselected</td>
<td>CI-1033 after chemotherapy</td>
<td>1-year survival rate, 26%–29%; ORR, 2%–4% ORR, 26%; median PFS, 5.1 months; median OS, 12.4 months</td>
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<td>N/A I/II</td>
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<td>Unselected</td>
<td>CI-1033 + paclitaxel + carboplatin</td>
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<td><strong>Third-generation irreversible TKIs</strong></td>
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<td>CO-1686 after TKIs</td>
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<td>AZD9291/EGFR (T790M mutation)</td>
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<td>NCT01802632</td>
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<td>AZD9291 after TKIs</td>
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<tr>
<td>NCT02094261</td>
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<td>Positive</td>
<td>AZD9291 in patients with T790M mutation after TKIs</td>
<td>Ongoing</td>
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</table>

**Abbreviations:** TKI, tyrosine kinase inhibitor; NSCLC, nonsmall cell lung cancer; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; PFS, progression free survival; ORR, overall response rate; OS, overall survival; HER4, human epidermal growth factor receptor 4.
to inhibit the tumor growth, but addition of the MET inhibitor crizotinib significantly shrinks the tumor.\textsuperscript{195,199} In HGF overexpressing lung cancer cell lines, crizotinib and gefitinib together suppress TKI-resistant tumor cell growth.\textsuperscript{200} Clinical evaluation of the effectiveness of dual MET/EGFR inhibition is currently undergoing.\textsuperscript{201,202} Moreover, HSP90, a protein-folding chaperone participating in EGFR stabilization in the tumor, may also emerge as a target for tumor control.\textsuperscript{203–205} Inhibition of HSP90 in lung cancer cell lines and animal models increases the sensitivity to TKIs and enhances tumor suppression.\textsuperscript{203,206,207} In an early clinical trial, HSP90 inhibitor ganetespib showed a notable disease control rate in patients refractory to chemotherapy and EGFR TKIs, and the adverse effects were acceptable.\textsuperscript{208} Further clinical cohorts are needed to evaluate the efficacy and safety of HSP90 inhibitors alone or in combination with other targeted treatments.\textsuperscript{209}

Abnormal activation of angiogenesis manifested by overexpression of VEGF is another hallmark of progressing NSCLC.\textsuperscript{210,211} In patients with advanced NSCLC, chemotherapy plus VEGF targeting monoclonal antibody bevacizumab has been shown to prolong progression-free survival (6.2 versus 4.5 months) and overall survival (12.3 versus 10.3 months) compared with chemotherapy alone.\textsuperscript{212} Close crosstalk between VEGF and EGFR signaling cascades provides a rationale to concurrently inhibit both pathways. The benefit of combining bevacizumab with erlotinib for the treatment of recurrent NSCLC after chemotherapy has been observed in Phase I/II studies, and the adverse effects are tolerable.\textsuperscript{213,214} However, subsequent Phase III trials comparing combined treatment of erlotinib and bevacizumab with erlotinib alone as the second-line treatment showed no superiority of the combination in terms of progression-free survival and overall survival.\textsuperscript{215,216} Notably, these trials are all conducted in unselected patients.\textsuperscript{210,211} VeriStrat-based proteomic analysis may effectively distinguish NSCLC patients who are likely to benefit from the dual inhibition. Patients with a good VeriStrat result had significantly longer progression-free and overall survival (18.9 versus 6.3 weeks and 71.4 versus 19.9 weeks, respectively) than the patients with a poor result.\textsuperscript{217} It is hoped that outcomes of the dual EGFR/VEGF inhibition may be improved in patients with activating EGFR mutations.\textsuperscript{218}

**Summary and perspectives**

Despite tremendous efforts made to improve outcomes of cancer care in the past decades, the death rate of patients with advanced NSCLC is still very high. Personalized cancer treatments targeting specific tumor-driving mutations provide new options for patients who failed chemotherapy, greatly transforming cancer care.\textsuperscript{219} However, despite an initial response, the patients eventually die from disease progression as a result of drug resistance. With a deeper understanding of tumor biology, we now realize this later-developed drug resistance may turn up as a result of tumor heterogeneity and clonal adaptation. In fact, complexity of the adaptive process is probably much beyond our imagination because CSCs, abnormalities in mitotic-maintaining genes, and epigenetic mechanisms may all contribute to the process. In terms of EGFR-targeted therapy, resistant mechanisms (eg, EGFR T790M mutation and MET amplification) emerge as a result of the tumor evolution after EGFR inhibition by gefitinib or erlotinib. Therefore, biomarkers that are sensitive to the resistant tumor clones and a new compound with specific effects on these resistant mechanisms have become the focus of research in this field (Figure 2). Moreover, instead of biopsy at a single time, next-generation personalization may involve multiple rounds of tumor biopsies, followed by changes of the treatment to overcome the dynamic tumor evolution and drug resistance.\textsuperscript{6,220,221} At present, laboratory findings are rapidly translated to clinical applications. Novel molecular targeted compounds and less invasive diagnostic tests are currently undergoing clinical evaluation, with some showing encouraging results. Cost/effectiveness evaluation is needed before these new tests and drugs can be spread for clinical use. Future studies should further elucidate the mechanism underlying tumor evolution, and measures to retard its process merit more investigation. With the advances seen in this highly active field, improvements in the clinical outcome of the patients with advanced NSCLC can be expected in the foreseeable future.

**Figure 2** Schematic summary of measures to overcome tumor heterogeneity and drug resistance in lung cancer.

**Notes:** Spatial heterogeneity indicates inter-/intra-tumour differences at the genomic, epigenetic, and proteomic levels, whereas temporal heterogeneity reflects dynamic tumor evolution over time. Some diagnostic and therapeutic approaches have been validated in patients for clinical translation.

**Abbreviation:** TKI, tyrosine kinase inhibitors.
Disclosure
The authors report no conflicts of interest in this work.

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