Tumor clonality and resistance mechanisms in *EGFR* mutation-positive non-small-cell lung cancer: implications for therapeutic sequencing

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While the development of *EGFR*-targeted tyrosine kinase inhibitors (TKIs) has revolutionized treatment of *EGFR* mutation-positive non-small-cell lung cancer, acquired resistance to therapy is inevitable, reflecting tumor evolution. Recent studies show that *EGFR* mutation-positive non-small-cell lung cancer is highly heterogeneous at the cellular level, facilitating clonal expansion of resistant tumors via multiple molecular mechanisms. Here, we review the mechanistic differences between first-, second- and third-generation *EGFR*-targeted TKIs and speculate how these features could explain differences in clinical activity between these agents from a clonal evolution perspective. We hypothesize that the molecular dissection of tumor resistance mechanisms will facilitate optimal sequential use of *EGFR* TKIs in individual patients, thus maximizing the duration of chemotherapy-free treatment and survival benefit.

First draft submitted: 25 September 2018; Accepted for publication: 19 October 2018; Published online: 8 November 2018

Keywords: acquired resistance • afatinib • clonal evolution • EGFR • erlotinib • gefitinib • NSCLC • osimertinib

Patients with *EGFR* mutation-positive non-small-cell lung cancer (NSCLC) are generally highly responsive to treatment with EGFR-targeted tyrosine kinase inhibitors (TKIs). Several TKIs are approved for the treatment of *EGFR* mutation-positive NSCLC, and are categorized as first-, second- and third-generation agents based on profound differences in pharmacological characteristics, mechanism-of-action and the order in which they were developed. First-generation TKIs, such as gefitinib and erlotinib, non-covalently and reversibly bind to EGFR (both wild-type and mutated forms). They interrupt EGFR signaling by competing with ATP at the intracellular kinase domain of the receptor, thus inhibiting dimer autophosphorylation and attenuating intracellular signaling [1,2]. Several seminal Phase III trials have demonstrated that first-line gefitinib and erlotinib prolong progression-free survival (PFS), but not overall survival (OS), compared with platinum-doublet chemotherapy in patients with *EGFR* mutation-positive NSCLC (median PFS of 9.2–13.1 months vs 4.6–6.3 months, respectively) [3–11]. Recent studies have demonstrated that the activity of first-generation TKIs is accentuated by combining them with anti-angiogenic agents such as bevacizumab [12].

The second-generation EGFR-targeted TKI, afatinib, works differently to erlotinib and gefitinib. It covalently and irreversibly binds not only to EGFR but also to other members of the ErbB family of receptors, HER2 and HER4, thus blocking transphosphorylation of tyrosine residues at the C-terminus of all three receptors [13,14]. It also blocks the transphosphorylation of the kinase inactive HER3 protein via its partner in HER3-containing heterodimers [14]. Therefore, afatinib represents an ErbB family blocker that covalently binds to, and irreversibly blocks, signaling from all possible ErbB homodimers and heterodimers [13,15]. It inhibits EGFR more potently than first-generation EGFR TKIs and has greater anti-NSCLC preclinical activity, both in cell-based experiments and in animal models [15,16]. Afatinib has demonstrated PFS benefit versus chemotherapy in two Phase III trials, LUX-Lung 3 and LUX-Lung 6 [17,18]. Also, in contrast to first-generation EGFR TKIs, afatinib was associated with significant improvements in OS versus chemotherapy in both trials, based on a prespecified analysis of patients with the most common category of *EGFR* mutation, deletions within exon 19 (Del19) [19]. In contrast to first- and second-generation agents, third-generation EGFR-targeted TKIs such as osimertinib were designed to covalently bind to only some mutated forms of EGFR, thus lowering the incidence of EGFR wild-type mediated toxicities. While such agents were developed in a second-line setting, osimertinib has recently demonstrated a median PFS benefit of 17.7 months (independent assessment) in previously untreated patients with *EGFR* mutation-positive (Del19 or L858R) NSCLC [20], and is now approved in this setting.

While EGFR-targeted agents are associated with robust clinical benefits, acquired resistance to treatment is inevitable. The most common resistance mechanism to first- and second-generation EGFR-targeted TKIs is the so-called ‘gatekeeper’ T790M mutation in exon 20 of *EGFR*, which has been identified in around 50–70% of cases [21–25]. This mutation increases the affinity of EGFR for ATP, thus reducing the potency of ATP-competitive TKIs [26]. The identification of T790M provided the original rationale for the development of osimertinib and other third-generation TKIs, which also inhibit T790M-positive tumors. Indeed, second-line osimertinib has demonstrated striking activity in this setting and is associated with high response rates (~60–70%) and impressive PFS (~10–12 months) in T790M-positive patients [23,27,28].

Several T790M-independent mechanisms of resistance to first-generation EGFR-TKIs have been identified including aberrations affecting other receptors (e.g., HER2, HER3, MET, RET and IGF-1 receptors) leading to compensatory signaling via intracellular pathways such as the PI3K/AKT/mTOR and JAK2/STAT3 cascades [29,30], transformation to small cell histology [22] and epithelial to mesenchymal transition [31]. Less data are available regarding T790M-independent resistance mechanisms to second-generation TKIs including afatinib. Unsurprisingly, given its mechanism of action, compensatory signaling via other ErbB family members has not been identified as a mechanism of resistance to afatinib. Rather, resistance mechanisms through non-target pathways such as MET amplification or histological transformation have been reported [32–34].

In contrast to first- and second-generation TKIs, no predominant mechanism of acquired resistance has been reported following first-line treatment with osimertinib, although few data are available at the moment. Resistance mechanisms identified to date appear to be highly heterogeneous [35,36]. In a cohort of 19 patients treated with first-line osimertinib, putative resistance mechanisms were only identified in nine cases and included mutations

in *MEK1*, *KRAS*, *PI3KCA*, *HER2* or *JAK2* or amplification of *KRAS*, *MET* or *EGFR*. Two patients acquired the tertiary *EGFR* resistance mutation, C797S. Consequently, possible targeted treatment options following first-line osimertinib remain unclear. More data are available regarding resistance to osimertinib in later-line settings. In general, such resistance mechanisms seem to differ depending on whether tumors maintain or lose the T790M mutation [37]. Mechanisms include the accrual of C797S in approximately 20–40% of cases, transformation to small cell histology, amplification of *HER2*, *MET* or *FGFR*, and mutations in *BRAF* [37–40]. *EGFR*-mediated resistance occurs more frequently in cases where T790M is preserved [37]. In patients with T790M-positive tumors treated with second-line osimertinib, C797S appears to predominantly occur in cells with a background Del19 mutation and *in cis* with T790M (i.e., on the same allele [39,40]). Hence, combinations of currently available *EGFR* TKIs are not expected to be effective against triple *EGFR* mutants. However, such combinations could be effective in a first-line setting where preventing emergence of resistance is the major goal. Other *EGFR* mutations (e.g., L792X and L718Q) have also been associated with resistance to osimertinib [41]. *In vitro* studies indicate that L792X mutants may retain sensitivity to first- and second-generation *EGFR* TKIs when T790M is absent, whereas L718Q mutants are sensitive to afatinib but not gefitinib [42]. Such observations may be valuable for clinical practice.

Therefore, understanding the clonal evolution of tumors, in other words, the proliferation of certain cell types, with certain molecular characteristics, in response to the selective pressures conferred by treatment is of utmost importance. Ultimately, an understanding of how tumors develop at the cellular level in individual patients will be crucial in improving clinical outcomes by guiding rational therapeutic decisions with a view to delay resistance, and the identification of the most appropriate treatment options following the development of resistance. Recent progress with cutting-edge genomic technologies have demonstrated that *EGFR* mutation-positive tumors can be complex and highly heterogeneous. Accordingly, there is a high risk of background genomic aberrations driving clonal expansion of a resistant, and potentially difficult-to-treat tumor, even if such aberrations are present in a very small number of tumor cells prior to initiation of therapy.

This review article comprises five sections. First, we briefly discuss recent developments in the general understanding of tumor clonality and the implications of clonal divergence on the diagnosis, clinical course and resistance to treatment of human cancers. Second, with these concepts in mind, we give an overview of recent findings that demonstrate considerable molecular complexity of *EGFR* mutation-positive NSCLC tumors and how this relates to clonal divergence, in other words, acquired resistance mechanisms, observed in patients with *EGFR* mutation-positive NSCLC. Third, given the structural and mechanistic differences between first-, second- and third-generation *EGFR*-targeted TKIs, we compare and contrast the activity of these agents against cell models of *EGFR* mutation-positive tumor development, focusing on their activity against different *EGFR* mutations and cells with co-occurring genetic aberrations. Fourth, we discuss recent clinical data comparing different generations of *EGFR*-targeted TKIs. Finally, we discuss how emerging knowledge of resistance mechanisms over the course of tumor evolution may help drive the therapeutic sequencing of *EGFR* TKIs, with the aim of maximizing the time patients can remain chemotherapy-free and optimizing survival outcomes.

The general implications of tumor heterogeneity on the diagnosis & treatment of cancer

It is generally accepted that tumors undergo complex Darwinian evolution over the course of their development, and during therapy [43]. This concept was first proposed in 1976 by Peter Nowell, who outlined a stepwise process by which tumor cell populations descended from a single progenitor or ‘clone’ acquire successive somatic mutations that confer a selective advantage [44]. Genomic instability of developing tumors creates a genetically diverse cell population that is subject to selective forces, as well as neutral changes over time (genetic drift) [45]. Eventually, selective outgrowth of clones that have a phenotypic advantage within a given microenvironment or therapeutic context results in the evolution of genetically distinct subclonal populations [44,46].

While the concept of tumor evolution has been accepted for a long time, it is only with recent technological advances, such as next-generation sequencing (NGS) platforms, that it has been possible to dissect the development of tumors at sufficient resolution to enable the construction of divergence-based phylogenetic trees to illustrate tumor development, including NSCLC [47,48]. Moreover, new liquid biopsy technologies have facilitated the tracking of mutations over time to determine whether they are clonal or subclonal events [49,50]. This has allowed the definition of different categories of mutational events during the evolutionary process (Figure 1) [51]. ‘Truncal’ mutations are clonal mutations that are present in all tumor cells and regions, indicating a potential early role in driving oncogenesis. ‘Branch’ mutations are later subclonal events that are present in only a small proportion of

Figure 1. Phylogenetic tree analysis. Truncal mutations (A) occur early during tumorigenesis and are present in most tumor cells/regions. Branch mutations (B & C) occur later in tumorigenesis and are present in some, but not all, tumor regions. Later branch mutations (D–F) tend to be highly localized within developing tumors.

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tumor cells and may emerge in subpopulations within discrete tumor locations (including in lung cancer), resulting in intratumoral clonal diversity [43,48].

The clonal nature of tumor evolution underpins the difficulty of long-term treatment of tumors. Any treatment modality, be it traditional chemotherapeutics or targeted therapies, may impose strong selective pressure on tumor cells allowing resistant cells to survive which, under the right circumstances, may develop into a tumor which is resistant to treatment and, in terms of molecular characteristics, different to the original malignancy. This has obvious implications when considering treatment strategies. For example, ‘truncal’ mutations are often targets of established therapies, including those directed against *EGFR* mutations in NSCLC. Given the role of ‘truncal’ mutations as oncogenic drivers, such treatment is usually highly effective initially, but can drive the clonal development of ‘branch’ mutations. Indeed, substantial changes in clonal dominance over time have been observed in a number of human malignancies including NSCLC, breast cancer, multiple myeloma and acute lymphoblastic leukemia, whereby the mutational profile at disease progression or death can be markedly different to that at diagnosis [43,46,49]. The selective pressure of multiple lines of drug treatment can increase the complexity of tumors by facilitating the selection, and propagation, of cells that have intrinsic or acquired resistance to targeted treatment, thus influencing disease progression [51].

Given the impact of clonal evolution on the development of acquired resistance to treatment, it would be highly advantageous to understand the clonal architecture of tumors prior to initiation of, and during, therapy. In practice, however, this has proved difficult for technological and practical reasons. For example, subclonal populations may intermingle or be spatially separated within tumor lesions or between primary and metastatic sites, such that sampling bias can confound resolution of the clonal status of mutations. Therefore, a single biopsy in one tumor region might identify multiple subclones, while a biopsy in another region could give a false indication of clonality if dominated by an individual subclone. Of note, even in cases where one clone dominates the physical entity of a tumor, low frequency and often undetectable subclones can determine the clinical course and, depending on treatment sequence, affect therapy outcomes [49]. Indeed, multiple different subclones may harbor distinct resistance-conferring mutations rather than there being a single dominant resistance clone [43]. This spatial and temporal heterogeneity of tumors underpins the need for studies that use longitudinal tissue sampling. To this end, the use of ‘liquid biopsies’, analyzing cell-free (cf) DNA or circulating tumor cells in peripheral blood, provides a relatively noninvasive method for monitoring genomic alterations leading to disease progression [51]. However, such methodology will not provide any information on intra- or inter-tumor spatial heterogeneity. Liquid biopsy is a representation of the total tumor burden (primary and secondary tumors) and some tumors may shed

DNA more than others. Furthermore, although cfDNA tests for the detection of activating and resistance *EGFR* mutations have already entered clinical practice, further validation of the concept and sensitivity/specificity of such platforms is required. Concordance rates between paired plasma and tumor samples demonstrate high specificity but low sensitivity (<50%), so false-negative results are possible [52]. Moreover, comparison of different platforms demonstrated concordance rates of 57–90% for the detection of T790M [52]. This is consistent with experience of cfDNA tests in other tumor types [53], demonstrating that the congruence rates of commercially available tests can be suboptimal.

The complexity of *EGFR* mutation-positive NSCLC tumors

Emerging data indicate that the concepts described above are relevant to *EGFR* mutation-positive NSCLC tumors. Indeed, it is becoming increasingly clear that such tumors are genetically heterogeneous and undergo clonal evolution. Two sources of heterogeneity, which have possibly been underappreciated in the past, are: the level of co-occurring genetic alterations that are present in the background of *EGFR* mutation-positive tumors and the potential existence of several different types of specific *EGFR* mutation within a single tumor. Recent evidence uncovering these phenomena, and their likely clinical significance, are discussed below.

Co-occurring genetic alterations

Until recently, there were very few data regarding co-occurring genetic mutations in *EGFR* mutation-positive tumors, and how such mutations may evolve over the course of tumor growth, and in response to treatment with *EGFR*-targeted TKIs. This paucity of information was largely due to technological limitations that precluded the possibility of longitudinal genetic analysis over multiple time points. Initial large-scale sequencing analysis of NSCLC tumors focused on a single time-point (i.e., at tumor biopsy) at an early stage of tumor detection prior to initiation of treatment [54]. Such analysis indicated that *EGFR* mutations are probably early clonal events that drive oncogenesis. However, the potential role of co-occurring aberrations in driving tumor progression and treatment resistance remained an open question. These questions are now being addressed, fostered by the development of validated blood-based cfDNA assays. In a recent study, Blakely *et al.* undertook comprehensive genomic analysis of 68 relevant cancer genes using a validated cfDNA exome platform on 1122 consecutive DNA samples from 1006 patients with advanced stage *EGFR* mutation-positive NSCLC [49]. This analysis highlighted that additional genetic mutations are very common in late-stage tumors. In addition to *EGFR*, there was an average of 2.6 ± 1.7 co-occurring genetic alterations in the 68 cancer relevant genes in the samples tested; 93% of patients had at least one variant of known or likely functional importance. These additional aberrations included known oncogenic drivers in intracellular signaling and cell cycle genes such as *PIK3CA*, *BRAF*, *MET*, *MYC*, *CDK6* and *CTNNB1*. The analysis also indicated that co-occurring mutations are very common in T790M-positive tumors. In 440 T790M-positive cases, there was an average of 2.4 ± 1.9 co-occurring genetic alterations in the cancer relevant genes. Frequent alterations were observed in the cell cycle pathway genes (24.1%), DNA repair genes (10.9%), epigenetic pathway genes (14.1%), WNT pathway genes (15.5%) and hormone signaling genes (6.8%).

As expected, activating *EGFR* mutations were generally truncal (in ~90% of samples) in the Blakely *et al.* study, whereas T790M was more frequently restricted to branches (~30% of samples) [49]. Approximately 40% of both Del19/L858R and T790M-positive tumors harbored additional branchial genetic aberrations, indicating that additional mutations evolve and develop over the course of the disease. Moreover, notwithstanding the limitations of liquid biopsies (which cannot detect spatial inter- or intra-tumor heterogeneity), data from patients who underwent longitudinal cfDNA analysis indicate that the genetic complexity of tumors probably increases over the course of treatment. In the 97 patients for whom longitudinal cfDNA, clinical context and treatment response data were all available, the frequency of additional somatic alterations increased with each line of therapy. Furthermore, the mean number of functional alterations was lower in patients who responded to *EGFR*-targeted TKI treatment (of any generation) than in patients who did not respond, possibly indicating lower tumor mutational burden in these patients [49]. These data indicate that *EGFR* mutation-positive tumors actually evolve to become more genetically complex during treatment and this complexity may be associated with primary and acquired resistance to successive lines of therapy. Therefore, understanding the baseline molecular makeup of the tumor and the mechanisms of resistance to particular agents may help drive clinical decision making in terms of selection of the most appropriate agents and sequence of therapies.

While the identification of T790M as the predominant mechanism of acquired resistance, and the development of third-generation *EGFR* TKIs, such as osimertinib, to target this aberration, were major advances in the treatment

of *EGFR* mutation-positive NSCLC, it is open to debate how, and when, this aberration evolves during first-line treatment with first- or second-generation *EGFR* TKIs. It is unclear whether small numbers of T790M-positive cells pre-exist within the tumor prior to treatment initiation or, alternatively, whether T790M arises as a *de novo* branching event during treatment. This is an important question because the level of clonality of T790M at the point of acquired resistance may have implications in terms of clinical response, and the duration of response, to third-generation *EGFR* TKIs, in other words, the greater the uniformity of T790M-driven resistance at progression, the higher the likelihood of enduring response to agents that specifically target this aberration. Again, this question has proved to be very difficult to directly address. Even highly sensitive technologies, including NGS and droplet-digital PCR may not have the resolution to detect rare pre-existing T790M-positive clones at the cellular level [50]. While several studies have reported the detection of low-frequency T790M clones in tumors prior to the initiation of first-line therapy [55,56], doubts have been raised regarding the validity of these findings due to the possibility of sequencing artifacts related to tissue fixation methodology [57].

Recent analysis of *EGFR* mutation-positive cell lines provided compelling evidence that T790M can arise from pre-existing clones prior to the initiation of first-line therapy, but can also develop during the course of treatment from a pool of drug-resistant ‘persister’ cells [58]. These cells have been observed in several studies [59–61], and survive treatment, possibly due to epigenetic adaptations, but do not expand until they acquire additional resistance mechanisms. Hata *et al.* undertook a series of experiments where *EGFR* mutation-positive cells were cultured in the presence of escalating concentrations of gefitinib [50]. Two different categories of resistant clones developed; those that displayed early resistance (i.e., within 6 weeks) and those that emerged later (within 24 weeks). All early resistant clones were T790M-positive, and by utilizing a recently developed DNA barcoding technology (ClonTracer) [62], it was demonstrated that these clones were derived from pre-existing T790M-positive cells. In contrast, late resistant clones were initially T790M-negative; T790M arose in drug-tolerant persister cells, and gradually expanded to become the dominant clone. Resistant cells that remained T790M negative had a plethora of other mutations at low allele frequencies including mutations in *NRAS*, *KRAS*, *BRAF* and *RET*. These findings indicate that T790M may gradually evolve from *de novo* events occurring within persister cells, within a background of complex genetic heterogeneity. Of note, late resistant clones appeared to be less sensitive to third-generation *EGFR* TKIs than early resistance clones, presumably reflecting the distinct evolutionary paths by which T790M arose. These preclinical observations have clinical ramifications and suggest that first-line treatment approaches that target the predominant oncogenic driver (i.e., *EGFR* activating mutations) but also limit the development of persister cells, with complex mutational profiles, would be beneficial, as these cells appear to develop in difficult-to-treat tumors.

EGFR mutation heterogeneity

The most frequently identified *EGFR* mutation category are Del19 mutations (~45% of all *EGFR* mutations), which represent at least 30 variants. The next most prevalent mutation is the L858R point mutation in exon 21 (~40% of *EGFR* mutations). Together Del19 and L858R are termed ‘common’ mutations, which are known to be highly sensitive to all generations of *EGFR*-targeted TKIs, although collectively Del19 mutations are typically regarded as being more sensitive than L858R [63]. Recent improvements in detection methods have indicated that ‘uncommon’ *EGFR* mutations are more prevalent than previously thought. According to the catalogue of somatic mutations in cancer (COSMIC) database, in 2016, 594 types of *EGFR* mutation had been reported [64]. Categories of uncommon mutation include insertions in exon 19 (Ins19; 0.6% of all *EGFR* mutations) or 20 (Ins20; ~6%), as well as point mutations in exon 18 at position G719 (G719X; ~3%), exon 21 L861Q mutations (~1%) and exon 20 S768I mutations (~1% of *EGFR* mutations) [64]. Despite being present in at least 10% of patients with *EGFR* mutation-positive NSCLC [65], most Phase III trials excluded patients with ‘uncommon’ mutations (LUX-Lung 3 and 6 being notable exceptions).

In addition, it is becoming increasingly clear that some patients with *EGFR* mutation-positive NSCLC have tumors that harbor more than one type of *EGFR* mutation, predominantly *in cis*, in other words, within the same *EGFR* allele (so-called ‘compound’ mutations) [64,66]. Compound mutations are associated with poorer clinical outcomes compared with simple mutations [67]. Recent NGS analysis indicates that compound mutations are present in up to 25% of patients with *EGFR* mutation-positive NSCLC [66,67]. Most commonly, compound mutations consist of an *EGFR* TKI-sensitizing mutation such as Del19, L858R or G719X, together with a rare mutation of unknown clinical significance [68]. A minority of compound mutations comprise only uncommon atypical mutations [66,67,69].

Figure 2. T790M gene dosage in Ba/F3 *EGFR* del19 cells resistant to erlotinib and afatinib. Ba/F3 cells expressing *EGFR* del19 were mutagenized with N-ethyl-N-nitrosourea and subsequently treated with erlotinib or afatinib to isolate drug-resistant clones. The *EGFR* sequence at residue T790 was analyzed in 20 wells displaying erlotinib- or afatinib-resistant cell growth by DNA extraction followed by PCR and Sanger sequencing (chromatograms, left). The peak heights of C and T at codon position 2 of T790 were calculated for each drug-resistant well and plotted (graph, right). Bar, mean and p-value, unpaired t-test.

Preclinical differences in activity of *EGFR*-directed TKIs against NSCLC tumor cells

Cells with co-occurring genetic alterations

Ideally, given the high prevalence of co-occurring genetic alterations in *EGFR* mutation-positive NSCLC, TKIs should have a broad inhibitory profile to mitigate for the heterogeneity of tumors and the risk of rapid expansion of resistant subclones. Also, if T790M-mediated acquired resistance occurs, it would be advantageous that the T790M clone is as homogeneous as possible to maximize the chances of a prolonged response to third-generation TKIs like osimertinib. Furthermore, low levels of T790M clonality may compromise the ability of tissue or liquid assays to detect the mutation.

As co-occurring aberrations in *EGFR* mutation-positive tumors are highly heterogeneous, and usually occur in only a small number of cells, it is technologically very difficult to compare the activity of *EGFR*-directed TKIs against them. Nevertheless, we have undertaken experiments that have specifically assessed the clonality of tumor cells following acquired resistance. We cultured Ba/F3 cells that expressed Del19 *EGFR*. The cells were subjected to N-ethyl-N-nitrosourea mutagenesis and cultured in the presence of afatinib or erlotinib (50-fold IC_{50} concentration) for 3 weeks. Resistant wells were selected and genomic DNA was extracted. Subsequently, the tyrosine kinase domain of *EGFR* was amplified by PCR and sequenced. The majority of resistant wells for both afatinib and erlotinib were T790M-positive. Interestingly, the frequency of T790M alleles was higher in afatinib-treated cells than erlotinib-treated cells as determined by measuring sequencing peak heights, indicating that T790M clones were more homogeneous following afatinib treatment (Figure 2). This observation possibly suggests that afatinib, due to its broader inhibitory profile, is active against a greater proportion of co-occurring aberrations than erlotinib in Del19 cells, preventing their clonal expansion. Another possibility is that, due to its modest activity against T790M, a higher allele frequency of T790M is required to generate resistance to afatinib compared with erlotinib or gefitinib. These inferences are speculative and require further investigation.

Figure 3. Assessment of drug sensitivity of uncommon single and compound *EGFR* mutations using the mixed-all-nominated-mutants-in-one (MANO) method in Ba/F3 cells.
Brown: Deletion; green: Missense mutation; red: Insertion.
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Cells with uncommon or compound EGFR mutations

The heterogeneity of *EGFR* mutations in *EGFR* mutation-positive NSCLC has potential clinical implications because different *EGFR*-targeted TKIs can have different activities against specific mutations. Therefore, treatment with *EGFR* TKIs that have limited efficacy against certain mutations may drive the clonal expansion of those insensitive mutations. Moreover, it is possible that exposure to drugs with a limited spectrum of activity against different mutations within a single oncogene may actually drive the selection of cells with compound mutations, leading to the development of difficult-to-treat resistant tumors. This phenomenon has been identified in patients with BCR–ABL1 mutation-positive chronic myeloid leukemia [70]. It may be important, therefore, that *EGFR* TKIs have as broad an inhibitory profile as possible in terms of activity against specific *EGFR* mutations.

Emerging preclinical evidence indicates that second-generation TKIs may have a broader inhibitory profile against *EGFR* mutations than first- or third-generation TKIs [64,66]. In a recent study, Kobayashi and Mitsudomi collated available preclinical data assessing the activity of first-generation (gefitinib and erlotinib), second-generation (afatinib, dacomitinib, neratinib) and third-generation TKIs (osimertinib, rociletinib) in Ba/F3 cells expressing specific *EGFR* mutations [59]. This analysis demonstrated that second-generation TKIs have a broad inhibitory profile with afatinib being particularly active against exon 18 mutations, Del19 mutations, Ins19 mutations and S768I.

Another recent preclinical study assessed the activity of different *EGFR*-directed TKIs against uncommon *EGFR* variants of unknown significance. By utilizing a high-throughput functional assay in Ba/F3 cells, Kohsaka *et al.* evaluated the sensitivity of over a hundred different variants of unknown significance, including compound mutations [66]. Interestingly, afatinib showed activity against almost all mutations tested and, in many cases, was more potent than erlotinib and gefitinib (Figure 3). Afatinib also showed a broader spectrum of activity against uncommon *EGFR* variants compared with osimertinib, although, as expected, afatinib was less effective against T790M than osimertinib [66].

A further interesting finding from the Kohsaka *et al.* study was that the emergence of certain compound mutations (e.g., L858R and E709A/G) was associated with diminished response to gefitinib and erlotinib. However, the sensitivity of L858R_E709 compound mutations to afatinib was not remarkably different to that observed for

L858R alone ($IC_{50} < 0.1$ nM). Indeed, afatinib reduced the viability of cells expressing numerous compound mutations, including those involving Del19, G719X and/or S768I mutations (Figure 3); only compound mutations involving T790M were considered to be resistant to afatinib [66]. Of note, cDNA-based amplicon sequencing and droplet digital PCR indicated that many compound mutations detected occurred *in cis*. This means that the co-existing mutations are likely to have an additive impact on the tertiary structure of EGFR, which could underlie the relevance of compound mutations in terms of acquired resistance. Importantly, preclinical data described above indicate that afatinib, possibly due to its alternative mechanism of action, may reduce the selection for compound mutations as a mechanism of acquired resistance compared with gefitinib, erlotinib or osimertinib.

Clinical data comparing different generations of EGFR-targeted TKIs

Head-to-head clinical studies

While there is a plethora of clinical data validating the first-line use of EGFR-targeted TKIs in patients with *EGFR* mutation-positive NSCLC, until recently, there have been very few head-to-head comparisons between the different generations of agent. However, in the last few years, several trials (LUX-Lung 7, FLAURA, ARCHER-1050) have demonstrated that second- and third-generation TKIs are more efficacious than first-generation TKIs in a front-line treatment setting.

LUX-Lung 7 was a global Phase IIb trial that compared afatinib ($n = 160$) versus gefitinib ($n = 159$) in patients with *EGFR* mutation-positive (Del19 or L858R) NSCLC [71]. The study had three co-primary end points; PFS, OS and time to treatment failure (TTF). TTF was chosen as an end point to reflect the fact that treatment with TKIs is often continued beyond radiological progression in 'real-world' clinical practice. Both PFS (median: 11.0 vs 10.9 months; hazard ratio [HR]: 0.73 [95% CI: 0.57–0.95], $p = 0.017$) and TTF (median 13.7 vs 11.5 months; HR: 0.73 [95% CI: 0.58–0.92], $p = 0.0073$) were significantly improved with afatinib compared with gefitinib. There was a trend toward improved OS (median: 27.9 vs 24.5 months; HR: 0.86 [95% CI: 0.66–1.12], $p = 0.2580$) [72]. The response rate with afatinib was higher than with gefitinib (70 vs 56%; odds ratio: 1.87 [95% CI: 1.18–2.99], $p = 0.0083$) [71]. Similar response rates were observed in patients with Del19 mutations (73% with afatinib and 66% with gefitinib). However, in patients with tumors harboring L858R mutations, the response rate was 66% with afatinib versus 42% with gefitinib.

Given its limited availability at the time of the study, only 50 patients went on to receive osimertinib following afatinib ($n = 27$) or gefitinib ($n = 23$). However, clinical outcomes in the patients were encouraging. At the time of analysis, median OS had not been reached in either group. A further analysis, including patients from LUX-Lung 3 and LUX-Lung 6 who received sequential afatinib and osimertinib ($n = 10$), demonstrated prolonged duration of therapy with osimertinib (median: 20.2 months) [73].

The recent Phase III ARCHER-1050 trial compared dacomitinib ($n = 227$), another ErbB family blocker, to gefitinib ($n = 225$) in patients with *EGFR* mutation-positive (Del19 or L858R) NSCLC [74]. Although there were differences in trial populations between LUX-Lung 7 and ARCHER-1050 (e.g., patients with stable brain metastases were included in the former but excluded in the latter), ARCHER-1050 provided further evidence that second-generation EGFR-directed TKIs are more efficacious than first-generation TKIs. Dacomitinib significantly prolonged PFS compared with gefitinib (14.7 vs 9.2 months; HR: 0.59 [95% CI: 0.47–0.74], $p < 0.0001$). Notably, it also significantly improved OS versus gefitinib (median: 34.1 vs 26.8 months; HR: 0.76 [95% CI: 0.58–0.99], $p = 0.044$) [75]. The response rate was 75% with dacomitinib and 72% with gefitinib. Response rates were similar regardless of mutation type. The duration of response was longer with dacomitinib than gefitinib (median: 14.8 vs 8.3 months; HR: 0.40 [95% CI: 0.31–0.53], $p < 0.0001$).

Osimertinib has also demonstrated superior clinical benefits versus first-generation EGFR TKIs. The Phase III FLAURA trial compared osimertinib ($n = 279$) versus gefitinib or erlotinib ($n = 277$) in patients with *EGFR* mutation-positive (Del19 or L858R) NSCLC [20]. PFS was significantly improved in the osimertinib arm (by investigator assessment, median: 18.9 vs 10.2 months; HR: 0.46 [95% CI: 0.37–0.57], $p < 0.001$). Response rate was similar between treatment arms (80% with osimertinib and 76% with erlotinib/gefitinib) but duration of response was higher with osimertinib (17.2 vs 8.5 months). OS data were immature at the time of publication (25%). The crossover rate to osimertinib for patients discontinuing study treatment in the erlotinib/gefitinib arm was only 23%. Of further note, afatinib was not included in the comparator arm of FLAURA, so while the outcome data are very encouraging, it is not possible to draw any conclusions regarding the relative efficacy of second- and third-generation EGFR-directed TKIs based on FLAURA.

Activity against uncommon EGFR mutations

Clinical data on the activity of EGFR-targeted agents against uncommon mutations are limited. The general consensus in the literature indicates that outcomes are generally unfavorable in patients with tumors harboring uncommon mutations [19,76,77]. *Post-hoc* analysis of the Phase III NEJ002 trial demonstrated that OS with gefitinib was significantly shorter in patients with uncommon *EGFR* mutations (G719X or L861Q; $n = 10$) versus those with common mutations (median: 11.9 vs 29.3 months; $p < 0.001$) [78]. Other clinical studies are in line with the observations in NEJ002 and demonstrated poor outcomes with first-generation EGFR TKIs in patients with uncommon mutations [76,77]. Furthermore, the outcomes appear to be heterogeneous depending on the type of uncommon mutation. Certain mutations, for example, exon 18 G719X mutations ($n = 10$), exon 20 S768I ($n = 6$) and exon 19 K757R ($n = 1$) and E746G ($n = 1$) mutations all appeared to confer a good outcome with erlotinib treatment, whereas exon 18 S720 mutations ($n = 3$) was associated with a particularly poor outcome [77].

Available clinical data with afatinib in patients with uncommon *EGFR* mutations appear to corroborate preclinical findings of broad activity. A *post-hoc* analysis of the LUX-Lung 2, 3 and 6 trials identified 75 afatinib-treated patients with tumors harboring uncommon mutations, including G719X ($n = 18$), L861Q ($n = 16$) and S768I ($n = 8$) single/compound mutations [79]. In this analysis, median PFS among 38 patients with uncommon mutations/duplications in exons 18–21 was 10.7 months (95% CI: 5.6–14.7) and median OS was 19.4 months (95% CI: 16.4–26.9). Based on these findings, the US FDA recently expanded the front-line indication for afatinib to include NSCLC harboring uncommon *EGFR* alterations in L861Q, G719X and/or S768I. Outcomes in patients with Ins20 mutations ($n = 23$) were poor (PFS 2.7 months; 95% CI: 1.8–4.2), reflecting the observation that while afatinib had moderate *in vitro* activity against these mutations, sufficient inhibition was not achieved in patients with standard dosing.

Additional evidence for afatinib's clinical activity against uncommon *EGFR* mutations comes from a Phase IIb single-arm open-label study (1200.66; NCT01953913) which included 67 TKI-naïve patients with NSCLC tumors harboring uncommon (Ins20, L861Q; G719S/A/C, T790M, S768I) mutations; median PFS in these patients was 9.1 months (95% CI: 5.6–13.6) [80]. Finally, an analysis of 165 patients with *EGFR* mutation-positive NSCLC who received first-line afatinib in real-world practice in South Korea showed that tumors harboring uncommon mutations other than T790M were sensitive to afatinib. Median PFS was not reached for those with uncommon mutations excluding T790M ($n = 10$) versus 4.7 months in those with *de novo* T790M ($n = 4$; $p = 0.01$); median PFS in patients with Del19- and L858R-positive tumors was 19.1 and 15.1 months, respectively [81]. The potential advantages of afatinib versus erlotinib in compound mutations have also been recognized clinically. In a case study of a patient with a compound L858M/L861Q *in cis* mutation, treatment with afatinib overcome primary resistance to erlotinib [82].

Could differences in clinical activity with EGFR TKIs reflect differences in the impact on clonal evolution?

We speculate that the results from LUX-Lung 7, ARCHER-1050 and FLAURA could potentially be explained from a clonal evolution perspective. As discussed, *EGFR* mutation-positive tumors can be highly heterogeneous. Therefore, the broader inhibitory profile of second-generation TKIs against other ErbB family receptors or particular mutants could prevent emergence of tumor cells that would otherwise survive treatment with first-generation TKIs (Figure 4). This, in turn, could delay the onset of clonal expansion and acquired resistance to therapy, which is possibly reflected in the observed improvements in PFS with afatinib and dacomitinib versus the first-generation TKI, gefitinib. Moreover, the encouraging clinical benefit with sequential afatinib and osimertinib observed in recent retrospective studies [73,83,84], could indicate that resistant T790M-positive clones are more homogeneous than those that develop during treatment with first-generation TKIs (Figure 4). For example, one study demonstrated that response rates and PFS were significantly better in patients who received sequential afatinib and osimertinib than patients who received sequential gefitinib/erlotinib and osimertinib [84]. This hypothesis is supported by our preclinical data and warrants further investigation. The impressive clinical benefits with osimertinib in FLAURA probably reflect its strong inhibitory activity against T790M, the major resistance mechanism to first- and second-generation EGFR TKIs, thus preventing the clonal expansion of pre-existing T790M cells or the development of *de novo* T790M-positive clones. Indeed available evidence indicates that *de novo* T790M mutations do not occur in patients treated with first-line osimertinib [36]. As previously mentioned, it appears that acquired resistance mechanisms to first-line osimertinib are heterogeneous, though data are limited [36]. More genomic data are required

Figure 4. Hypothetical changes in clonal composition over time of non-small-cell lung cancer, tumors treated with sequential EGFR tyrosine kinase inhibitors.

TKI: Tyrosine kinase inhibitor.

to help define potential drug combinations, and drive the development of additional agents, that may be active in the post-osimertinib setting.

Conclusion & future perspective

The availability of several EGFR-targeted agents has revolutionized treatment for patients with *EGFR* mutation-positive NSCLC. However, these developments pose complex questions in terms of how best to utilize available agents, and how to sequence therapy to maximize clinical benefit and prolong the time patients remain free from chemotherapy. Clearly, these are multifaceted questions and depend on a number of factors including first-line efficacy and tolerability of different agents, activity against specific *EGFR* alleles, impact on quality of life, physician experience and preference as well as cost and reimbursement considerations. A key factor, however, is which treatment regimen is most likely to maximize OS, which in turn depends on the availability of subsequent therapies beyond first-line EGFR-directed TKIs. A greater understanding of the clonality of *EGFR* mutation-positive tumors, and how tumors evolve in response to different agents, is integral to address this question.

Head-to-head clinical trials clearly demonstrate the superiority of second- and third-generation TKIs compared with first-generation TKIs in a first-line treatment setting. However, second- and third-generation TKIs have not been directly compared. Therefore, the optimal first-line treatment for patients with *EGFR* mutation-positive NSCLC remains an open question, especially given the current paucity of mature OS data with different agents. We hypothesize that broad ErbB family inhibition could be a preferable first-line treatment choice given emerging knowledge about the heterogeneity and clonal evolution of *EGFR* mutation-positive tumors. Moreover, given the preponderance of T790M after first- and second-generation EGFR TKIs, in principle, more than half of all patients with *EGFR* mutation-positive NSCLC could be eligible for, and benefit from, second-line osimertinib. In

this regard, one needs, of course, to acknowledge the challenge of a second biopsy for the patient, the technological and conceptual limitations of liquid biopsies, and the limited availability of sensitive test platforms, like NGS, in ‘real-world’ clinical practice [85].

While data from the recent FLAURA trial are encouraging and position osimertinib as a first-line treatment option in patients with *EGFR* mutation-positive NSCLC [20], targeted treatment options following osimertinib resistance remain poorly defined at this time because of the heterogeneity of resistance mechanisms [36]. Therefore, at the moment, the most likely treatment option beyond osimertinib is chemotherapy. Accordingly, further clinical trial data are needed to assess optimal sequence of therapy with *EGFR* TKIs in individual patients over the course of their treatment, with OS as the key end point. Detailed analysis of the changing molecular features of tumors, as they continually evolve in response to treatment, will be a fundamental aspect of future clinical studies [86]. This will require further improvements, and better access to, molecular genomic technologies. Ultimately, monitoring of tumor clonality will help drive rational treatment choices with the ambition of turning *EGFR* mutation-positive NSCLC into a chronic disease in many patients.

Executive summary

The general implications of tumor heterogeneity on the diagnosis & treatment of cancer

- Tumors evolve over the course of their development, and during therapy. Genomic instability of developing tumors creates a genetically diverse cell population that is subject to selective forces; clones that have a phenotypic advantage within a given microenvironment or therapeutic context result in the evolution of genetically distinct subclonal populations.
- Clonal evolution plays a key role in acquired resistance of tumors to treatment. Therefore, it would be highly advantageous to understand the clonal architecture of tumors prior to initiation of, and during, therapy. Despite technological advances, for example, next-generation sequencing and liquid biopsy techniques, this still represents a major challenge.

The complexity of *EGFR* mutation-positive non-small-cell lung cancer tumors

- It is becoming increasingly clear that *EGFR* mutation-positive non-small-cell lung cancer (NSCLC) tumors are genetically heterogeneous and undergo clonal evolution. Two sources of heterogeneity are: co-occurring genetic alterations within other genes that are present in the background of *EGFR* mutation-positive tumors and different *EGFR* mutations within a single tumor.

Preclinical differences in activity of *EGFR*-directed tyrosine kinase inhibitors against NSCLC tumor cells

- Approved first- (erlotinib, gefitinib), second- (afatinib) and third-generation (osimertinib) tyrosine kinase inhibitors (TKIs) are available for the first-line treatment of *EGFR* mutation-positive NSCLC. These agents have different pharmacological profiles: erlotinib and gefitinib are *EGFR*-selective reversible inhibitors; afatinib is an irreversible ErbB family blocker; osimertinib is an *EGFR* selective wild-type sparing inhibitor. Owing to differences in pharmacological profile, different *EGFR* TKIs are likely to confer different selective pressures on developing *EGFR* mutated tumors.
- Compared with first-generation *EGFR* inhibitors, afatinib (and possibly dacomitinib), besides the broader ErbB inhibitory spectrum, is also active against several *EGFR* mutations, including compound mutations and uncommon *EGFR* mutations. This broad inhibitory profile may help delay the expansion of particular resistant subclones, resulting in more homogeneous subclones with high T790M gene dosage.

Clinical data comparing different generations of *EGFR*-targeted TKIs

- Recent head-to-head clinical trials (LUX-Lung-7, ARCHER-1050) have demonstrated that second-generation *EGFR* TKIs are superior to first-generation *EGFR* TKIs in patients with *EGFR* mutation-positive NSCLC. The recent FLAURA trial demonstrated that osimertinib is superior to first-generation *EGFR* TKIs; there are no prospective data comparing second- and third-generation *EGFR* TKIs. Differences in clinical activity could reflect differences in the impact of clonal evolution of tumors.

Conclusion & future perspective

- At present, the optimal use of *EGFR* TKIs has not been defined. In the clinical setting, T790M-positive clones represent more than half of the resistant clones after erlotinib or afatinib treatment; in the preclinical setting, our data suggest that afatinib-resistant subclones are relatively homogeneous, display high T790M gene dosage and are likely to be very sensitive to second-line osimertinib.
- Emerging, albeit, limited clinical data have shown encouraging survival outcomes in patients treated with sequential afatinib/osimertinib.
- In the future, further technological developments, and the monitoring of the evolution of tumors during treatment, will be instrumental in guiding the optimal treatment strategy of *EGFR* mutation-positive NSCLC patients.

Author contributions

The authors were fully responsible for all content and editorial decisions, were involved at all stages of manuscript development and have approved the final version.

Financial & competing interests disclosure

S Kohsaka has received research support from AstraZeneca and Boehringer Ingelheim. F Solca and M Petronczki are employees of Boehringer Ingelheim. M Maemondo has received honoraria from Chugai Pharmaceutical, AstraZeneca and Boehringer Ingelheim. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Medical writing assistance, supported financially by Boehringer Ingelheim, was provided by Lynn Pritchard of GeoMed, an Ashfield Company, part of UDG Healthcare plc, during the preparation of this article.

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