Novel Third-Generation EGFR Tyrosine Kinase Inhibitors and Strategies to Overcome Therapeutic Resistance in Lung Cancer

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Abstract

EGFR-activating mutations are observed in approximately 15% to 20% of patients with non–small cell lung cancer. Tyrosine kinase inhibitors have provided an illustrative example of the successes in targeting oncogene addiction in cancer and the role of tumor-specific adaptations conferring therapeutic resistance. The compound osimertinib is a third-generation tyrosine kinase inhibitor, which was granted full FDA approval in March 2017 based on targeting EGFR T790M resistance. The compound has received additional FDA approval as first-line therapy with improvement in progression-free survival by suppressing the activating mutation and preventing the rise of the dominant resistance clone. Drug development has been breathtaking in this space with other third-generation compounds at various stages of development: rociletinib (CO-1686), olmutinib (HM61713), nazartinib (EGF816), naquotinib (ASP8273), mavelertinib (PF-06477775), and AC0010. However, therapeutic resistance after the administration of third-generation inhibitors is complex and not fully understood, with significant intertumoral and intratumoral heterogeneity. Repeat tissue and plasma analyses on therapy have revealed insights into multiple mechanisms of resistance, including novel second site EGFR mutations, activated bypass pathways such as MET amplification, HER2 amplification, RAS mutations, BRAF mutations, PIK3CA mutations, and novel fusion events. Strategies to understand and predict patterns of mutagenesis are still in their infancy; however, technologies to understand synthetically lethal dependencies and track cancer evolution through therapy are being explored. The expansion of combinatorial therapies is a direction forward targeting minimal residual disease and bypass pathways early based on projected resistance.

Introduction

EGFR-mutant lung cancer has been a model of drug development for oncogene addiction in cancer. The receptor belongs to the ERBB family of receptors that bind ligands including EGF, TGFα, amphiregulin, epigen, betacellulin, heparin-binding EGF, and epiregulin. Upon ligand binding to EGFR, homodimerization or heterodimerization with other ERBB family members triggers receptor phosphorylation and activation of downstream signaling through RAS-RAF-MEK-ERK and RAS-PI3K-PTEN-AKT-mTOR activation, leading to proliferation, differentiation, and migration of cancer cells (Supplementary Fig. S1; ref. 1). Herein, we describe some of the important lessons learned from an over a decade of experience with EGFR inhibitors in lung cancer, drug resistance, and some of the projections forward with new strategies in first-line clinical management.

Historical Perspective

Initially approved as second line therapy in advanced non–small cell lung cancer (NSCLC) after chemotherapy, first-generation reversible anti-EGFR tyrosine kinase inhibitors (TKI) erlotinib and gefitinib entered clinical guidelines based on a body of clinical trials that demonstrated efficacy in some patients after initial lines of chemotherapy (2–6). In 2004, three groups of researchers identified a subset of patients with NSCLC with activating mutations in the EGFR tyrosine kinase domain (2, 5, 6). The most common EGFR mutations are exon 19 deletions (54%), comprising of five amino acids from codons 746–750, and substitution of leucine with arginine at codon 858 (L858R, 41%; ref. 7). Other less common aberrations include G719X (4%), L861X (1%), S768I (1%) mutations, and exon 20 insertions (2%–9%; ref. 8). Patients with activating EGFR mutations (except exon 20 insertions) have been exquisitely sensitive to first-generation reversible EGFR TKIs, and a true paradigm shift emerged when these compounds showed improved efficacy to chemotherapy as initial therapy. For the first time, a solid tumor was treated with oral anticancer therapy in the first-line and improved PFS from approximately 5 months with chemotherapy to 11 months (3–4). The lung cancer clinical community was intellectually inspired, and the adoption of tolerable, precision oriented, oral anticancer therapy emerged to replace standard intravenous chemotherapy for a subset of patients. Despite revolutionary advancements, patients who were sensitive to EGFR TKIs eventually progressed after approximately 9 to 11 months. More than 60% of patients develop resistance to...
first- or second-generation inhibitors with an acquired second site mutation in the ATP binding pocket of EGFR exon 20 leading to a threonine-to-methionine substitution at the amino acid position 790 (T790M; ref. 7). This mutation prevents drug binding by increasing the affinity of the ATP binding site for ATP. Second-generation, irreversible TKIs including afatinib and dacomitinib were developed with intent to overcome these resistant tumor clones (9, 10). However, despite encouraging in vitro data, clinical trials with these compounds did not improve overall survival in T790M mutant patients likely due to the inability to escalate dosing because of potent effects on wild-type EGFR at the therapeutic window (9). Third-generation inhibitors with pyrimidine core structures were created to target the T790M clone with maintained activity against the original exon19del and L858R mutations. Binding at the C797 locus and having selectivity against the T790M mutation, third-generation inhibitors have had responses for patients with T790M resistance after first-generation inhibitors (Fig. 1A–E). First-line data have demonstrated superior clinical outcomes compared with first-generation inhibitors with trends toward overall survival improvement (11).

### Third-Generation TKIs

The race to discover a next-generation EGFR TKI effective against resistant tumors has been both illustrative and ground breaking. Osimertinib initially gained full FDA approval for patients with metastatic EGFR T790M mutant NSCLC after progressing on first- or second-generation EGFR TKIs and has subsequently gained approval in the first-line treatment of EGFR-mutant lung cancer (11, 12). Other third-generation inhibitors designed to target the T790M clone have been at various stages of development and include nazaritinib (EGFR 816), rociletinib (CO-1686), olmutinib (HM61713), naquotinib (ASP8273), mavelertinib (PF-06747775), and AC0010 (Supplementary Fig. S2; Supplementary Table S1). Following over a decade of active drug development, osimertinib is the only third-generation inhibitor that is currently approved by the FDA, with data that demonstrate significant improvement in clinical endpoints, including PFS, tolerability with limited wild-type inhibition, and response with central nervous system (CNS) disease (11, 12). Other third-generation inhibitors as of yet have not demonstrated superiority to osimertinib despite antitumor efficacy, and at this time it remains unclear where they will be clinically positioned.

#### Osimertinib

Osimertinib, a pyrimidine-based compound that binds irreversibly to the cysteine-797 residue at the ATP binding site of the growth factor receptor, preclinically displayed significant tumor cytoreduction in xenograft and transgenic mice with activity against activating mutations exon19del and L858R with and without detectable T790M mutations and less activity for the wild-type EGFR target (13). Clinically, the phase II AURA2 trial was restricted to EGFR T790M-positive NSCLC patients with disease progression after initial first line therapy and showed a 71% (140/199) objective response with six patients (3%) having complete response and 134 (67%) with partial response (14). Full FDA approval was given after the phase III AURA3 study demonstrated an overall response rate (ORR) of 71% compared with 31% with platinum-pemetrexed therapy (OR 5.39; *P* < 0.001) and a median PFS of 10.1 months versus 4.4 months (HR 0.30; *P* < 0.001; ref. 12). The most common all grade drug-related adverse

![Figure 1](https://example.com/figure1.png)

**Figure 1.**

In silico modeling of EGFR C797S interaction with osimertinib in the complex of EGFR derived from its crystal structure (PDB4ZAU) using Chimera software. **A**, Space filling hydrophobicity surface model of EGFR with osimertinib. **B** and **C**, Osimertinib-EGFR interaction with C797wt (**B**) or C797S-mutant (**C**) residues pan-view with relevant residues to TKI resistance G719, T790, and L858 indicated on the model. **D** and **E**, Osimertinib covalent binding to the SH-group on C797 showing both the covalent binding (red) and the separation distance to C797 wild type. **E**, In silico modeling of the most stable rotamer substitution by the less nucleophilic serine side chain at position 797, which cannot undergo covalent bond formation. A higher distance between the C797S and osimertinib closest carbon atom is indicated in yellow.
events included diarrhea (43%) and rash (40%), however impressively less than 1% were grade three or higher. Interstitial lung disease (ILD) was observed in eight patients and, although rare with a mechanism that has not yet been described, did have at least one fatal event (12, 14). In a larger study of 1,217 patients (ASTRIS, NCT02474355), the investigator-assessed response rate was 64% at 4 months and safety parameters reflected the original study (15). Pooled analysis has demonstrated a median survival of 26.8 months with second-line osimertinib (16). The success of the compound in the second line prompted evaluation in the first-line therapy for EGFR-mutant disease (11).

First-line data

The evaluation of osimertinib in the first-line space hinged on the hypothesis that suppressing the T790M clone early in treatment course may improve clinical outcomes. The phase III FLAURA trial (NCT02296125) directly compared osimertinib (80 mg daily) with first-generation therapy (either gefitinib 250 mg or erlotinib 150 mg daily) in a total of 556 patients with NSCLC with newly diagnosed EGFR exon 19 deletions or L858R mutations. Osimertinib had a superior PFS relative to the gefitinib or erlotinib arm with median progression-free survival of 18.9 months compared with 10.2 months for standard therapy [HR 0.46; 95% confidence interval (CI), 0.37–0.57; P < 0.0001; ref. 11]. The median duration of response was 17.2 months in patients treated with osimertinib compared with 8.5 months in the first-generation TKI cohort. The incidence of grade three or higher toxicities was lower with osimertinib (32%) than standard treatment (41%; ref. 11). Although OS data have not yet matured, the compound has been adopted into first-line with FDA approval.

Role in brain metastasis

Brain recurrence for patients on therapy is an unmet need with limited therapeutic options and contributed by challenging pharmacokinetics, drug efflux transporters, and molecular weight (17). In preclinical models, osimertinib demonstrated the ability to cross the blood–brain barrier (BBB) with a higher ratio of unbound tissue concentration to unbound plasma concentration and higher substrate activity against permeability glycoprotein (Pgp) and breast cancer resistance protein (BCRP) efflux transporters as compared with gefitinib (17, 18). Measurement of AUC unbound tissue to plasma ratios showed osimertinib more highly distributed in the brain (1.7–2.0) than gefitinib (0.21), rociletinib (<0.08), and afatinib (<0.36). Data from the BLOOM study (NCT02228369) suggested encouraging activity with osimertinib (160 mg daily) with a median treatment duration of 6 months and manageable toxicity in patients with leptomeningeal disease; 10 patients (43%) had had intracranial radiologic response and 13 patients (56%) with stable disease (SD; ref. 18). Among patients in the AURA3 randomized phase III study, the median CNS PFS was significantly longer with osimertinib than with chemotherapy (11.7 months vs. 5.6 months; HR 0.32; P = 0.004). CNS ORR was 70% (21/30) with osimertinib compared with 31% ORR with chemotherapy pointing to the intracranial efficacy of the compound (12). In the first-line FLAURA study, events of CNS progression were observed in 12 of 61 patients (20%) in the osimertinib group and 26 of 67 (39%) in the standard EGFR TKI group (11). CNS response rates in measurable brain metastases were 91% with osimertinib and 68% with first-generation EGFR TKIs (19).

Combinational strategies with osimertinib

Studies evaluating the safety and efficacy of additional targeted therapies added to osimertinib are underway and include combinations with agents that target pathways including MET, MAPK, BCL-2, and JAK activation among others. Based on kinase screens and given that upregulation of other oncogenic pathways is a common resistance mechanism to tyrosine kinase inhibition, multiple clinical studies are evaluating targets in combination with osimertinib (20, 21). The TAITON phase Ib study (NCT02143466) involves combinations of osimertinib at increasing doses in combination with either savolitinib (AZD6094, MET inhibitor), selumetinib (MEK inhibitor), or duvelisibumab (anti-PD1i antibody; ref. 20).

Early work with a gefitinib-sensitive lung cancer cell line revealed focal amplification of the MET proto-oncogene in cells that developed resistance to gefitinib. Inhibition of MET signaling in these cells restored their sensitivity to gefitinib and MET amplification was detected in 4 of 18 (22%) lung cancer specimens that had developed resistance to gefitinib or erlotinib. It was found that MET amplification causes gefitinib resistance by driving ERBB3 (HER3)–dependent activation of PI3K (21). In clinical samples obtained after treatment with the third generation inhibitor osimertinib, plasma circulating tumor DNA analyses have identified MET copy number gain after first line osimertinib in approximately 19% of patients on the FLAURA trial and 15% after second-line osimertinib (22, 23). Updated analysis of TAITON phase Ib study combining osimertinib and the MET TKI savolitinib showed an ORR of (i) 33% in prior third-generation TKI exposed and (ii) 61% in patients with T790M mutation (24). No prior third-generation EGFR-TKI exposed cohorts (24). Second site mutations in the MET oncogene (D1228V) have been seen at resistance on the osimertinib and savolitinib trial demonstrating the dependency on the pathway (25). Grade ≥3 adverse events were experienced by 50% of patients, with the most common all-cause events being vomiting (8%), AST/ALT increase (6%), and rash (6%). Savolitinib was discontinued for 30% of patients because of adverse events (24).

Osimertinib is also being combined with inhibitors in the MAPK pathway. Resistance to the third generation EGFR inhibitor tool compound WZ4002 has been seen through amplification of ERK through activation of the MAPK1 pathway or downregulation of negative regulators of ERK signaling (26). The MEK inhibitor CI-1040 downregulates ERK1/2 phosphorylation and, when combined with WZ4002, PC9 WZ4002-resistant cells were responsive to the combination in vitro (27). In post-progression patient samples, resistance involving the RAS–MAPK pathway has been seen in patients treated with osimertinib, with acquired KRAS mutations and V600E BRAF mutations observed after acquired resistance (28). Furthermore, preclinical work demonstrated that the concomitant inhibition of EGFR and ERK1/2 by the MEK inhibitor trametinib prevents ERK1/2 reactivation, enhances anti-EGFR–induced apoptosis, and inhibits the emergence of resistance in EGFR-sensitive models known to acquire resistance via both T790M-dependent and T790M-independent mechanisms. Resistance to the combination with trametinib may emerge due to AKT/mTOR reactivation (29). In another study combining osimertinib with selumetinib in ex vivo primary cell cultures obtained from osimertinib plus selumetinib-resistant tumors, the Hedgehog pathway was activated and a smoothened (SMO) inhibitor plus osimertinib and selumetinib inhibited proliferation and migration of resistant cells (30). These collective
data form the basis of the study of osimertinib and selumetinib (NCT02143466) with some responses seen at interim data analysis in the phase I study. The most commonly reported grade 3 adverse events were diarrhea (21%), dermatitis acneiform (15%), and hypokalemia (10%; ref. 20).

Osimertinib in combination with navitoclax is an ongoing phase Ib trial in patients with disease progression on an anti-EGFR TKI (NCT02520778). Inhibition of Bcl-2, a prosurvival protein, prompts cells to undergo apoptosis by upregulating and dephosphorylating BIM, a proapoptotic protein from Bcl-2 family components. ABT263 (navitoclax) is a dual inhibitor of BCL-xL and Bcl-2, which induces BIM by decreasing the capacity of BCL-xL to neutralize BIM and facilitates Bax and Bak to initiate caspases, leading to cell death. In a drug screen with EGFR T790M patient derived cell lines, navitoclax in combination with the tool compound WZ4002 induced significantly more apoptosis compared with the EGFR inhibitor WZ4002 alone (31).

Downstream targeting of the mTOR pathway is being studied in a phase II trial of sapanisertinib (INK128), a potent mTORC1/2 inhibitor, in combination with osimertinib for patients with advanced EGFR-mutated NSCLC (NCT02503722). Preclinically, inhibition of TORC1 and TORC2 decreased cell growth, metabolism, and angiogenesis. mTOR kinase inhibition via INK128, which involves ATP-dependent phosphorylation of proteins S6 and 4EBP1 downstream of TORC1, leads to selective inhibition of Akt phosphorylation (32). Novel mTOR mutations have been seen on EGFR-resistant patient samples, and in mutant cells continuation of EGFR and mTOR inhibition fully abrogated EGFR downstream signaling (33). Additionally, osimertinib has been combined with JAK inhibitors interrupting signaling of the JAK/STAT pathway in an ongoing second-line study in T790M mutant patients (INCB39110, NCT02917993 and AZD4205, NCT03450330).

A phase I dose-escalation study of osimertinib combined with necitumumab, a humanized EGFR monoclonal antibody, is underway for patients who progressed on previous first-line TKI therapy (NCT02789345). Recent work demonstrates that amplification of EGFR wild-type alleles can confer resistance to EGFR TKIs (34). By binding to extracellular domain of the EGFR receptor and competing with ligand binding, downstream signaling leads to impaired tumor cell growth.

Nazaritinib (EGF816)

EGF816 is an irreversible, mutant-selective EGFR inhibitor that targets L858R, exon 19 deletion, T790M mutations, and is also being explored for efficacy against exon 20 insertion mutations, which are intrinsically resistant to first- and second-generation inhibitors (35). A phase I study of EGF816 showed an ORR of 44% and DCR of 91% with a median duration of response of 9.2 months and grade 3 or 4 adverse events including diarrhea (6%), rash (14%), and anemia (6%; ref. 36). In 14 patients who received EGF816 and then osimertinib, the ORR to osimertinib was 14% (one CR, one PR, eight SD, four PD) with a median duration of response 8.3 months; ref. 43). The side-effects included diarrhea (59%), rash (41%), pruritus (42%), and nausea (39%). Further ELUXA trials have been stopped due to incidence of skin toxicity including toxic epidermolysis necrosis (n = 2, one fatal) and Stevens-Johnson syndrome (n = 1; ref. 43). The side-effect profile of this compound is unfavorable relative to osimertinib, which has affected its use although the compound had been approved in South Korea.

Olamutinib (HM61713)

Olamutinib (HM61713) began clinical evaluation in May 2015 for T790M-positive mutant NSCLC and was approved for treatment of in South Korea. In phase II ELUXA trials, 76 patients included in the phase II expansion after first- or second-generation TKIs received 800 mg/day with median treatment duration of 7.0 months. Of the 71 evaluable patients, 54% demonstrated an objective response, 90% achieved disease control, mean duration of response was 8.3 months, and median PFS of 6.9 months (95% CI, 5.5–8.3 months; ref. 43). The most common adverse effects included diarrhea (59%), rash (41%), pruritus (42%), and nausea (39%). Further ELUXA trials have been stopped due to incidence of skin toxicity including toxic epidermolysis necrosis (n = 2, one fatal) and Stevens-Johnson syndrome (n = 1; ref. 43). The side-effect profile of this compound is unfavorable relative to olamutinib, which has affected its use although the compound had been approved in South Korea.

Naquotoninib (ASPB273)

Not all third-generation compounds have shown superiority to first- and second-generation inhibitors in the first-line. ASPB273 is a small irreversible TKI that binds at C797 and targets the common EGFR mutations including T790M while sparing wild-type EGFR. First-line ASPB273 resulted in an ORR of 52%, disease control rate (DCR) of 94% and PFS of 11.3
months [44]. Among patients treated as second-line therapy, the confirmed ORR was 30% and median PFS for T790M-positive patients treated was 6.0 months (95% CI, 5.3–9.8 months; ref. 45). Grade 3 adverse events included diarrhea (4%), hypotension (1%), and grade 2 peripheral neuropathy (n = 1) was noted (45). The decision to discontinue development in the SOLAR first-line study was based on the recommendation of the Independent Data Monitoring Committee comparing lower PFS and ORR to the comparators, erlotinib or gefitinib. Differential selectivity with ASP8273 against the original EGFR activating mutations compared with T790M may have contributed to the efficacy results compared with first- or second-generation TKIs in the first-line.

Mavelertinib (PF-0647775)

PF-0647775 is a third-generation EGFR TKI currently being studied in patients with mutated EGFR ex19del or L858R with or without T790M mutation and has completed dose escalation from 25 to 600 mg. RECIST responses observed include 11 PRs from 25 to 600 mg. RECIST responses observed include 11 PRs, 11 MRs, and 16 SDs (43). RECIST ORR was 42.3% (7/17 pts), with 6/17 pts achieving SD lasting greater than 150 mg dose and included diarrhea (12.4%) and skin toxicities (30.8%; ref. 46). Further analyses will evaluate the efficacy and tolerability of the compound in combination with paclitaxel, an inhibitor of the cyclin-dependent kinases CDK4 and CDK6 based on preclinical work demonstrating synergy (NCT02349633).

Mechanisms of TKI Resistance with Second Site Mutations in EGFR

Given that third-generation EGFR inhibitors irreversibly bind at the C797 site, mutations at this locus within the EGFR gene confer resistance and estimated to be present in 7% to 20% of cases (Fig. 1A–E; refs. 22, 23). In patients who progress on osimertinib circulating tumor DNA analyses have shown that T790M may be retained with C797S acquisition (47). When C797S and T790M are in a cis conformation on the same allele (82%), EGFR TKI single agents or combinations are unable to suppress activity. When the mutations are in the trans conformation on different alleles, patients have seen responses to the combination of first- and third-generation EGFR TKIs (48, 49). C797S and T790M mutations were sensitive in preclinical models to a combination of gefitinib with WZ4002, a tool compound developed as a third-generation TKI (49). There have been case reports of clinical efficacy with third- and first-generation inhibitors with clinical trials ongoing (NCT03122717). Furthermore, as third-generation compounds have moved to the first line, clinical trials revisiting reversible first-generation inhibitors to combat C797 resistance after third-generation inhibitors are being explored.

Additional novel drug combinations are being explored to overcome EGFR C797S resistance. EA1045 is an allosteric EGFR inhibitor that in combination with cetuximab prevents effective EGFR dimerization. This compound has been shown to be effective in mouse models of NSCLC with EGFR (L858R/T790M/C797S) mutation by displacing the regulatory C-helix inactive conformation of the kinase (50). The allosteric pocket is accessible in two subunits of the dimer, and the compound inhibits activation by displacing the C-helix. With the addition of cetuximab, dimerization is inhibited creating uniform vulnerability within the kinase. EGFR del 19 or L858R/T790M/C797S cells are resistant to gefitinib, afatinib, WZ4002, rociletinib, and osimertinib; however, triple mutant tumor cells were partially sensitive to cetuximab (50). These data provide preclinical rationale for the combination of a next-generation inhibitor in combination with an EGFR monoclonal antibody for patients with NSCLC with the triple mutation profile. The development of inhibitors that do not bind C797 will be important for patients who develop this resistance. One such compound ADCC010 is a structurally distinct pyrrolopyrimidine-based irreversible inhibitor, and in a xenograft model oral administration of ADCC010 at a daily dose of 500 mg/kg resulted in remission of tumors with EGFR-active and T790M mutations for over 143 days with no weight loss (51). Objective responses have been observed in patients with NSCLC with EGFR T790M mutation on this compound, although it has of yet to be tested after osimertinib for C797S resistance.

Additional EGFR second site mutations have been detected on therapy. EGFR-mutant cells in vitro exposed to increasing quantities of WZ4002 acquired resistance through several additional second-site EGFR mutations including EGFR L718Q, L844V, L798I, L692V, E709K. The full impact and biologic consequences of the second site mutations is not clear at this time. EGFR L718Q and L844V have been seen after pyrimidine based inhibitors, however, have occurred more frequently after rociletinib and WZ4002 than osimertinib (52). In the clinic, resistance due to the L718Q was noted after a patient with a coexisting EGFR L858R mutation was treated with gefitinib and subsequently with osimertinib for T790M progression (53). Additional case reports describe other second site mutations such as G724S, G796D, and L792H on osimertinib therapy. The second-generation inhibitor afatinib has been responsive to the acquired G724S mutation (54). E709K and L682V, both activating missense substitutions, were seen after exposure to rociletinib. EGFR L798I is a novel mutation observed in cis conformation with T790M that affects the covalent bond formation of rociletinib at C797 (55). Although sensitivity of EGFR TKIs to some uncommon mutations are known (L861Q, G719X, S768I), further work will be needed to determine the sensitivity of different anti-EGFR TKIs across a diversity of second-site EGFR TKI mutations and the impact of sequence of therapy on outcome (56). Genome editing using CRISPR-Cas9 technologies has been used to create preclinical models for drug testing and will improve our ability to investigate effective therapy combinations (57).

Mechanisms of Resistance in Alternate Bypass Pathways

A recent neoadjuvant study of erlotinib for locally advanced resectable EGFR-mutant patients has demonstrated that only a few patients (3/25 pts) had a major pathologic response (<10 viable tumor cells) after first-generation inhibitors (58). This highlights that resistance may occur early with mechanisms of resistance that are diverse and independent of the EGFR pathway. Continued cell growth and proliferation despite the reduction and disappearance of T790M mutant clones may signify reliance on a different mechanism of resistance or bypass pathways apart from EGFR (Fig. 2; refs. 28, 55). Escape mechanisms following therapy with third-generation EGFR TKIs are heterogeneous and
Clonal Evolution of EGFR Mutant Lung Cancer on Therapy

Figure 2.
Clonal heterogeneity through EGFR-targeted therapy. Visual representation of minimal residual disease and malignant cell progression after anti-EGFR TKIs demonstrating genomic patterns of selection observed in clinical samples through different lines of therapy.
inhibitor being explored with first-generation EGFR TKIs (NCT02424617).

In addition to the careful molecular characterization of resistant tumors, high-throughput screening with CRISPR-Cas9 technology is a means to discover additional potential resistance mechanisms (69). The nuclease Cas9 can be directed with great specificity using a short RNA barcode allowing for the rapid knockout of any gene. These barcodes can be read using next-generation sequencing, and CRISPR knockout screens can be highly multiplexed allowing for genome-wide analyses (70). Synthetic lethal approaches have revealed mechanisms that can drive both oncogene and non-oncogene EGFR-resistant NSCLC. Non-oncogene–dependent mechanisms include a host of factors including NF-κB, STAT5, Wnt, src family kinases, and chromatin remodeling proteins (71). Genome-wide knockout screens in sensitive and resistant NSCLC cell lines can be conducted with various EGFR inhibitors to identify de novo and acquired resistance mechanisms that generalize and that are specific to a particular compound.

Immune Combination Therapies

Anti-PD-1:PD-L1 therapies appear to be of limited benefit in patients with EGFR-mutant NSCLC, and the role of checkpoint inhibitors in EGFR-mutant disease is poorly understood (72). Larger studies with anti-PD1:PD-L1 antibodies have not shown high response rates or improved overall survival in the subset of EGFR-mutant patients. In a meta-analysis in NSCLC, immune checkpoint inhibitors did improve OS over docetaxel in the EGFR wild-type subgroup \( (n = 1362; HR = 0.66; P < 0.0001) \) but not in the EGFR-mutant subgroup \( (n = 186; HR = 1.05; P = 0.03; \text{ref. 72}) \). Preclinical data have shown that EGFR activation can upregulate PD-L1 expression (73). However, even in high expressing PD-L1 EGFR-mutant patients, checkpoint inhibitors have had limited clinical benefit and have been associated with toxicity with pneumonitis and transaminitis (NCT02879994). A trial of osimertinib combined with durvalumab (NCT02143466) has been halted due to an increase incidence of ILD (26% in pretreated patients and 64% in treatment naïve patients; ref. 20).

Hypotheses exploring the lack of response allude to lower tumor mutation burden (TMB) and a non-PDL1–dependent immunosuppressive tumor microenvironment. A recent publication has demonstrated that EGFR-mutated tumors generally have a low TMB, and higher TMB predicts worse response to anti-EGFR inhibitors (74). Mutations in p53 have been more common in EGFR-mutant patients with high TMB, and EGFR-mutant tumors have higher TMB at the time of progression on EGFR-TKI compared with pretreatment. Increased TMB may be common in EGFR-mutant patients with high TMB, and higher TMB predicts worse response to anti-EGFR inhibitors (74). Mutations in p53 have been more common in EGFR-mutant patients with high TMB, and EGFR-mutant tumors have higher TMB at the time of progression on EGFR-TKI compared with pretreatment. Increased TMB may be due to the emergence of subclonal mutations, and these mutations may increase overall TMB but may not confer effective immunogenicity (74).

Using flow cytometry and/or quantitative PCR in cell lines, median CD73 expression was increased 10-fold in EGFR-mutant NSCLC. Expression of the immunosuppressive molecule CD73 and reduced expression of the IFNγ mRNA signature has been hypothesized to contribute to an immune suppressive environment in this subset of NSCLC (75). A trial is currently underway evaluating an anti-CD73 molecule (oleclumab, MED194477) in combination with osimertinib and the adenosine receptor antagonist (AZD4635) in EGFR-mutant NSCLC (NCT03381274).

Immune combinations with antiangiogenic strategies have been undertaken and further data is awaited to determine if there is true synergy with this approach. Overall, antiangiogenic therapies in combination with EGFR-TKIs have shown encouraging synergy but limited overall survival benefit. Preclinical work with VEGF inhibitors including vandetanib or erlotinib/bevacizumab were more efficacious than gefitinib or erlotinib alone (76). The addition of bevacizumab to erlotinib resulted in an improved PFS compared with erlotinib alone as initial treatment (16 months vs. 10 months; HR 0.41; ref. 77). However, the phase II study ACCRU (NCT01532089) did not report an improvement in PFS and OS, and a phase 1/2 study (NCT02803203) is currently assessing osimertinib and bevacizumab as an initial treatment for patients with EGFR-mutant lung cancers. First-line osimertinib and chemotherapy combinations have been brought forward with the goal of eradicating minimal residual disease based on encouraging OS data with first-generation inhibitors (NEJ009).

Unanswered questions

The pattern of drug development in EGFR-mutant lung cancer has been a model for tumors driven by dominant oncogenic driver genes, changing multiple paradigms in clinical care. The FDA approval of osimertinib and impressive median overall survival marks an important advance with simultaneous targeting of the EGFR activating mutation and the dominant resistance clone. Clinical trials are exploring targeted therapies in early stage neoadjuvant and adjuvant settings (NCT 03433469 and NCT 02511106). Advances in genome sequencing technologies provide an opportunity for the serial measurement of circulating tumor DNA to allow for detailed characterization of EGFR inhibitor resistance through time and on therapy. The optimal order to sequence EGFR inhibitors and whether combinations of EGFR inhibitors or combinations with other targeted therapies can prevent the development of resistance clones is an active area of investigation. Effective and tolerable combinations that target EGFR independent bypass pathways have been informed by preclinical data from in vitro and patient derived xenograft experimentation and data on resistance patterns collected from clinical trials. Well-defined pre-clinical, pharmacokinetic, and pharmacodynamic testing has been employed with novel drug combinations to identify dose scheduling that allows for tolerable side effect profiles of combination therapy.

Additional third-generation TKIs have been evaluated with mixed success, and current strategies are evaluating the first-line utility of these compounds, sequencing strategies, drug combinations to target cancer dependencies, and effects on brain metastases. Accounting for the complexity of resistance pathways, the ability to characterize resistance clones through plasma-based genomic strategies may assist in tracking cancer evolution through therapy. Novel technologies such as CRISPR-Cas9 gene editing and integration of RNA sequencing analyses to identify bypass pathway activation will be important to better characterize the diversity of acquired resistance. Further understanding the biology of the disease, the evolution and progression of clones on therapy, the pharmacology of each inhibitor, polymorphisms, which affect selection and third-generation TKI drug sensitivity, and the resistance profiles seen with each compound will inform further clinical trials and may lead to more durable responses to targeted therapy.
Search strategy and selection criteria

We identified references for this Review through search of PubMed (MEDLINE, Web of Science, Cochrane Library, etc.) from 2001 to 2018, using the search terms NSCLC, EGFR, TKI, osimertinib, resistance, heterogeneity in various combinations. We only reviewed papers and abstracts in English and primarily focused on recent clinical trials. The final reference list was based on originality and relevance to this Review’s scope.

Disclosure of Potential Conflicts of Interest

J. Paul Shen reports receiving other commercial research support from Idyea Biosciences and has ownership interest (including stock, patents, etc.) in Tetraphase Pharmaceuticals and Illumina. H. Husain reports receiving other commercial research support from Pfizer, AstraZeneca, and Mirati; has received speakers bureau honoraria from AstraZeneca, Merck, and Bristol Myers Squibb; and is a consultant/advisory board member of AstraZeneca, Boehringer Ingelheim, Foundation Medicine, and Abbvie. No potential conflicts of interest were disclosed by the other authors.

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