Introduction

The use of small molecule inhibitors directed at specific oncogene targets, including epidermal growth factor receptor (EGFR) mutations, has improved outcomes and defined precision medicine for non-small cell lung cancers (NSCLC). However, despite the impressive responses and improvements in survival seen with these agents, they are rarely curative, and even in patients who initially achieve complete responses to therapy, resistance inevitably develops, and disease progression occurs.

For EGFR mutant NSCLC, targeting the T790M resistance mutation defined the role of osimertinib (1), an agent with substantial efficacy but limited toxicities, and resulted in increasing efforts to define resistance mechanisms in the hope that these too could be targeted with further gains in survival.

While initially clonal heterogeneity may be limited early, selection pressures induced by treatments enables the growth of preexisting or new tumor cell clones that are resistant to therapy (2). The varied mechanisms by which resistance can occur, coupled with the co-occurrence of multiple resistance mechanisms within one patient, constitute a major challenge in developing an efficient treatment strategy to counteract tumor progression. The clonal evolution of oncogene-addicted NSCLC can give rise to different molecular aberrations both spatially (between primary and metastasis) and temporally (after treatment failure), further contributing to the complexity of the molecular resistance.

Resistance to EGFR TKIs

Resistance to EGFR TKIs broadly falls into ‘EGFR-dependent’ and ‘EGFR-independent’ mechanisms. The first group results in EGFR alterations, such as the T790M and C797S mutations; the latter addresses other methods that divert signaling dependence, such as activation of other downstream pathways such as RAS, gene fusions, BRAF, or even histologic transformation. Resistance to first and second generation EGFR TKIs are most often EGFR-dependent, with the commonest mechanism being the EGFR T790M occurring in 50–60% (3,4). Other EGFR-dependent mutations such as EGF D761Y have been described but occur much less frequently (5). EGFR-independent mechanisms most frequently involve MET (5% to 20%) and HER2 amplification (8%) (3,4). Histologic transformation to small cell is another cause of resistance (5–14%) (3,4) and de novo RB1/TP53 mutations enrich for tumors likely to develop such transformation (6).

The spectrum of resistance to osimertinib is distinct to that seen with first or second generation EGFR TKIs and varies according to whether the drug is used in the first-line

^ ORCID: 0000-0002-7369-8150.
or later-line context. When used to treat acquired T790M mutation, between 20% and 30% develop resistance via an EGFR-dependent tertiary EGFR change, most commonly in the specific binding site of osimertinib, EGFR C797 (7,8). Mutations at other sites of the EGFR such as G724, L718, G719 and L792, and EGFR amplification have also been described (7-10). Resistance due loss of T790M develops in around half the cases, but remarkably in most of these cases is also associated with the emergence of other bypass mutations such as KRAS mutations, MET amplification, gene fusions or small-cell transformation, thus EGFR-independent (7,8). Therefore, in contrast with earlier-generation TKIs, around 60% of Osimertinib treated T790M positive cases develop resistance in an EGFR-independent fashion, namely amplification of MET (6% to 26%) and HER2 (up to 8%). Other less frequent, but still EGFR-independent and potentially targetable mutations have been described: HER2 insertions, KRAS and BRAF V600E mutations, NTRK, RET, ALK and FGFR fusions, and MET exon 14 alterations (7,9,10). Similarly to resistance to earlier-generation TKIs, histologic transformation (to small-cell or squamous) is recognized to occur in between 4% and 15% of osimertinib-resistant cases in the later-line setting (7,10).

Resistance patterns to first-line osimertinib, as observed in the FLAURA trial occurred via EGFR-independent mechanisms in 32%, but surprisingly only 8% developed EGFR C797S mutations (11). More frequent EGFR-independent mechanisms included MET amplification (15%), cell cycle gene alterations (10%), PIK3CA mutations (7%), BRAF or KRAS mutations (3% each) and HER2 amplifications (2%) (11). In later-line osimertinib trials, it is possible that addiction to the EGFR pathway through T790M development is predicated, which may explain why more EGFR-dependent resistance pathways are activated. Since the resistance data from FLAURA comes only from plasma genotyping, histologic transformation could not be identified; moreover, other resistance mechanisms could be higher, due to underestimation of gene amplification in plasma. This may explain why a large proportion (40–50%) have unknown resistance mechanisms (10); tissue biopsies may still be important to clarify osimertinib resistance mechanisms in the first-line context.

**HER2D16: a potential novel resistance pathway**

In this context, Hsu et al. (12) describe a patient who developed resistance to osimertinib after multiple other therapies. The resistance mechanism detected was complex; defined by T790M loss plus two EGFR-independent causes: a HER2 amplification and a novel HER2 ex 16 skipping (HER2D16). The latter has been previously reported in breast cancer only and constitutes one of the three splice variants of HER2, but results in addiction to HER2 signaling (13). Its clinical significance warrants further clarification, but is thought to explain some of the variability in the response to HER2 blockade (13). In breast cancer, the oncogenic properties of HER2D16 are mediated through direct coupling with Src kinase (13,14).

Interestingly, in the article by Hsu et al. (12) the HER2D16 mutation was detected in the plasma prior to commencing Osimertinib, but the allelic fraction increased with disease progression, leading to the hypothesis that this novel mutation mediated resistance. To further this, the authors use an EGFR-T790M/L858R positive cell line (H1975), to stably express HER2D16 and demonstrate elegantly that this novel protein cooperates with the EGFR, in both wild-type (WT) and mutant cells to allow constitutive activation despite osimertinib inhibition. Remarkably, Src levels did not alter after treating cells with dasatinib (a known Src inhibitor), and therefore failed to suppress cellular proliferation, with or without the presence of osimertinib. However, in an attempt to better suppress HER2 signaling in vitro, the authors combined osimertinib with afatinib (a known pan-HER2 TKI). The combination of afatinib and osimertinib was indeed synergistic in vitro, but this was in a construct where both mutations are present in the one cell. Whether this is what actually occurs in vivo is difficult to know.

**Discussion**

HER2 alterations include amplifications and mutations, but are most commonly in-frame exon 20 insertions and occur de novo in about 1% to 5% of lung adenocarcinomas (15). They have been previously reported as osimertinib resistance mechanisms; in FLAURA, HER2 amplifications were detected in 2%, and HER2 mutations in 1% (11); in the later-line context, up to 5% of HER2 amplifications have been described, but no HER2 mutations (8,10,15). A recent publication described the in vitro use of trastuzumab-emtansine (TDM-1) combined with osimertinib to overcome HER2 amplification-mediated resistance in EGFR-T790M-positive NSCLC cell lines, another example that combination strategies could be used to overcome resistance (16). A phase I–II trial is testing this combination
in patients with EGFR-mutant NSCLC, progressing after standard EGFR treatment who developed a HER2 bypass track mechanism of resistance (NCT03784599).

The real question from these data is whether this novel resistance mechanism and the in vitro targeting will translate in the in vivo context. Afatinib binds to Cys797, and preclinical evidence suggested effective inhibition in several EGFR activating mutations including T790M (17), but its clinical performance in patients with erlotinib-resistant cancers harboring T790M was minimal (18). Afatinib is equally potent against WT EGFR and EGFR T790M, so the toxicity resulting from inhibiting WT EGFR precludes the use of doses that would be needed to effectively suppress T790M. This same caveat was reported by the authors (12), since the drug concentration of afatinib which is effective with osimertinib may not be deliverable due to toxicity. Moreover, there is very limited experience with the combination of afatinib-osimertinib in literature, with debatable benefit (19). Similarly, while there was clear preclinical evidence suggesting activity of afatinib on HER2 mutant NSCLC, the largest prospective trial that attempted to test its efficacy was stopped due to futility (20).

In the same context, preclinical data in a T790M-positive cell line suggested that the configuration of the T790M and C797S affected the response to therapy (21): if the two EGFR mutations were in cis (same DNA strand), the cells were refractory to combination first and third-generation TKIs; on the contrary, when the two mutations were in trans (on different DNA strands), a combination of EGFR inhibitors showed clear evidence of in vitro response (21). While this combination has shown some efficacy, unfortunately it is limited, making routine C797S/T790M testing less clinically meaningful (22).

The patient detailed by Hsu et al. (12) was heavily pretreated, with systemic treatment including chemotherapy, TKIs and even with radiofrequency ablation. The exposure to several treatments is likely to have imposed different selection pressures, leading to multiple different clones. The mixed response to osimertinib, is suggestive of tumoral heterogeneity and is known to be poorly prognostic (23). Since these mutations were found in separate blood samples and the response was heterogeneous, raises the question whether these mutations coexisted in the one site or is the result of spatial heterogeneity. This then draws into challenge whether targeting this resistance pathway would be likely to be broadly effective.

The importance of identifying resistance mechanisms is based on the principle that further specific targeting may evoke durable benefit and minimal toxicity. Considering that resistance to osimertinib usually involves combined mechanisms, such as the activation of alternative cellular pathways and/or aberrant downstream signaling, osimertinib-based combination therapies are currently being investigated (10). Moreover, several case reports and small clinical series including novel combinations with other TKIs against EGFR, RET, ALK, ROS, MET and BRAF inhibitors have been reported (10,22,24-26). However, again, all this initial evidence requires further confirmation. An interesting approach is the recently launched ORCHARD Phase II trial (NCT03944772) which will explore treatment options after disease progression on first-line osimertinib according to the onset of acquired resistance mechanisms.

In this innovative platform trial, patients will be allocated to a biomarker-matched study treatment: osimertinib plus gefitinib - osimertinib plus savolitinib (a novel MET inhibitor)—osimertinib plus nectumumab or platinum-based doublet plus durvalumab—within each group based on tumour molecular profile.

**Conclusions**

Since no clear mechanism of resistance is identified in between 30–40% of patients treated with later-line osimertinib and up to 50% of patients treated with first-line osimertinib (10), studies like Hsu et al. (12) are key to identify targetable alterations. The combination TKI approach is familiar and easy to implement although toxicity would need to be considered and needs confirmation of benefit. But more importantly, this study shows us that resistance is a complex process. It may incorporate both de novo clonal heterogeneity and clonal selection, but also may be the result of mutagenesis with single cells developing multiple resistance mechanisms.

The data from the cell line construct suggest that the HER2D16 mutation is targetable and the authors argue that it should be included as standard testing for reversible mechanisms of osimertinib resistance. However, it is important to acknowledge that while it is useful to identify potential mediators of resistance, the main impetus to implementing such testing is if this mutation is targetable in vivo with an effective yet non-toxic regimen. Until then, while these results are fascinating and important in better understanding the biology of this disease, they remain primarily of academic interest.
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