Review Article

Emerging uses of biomarkers in lung cancer management: molecular mechanisms of resistance

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Abstract: Management of patients with advanced non-small cell lung cancer (NSCLC) has recently been transformed by molecularly targeted and immunotherapeutic agents. In patients with EGFR/ALK/ROS mutated NSCLC, first line molecular therapy is the standard of care. Moreover, immune checkpoint inhibitors are revolutionary treatment options for advanced NSCLC and are now the standard of care in front-line or later line settings. Both classes of agents have led to improved patient outcomes, however, primary resistance and development of acquired resistance to both targeted and immunotherapeutic agents is commonly observed, limiting the use of these agents in clinical settings. In this review, we will discuss the most recent advances in understanding the mechanisms of primary and acquired resistance, progress in the spectrum of assays detecting causative molecular events and the development of new generations of inhibitors to overcome acquired resistance.

Keywords: Resistance; molecular mechanisms; targeted therapy; epidermal growth factor receptor (EGFR); T790M; ALK; MET; ROS; immune checkpoint inhibitor

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Introduction

Research over the last decade has transformed the management of patients with advanced non-small cell lung cancer (NSCLC) with the recognition of molecularly defined subsets of tumors with unique sensitivities to targeted therapeutics, such as patients with EGFR/ALK/ROS-mutated lung adenocarcinoma. Upfront molecular therapy is now the standard of care for optimization of treatment, and patient outcomes have greatly improved. In addition, an expanding group of other molecular alterations are continuing to be recognized and biomarker-driven immunotherapeutic strategies have yielded dramatic advances in patient management. However, acquired resistance to both targeted and immunotherapeutic agents have become a pivotal issue limiting the long-term benefit of such therapies. In the current review, we will highlight the most significant advances in our understanding of mechanisms of primary and acquired drug resistance, strategies to detect secondary molecular events, and drug development strategies yielding new generations of inhibitors with increasing success to overcome acquired resistance (*Figure 1*).

Epidermal growth factor receptor (EGFR)

The *EGFR* gene, located on chromosome 7p12-13, encodes for a HER family receptor tyrosine kinase (RTK) that upon activation, will "switch on" several downstream signaling pathways important in cell survival and proliferation (1). Activating mutations in the tyrosine kinase domain of the EGFR gene are seen in 10–15% of non-squamous NSCLC and are responsible for tumor growth, proliferation,

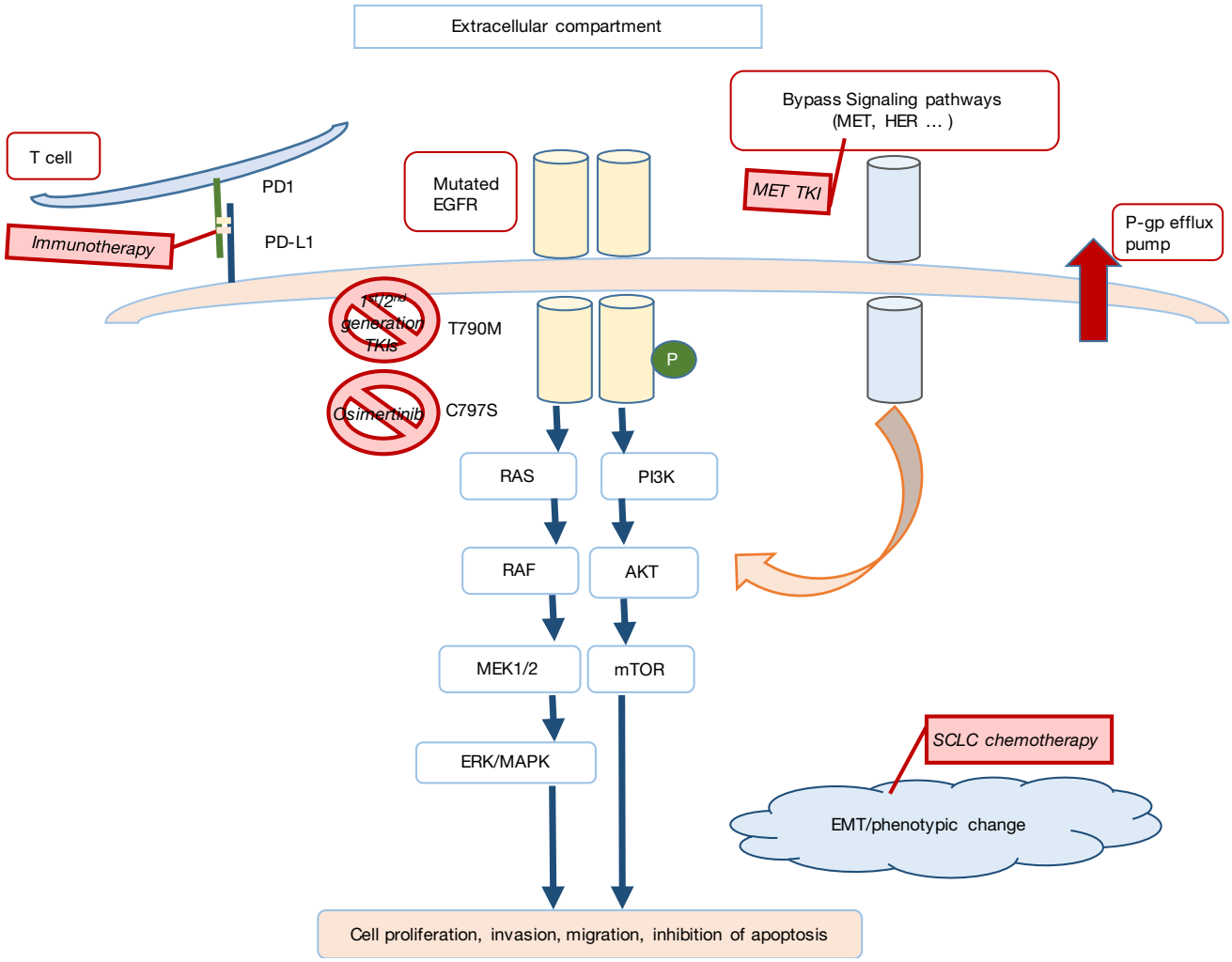

Figure 1 General mechanisms of resistance.

invasion and metastases by promoting pro-proliferative and anti-apoptotic effects. These mutations occur more commonly in tumors in women, non-smokers and patients of Asian ethnicity and can be as frequent as 50–60% in lung adenocarcinomas in Asian women (2,3).

Two so-called "classic" mutations account for >90% of the known activating EGFR mutations: L858R point mutation on exon 21 and in-frame deletions around the conserved LREA motif of exon 19 (2,3). The presence of such activating EGFR mutations serves as a biomarker as well as a target for therapy with EGFR-tyrosine kinase inhibitors (TKI). Several clinical studies revealed that response rates (RRs) to first generation EGFR-TKIs, such as gefitinib and erlotinib, in patients harboring an EGFR-activating mutation are 50–80% and can lead to durable responses with progression free survival in the 8–12 months range (4-8). In 2012, a meta-analysis of six randomized controlled trials confirmed significant improvement in overall response rate (ORR) and doubling of progression free survival (PFS) in patients with advanced EGFR mutated NSCLC receiving first-line EGFR-TKI, as compared to conventional chemotherapy (9). Therefore, EGFR-TKIs are the current standard treatment for these patients and frontline molecular testing is now part of routine management (4,9).

However, resistance to EGFR-TKIs poses a major clinical problem, and can be categorized as primary or acquired. Primary resistance refers to *de novo* lack of response to the targeted therapy, while acquired resistance is defined as progression of disease after an initial period of

clinical response (10,11).

Primary resistance

Several mechanisms of primary resistance to EGFR-TKIs have been described.

First generation EGFR-TKIs are not effective in patients with the gatekeeper EGFR T790M mutation which can be present as a germline mutation in rare familial clusters. This mutation will be discussed in detail later in this review (10).

Another example of primary resistance to EGFR TKIs is the EGF exon 20 insertion mutation. The RR of NSCLCs with EGFR exon 20 insertion mutation to EGFR-TKIs is below 5% (12). The mechanism for this primary resistance has been described by Yasuda *et al.* (12), 80–90% of activating EGFR exon 20 mutations cause insertion of one to four amino-acids beyond the C-helix of the tyrosine kinase domain, forming a wedge at the end of the C helix that promotes the active kinase and leaves the adenosine triphosphate binding pocket unaltered. Interestingly, an EGFR exon 20 insertion mutation sensitive to the EGFR-TKIs was also identified (EGFR A763_Y764insFQEA). Structurally, this mutation is very different from other exon 20 insertions and more closely resembles the L858R and exon 19 deletion mutations. At present, the standard of care for TKI-resistant exon 20 mutated EGFR tumors is conventional chemotherapy (13,14). Efforts are being made to develop EGFR targeting agents with activity against this important class of mutation (e.g., AP32788 NCT02716116).

Another mechanism of primary resistance is conferred by polymorphisms in the Bim gene. Bim is a potent pro-apoptotic protein and its expression is suppressed in EGFR mutated lung cancers. TKI therapy can upregulate Bim expression and allow for cell death and tumor regression. In the case of certain inherited Bim gene polymorphisms, the pro-apoptotic domain is not present and TKI therapy is less successful with shorter duration of response than in other genotypes (15).

Finally, as expected, EGFR-TKI therapy is not effective against tumors with concurrent activating mutations in genes downstream of EGFR such as K-ras and BRAF or against tumors harboring other oncogenic gene alterations (10). Canale *et al.* (16) observed that TP53 tumor suppressor gene mutations, especially exon 8 mutations, reduce the RR to TKIs. Inferior outcomes in the presence of p53 mutations had been similarly noted by the Lung Cancer Mutation Consortium (17). TP53 mutations occur in about 30%–40% of NSCLCs and while TP53 mutations might not be actionable, this may be important in order to risk stratify patients.

Acquired resistance

Acquired resistance to EGFR-TKIs typically develops within 6–18 months of starting therapy (11). Biopsy and sequencing of tumors in patients with disease progression on an EGFR TKI as well as *in vitro* and mouse studies have led to the discovery of potential mechanisms of acquired resistance as well as novel and effective ways of overcoming certain resistance mechanisms (18).

T790M mutation

The most common mechanism of acquired resistance, accounting for up to 60% of cases, is a secondary mutation of EGFR gene, leading to the substitution of methionine for threonine at position 790 (T790M) (10). Methionine's large side chain causes steric hindrance and reduces the ability of 1st generation EGFR-TKIs such as erlotinib and gefitinib to bind to the ATP-kinase pocket. In addition, this mutation changes the dynamics at the binding site such that ATP, rather than the ATP-competitive EGFR-TKIs is the favored substrate. This leads to a 1,000-fold increased resistance against the EGFR-TKIs (10,18).

Several studies have been done to determine if tumor cells containing the T790M mutation are present prior to EGFR-TKI treatment initiation or if they develop from cancer stem cells during treatment. These studies have found very few TKI-resistant cells prior to treatment thereby suggesting that the T790M mutation more commonly arises due to selective pressure during EGFR-TKI therapy (10,19). Three other point mutations have been implicated in EGFR-TKI resistance (D761Y, L747S, T854A), however they have been reported only occasionally and their mechanism of resistance is less understood (10).

Activation of bypass signaling pathways

A second mechanism of acquired resistance is through the activation of bypass signaling pathways. For example, amplification of the MET gene has been seen in 5–22% of NSCLC patients who develop acquired resistance to EGFR-TKIs. These amplifications are seen in very low rates in tumors that have not yet undergone treatment with an EGFR-TKI. Thus, it has been postulated that under the selective pressure of EGFR-TKI therapy, cells

with dual EGFR and MET activation can undergo clonal selection. Ultimately, these EGFR + MET mutated cells form the bulk of the tumor leading to clinical resistance against EGFR-TKIs. *In vitro*, MET amplification has been reported in gefitinib-resistant cell lines and dual EGFR and MET inhibition has been successful in inducing apoptosis in this case (20,21).

MET activation by its ligand HGF may also be another mechanism contributing to acquired resistance. In a few studies, it has been noted that HGF is expressed in high levels in 29% of patients with primary resistance and 61% of patients with acquired resistance (22). If future studies validate this mechanism *in vivo*, targeted therapy against HGF can be a potential strategy to treat this subset of patients (10).

HER2 amplification is another alternative signaling pathway that plays a role in acquired resistance to EGFR-TKIs and has been noted in up to 13% of patients (10). In a phase Ib study of patients with NSCLC resistant to erlotinib/gefitinib, dual inhibition of EGFR and HER2 with afatinib and cetuximab demonstrated promising activity leading to median PFS of 4.7 months (23). Another bypass signaling mechanism that has been recently implicated in afatinib-resistant PC9 cells is the IGF1R pathway—increased expression of insulin-like growth factor binding protein 3 (IGFBP-3) enhances IGF1R activity leading to increased AKT phosphorylation and subsequent cell cycle progression (24).

Phenotypic changes

Yet another mechanism of acquired resistance is through phenotypic alterations. Epithelial mesenchymal transition (EMT) is defined as the loss of epithelial markers and a subsequent gain of mesenchymal features (10). NSCLC cells that undergo EMT lose sensitivity to EGFR-TKIs (25). Furthermore, when the epithelial phenotype is restored in these EGFR-mutated NSCLC cell lines, they become sensitive again to EGFR-TKIs (26). The transition between epithelial and mesenchymal phenotypes is likely influenced by the AXL RTK. A high level of expression of AXL RTK has been implicated in acquired resistance to erlotinib, and over-expression of this same RTK has been noted in NSCLC cells with the mesenchymal subtype (10,27). Taken together, it seems as though AXL plays a significant role in the acquired resistance to EGFR-TKIs and may one day be an effective pharmaceutical target. In fact, a recent report of tumor genomic profiling cites the case of a patient with lung adenocarcinoma and pleural carcinomatosis whose genome analysis showed focal gain of chromosome 19q12-13.11, including AXL. He was enrolled in a Phase I trial of MGCD265, a TKI targeting MET and AXL with a dramatic response and near resolution of lung infiltrates on imaging in just 2 months (28).

A different kind of phenotypic alteration happens when the NSCLC cells undergo histological transformation to small cell lung cancer (SCLC). This has been reported in 3–14% of patients with acquired resistance to EGFR-TKI who underwent re-biopsy. In a recent study Lee *et al.* (29) investigated 21 patients with advanced EGFR-mutated NSCLC that transformed into SCLC. Whole genome sequencing was performed at different time points during disease evolution. It was concluded that TKI-resistant EGFR-mutated NSCLC and SCLCs share a common clonal origin and the clonal divergence occurs before the EGFR TKI therapy. Complete inactivation of RB1 and TP53 were observed from the early NSCLC stages in these tumors. There have been multiple cases that reported good response to SCLC chemotherapy regimens once the NSCLC undergoes this histological transformation underlining the importance of testing for such alterations (10,30).

Pharmacodynamic limitations

Another limitation to the use of first and second generation TKIs is the inability of these drugs to fully penetrate the blood brain barrier (BBB) and enter the central nervous system (CNS). Patients can often develop CNS metastases during treatment, even when their extracranial tumors are still under control (31). CNS concentrations of all currently available agents are lower than plasma [gefitinib has a CSF concentration approximately 1% of serum and erlotinib has a CSF concentration 5% of serum (32,33)]. Many EGFR TKIs that have been designed for improved CNS penetration are currently under investigation. One such drug, AZD3759 has shown promise with CNS penetration adequate enough to promote tumor shrinkage in patients with brain and leptomeningeal metastases in addition to a tolerable side-effect profile (34).

Treatment approaches to overcome resistance

Third generation EGFR TKIs

Knowledge of the biological mechanisms behind acquired resistance to EGFR-TKIs has led to the development of 3rd

generation TKIs that specifically target the EGFR T790M mutant cells. The side-effect profile of these new TKIs is more favorable given that they spare WT EGFR (10,11).

Of the 3rd generation TKIs, osimertinib has progressed the furthest in clinical development. Osimertinib, an oral irreversible agent with CNS penetration, had initially been approved by the FDA under the Breakthrough Therapy Designation Program based on outstanding results from the AURA-1 and AURA-2 studies suggestive of great efficacy with RRs in the 50–60% range in EGFR T790M-mutated cases as well as favorable toxicity profiles. Recently, Mok *et al.* published the results of a confirmatory, randomized, open-label, international, phase 3 (AURA 3) trial to show the superiority of osimertinib over platinum plus pemetrexed chemotherapy in patients with confirmed T790M-positive advanced NSCLC after first-line EGFR-TKI therapy. Median PFS was significantly longer with osimertinib as compared to chemotherapy (10.1 *vs.* 4.4 months) and objective RR was significantly better with osimertinib (71% *vs.* 31%). Even among the 144 patients in the trial who had CNS disease, osimertinib performed better, PFS was 8.5 months with osimertinib *vs.* 4.2 months with chemotherapy. Adverse events of grade 3 or higher were more common in the chemotherapy group as compared to osimertinib (47% *vs.* 23%) (35). These results led to full FDA approval of osimertinib in 2017.

Unfortunately, tumor samples from patients treated with osimertinib have already revealed that emerging resistance will remain a key shortcoming of this class of drugs. The EGFR C797S mutation appears to be the most dominant resistance mechanism, with frequency as high as 20–40% (36). This mutation is very logical from a biochemical perspective as osimertinib and other irreversible EGFR inhibitors covalently bind to C797 of EGFR gene and this mutation interferes with such binding, thus drastically diminishing activity. Interestingly, the allelic context of C797S seems to have treatment implications—when the T790M and C797S mutations occur in *trans* (on separate alleles), a combination of first and third generation TKIs can restore EGFR inhibition. But, if the mutations are in *cis* (on same allele), the cells will not be sensitive to any available EGFR TKI (37). Understanding of such novel resistance mechanisms is already leading to innovative approaches of preventing/overcoming such resistance.

For example, a new compound called EAI045, which acts as an allosteric inhibitor of the TK domain of EGFR, could be a promising agent in overcoming resistance to 3rd generation TKIs. In mouse models, EAI045 in combination with cetuximab, an antibody that stops EGFR dimerization, has been shown to be effective against the L8585R/T790M and L858R/T790M/C797S EGFR mutants (38). Another drug regimen that is a powerful candidate to overcome triple-mutant EGFR is the combination of cetuximab with brigatinib, a potent and selective ALK/EGFR T790M inhibitor. This regimen showed excellent efficacy in del19/T790M and del19/T790M/C797S cell lines as well as in del19/T790M/C797S xenograft mouse models (39).

MET inhibition for bypass signaling

Dual inhibition of EGFR and MET has been studied in two Phase III trials: the MET-Lung trial that investigated the combination of erlotinib ± onartuzumab, a monoclonal antibody against MET, as well as the MARQUEE trial that compared erlotinib ± tivantinib, a MET TKI. However, neither of these studies met their primary endpoint of improving OS, maybe due to the fact that the studies did not focus on the EGFR-mutated population (10,40).

Since successful treatment with erlotinib and crizotinib (a potent MET and ALK inhibitor) has been described in case reports (41,42), a phase I trial was designed to study this combination. However, in the 26 patients in the trial, the MTD for the two drugs was lower than the approved dose of the individual drugs, so phase II trials have not been initiated yet (43). Further trials have also been halted for the combination regimen of erlotinib ± the anti-MET antibody LY2875358 because of initial disappointing results (10).

There are more promising biomarker-driven studies that are currently being pursued. Preliminary studies of another MET inhibitor, capmatinib revealed that patients with *cMET* gene copy number ≥5 had an ORR of 40% (44). At present, a three-arm study has been designed to study capmatinib by itself and in combination with erlotinib as compared to standard chemotherapy in EGFR-mutated NSCLC patients specifically with c-MET copy number >6 (NCT02468661).

Another study will compare the combination of gefitinib and a MET inhibitor named tepotinib versus chemotherapy as second line therapy in patients with MET + EGFR mutant tumors with acquired resistance to gefitinib (NCT 01982955). The phase IB data from this study revealed 4/18 partial responses and a good side-effect profile, and phase II is currently in process (45).

Volitinib is another MET inhibitor that is presently being studied. One trial explores the safety of the volitinib + gefitinib combination in EGFR-mutated NSCLC

(NCT02374645), while the TATTON trial is investigating volitinib in combination with different doses of osimertinib in EGFR-mutant NSCLC that has progressed on prior EGFR-TKI therapy (NCT02143466). Phase Ib data from the TATTON study was promising, demonstrating partial responses in 6/11 patients (46).

In all these studies that involve MET-targeting drugs, it is imperative to have assays that reliably detect MET pathway activation and alterations and serve as biomarkers that predict response to MET inhibitors. Currently, there are a number of methods that can be used to determine MET expression, however not all of them are equal. For example, immunohistochemistry (IHC) is an easy assay but studies have revealed that it is a poor marker of predicting sensitivity to MET inhibitors (47). Fluorescent *in situ* hybridization (FISH) and rtPCR are reliable methods for detecting copy number alterations in the MET gene, although the latter encounters problems in distinguishing polysomy from true copy number changes (48). One drawback with FISH was the question of what cutoff should be used to predict sensitivity to MET inhibitors. A recent study by Noonan et al sheds light on the matter as it showed that a FISH MET/CEP7 ratio of ≥5 was associated with the most sensitivity to MET inhibition (49). While circulating-tumor DNA (ctDNA) testing might be a good option for capturing MET point mutations, however it is less sensitive to detect copy number changes and gene rearrangements. Perhaps the most effective method of detecting MET gene alterations and predicting response to MET inhibitors is next-generation sequencing (NGS). This is because NGS has the ability to reveal the multitude of genomic changes impacting the MET gene.

A range of studies focusing on novel combination treatments, in an effort to develop appropriate synergistic treatment strategies, for example combinations of immunotherapeutic, anti-angiogenic and Met/AXL/MEK targeting agents, are ongoing (*Table 1*).

Other oncogenes

Anaplastic lymphoma kinase (ALK)

ALK gene chromosomal rearrangements define another distinct molecular subtype of NSCLC. Therapeutic options for the treatment of advanced ALK-positive NSCLC have expanded rapidly through the development of increasingly potent and selective ALK inhibitors, however the problem of developing resistance to these therapies have emerged as a key clinical issue. Efforts to understand the mechanisms of resistance to ALK inhibitors have led to recognition of many different mechanisms of resistance.

Crizotinib, the first generation ALK inhibitor, significantly improves the objective response rates (ORRs) and progression-free survival (PFS) compared to cytotoxic chemotherapy in advanced ALK-positive lung cancer and has become the standard front-line therapy for ALK-positive patients. However, most patients who initially respond to crizotinib develop progressive disease within 1 year (50-52). Initial studies examining biopsies obtained at the time of resistance revealed multiple potential resistance mechanisms. Mechanisms of resistance to ALK inhibitors are divided into two categories, on target or ALK dependent alterations and off target or ALK independent alterations. On target alterations include *mutations in the ALK tyrosine kinase domain* and *amplification of ALK* (53,54).

Mutations in the ALK tyrosine kinase domain can cause resistance to ALK inhibitors by directly blocking the TKI binding to the target kinase, altering the kinase's conformation, or altering the ATP-binding affinity of the kinase. Many mutations in the ALK tyrosine kinase domain have been identified. For example, the L1196M gatekeeper mutation alters the gatekeeper residue at the bottom of the ATP-binding pocket and impairs TKI binding; and solvent-front mutations impair drug binding likely through steric hindrance. Interestingly each ALK TKI appears to be associated with a distinctive pattern of *ALK* resistance mutations (53,54). *Amplification of* ALK occurs less frequently and causes resistance to crizotinib. It has been reported in 6.7% to 18.2% of cases of resistance to crizotinib in different series (51,55). ALK amplification has not yet been detected as a resistance mechanism after second generation ALK TKIs (53).

Similar to the case of acquired EGFR TKI resistance, off target alterations that cause resistance to ALK inhibitors include *activation of bypass signaling pathways*, *lineage changes* and *drug efflux pump* (53,54). Numerous examples of bypass signaling activation have been discovered. For example, gene expression profiling of crizotinib-resistant versus crizotinib-naive NSCLC tumor samples identified EGFR and HER2 signatures as two of the most enriched gene expression signatures in resistant tumors (51,56,57). In one study, increased EGFR activation was identified in 4 of 9 cases (44.4%) when biopsy samples prior to crizotinib initiation and after development of resistance were compared (51). This was associated with higher EGFR mRNA expression and persistent activation of downstream ERK and AKT

Table 1 Key current studies exploring novel treatment options

Study	Phase	Drug	Treatment combination	Patient population
EGFR targeted drugs				
NCT01532089	II	Erlotinib	Alone or with Bevacizumab	Stage IV NSCLC with activating EGFR mutations without prior treatment
NCT01859026	I/IB	Erlotinib	MEK162 (MEK inhibitor)	NSCLC harboring KRAS or EGFR mutation
NCT02438722	II/III	Afatinib	Alone or with cetuximab	Newly diagnosed or recurrent EGFR mutant NSCLC
NCT03054038	I	Afatinib	Necitumumab (anti-EGFR)	EGFR mutant NSCLC with acquired resistance to 1st and 3rd generation EGFR TKI
NCT02454933	III	Osimertinib	Alone or with Durvalumab (anti-PD-L1)	Locally advanced or metastatic EGFR T790M positive NSCLC with prior EGFR TKI therapy
NCT02803203	I/II	Osimertinib	Bevacizumab	Metastatic NSCLC due to activating EGFR mutation and no prior treatment with EGFR TKI or VEGF inhibitor
NCT02789345	I	Osimertinib	Ramucirumab (anti-VEGF) or necitumumab	Advanced T790M positive EGFR mutant NSCLC after progression on 1st line EGFR TKI therapy
NCT02520778	Ib	Osimertinib	Navitoclax (Bcl-2 inhibitor)	EGFR mutant NSCLC with progression after EGFR TKI
NCT02917993	I/II	Osimertinib	INCB039110 (Jak1 inhibitor)	EGFR mutant T790M positive NSCLC with prior EGFR TKI therapy
NCT02503722	I	Osimertinib	INK128 (TORC 1/2 inhibitor)	EGFR mutant NSCLC with progression after EGFR TKI
NCT02143466	Ib	Osimertinib	Ascending doses of volitinib (MET inhibitor) or Selumetinib (MEK 1/2 inhibitor)	EGFR mutant advanced NSCLC with progression after prior EGFR TKI therapy
NCT02616393	II	Tesevatinib (EGFR TKI)	Alone	EGFR mutant NSCLC with prior EGFR TKI therapy and brain or leptomeningeal metastases
MET/AXL targeted drugs				
NCT02468661	Ib/II	Capmatinib (MET inhibitor)	Alone or with erlotinib compared to platinum + pemetrexed	EGFR mutated and MET amplified locally advanced or metastatic NSCLC with acquired resistance to prior EGFR TKI
NCT02335944	Ib/II	Capmatinib	EGF816	EGFR mutant NSCLC with progression of disease on 1st and 2nd gen EGFR TKI
NCT 01982955	Ib/II	Tepotinib (MET inhibitor)	Gefitinib	EGFR mutant, T790M-negative, MET amplified locally advanced or metastatic NSCLC with acquired resistance to prior EGFR TKI
NCT02374645	Ib	Volitinib (MET inhibitor)	Gefitinib	EGFR mutated NSCLC after progression on EGFR TKI
NCT02424617	I/II	BGB324 (AXL TKI)	Alone or with Erlotinib	Locally advanced or metastatic NSCLC
ALK targeted drugs				
NCT02511184	Ib	Crizotinib	Pembrolizumab	Untreated advanced ALK-rearranged NSCLC
NCT02521051	I/II	Alectinib	Bevacizumab	Advanced ALK positive NSCLC
NCT03087448	I/II	Ceritinib	Trametinib (MEK inhibitor)	ALK positive NSCLC in 3 different cohorts: ALK-inhibitor naïve patients, patients who progressed on Crizotinib, and patients who progressed on other ALK TKI
NCT02321501	I/Ib	Ceritinib	Everolimus	Advanced ALK positive NSCLC

Table 1 *(continued)*

Table 1 *(continued)*

Study	Phase	Drug	Treatment combination	Patient population
Immunotherapy				
NCT02364609	I/Ib	Pembrolizumab	Afatinib	EGFR mutant NSCLC with resistance to Erlotinib
NCT02584634	Ib/II	Avelumab	Crizotinib (Group A) or Lorlatinib (Group B)	Group A: ALK negative NSCLC with progression after 1 prior treatment
				Group B: ALK positive NSCLC
NCT02013219	Ib	Atezolizumab	Erlotinib or alectinib	Erlotinib group: EGFR TKI naïve pts with EGFR mutant NSCLC
				Alectinib group: treatment naïve pts with ALK-positive NSCLC
NCT02805660	I/II	Durvalumab	Mocetinostat (HDAC inhibitor)	Phase I: solid tumors; phase II: NSCLC
NCT01968109	I/IIa	BMS-986016 (Anti-LAG3)	Alone or with Nivolumab	Advanced solid tumors including NSCLC

NSCLC, non-small cell lung cancer; ALK, anaplastic lymphoma kinase.

signaling. EGFR activation may result from receptor and/or ligand upregulation rather than *EGFR* mutations or amplification (51,58). MET amplification is another example of bypass signaling. Crizotinib is a potent ALK and MET inhibitor however most other ALK inhibitors do not have anti-MET activity. MET amplification has recently been reported as a bypass mechanism in patients who have failed second-generation inhibitors like alectinib (59-61). Direct activation of downstream signaling pathways can also enable acquired resistance to ALK inhibitors. MEK activating mutations, *PIK3CA* mutations, *KIT* amplification, IGF1R activation, SRC activation and *TP53* mutations have all been identified, among others (62-64). Of note, ALK/MEK dual blockade may be effective not only in overcoming but also in delaying ALK TKI resistance and are being tested in early phase clinical trials (63,65). Similar to EGFT TKI resistance, phenotypic changes such as EMT and SCLC transformation might cause ALK TKI resistance (53,66).

Lastly, P-glycoprotein (P-gp) is an ATP-dependent efflux pump encoded by the multidrug resistance 1 (*MDR1*) gene (67) and might mediate cases of pharmacodynamics resistance. P-gp is a substrate for crizotinib and ceritinib and its overexpression can lead to acquired ceritinib and crizotinib resistance by exporting the drug outside of the cancer cells. P-gp has also been implicated in decreased penetration of the blood–brain barrier by P-gp. Alectinib is not a P-gp substrate (54,68).

Second-generation ALK inhibitors, ceritinib and alectinib, were rapidly developed and showed excellent activity in the second line setting, leading to FDA approval of both compounds (69,70). ORRs to second-generation ALK inhibitors are reported to be 48% to 71% following progression on crizotinib (71,72). A third agent, brigatinib was recently granted accelerated approval for the treatment of patients with ALK positive NSCLC who have progressed on or are intolerant to crizotinib (73,74). Intriguingly, the activity of these agents seems to be similar regardless of the presence or the absence of secondary ALK mutations and therefore repeat mutation testing at the time of crizotinib resistance has less clear value as compared to for example EGFR T790M mutation testing. It appears that part of the reason for this is the relatively weak ALK inhibitory potency of crizotinib as compared to these novel agents.

Recent data is more supportive of the need for molecular testing at the time of acquired resistance with the availability of several more potent agents as the type of emerging mutation might be a key determinant of efficacy of other alternate ALK inhibitors.

In a recent article, Gainor *et al.* (53) reported mechanisms of resistance in 83 ALK-positive patients who underwent repeat biopsies following disease progression on first or second generation ALK inhibitors. One hundred and three repeat biopsy samples from 83 patients were evaluated using genetic and cell based assays. ALK kinase domain mutations were found in 20% (11/55) of crizotinib resistant samples. The two most common mutations were the L1196M (gatekeeper mutation) in 7% and the G1269A in 4% of patients. ALK kinase domain mutations were detected in

54% (13/24) of ceritinib resistant specimens, 53% (9/17) of alectinib resistant samples, and 71% (5/7) of brigatinib resistant samples. Distinct patterns of mutations were found for each of the three second-generation ALK inhibitors. The G1202R solvent front mutation, which produces resistance to 1st and 2nd generation ALK inhibitors, was found in 21% ceritinib, 29% alectinib, and 43% of brigatinib resistant samples. This has clinical implications as the novel ALK inhibitor, lorlatinib can overcome this mutation. Compound mutations were identified in 12.5% (6/48) of patients resistant to second-generation ALK TKIs. *In vitro*, lorlatinib was the only agent to retain potency against the compound mutations evaluated. EMT was observed in 42% (5/12) of cases. The complex evolution of resistance mutations over multiple lines of ALK TKI therapy and the potential for optimizing drug selection based on the understanding of secondary ALK mutations reinforces the need for repeat biopsies to tailor therapeutic selection and ctDNA is also being developed as a robust platform for molecular monitoring (75).

ROS/MET/BRAF

ROS

Patients with ROS proto-oncogene 1 RTK (*ROS1*) rearranged NSCLC in general respond to treatment with the ROS1 inhibitor-crizotinib very well with quite durable remissions being the norm, however these tumors eventually become resistant as well (76,77). There is limited data available on the mechanisms of acquired resistance to crizotinib in ROS1 positive NSCLC, however several secondary ROS1 mutations that can cause resistance have been identified, for example the G2026M mutation (gatekeeper) or the L2155S solvent-front (D2033N) mutation which can still respond to the multi-targeted TKI, cabozantinib which has ROS inhibitory activity as well (78-80). Other mechanisms of resistance might include gain of function mutations of KIT (81), activation of the RAS pathway due to either KRAS/NRAS mutations or to KRAS amplification (82), rat sarcoma viral oncogene homolog, EGFR activation (83), and epithelial-to-mesenchymal transition (78,84).

MET

NSCLC harboring MET mutations such as MET exon 14 skipping mutations or focal amplification can respond to treatment with MET TKIs, such as crizotinib and cabozantinib. Acquired resistance to MET TKIs have been reported, however the molecular mechanisms are not yet well defined (85). In a recent study, Li *et al.* (86) reported two acquired MET mutations, Y1248H and D1246N, which can cause resistance against Type I MET-TKIs. It was also noted that EGFR amplification may act as an alternative MET-TKIs bypass resistance mechanism (86). Amplification of MET, amplification of K-RAS and activation of B-RAF or EGFR pathways have also been proposed as mechanisms of acquired resistance to MET TKIs (87-89). Knowledge of specific molecular mechanisms might yield treatment benefits as some secondary MET mutations might respond to for example type II MET TKIs.

BRAF

In V600 BRAF mutated NSCLC, duration of response to BRAF TKIs are in general relatively short due to the development of resistance. Resistance patters are diverse and can occur due to a secondary MAPK alteration or due to activation of bypass tracks (90,91). Non-V600E BRAF mutations demonstrate limited sensitivity to treatment with BRAF TKIs and studies are ongoing to assess the utility of MEK inhibition in such cases (92).

Mechanisms of resistance to BRAF are much better studied in patients with malignant melanoma.

For instance, Johnson *et al.* (93) studied 132 tumor specimens from 100 patients with malignant melanoma who had developed resistance to BRAF inhibition, the mechanisms of resistance included NRAS or KRAS mutations in 20%, BRAF splice variants in 16%, BRAF amplifications in 13%, MEK1/2 mutations in 7%, and non-mitogen-activated protein kinase pathway alterations in 11%. While these resistance mechanisms are likely shared with V600-mutated lung cancers, this will still certainly need to be demonstrated.

ctDNA as a novel testing platform in the monitoring of acquired resistance

CtDNA analysis has emerged as a powerful, and in certain settings validated, tool that allows clinicians to non-invasively detect actionable mutations (94,95). EGFR T790M testing provided the first validated usage for ctDNA testing and while tissue biopsy remains the gold standard for EGFR T790M genotyping, ctDNA is emerging as a valuable alternative and/or complementary tool. Zheng *et al.* reported 81.25% sensitivity and 100% specificity of plasma

T790M testing by dPCR assays in a study of 117 patients with TKI resistance (96). Oxnard *et al.* demonstrated that the sensitivity of ctDNA detection of T790M was 70% and the objective RR and median PFS with osimertinib were similar in patients with T790M-positive plasma or T70M-positive tumor results (97). It appears that the positive predictive value of detecting EGFR mutations in ctDNA is high enough to justify initiation of 3rd generation TKI therapy, but the negative predictive value is less robust. Currently, studies are in progress to test the efficacy of ctDNA in detecting ALK resistance mutation (98).

Acquired resistance to immunotherapeutic agents

Immune checkpoint inhibitors are now widely used as single agents for treatment of patients with advanced NSCLC in front-line or later lines of therapy. PD-1/PD-L1 blockade is a revolutionary treatment option for advanced NSCLC, however there is a high rate of primary resistance, and furthermore most patients develop acquired resistance to PD-1/PD-L1 blockade (99).

Mechanisms of primary resistance to immunotherapeutic agents include lack of PD-L1 expression on tumor cells, insufficient tumor-infiltrating lymphocytes or severely exhausted $CD8^+$ T cells (100). Another primary resistance mechanism might be the scarcity of neo-antigens in tumors with a low mutation burden, such as EGFR/ALK/ROS-mutated lung cancers where the average tumor mutation burden is a magnitude or so lower than in smoking-associated lung cancers and indeed low RRs of 4% had been reported in this subset of patients (53).

Acquired resistance, defined by initial response to PD-1 blockade followed by progression of disease, is observed commonly. Few mechanisms for acquired resistance have been clearly identified as of yet but better understanding of determinants of efficacy and resistance will be pivotal to allow optimal utilization of these novel agents. Candidate resistance mechanisms might include upregulation of alternate immune checkpoints (101), somatic mutations in *HLA* or *JAK1/JAK2* genes (102,103) and genomic changes resulting in loss of mutation-associated neoantigens in resistant clones (104).

Koyama *et al.* (101) analyzed the tumor immune microenvironment in two fully immunocompetent mouse models of lung adenocarcinoma. In tumors with acquired resistance to PDL-1 blockade, upregulation of alternative immune checkpoints, notably T-cell immunoglobulin mucin-3 (TIM-3) in PD-1 antibody bound T cells was identified.

Zaretsky *et al.* (103) demonstrated that acquired resistance to immunotherapeutic agents is possible through genetic aberrations translating into immune escape mechanisms in tumor cells which are then clonally expanded, leading to disease relapse in initially responding patients. Whole exome sequencing of 4 paired melanoma specimens at baseline and at relapse revealed loss of function mutations in the genes encoding interferon receptor associated Janus kinase 1 (JAK1) or Janus kinase 2 (JAK2), concurrent with deletion of the wild-type allele in 2 of 4 patients. JAK1 and JAK2 mutations resulted in a lack of response to interferon gamma, including insensitivity to its anti-proliferative effects on cancer cells. A truncating mutation in the gene encoding the antigen-presenting protein beta-2-microglobulin (B2M) was identified in a third patient. The B2M truncating mutation led to loss of surface expression of major histocompatibility complex class I, which is a previously identified mechanism for cytotoxic T-cell escape. Similar results have yet to be reported in lung tumors.

Anagnostou *et al.* (104) performed whole exome sequencing in four patients with NSCLC who developed acquired resistance to PD-1 blockade and compared the results in pre-treatment and post-treatment specimens and identified genomic changes resulting in loss of 7 to 18 mutation associated neoantigens in resistant clones. Peptides generated from the eliminated neoantigens elicited clonal T-cell expansion in autologous T-cell cultures, suggesting that they generated functional immune responses.

More studies are needed and efforts need to be expanded to better understand mechanisms of resistant and to be able to develop more effective drugs or combination strategies to enhance efficacy of immunotherapies.

Summary

The introduction of molecular targeted and immunotherapeutic agents have completely transformed the landscape of NSCLC management, dramatically improving patient outcomes. Molecular and immune biomarker testing is paramount in optimal treatment selection and acquired resistance is a pivotal clinical issue with an increasing understanding of a range of mechanisms (*Figure 2*).

Molecular monitoring utilizing tissue biopsy or plasma is now a routine element of patient management with the availability of second and third-generation agents. Continued efforts towards novel combination studies to

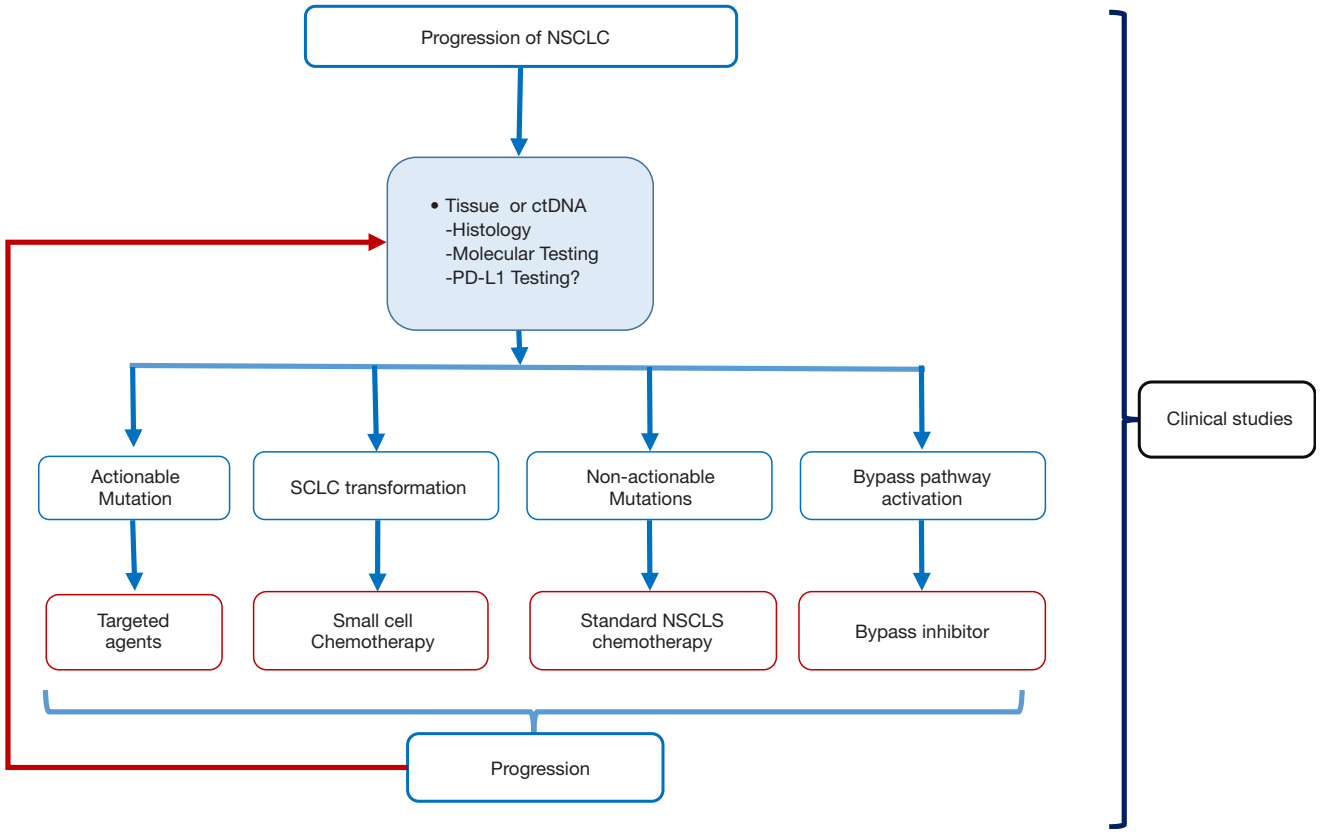

Figure 2 Treatment approach after progression of NSCLC. NSCLC, non-small cell lung cancer.

overcome and possibly prevent the emergence of acquired resistance remain the most critical experimental need.

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Footnote

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