Review

Molecular Cancer Therapeutics

Strategies to Overcome Bypass Mechanisms Mediating Clinical Resistance to EGFR Tyrosine Kinase Inhibition in Lung Cancer

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Abstract

The vast majority of patients with metastatic lung cancers who initially benefit from EGFR-targeted therapies eventually develop resistance. An increasing understanding of the number and complexity of resistance mechanisms highlights the challenge of treating tumors resistant to EGFR inhibitors. Resistance mechanisms include new, second-site mutations within *EGFR* (e.g., T790M and C797S), upregulation of MET kinase, upregulation of insulin growth factor receptor (IGFR), *HER2* amplification, increased expression of AXL, BIM modulation, NF-κB activation, histologic switch to small-cell cancer, epithelial-to-mesenchymal transition, PDL1 expression with subsequent immune tolerance, and release of cytokines such as TGFβ and IL6. Herein, we review the growing body of knowledge regarding EGFR bypass pathways, and the development of new drugs and combination treatment strategies to overcome resistance. *Mol Cancer Ther; 16(2); 265–72. ©2017 AACR.*

Introduction

Receptor tyrosine kinases (RTK) function as key regulators of cell growth, proliferation, and survival by transducing signals initiated by growth factors to the MEK/ERK, PI3K/AKT, and STAT pathways.

Activation of RTKs is reversible and tightly regulated, and the cells are dependent on extracellular cues from the environment. However, in cancer cells, these pathways are often constitutively activated due to genetic alterations in either RTKs themselves or components of the downstream signaling pathways (1). These alterations can result in constitutive signaling that lead to increased cell growth and survival, all of which are hallmarks of cancer (2). Inhibition of the mutant kinases by EGFR tyrosine inhibitors leads to simultaneous suppression of multiple pathways, frequently resulting in cell growth arrest and death (3, 4). A number of different pathways to acquired resistance have been described, and include development of resistant second-site *EGFR* mutations, or activation of alternative pathways through mutation, amplification, or other mechanisms (Table 1; refs. 3, 5–15).

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EGFR Signaling and Aberrations

EGFR (also known as *ERBB1* or *HER1*) belongs to the ERBB family of cell-surface RTKs that also includes *HER2* (also known as *NEU* or *ERBB2*; ref. 16). EGF binding to EGFR triggers homodimerization or heterodimerization of this receptor with other ERBB members, namely HER2, receptor phosphorylation, and activation of downstream effectors such as RAS–RAF–MEK–ERK and PI3K–AKT–mTOR, leading to cell proliferation (16). Other EGFR ligands include TGFα, amphiregulin, β-cellulin, heparin-binding EGF, and epiregulin (17).

The common EGFR mutations, such as exon 19 deletions and the exon 21 L858R, which account for about 85% of all *EGFR* mutations, predict sensitivity to afatinib and dacomitinib (irreversible EGFR TKI) as well as gefitinib and erlotinib (reversible EGFR TKIs) in patients with lung cancer (18). Less common aberrations besides exon 19 deletions and L858R include G719X and L861X that together make up 5% of *EGFR* mutations (ref. 19; Table 1). In contrast, exon 20 insertions or T790M mutations are resistant to most first-generation EGFR TKIs given as monotherapy. Data suggest that there may be two routes to developing T790M resistance: one that is selected for over time from preexisting T790M mutation in cells and a second that is acquired at a later time in initially T790M-negative *EGFR*-mutated cells (20). Strategies are underway to evaluate combinations of anti-EGFR tyrosine kinase inhibitors and mAbs that may be used for patients with T790M mutations or exon 20 insertions. Of interest in this regard, exon 20 insertions may increase EGFR dimerization, creating a susceptibility to EGFR antibodies (6). Third-generation anti-EGFR TKIs are irreversible inhibitors and include, but are not limited to, osimertinib (AZD9291; approved by the FDA), rocelitinib (CO-1686; $C_{27}H_{28}F_3N_7O_3$), ASP8273, PF7775, and EGF816 ($C_{26}H_{31}ClN_6O_2$; refs. 21, 22; Supplementary Fig. S1). These molecules were developed to have high potency against the first-generation EGFR TKI-resistant mutation

Table 1. EGFR-activating and resistance mutations in adenocarcinoma of the lung

Mutation	Frequency in EGFR-mutant lung adenocarcinoma (%)	Response rate to first-generation EGFR TKIs (%)	Median PFS after first-generation TKIs (months)	Median OS after first-generation TKIs (months)	Comment	References
Exon 19 deletions	∼45	∼60–85	∼9–15	∼24–34		5, 7, 9, 10
L858R (exon 21)	∼40	∼50–67	∼8–11	∼21		5, 7, 10
Exon 20 insertions	∼2–9	NA	NA	Varies widely 4–16 months	May increase dimerization and sensitize to EGFR antibody	6, 7, 8
G719X (Exon 18)	3	∼37	NA	NA		11
L861X (exon 21)	2	∼40	NA	NA		11
Exon 19 insertions	1	NA	NA	NA	Case series report responsiveness to erlotinib	12
T790M (exon 20)	∼25% in *de novo* patients	Resistant to first-generation TKIs Response rate to third-generation EGFR TKI AZD9291 is ∼61%	NA	NA	About half of the patients with acquired resistance develop T790M	3, 13–15

Abbreviations: Mo, months; NA, not available; OS, overall survival; PFS, progression-free survival.

T790M, and active areas of research are focusing on identifying mechanisms of resistance to these compounds.

Acquired Resistance to EGFR-targeted Therapies

Tumor progression on targeted therapy may be the product of both intrinsic and acquired resistance (20). In addition, drug dosing, pharmacokinetics, and administration may explain why some patients do not respond to anti-EGFR TKI therapies. When EGFR TKIs are coadministered with drugs such as the cholesterol-modifying medication fenofibrate that induce CYP3A4, erlotinib metabolism is increased (23). In contrast, proton pump inhibitors and H2-receptor antagonists decrease pH-dependent drug solubility, and erlotinib drug levels may be decreased and responses attenuated (24). Although pharmacokinetic factors are important as we consider combinatorial strategies, we will focus on the cell-intrinsic bypass mechanisms of acquired resistance below (Fig. 1).

MET

One of the earliest suggestions that RTK bypass signaling could promote resistance to targeted therapies came in the setting of *EGFR*-mutant non–small cell lung cancer (NSCLC). In lung adenocarcinoma treated with anti-EGFR TKI therapy, amplification of the *MET* gene encoding the MET kinase has been observed in cancers with acquired resistance to EGFR TKIs, but not in the pretreatment samples (25, 26). *MET* amplification was initially discovered in an *EGFR*-mutant cell line that was cultured in the presence of gefitinib until resistance developed. MET was found to promote resistance by reactivating both PI3K/AKT and MEK/ERK signaling, despite the inhibition of EGFR (27). The combination of a MET and an EGFR inhibitor was both necessary and sufficient to block downstream signaling and induce marked tumor regressions *in vitro* and *in vivo*. A subsequent study revealed that there were rare cells (less than 1%) with *MET* amplification in pretreatment samples from several patients with lung cancer whose resistant tumors ultimately developed overt *MET* amplification. This raises the possibility that these resistant cells exist at low frequency before treatment (27).

Although early-phase studies of the combination of MET and EGFR inhibitors held promise for this combination, larger phase III studies showed less clear signals of efficacy (28, 29). A recently reported phase III study of the MET kinase inhibitor tivantinib (ARQ197) and erlotinib versus erlotinib and placebo recruited 1,048 patients, but failed to meet its primary objective, with a HR of 0.98 for overall survival. This result is perhaps related to the fact that the patients with lung cancer on the trial were not selected for *EGFR* mutations or MET aberrations (28). A phase II study of the MET inhibitor onartuzumab in combination with erlotinib in *MET*-amplified, *EGFR*-uncharacterized patients showed improvement in progression-free survival (PFS; HR 0.53; $P = 0.04$) and overall survival (OS; HR 0.37; $P = 0.002$), and a companion biomarker analysis was done comparing MET IHC, FISH, PCR, and ELISA, and found that IHC was the only significant predictor of OS and PFS benefit (30). A phase I study of INC280 (an oral MET inhibitor with preclinical activity in *EGFR*-mutant/MET-activated NSCLC) had 6 of 41 patients respond when selected on the basis of *MET* amplification (FISH $\geq$ 5 copy number) or IHC of 2/3+ (31). A dose-finding phase I/II study of 25 patients receiving crizotinib and erlotinib demonstrated that one patient achieved PR and nine attained stable disease. Coadministration of both drugs increased erlotinib AUC by 1.8-fold and a MTD of erlotinib 100 mg daily and crizotinib 150 mg twice daily was defined (32). Resistance to the third-generation anti-EGFR compounds has also been associated with *MET* amplification (33). Studies have started to evaluate the combination of MET-directed therapy and third-generation anti-EGFR TKI therapy post progression on first- or second-generation anti-EGFR TKI therapy (TATTON – NCT021434466).

HER2

Her2 has no known ligand, favors dimerization, and is part of the EGFR family (EGFR being HER1; ref. 34). *ERBB2* (Her2) anomalies may be important in patients with NSCLC regardless

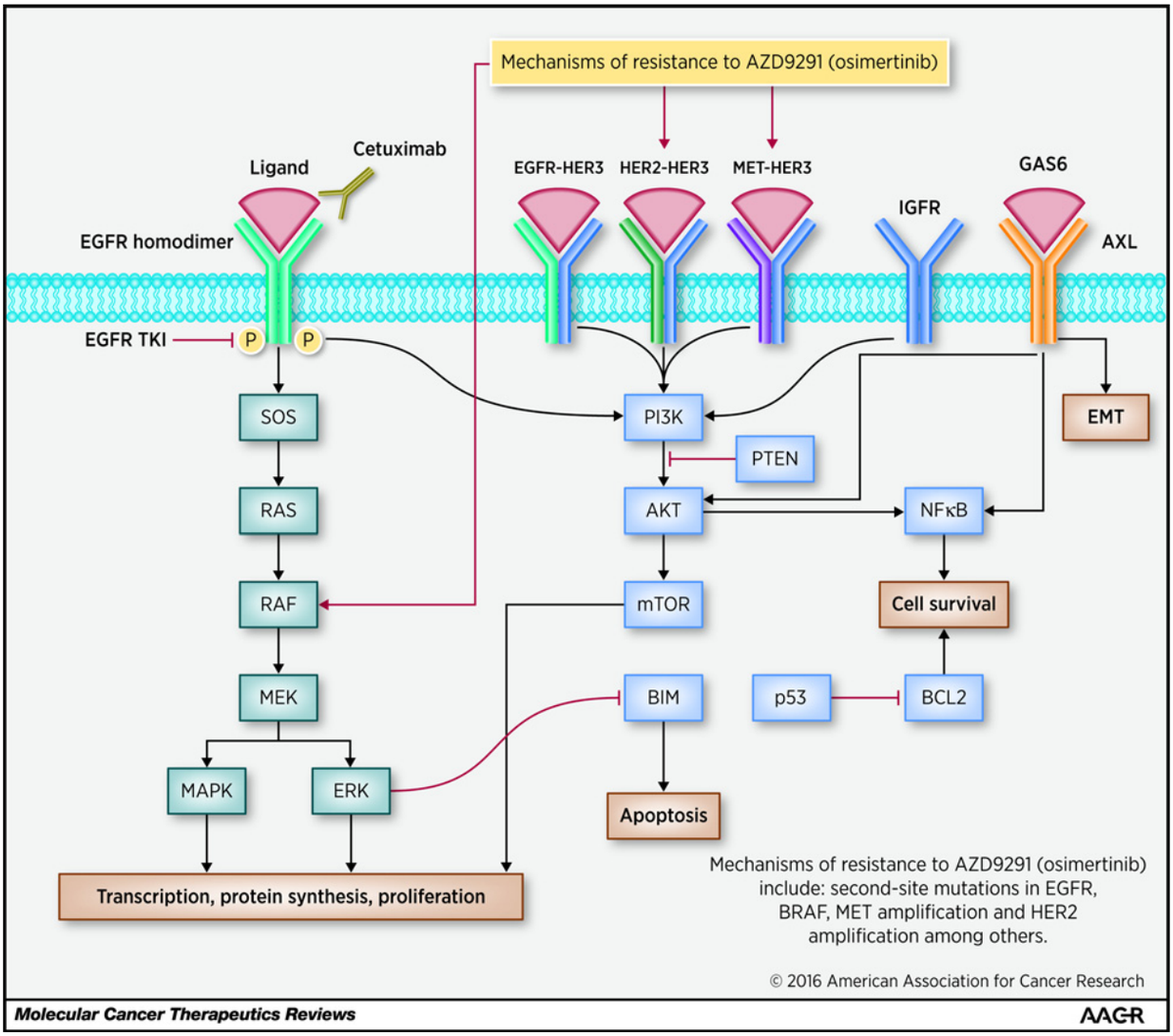

Figure 1.
EGFR response and resistance pathways.

of *EGFR* mutation status (35, 36). Indeed, *ERBB2* (*HER2*) mutations and amplifications have been described in a small subset of patients with lung cancer who do not have *EGFR* mutations; patients with tumors harboring some of these alterations may respond to Her2-targeting agents (36). *ERBB2* amplification, as identified by FISH, has also been observed in 12% of drug-resistant, *EGFR*-mutant lung cancers (35). These patients did not harbor T790M-resistant mutations, suggesting that the *ERBB2* abnormalities may have mediated resistance. Increased ErbB2 protein was also detected in a cell line model of acquired resistance to EGFR TKIs (35). Patients who had progressed while on prior therapy and went on to receive afatinib had a response rate of 7%, and, in aggregate across genotype, no significant survival benefit (10.8 months vs. 12.0 months; ref. 37). It is not known if those patients who had objective responses had *Her2* amplification. HER2/EGFR inhibitors, such as lapatinib or afatinib, merit more rigorous investigation in a molecularly defined cohort. Patients treated with the second-generation EGFR inhibitor afatinib have shown increased PFS and time-to-treatment failure compared with gefitinib in a first-line trial of patients with *EGFR*-mutant NSCLC (LUX-Lung 7 trial; ref. 38). The enhanced efficacy endpoint could be due to the more potent effects of these second-generation inhibitors (irreversible binding) as opposed to specific activity against Her2.

Insulin-like growth factor receptor

The activity of the insulin-like growth factor 1 receptor (IGF1R) can promote acquired resistance to gefitinib in *EGFR*-amplified and *EGFR*-mutant cancer cell line models. In gefitinib-resistant A431 epidermoid carcinoma cells, loss of expression of IGFBP3 and IGFBP4, which encode insulin-like growth factor binding proteins 3 and 4, respectively, leads to increased IGF1R/PI3K/AKT pathway activity and

maintenance of PI3K/AKT signaling despite EGFR inhibition (39). Likewise, PC9 NSCLC cells incubated in the presence of next-generation EGFR inhibitors (which have the capacity to suppress EGFR-T790M activation) showed decreased IGFBP3 abundance and IGF1R-dependent maintenance of PI3K/AKT signaling (40). Inhibition of IGF1R by a kinase inhibitor was sufficient to restore sensitivity to EGFR inhibition, and the combination was necessary to suppress PI3K/AKT signaling (40). IGF1R expression is detected in a majority of NSCLC tumors by histologic analysis, lending credibility that this mechanism could mediate resistance in appropriately defined patients (41).

Phase I/II studies with the IGFR inhibition either alone or in addition to chemotherapy exhibited tolerability (42). However, phase III studies of figitumumab in unselected populations of squamous NSCLC were terminated early as the compound failed to show a survival advantage as compared with standard chemotherapy in the first-line setting (43). The EGFR molecular status was not interrogated in these patients, and the population was unlikely to harbor activating mutations based on their non-adenocarcinoma histology. An additional study of cixutumumab combined with cetuximab and chemotherapy in unselected NSCLC was stopped early because of grade 5 events and limited efficacy (44). The need to develop biomarkers and a clearer understanding of insulin receptor function will help define whether or not there is a clinical role for IGFR inhibition in EGFR TKI–resistant patients (45).

AXL

AXL is a RTK whose role is poorly understood. Studies indicate that it may participate in inducing epithelial-to-mesenchymal transition and has been implicated preclinically in acquired resistance to TKIs in patients with *EGFR*-mutant NSCLC (46, 47). The expression of AXL and its ligand GAS6 is increased in a subset of lung tumors from EGFR TKI drug-resistant patients. Large panels of genetically diverse cell lines have shown that both AXL and epithelial-to-mesenchymal transition are associated with intrinsic resistance to EGFR inhibitors (48). The small-molecule multikinase AXL inhibitors MP-470 and XL-880 restore sensitivity to erlotinib in *in vitro* preclinical models (49, 50). BGB324 has been launched into a phase I/II clinical trial for patients in the second line after initial anti-EGFR tyrosine kinase therapy (NCT02424617). Inhibitors of this pathway are entering the clinic with combination of cytotoxic agents, anti-EGFR drug therapies, and combined with immunotherapy in a molecularly stratified cohort (51).

Fibroblast growth factor receptor

Activation of fibroblast growth factor receptor 1 (FGFR1) through an FGF2–FGFR1 autocrine loop was also identified as a mechanism of resistance in a PC9 lung cancer cell line model (52). Inhibition of FGFR1 or FGFR2 by PD173074 restores sensitivity to gefinitib in the PC9 gefitinib-resistant cell line. Clinically, a study of erlotinib and dovitinib, a small-molecule inhibitor of FGFR and VEGFR, was terminated after two dose cohorts because of toxicity and pharmacokinetic interaction with markedly decreased erlotinib exposure, likely mediated through CYP1A1/1A2 induction (53). Several potent FGFR inhibitors are approved or in clinical development, but their activity in the context of EGFR resistance has not been described.

EGFR mutations mediating resistance

EGFR mutations in exon 19 and 21 (L858R) are generally sensitive to first- and second-generation EGFR TKIs (54). However, *EGFR* exon 20 mutations (such as insD770, A767, S768, H773) and T790M are resistant (55). Currently, there are no approved targeted therapies for EGFR exon 20 insertions; however, clinical trials with Hsp90 inhibitors (NCT01854034) and EGF816 have cohorts specifically evaluating this population of patients (NCT02108964). Recent phase I/II trials with the HSP90 inhibitor AUY922 in unselected patients have shown limited responses. Toxicities included night blindness, diarrhea, and rash (56).

Third-generation inhibitors that are T790M specific (osimertinib (AZD9291), rocelitinib (CO-1686), EGF816, PF7775, and ASP8237) are at various stages of approval or development for patients resistant to first-line anti-EGFR-TKIs (refs. 21, 22 and Supplementary Fig. S1). Osimertinib (AZD9291) was approved by the FDA in November 2015 based on objective response rates of approximately 60% in EGFR T790M-mutant NSCLC with a median PFS of 9.6 months (57). Combination studies of AZD9291 are underway after progression on an anti-EGFR TKI, with a multi-arm phase 1b study incorporating arms with agents targeting the c-MET pathway (AZD6094), MEK pathway (selumetinib), or anti-PDL1 pathway with durvalumab (TATTON study: NCT021434466). In addition, AZD9291 is being evaluated with the mTORC 1/2 inhibitor INK128 (NCT02503722), EGFR mAb necitumumab (NCT02496663), and bcl-2 inhibitor navitoclax (NCT02520778). Rociletinib showed response rates in in T790M-positive patients, although many responses previously reported were unconfirmed and the confirmed response rate has been subsequently reported at 30% down from previously stated (58, 59). A recent press release reports that there will be no more enrollments onto the TIGER studies with rocelitinib based on strategic review of recent data within the company (60). Recently presented work demonstrates that, after exposure to AZD9291, 6 of 15 patients demonstrated emergence of a new second-site mutation in EGFR (C797S) that may confer resistance to the third-generation anti-EGFR TKIs (61).

Dual EGFR inhibition combining an EGFR TKI and mAbs

Several combinations of dual EGFR TKIs and mAbs have been explored. EGFR TKIs have shown limited effectiveness against EGFR exon20 insD770 alterations. However, *in silico* modeling predicted that these mutations would increase dimerization and sensitize tumors to anti-EGFR mAbs such as cetuximab, and responses to the combination of EGFR TKI and mAb have been reported (6, 7). A retrospective analysis across nonmolecularly defined NSCLC showed that erlotinib and cetuximab was active in patients, with 5 of 20 patients (25%) having stable disease for 6 months or longer and or partial/complete remission, including individuals that had squamous histology, brain metastases, resistant EGFR mutations, and or wild-type EGFR (62). A prospective single-arm study of afatinib in combination with cetuximab resulted in about a 32% response rate in an EGFR-mutant, T790M-positive population, and a 25% response rate in T790M-negative patients (63). When evaluated across studies, it is important to note that there was a 7% response rate with afatinib alone (37). Preclinical studies demonstrate that EGFR may have both a kinase-dependent and kinase-independent effects, perhaps

explaining synergy for kinase inhibitors and antibodies (64). Ongoing studies including AZD9291 and necitumumab (NCT02496663) or afatinib and nimotuzumab (humanized EGFR antibody) are underway, with manageable adverse toxicities including skin rash and diarrhea (NCT01861223).

A study of erlotinib and bevacizumab (BeTa) in an unselected patient population demonstrated that the population that received the most benefit from this combination may be the *EGFR*-mutant population (65). An ongoing phase III study is evaluating the efficacy of this combination in a selected, *EGFR*-mutant population compared with erlotinib alone (NCT01532089).

Histologic transformation

There is a subset of patients (5%–10%) with mutated *EGFR* that transform from NSCLC to small-cell lung cancer under the selective pressure of anti-EGFR TKI therapy (55, 66). Some of these tumors harbor retinoblastoma gene (RB) loss, which can also be seen in *de novo* small-cell lung cancer (66). One mechanism contributing to this transformation includes the epithelial-to-mesenchymal transition, as manifested by the loss of E-cadherin expression and increased expression of fibronectin and vimentin. The role of the AXL kinase in mediating this process is not entirely clear (55, 67). Other pathways also involved in the histologic transformation of EGFR TKI–resistant tumors may include Notch-1 and TGFβ (68, 69).

The microenvironment and the immune system

It has been shown in preclinical models that EGF pathway activation contributes to PDL1 upregulation, which in turn promotes immune tolerance (70). Ongoing studies are testing the clinical utility of combinations of targeted therapy with immuno-oncology agents such as nivolumab, pembrolizumab, and durvalumab. Combination of osimertinib with durvalumab in a Chinese patient population was noted to have increased incidence of pneumonitis, and clinical trials were halted in Asia with these specific agents (71).

Secreted factors in the tumor microenvironment may be important for resistance. Cytokines such as TGFβ promote tumor escape from immune-surveillance, and high plasma levels of TGFβ correlate with a negative prognosis in a variety of cancers (72). The production of TGFβ can lead to the accumulation of CD3+, CD4+, CD25+, FOXP3+ Treg cells (72). Studies have shown that secretion of IL6 by stromal cells into the tumor microenvironment may also promote tumor survival and block apoptosis, thereby resulting in chemotherapeutic resistance. These exogenous effects are mediated via endogenous JAK/STAT and DNMT1 methylation pathways (73, 74). In a preclinical model, induction of inflammation stimulated IL6 secretion and resulted in decreased tumor response to erlotinib (69). A phase I study of a humanized anti-IL6 (ALD518) showed it was well tolerated and had effects against cancer-related fatigue and anemia (75). Evidence for efficacy requires additional studies (74).

Reprogramming events within the tumor and the microenvironment may also be involved in mediating drug resistance. Cell-intrinsic factors including Bcl-2 family members have been studied in EGFR resistance and, in drug-sensitive, EGFR-mutant lung cancer cells, induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors (76). An inhibitor of anti-apoptotic proteins, ABT-737, enhances erlotinib-induced cell death *in vitro* (76), and these observations have inspired trials with the drug ABT-263 (navitoclax) as a potent small-molecule inhibitor of BCL-2, BCL-XL, and BCL-w (NCT02520778). Preclinical work demonstrates that integrin $\alpha_{(v)}\beta_3$ may serve as a marker of breast, lung and pancreatic carcinomas with stem-like properties that are highly resistant to receptor TKIs such as erlotinib (77). Mechanistically, integrin $\alpha_{(v)}\beta_3$ recruits K-Ras and RalB to the tumor cell plasma membrane, leading to the activation of the NF-κB transcription factor (77). Pharmacologic targeting of this pathway could conceivably alter the plasticity of tumor stemness and TKI resistance, and studies are underway to evaluate this strategy.

Novel technologies to detect and monitor resistance—noninvasive liquid biopsies

Monitoring patients with NSCLC for disease response and evolution is crucial for their effective management on anti-EGFR–directed therapies. This can be done through repeat tissue biopsies, although these can be associated with complications, morbidity, and significant resources. Noninvasive plasma circulating tumor DNA (ctDNA) analyses have been explored in this setting to qualitatively evaluate DNA shed by the tumor into the circulation (78). DNA from circulating tumor cells may also be analyzed to detect resistance within live cells, and recent work suggests some concordance between circulation tumor cell DNA and circulating tumor DNA analyses in *KRAS*-mutated NSCLC (79). Tumor DNA may be isolated from a variety of other fluids: saliva, pleural effusions, ascites, and urine. These tests can detect and quantitate EGFR aberrations (80, 81). The emergence of resistance mutations such as EGFR T790M may be noted in the urine months before imaging shows progression. Response to EGFR inhibitors may be associated with distinct patterns of change of *EGFR* mutations levels in the urine and can be seen within 72 hours of drug administration (81).

Discussion

Preclinical studies identifying mechanisms of resistance to EGFR TKIs have been translated into several completed or ongoing clinical trials of combinatorial therapy in the attempt to offset resistance. Some clinical trials have had limited therapeutic efficacy, possibly because many were explored in non-molecularly defined patient populations. With the incorporation of third-generation compounds that target acquired resistance mutations, a heightened appreciation of the need for rationally designed combinations impacting bypass pathways is becoming increasingly apparent. Further work to clarify the dose-limiting toxicities encountered with combinational therapies is needed, and an ideal partner for an EGFR TKI may be a drug that targets completely different cancer vulnerability. Novel technologies such as liquid biopsies may enhance our ability to detect resistance and/or response early and allow the opportunity to nimbly adjust therapy depending on markers of response. Comprehensive genomic evaluation provides information about novel mechanisms of resistance to the third-generation EFGR inhibitors and insights into sequencing combinatorial therapies. New drug development in this space has created favorable prospects for characterizing and targeting the

biologic basis of resistance and employing combinatorial strategies to improve patient outcomes.

Disclosure of Potential Conflicts of Interest

Razelle Kurzrock has ownership interest in Novena, Inc. and Curematch, Inc., reports receiving a commercial research grant from Genentech, Merck Serono, Pfizer, Sequenom, Foundation Medicine, and Guardant, and is a consultant/advisory board member of Sequenom, Actuate Therapeutics and Xbiotech. No potential conflicts of interest were disclosed by the other authors.

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