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REVIEW

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Mutation testing for directing upfront targeted therapy and post-progression combination therapy strategies in lung adenocarcinoma

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ABSTRACT

Introduction: Advances in the biology of non-small-cell lung cancer, especially adenocarcinoma, reveal multiple molecular subtypes driving oncogenesis. Accordingly, individualized targeted therapeutics are based on mutational diagnostics.

Areas covered: Advances in strategies and techniques for individualized treatment, particularly of adenocarcinoma, are described through literature review. Approved therapies are established for some molecular subsets, with new driver mutations emerging that represent increasing proportions of patients. Actionable mutations are *de novo* oncogenic drivers or acquired resistance mediators, and mutational profiling is important for directing therapy. Patients should be monitored for emerging actionable resistance mutations. Liquid biopsy and associated multiplex diagnostics will be important means to monitor patients during treatment.

Expert commentary: Outcomes with targeted agents may be improved by integrating mutation screens during treatment to optimize subsequent therapy. In order for this to be translated into impactful patient benefit, appropriate platforms and strategies need to be optimized and then implemented universally.

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1. Introduction

Lung cancer is the most frequently diagnosed cancer and a leading cause of cancer death worldwide [1]. Non-small-cell lung cancer (NSCLC) is the most commonly diagnosed form of the disease (>85% of cases) and includes a heterogeneous group of histologies, the most common being adenocarcinoma, then squamous cell carcinoma, and less so large-cell carcinoma [2]. These histologies possess different clinical characteristics, and there are potential differences in response to cytotoxic chemotherapies. Approximately 40–50% of patients with NSCLC will be diagnosed with advanced or metastatic disease and are not candidates for curative therapy. Systemic chemotherapy, once the treatment of choice for all patients, is no longer universally used following the advent of targeted therapy. For example, randomized Phase III trials showed a significant benefit (response rate and progression-free survival [PFS]) in patients with epidermal growth factor receptor (*EGFR*)-mutant disease treated with tyrosine kinase inhibitors (TKIs) versus those treated with standard chemotherapy [3]. Further advances in the underlying biology of NSCLC have revealed multiple distinct molecular subtypes, increasingly supporting a model in which NSCLCs depend on oncogenic 'driver mutations' for the malignant phenotype [4]. Along with mutations in *EGFR*, gene fusions involving rearrangements of the anaplastic lymphoma kinase (*ALK*) gene are prominent genetic markers. Personalized therapy aims at matching these genotyped lung adenocarcinomas with effective targeted therapies such as specific TKIs and is currently utilized; EGFR TKIs are US FDA approved for the first-line treatment of *EGFR*-mutant NSCLC, and *ALK*-rearranged NSCLC may be treated first-line with the multi-targeted ALK/MET/ROS1 (ROS proto-oncogene 1, receptor tyrosine kinase [RTK]) TKI crizotinib [5,6] and second-line with ceritinib [7].

Molecular testing for *EGFR* and *ALK* is now considered standard of care [2,4], with other driver mutations in oncogenes such as *ROS1*, *BRAF* (v-Raf murine sarcoma viral oncogene homolog B), *RET* (rearranged during transfection), *MEK1* (mitogen-activated protein kinase kinase 1), *NTRK* (neurotrophic tyrosine kinase receptor), *MET*, and *KRAS* (Kirsten Rat Sarcoma viral oncogene homolog), also increasingly being incorporated into the diagnostic workup of adenocarcinoma patients to determine eligibility for enrollment in diverse clinical studies on appropriate targeted agents. Reflecting these developments, current guidelines for advanced NSCLC treatment from the American Society for Clinical Oncology (ASCO) [8], College of American Pathologists (CAP)/International Association for the Study of Lung Cancer (IASLC)/Association for Molecular Pathology [3], and the US National Comprehensive Cancer Network [2] support testing on tumor tissue to determine any genetic alterations and choose an appropriate therapy. Molecular testing for *EGFR* mutations and *ALK* rearrangement are recommended in the treatment guidelines, and further molecular testing may be appropriate

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depending on tissue availability and clinical criteria. As the number of molecular subgroups of NSCLC continues to grow and the methods for their detection improve, there is a need to review recent developments. This review gathers together recent data on driver mutations, discusses their characterization in the clinical diagnostic setting, and their impact on potential first- and second-line monotherapy and combination therapy decisions for patients with NSCLC.

This article will summarize some of the mutations that are 'actionable' in NSCLC. Certainly, there is a large momentum for immunotherapy in NSCLC; however, the reader is referred elsewhere for further understanding of this.

2. Initial testing for mutations and expression patterns

In addition to *EGFR* and *ALK*, important oncogenic driver mutations/rearrangements that may be considered for diagnostic screening include *KRAS, MET, PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha), *HER2* (human epidermal growth factor receptor 2), *BRAF, NTRK, ROS1, RET*, and *MEK*. Most driver oncogenes tend to occur in ≤25% of individual tumors (see Figure 1 for a chart showing some of the more frequent actionable mutations) and singly in tumor samples; for example, clinical data show that overlapping *EGFR* and *KRAS* mutations occur in <1% of patients with lung cancer [2] and *ALK* rearrangements are usually mutually exclusive with mutations in *EGFR* or *KRAS* [9].

2.1. ERBB family RTKs

Sensitizing *EGFR* mutations are found in around 10% of Caucasian patients and up to 50% of Asian patients with NSCLC [2,10]. The most frequent *EGFR* mutations result in substitution at amino acid 858 in exon 21 (Leu858Arg [L858R]) and in-frame deletions at exon 19, which alter the configuration of the kinase to preserve an activated state. Patients whose tumors have exon 19 deletions or exon 18 (G719X, G719A, G719S, G719C, G719D), exon 20 (S768I), or exon 21 (L858R, L861Q, L861R) mutations are sensitive to EGFR–TKI therapy [11–13]. Erlotinib is approved by the US FDA (2013) for the first-line treatment for patients with metastatic NSCLC harboring *EGFR* exon 19 deletions or exon 21 (L858R) substitution mutations based on a response rate of 65% compared with 16% for platinum-based chemotherapy and a median PFS of 10.4 versus 5.2 months [14]. Erlotinib is also approved for maintenance treatment of locally advanced or metastatic NSCLC after platinum-based chemotherapy. Afatinib and gefitinib are now also fully US FDA-approved (2013 and 2015, respectively) for the first-line treatment of patients with the same types of *EGFR*-mutant NSCLC [15,16].

De novo mutations in *HER2* typically occur in 3–5% of NSCLC (predominantly exon 20 insertions) and are usually mutually exclusive with *EGFR* and *KRAS* mutations [17,18]. Clinical trials have not yet demonstrated a clear benefit, but HER2-targeted therapies such as afatinib have demonstrated signs of clinical activity in heavily pretreated patients with *HER2*-mutated adenocarcinoma [19], including activity in patients with *HER2*-mutated lung cancers with exon 20 YVMA insertions, the most common variant [20].

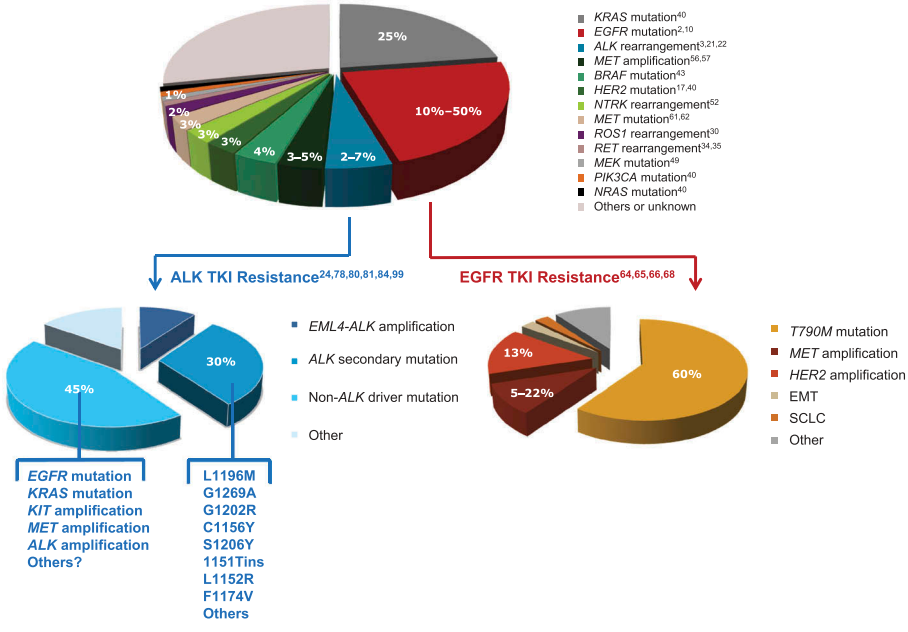

Figure 1. Frequency of mutations/genomic alterations in NSCLC (adenocarcinoma) in Caucasian populations, and known mutation profiles in ALK and EGFR TKI-resistant disease. ALK: anaplastic lymphoma kinase; BRAF: v-Raf murine sarcoma viral oncogene homolog B; EGFR: epidermal growth factor receptor; EML4: echinoderm microtubule-associated protein-like 4; EMT: epithelial-mesenchymal transition; HER2: human epidermal growth factor receptor 2; KRAS: Kirsten Rat Sarcoma viral oncogene homolog; MEK: mitogen-activated protein kinase kinase; NTRK: neurotrophic tyrosine kinase receptor; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; RET: rearranged during transfection; ROS1: ROS proto-oncogene 1, receptor tyrosine kinase; SCLC: small-cell lung carcinoma; TKI: tyrosine kinase inhibitor.

2.2. ALK

ALK gene rearrangements are present in approximately 2–7% of patients with NSCLC, typically fusions with other genes (most commonly, *EML4* [echinoderm microtubule-associated protein-like 4]) [3,21,22]. Patients diagnosed with lung tumors harboring *ALK* fusions can be effectively treated with ALK inhibitors, such as crizotinib [3,21,22]. Phase I and II studies of crizotinib in *ALK*-rearranged NSCLC demonstrated impressive activity and clinical benefit, leading to FDA approval in 2011 [23,24]. In addition, a subsequent Phase III trial showed that crizotinib was superior to standard first-line pemetrexed-plus-platinum chemotherapy in patients with previously untreated advanced ALK+ NSCLC [6]. However, despite high response rates (65–74%), most patients develop resistance to crizotinib within 2 years, and second-generation agents are now FDA approved (ceritinib, alectinib) or in advanced development as FDA Breakthrough Designated Therapy (brigatinib) [24–26]. It should be noted that as ALK+ patients are surviving longer, relapses within the central nervous system (CNS) are increasingly being diagnosed. The penetration of the blood–brain barrier by these therapeutic agents will therefore be important in controlling CNS metastases [27,28]. It is also interesting to note that there are unique patterns of metastases in ALK+ tumors, especially in women with metastases to the adnexa [29].

2.3. ROS1

Chromosomal rearrangements of the gene encoding the ALK-related tyrosine kinase *ROS1* have been identified in 1–2% of NSCLC cases that generate several distinct gene fusion partners, including *SLC34A2* (solute carrier family 34 [type II sodium/phosphate cotransporter], member 2), *TPM3* (tropomyosin 3), and others [30–32]. *ROS1* rearrangements are relatively more prevalent in patients with adenocarcinoma and advanced stage disease [33]. For patients with tumors with *ROS1* rearrangements, crizotinib is effective and is therefore a potential first-line therapy [8,32].

2.4. RET

Translocations of the RTK *RET* occur in approximately 1% of NSCLC patients, with relatively high frequencies in young, light/never smokers with adenocarcinoma and poorly differentiated tumors [34–36]. A number of fusion variants are now known, the most common of which is *KIF5B-RET* [34,37,38]. A Phase II trial of the RET inhibitor cabozantinib in patients with *RET*-positive NSCLC has shown preliminary efficacy [39]. Multiple clinical trials in NSCLC with *KIF5B-RET* rearrangements using existing RET inhibitors (including cabozantinib, lenvatinib, vandetanib, sunitinib, ponatinib, and AUY922) are underway [36].

2.5. KRAS

KRAS mutations are detected in approximately 25% of lung adenocarcinomas and 4% of lung squamous cell carcinomas, most often in codons 12 or 13 [40]. *KRAS* mutations are most common in non-Asians and smokers [41,42] and are associated with intrinsic EGFR TKI resistance. *KRAS* mutation testing may thus identify patients who may not benefit from further molecular diagnostic testing. No direct targeted therapy is available for *KRAS*-mutant NSCLC, and therefore, investigations have focused on targeting downstream signaling proteins of the RAS/RAF/MEK/ERK (extracellular signal-regulated kinase) pathway, such as BRAF and MEK. The incidence of synthetic lethality associated with *KRAS* mutation means that these pathways need to be explored further.

2.6. BRAF

BRAF is a serine–threonine kinase belonging to the RAF kinase family lying downstream of KRAS and directly interacts with the MEK–ERK signaling cascade. *BRAF* mutations are found in up to 4% of lung adenocarcinomas [43], half of them harboring the *V600E* mutation; other mutations occur within exons 11 and 15 [44–46]. Treatment of *BRAF V600E*-mutated NSCLC with BRAF inhibitor monotherapy, exemplified by dabrafenib, has demonstrated encouraging antitumor activity in early clinical trials [43,47]. Dabrafenib was granted FDA Breakthrough Therapy Designation (2014) for chemotherapy-pretreated *BRAF V600E* mutation-positive NSCLC [48].

2.7. MEK

MEK1 encodes a serine–threonine kinase and is mutated in about 1% of NSCLC, largely adenocarcinoma [49]. Several MEK inhibitors are in clinical development for NSCLC, including selumetinib (AZD6244) and trametinib (GSK1120212), with a focus on combination regimens that may prevent or combat resistance due to secondary *MEK* mutation and/or *KRAS*/*BRAF* amplification in *BRAF*- or *KRAS*-mutant NSCLC [50,51].

2.8. NTRK

NTRK1 fusions occur in around 3% of lung adenocarcinomas [52]. Amplifications of *NTRK1* and *NTRK2* (among other genes) encode Src kinases that can complement loss of EGFR activity across multiple EGFR-dependent models, via EGFR-independent activation of the MEK–ERK and phosphoinositide 3-kinase (PI3K)-AKT pathways, suggesting a range of kinases capable of overcoming dependence on EGFR [53]. Significant antitumor activity has been reported for the kinase inhibitor entrectinib (RXDX-101) in a patient with NSCLC harboring an *SQSTM1* (sequestosome 1)-*NTRK1* gene rearrangement, validating *NTRK* gene rearrangements as a potential clinical target in NSCLC [54].

2.9. MET

MET is an RTK that binds to hepatocyte growth factor. Activation of MET promotes signaling pathway activation, including the RAS-RAF-mitogen-activated protein kinase (MAPK) and PI3K-AKT-mTOR (mammalian target of rapamycin) pathways. *MET* amplifications are found in 3–5% of newly diagnosed NSCLC, predominantly in adenocarcinoma [55–58]. Exon 14 skipping *MET* mutations have also been identified as oncogenic drivers, as initially discovered by the author's laboratory in lung cancer [59,60], occurring in around 3%

of lung adenocarcinomas and in 1–2% of other NSCLC subsets [61,62], and respond to MET inhibitors such as crizotinib, cabozantinib, and capmatinib (INC280) [61–63]. *MET* amplification and overexpression may confer resistance to EGFR inhibitors in *EGFR*-mutant lung adenocarcinoma [64,65].

3. Mutation patterns in relapsed patients

Acquired resistance to targeted TKIs can occur by several mechanisms during treatment, and mutations that enable escape of dependence on the initial oncogenic driver present important second-line diagnostic targets [66]. Identification of resistance mutations or pathway activation upon progression is increasingly important as new and specific treatment options emerge.

3.1. EGFR

Most patients treated with EGFR TKIs will progress after about 1 year of therapy due to acquired resistance that is generally mediated through persistence of MAPK signaling and largely due to mutations in exons 19 and 20 (most commonly, the *T790M* mutation; Figure 1) [66–68]. Secondary mutations in EGFR may be targeted by treatment with second- and third-generation TKIs [68]. The FDA has recently granted accelerated approval to the EGFR TKI osimertinib (AZD9291) for patients with advanced *T790M* mutation-positive NSCLC based on response rates of 57–61% in two single-arm studies [13,69]. Other agents in clinical development include rociletinib (CO-1686) that is active against both the *T790M* mutation as well as the baseline activating *EGFR* mutations [70], and in a first-in-human study of the *EGFR*-mutant-specific TKI EGF816, an overall response rate (ORR) of 55% and disease control rate of 86% were reported [71]. Other potentially targetable EGFR TKI resistance mechanisms related to MAPK signaling that future re-biopsy diagnostics will need to detect to inform on salvage treatment strategies include amplification of *HER2* or *MEK1* and activating mutations in *RAS* or *BRAF* [72–74]. Bypass activation of other pathways also plays important roles in resistance, and includes amplification of *MET* [64,66], and acquired mutation of *PIK3CA* [74]. The *MET* gene is amplified in up to 21% of NSCLC cases with EGFR inhibitor resistance [65]. Acquired resistance to next-generation EGFR TKIs may emerge through increased ERK activation (via *MEK1* amplification or mutation), and downstream inhibitors of this pathway such as those already described may be effective in this setting when these aberrations are detected on progression [72]. *RET* rearrangement has also been implicated in *EGFR*-mutant NSCLC that has progressed on EGFR TKI therapy, and this genetic aberration should be added to the growing list of potential markers in genetic resistance screens [75]. There is also evidence that with TKI resistance, there can be change of histology (to small-cell lung cancer or squamous cell carcinoma) [76,77].

3.2. ALK

Although crizotinib provides impressive initial responses, resistance develops within 1–2 years [24]. Several bypass mechanisms have been implicated, including *ALK* amplification, *MET* activation, *KIT* amplification, and mutations in MAPK pathway signaling components (Figure 1) [78–81]. However, the major mechanism of resistance is through any one of a multitude of known secondary *ALK* mutations that either induce changes at the ATP-binding pocket and cause steric hindrance to binding of crizotinib, or destabilize the wild-type auto-inhibitory conformation of ALK to which crizotinib binds [80,82–86]. The most commonly occurring mutations associated with crizotinib resistance are L1196M (the 'gatekeeper' mutation) and G1269A (see Figure 1 for a more comprehensive list) [86,87]. Mutational screens involving Ba/F3 cells expressing native EML4–ALK have identified more resistance loci [88], and additional types of mutation may emerge as more patients are treated with ALK inhibitors. New-generation ALK inhibitors such as ceritinib can overcome *ALK* mutation-derived resistance to crizotinib [78,89]. In a Phase I study of ceritinib in ALK+ NSCLC, marked antitumor activity was seen in both crizotinib-relapsed and crizotinib-naïve patients (i.e. regardless of the presence of resistance mutations in ALK) [7], and based on these data, ceritinib received FDA approval in 2014; confirmatory Phase II data have now been reported, including an ORR of 36% and PFS of 7.2 months in crizotinib-pretreated patients [90,91]. In a retrospective study of a cohort of ALK+ patients treated with crizotinib and ceritinib, data showed that ceritinib had significant antitumor activity in ALK+ NSCLC, even when crizotinib immediately preceded treatment with ceritinib; the median combined PFS for sequential treatment with crizotinib and ceritinib was 17.4 months [92]. Other ALK inhibitors are in advanced clinical development with activity in crizotinib-resistant NSCLC patients; alectinib and brigatinib provide ORRs of 45–71% in patients who have progressed on crizotinib treatment [93–96]. However, new-generation ALK inhibitors may, in turn, induce secondary resistance mutations, for which new drugs will have to be designed [78,79,97,98]. Despite initial durable responses to ceritinib in crizotinib-resistant patients, tumors eventually develop resistance to ceritinib. Biopsies from crizotinib-resistant tumors that progressed on ceritinib showed eradication of ceritinib-sensitive mutations (S1206Y, G1269A) and the emergence of the cross-resistant G1202R mutation, which is also associated with clinical resistance to alectinib and crizotinib [78,87,99]. In a study of 11 patients with acquired resistance, 5 patient biopsies had either G1202 or F1174 mutations and the remaining 6 biopsies had wild-type *EML4–ALK* with no mutation [78]. The profile of ALK resistance mutations shifts depending on the ALK inhibitor, and accurate screening to match the mutational profile of tumors with the appropriate ALK inhibitor is likely to be important to maximize benefit for patients who relapse on ALK inhibitor therapy by directing subsequent sequential or combination therapy [100]. Rare and complex mutational profiles have also been encountered in ALK inhibitor drug-resistant ALK+ NSCLC, including *KRAS Q22K* mutation and *STK11* frameshift mutations; this highlights the importance of

comprehensive molecular testing in progressing patients [101]. The heterogeneity of ALK inhibitor resistance mechanisms means that effective monitoring of genetic changes during treatment will be important in treating or preventing the emergence of resistance.

3.3. ROS1

Crizotinib is a recommended treatment for *ROS1*-rearranged NSCLC [102]; however, secondary mutations in *ROS1* causing resistance to crizotinib have also been reported [103,104]. Other ALK/ROS1 inhibitors may prove effective as second-line options, although cell-based resistance profiling studies demonstrate that ROS1-selective inhibitors retain efficacy against the mutant, whereas the dual ROS1/ALK inhibitors are ineffective [105]. Lorlatinib (PF-06463922), a new dual ROS1/ALK inhibitor, blocks such crizotinib-resistant mutations in preclinical studies [106].

3.4. BRAF

Acquired resistance to BRAF inhibitors can occur through MAPK pathway reactivation due to a number of genetic aberrations, including *BRAF V600E* amplification, alternate splicing of *BRAF*, *NRAS* mutation, *KRAS* mutation, and *MEK1* mutation [107–109]. Co-inhibition of BRAF and MEK may overcome resistance, and the combination of dabrafenib and trametinib has provided a response rate of 68% in *BRAF*-mutant NSCLC, providing the basis for FDA Breakthrough Therapy Designation (2015) for this combination regimen in this indication [110]. Dual MEK–ERK inhibitors exhibit additive/synergistic effects and can delay the emergence of, and potentially overcome, acquired MEK inhibitor resistance [51]. *PIK3CA* mutations have also been implicated in resistance to BRAF inhibitors, and diagnostic detection of this mutation during therapy may thus direct decisions on subsequent PI3K inhibitor combination therapy [111].

3.5. MET

Preclinical models suggest that resistance to MET inhibitors may be mediated by *KRAS* amplification and overexpression that is potentially targetable with MAPK pathway inhibitors [112]. Secondary *MET* mutations may also be targetable in the event of effective appropriate diagnostic signals [113].

4. Optimal reanalysis of tumor genetic profiles

In a recent study of molecular and histologic changes in NSCLC tumors ($N = 50$) posttreatment, in the second tissue sampling, 54% of cases had additional genomic changes, including newly acquired alterations (81%) or losses (18%) [114]. As demonstrated by the breadth and heterogeneity of potential resistance mechanisms to diverse targeted therapies, re-biopsy of growing tumors following disease progression has become increasingly important for prognosis and to direct a change in therapy [66]. However, detecting mutations in resistant tumors can be challenging due to limited tissue availability; obtaining tissue from an outside institution in a timely fashion and delays in confirmation of tissue adequacy have been reported as important issues for oncologists [68]. Biopsies may be required during treatment, and both ethical and bureaucratic obstacles need to be removed so that molecular testing procedures can be carried out, where required, on very sick patients. Communication between disciplines, and particularly between pathologists and treating oncologists, is paramount to ensure that appropriate tests are carried out on precious and limited biopsy material [115,116]. Molecular testing should be prioritized and immunostaining limited where feasible, yet sampling procedures need to be minimally invasive while still providing sufficient material for both morphologic and mutational analysis [68,115]. Cell blocks prepared from malignant effusions can be a useful alternative to core biopsy [68]. Histologic/cytologic assessments may be required to locate tissue for macrodissection and ensure adequate tumor content above the sensitivity level of testing methods (sensitive testing methods are discussed below) [117]. Aspirates may be preferable to core biopsies for obtaining tumor material from bone metastases, due to the relative ease in obtaining DNA of adequate quality for analysis; alternative tissue sampling for genetic profiling may be desirable in many cases [68].

Liquid biopsies are assuming increasing importance as a noninvasive means of performing dynamic genetic surveillance in patients receiving targeted therapy treatment where acquired resistance may be expected and may overcome problems associated with tumor heterogeneity, as has been reported for ALK+ NSCLC small biopsy and excision samples [118]. During treatment and at the time of progression, circulating tumor (ct)DNA, circulating tumor cells (CTCs), and microRNAs (miRNAs) can be used to monitor treatment response noninvasively, and track and reveal molecular mechanisms of resistance [119]. This disease-related genetic information can be obtained through the analysis of ctDNA in the blood of NSCLC patients [120,121]. Real-world data from a large multicenter clinical study also suggest ctDNA samples are suitable for upfront *EGFR* mutation analysis when tumor samples are unavailable [122]. Nonetheless, robust and sensitive analysis methods are recommended to minimize false negative results. CTCs themselves may also be analyzed through liquid biopsy for individualizing and monitoring treatments and resistance [123]. Changes in miRNAs from liquid biopsies are predictive of response and may also help monitor resistance to treatment and detect progression early [124,125]. A number of methods are available for liquid biopsy analysis (there is currently no formal consensus on preferred techniques), and turnaround times need to be optimized so that treatment decisions can be made quickly when resistance to ongoing targeted therapy needs to be addressed urgently. Liquid biopsy allows detection of emerging mutations during treatment but cannot always be relied upon alone to identify all potential resistance mechanisms; for example, overexpression of potential bypass pathway driver proteins would not be identified and re-biopsy at progression would still be required in such cases where resistance mutations have not been detected in relapsed patients. Although having potential

utility in the dynamic monitoring for resistance mutations in previously diagnosed patients, liquid biopsy techniques are some way from routine clinical application as a primary diagnostic tool. Future developments will need to address the sensitivity of the method for application in asymptomatic or as yet undiagnosed patients.

5. Optimal diagnostics for routine mutation screening

A wide variety of commercially available molecular assays may be used to detect mutations in lung adenocarcinomas. An ideal assay is sensitive and specific enough to comprehensively cover all clinically relevant targets using limited samples, while being cost-effective and efficient. The method of choice depends on the type of mutation to be detected, the scale of throughput expected, and the type of sample available.

5.1. Direct Sanger sequencing

DNA mutational analysis via direct (traditional Sanger) sequencing is considered the gold standard for characterizing mutations and is generally performed on polymerase chain reaction (PCR) products using sequencing primers spanning the DNA region of interest [117]. Direct sequencing is the standard to detect *EGFR* mutations for determining whether patients are eligible for first-line EGFR–TKI therapy [3,126,127]. This can be targeted to specific mutations or aimed at screening or scanning larger regions [128]; direct sequencing of DNA corresponding to exons 18–21 of the *EGFR* gene is a reasonable initial approach [3]. Targeted assays are available from various manufacturers, for example the non-digital Cobas *EGFR* Mutation Test (Roche Molecular Systems) and the Therascreen EGFR Kit (Qiagen) [117,129].

Additional clinically relevant mutations implicated in resistance can be added to these assays, for example a new version of the Cobas test adds the *T790M* mutation to those detected in the original test and has been recently approved as a companion diagnostic test for osimertinib [130]. Digital PCR of plasma cell-free DNA has been successful for noninvasive detection of drug resistance mechanisms in *EGFR*-mutant NSCLC and detects the *T790M* mutation with 82% sensitivity and 86% specificity; the method was less successful in detecting *MET* gene copy number gain in plasma DNA [131]. Multiplex droplet digital PCR has been used to detect low-frequency mutations, such as *KRAS* point mutations, with a detection limit that compares favorably with next-generation sequencing (NGS; also known as massively parallel sequencing [MPS]) and Sanger sequencing [132]. Direct sequencing is limited by its low sensitivity, and it is estimated that a mutation should be present in ~20% of the sampled DNA to be reliably detected [128]. High-resolution melt analysis may be an alternative to direct sequencing that has provided 100% sensitivity and specificity in detecting *EGFR* mutations in surgically resected NSCLC [133].

5.2. Multiplex screening technologies

Screening technologies such as NGS and pyrosequencing have the potential to detect all *EGFR* mutations and allow detection of these and other mutations in tumor samples at levels as low as 5% [117]. Targeted NGS/exome sequencing enriches the target of interest and allows higher coverage, read depth, or simultaneous detection of mutations across different genes, using material from small biopsies and cytological samples. The CellSearch System coupled with NGS has proved successful in *EGFR* mutation analysis of CTCs in the Phase II erlotinib (TRIGGER) study [134]. More sensitive methods (supported by joint CAP/IASLC/ASCO guidelines) [3] are able to detect a range of 'actionable' mutations occurring with a frequency of ≥1%. These include the Sequenom MassARRAY system and SNaPshot Multiplex System (Life Technologies/Applied Biosystems), and these methods are supported by clinical guidelines [3,117,135]. In patients with NSCLC who experienced a treatment failure in response to EGFR–TKIs and had new biopsies, *T790M,* and other mutations outside EGFR have been successfully detected using NGS technology, by DNA sequencing on an Ion Torrent Personal Genome Machine (PGM) system (the Ion AmpliSeq Cancer Hotspot Panel version 2) [136]. Acquired *T790M* resistance mutations were detected in 60% of patients; other non-*EGFR* mutations identified included *TP53 P72R* mutations (87%), *KDR Q472H* (33%), and *KIT M541L* (13%). PCR-based NGS was thus able to detect *EGFR T790M* mutations in cases not readily diagnosed by other conventional methods. In addition, the detection of coexisting oncogenic mutations that may play a role in acquired EGFR–TKI resistance may help direct alternative treatment strategies in relapsed patients [136]. The Ion Torrent PGM system was clinically validated in a retrospective study of 39 NSCLC samples (and 51 colorectal cancer samples), interrogating 1850 hotspots in 22 genes [137]. Sensitivity and accuracy for detecting variants at an allelic frequency (AF) >4% was 100% for commercial reference standards, and the concordance between NGS and the reference test (*EGFR* mutation was used for the NSCLC samples) was >95%. The AmpliSeq panel was thus specific and sensitive for mutation analysis of gene panels and may be suitable for incorporation into clinical daily practice [137].

MPS methodologies are now being developed for the detection of gene rearrangements (e.g. *ALK, ROS1, RET*), as well as gene mutations in single-tube assays [138,139]. A novel multiplexed transcript-based assay, the Nanostring nCounter, detects overexpression of the 3′ end of transcripts versus the 5′ end, common in fusion genes [117]. Whole-transcriptome sequencing has been used to detect *RET* fusion oncogenes [38]. Microdissected tumor samples and cytology samples (including fine needle aspirates, pleural effusion) can be used to detect rare mutations if sensitive testing methods are used [128]. However, small tissue samples often preclude microdissection and may be prone to false negative results; equally, with high-sensitivity assays, clinical laboratories must be extremely careful to guard against false positive results [68]. NGS and parallel-sequencing technologies enable efficient, simultaneous detection of driver and drug-targeted mutations in NSCLC. GS Junior-based

sequencing has been used to screen samples for multiple mutations across several driver genes [140], and similarly, MiSeq™, GS Junior, and PGM Ion Torrent™ have been validated across several centers [141]. NGS applied to formalin-fixed paraffin-embedded tissue has become established as a routine diagnostic tool in some centers [142].

5.3. *In situ hybridization techniques*

For *ALK, ROS1*, and *RET* fusions, fluorescence *in situ* hybridization (FISH) is widely used in clinical laboratories and requires only a single paraffin section [117]. It is the current gold standard for the detection of *ALK* rearrangements regardless of the fusion partner, although it cannot identify the fusion, and is not feasible for large-scale screening of low-frequency rearrangements [117]. *ALK* gene rearrangements can be detected using the dual probe break-apart FISH assay, approved by the FDA as a prerequisite before treatment with crizotinib [3,143]. FISH has also been used to evaluate gene amplification in tissue microarray sections, including *MET* [57]. Immunohistochemistry (IHC) or reverse transcription (RT)-PCR can also be used for detection of specific fusion transcripts [22,143]. IHC offers an alternative and universally available option for pathology laboratories unable to carry out FISH. Fusion specific RT-PCR kits are commercially available. Combined with Sanger sequencing or NGS of PCR products, RT-PCR allows specific identification of the fusion partners; however, novel translocations may be missed, and RNA in clinical samples may be of poor quality [117].

6. Therapeutic approaches to combat resistance based on multiple mutation screening

With dozens of known targets and many agents approved or in development, there are a large number of possible combinations and sequences that can potentially be investigated or deployed in to treat or prevent drug-resistant patients in the clinic, often with support from preclinical models [144]. Several Phase III studies of combined targeted agents are underway. Patient-derived models of acquired resistance have provided valuable data that help identify effective drug combinations and could help direct combination therapy strategies in individual patients that are not predicted by genetic analysis alone [145]. Combinations of targeted therapies based on resistance mechanisms are currently being evaluated in a variety of lung cancer settings in Phase I and II studies (clinicaltrials.gov; January 2016). For example, cyclin-dependent kinases CDK4 and CDK6 are components of cell cycle control that switch on potential resistance-mediating RAS/RAF/MEK/ERK and PI3K/AKT/mTOR bypass signaling pathways in ALK+ NSCLC. The combination of ceritinib and the CDK4/6 inhibitor ribociclib (LEE011) is being evaluated in patients with ALK+ NSCLC who have progressed on an ALK inhibitor (including ceritinib) or who are ALK inhibitor-naïve. A study of the MEK inhibitor selumetinib (AZD6244) and gefitinib in patients with *EGFR*-mutated NSCLC and EGFR TKI resistance is ongoing (NCT02025114). The MET inhibitor capmatinib (INC280) is being evaluated in combination with gefitinib (NCT01610336) and erlotinib (NCT02468661) in patients with *EGFR*-mutated, *MET*-amplified NSCLC who have acquired resistance to an EGFR TKI, and with the *EGFR*-mutant-specific irreversible EGFR inhibitor EGF816 in *EGFR*-mutant NSCLC (NCT02335944). The combination of gefitinib and the PI3K inhibitor buparlisib (BKM120) is being evaluated in patients with NSCLC who have EGFR TKI resistance and molecular alterations in the PI3K pathway, such as *PIK3CA* mutation (NCT01570296). A study in patients with *EGFR*-mutated NSCLC who have EGFR TKI resistance will evaluate the TORC1/2 inhibitor INK128 in combination with the third-generation irreversible EGFR TKI AZD9291 (NCT02503722). Combination therapy is also being evaluated with the HER2-targeted therapy dacomitinib (NCT01918761). The increasing number of targetable mutations poses challenges for clinical study design to allow accurate efficacy assessment, particularly with respect to combination therapies of multiple targeted drugs (with or without conventional chemotherapy) and drug sequences [142]. The resulting high complexity, requiring unconventional study designs and analyzing small patient pools, may potentially limit approval [142].

For patients who have progressed without a defined mutation or one with no known targeted therapy, and/or the presence of other markers (e.g. programmed death-ligand-1), the advent of immunotherapy provides an alternative option to conventional chemotherapy. However, targeted therapies may increase sensitivity to immunotherapy and the combination of a targeted agent with immunotherapy may combat or prevent the emergence of resistance; this has been reviewed elsewhere [146,147]. Immunotherapy is outside the scope of this review, but a large number of clinical studies are now ongoing to evaluate how to optimize its merger with targeted therapy in lung cancer patients (Table 1) [148].

7. Expert commentary

Significant advances in molecular pathology in recent years have improved our understanding of NSCLC, with actionable oncogenic

Table 1. Ongoing clinical trials evaluating immunotherapy combined with targeted therapy in patients with NSCLC [148].

Combination	Phase	Trial ID	Partner target	Patient population
Ceritinib + nivolumab	1	NCT02393625	ALK	ALK+ NSCLC
EGF816 + nivolumab	2	NCT02323126	EGFR	*EGFR T790M*-mutant NSCLC
Capmatinib (INC280) + nivolumab			c-MET	c-MET+ NSCLC
Erlotinib + nivolumab	1	NCT01454102	EGFR	*EGFR*-mutant NSCLC
ACY-241 + nivolumab	1	NCT02635061	HDAC6	NSCLC
Erlotinib + ipilimumab	1	NCT01998126	EGFR	*EGFR*-mutant NSCLC
Crizotinib + ipilimumab			ALK	ALK+ NSCLC
Crizotinib + pembrolizumab	1	NCT02511184	ALK	ALK+ NSCLC
Necitumumab + pembrolizumab	1	NCT02451930	EGFR	*EGFR*-mutant NSCLC ALK+ NSCLC
Afatinib + pembrolizumab	1	NCT02364609	EGFR	*EGFR*-mutant NSCLC

ALK: anaplastic lymphoma kinase; EGFR: epidermal growth factor receptor; HDAC: histone deacetylase; NSCLC: non-small-cell lung cancer.

mutations in multiple signaling pathways identified, leading to the rational use of targeted agents tailored to tumor characteristics. Based on this approach, agents targeted to specific *EGFR* or *ALK* mutations have become widely used. Although reflex molecular testing for driver mutations is now commonplace, for those patients who have not been comprehensively screened or suboptimal diagnostics applied, no known driver mutation will be documented and routine treatment will be standard cytotoxic chemotherapy. Outcomes with targeted agents in patients with known actionable mutations have been impressive, but acquired resistance is our next major challenge – both in terms of treatment and effective monitoring. Outcomes with targeted agents will likely be improved by integrating increasing numbers of mutation screens at the point of treatment failure or even during treatment through repeated liquid biopsies, to allow subsequent therapy to be optimized. A recommended potential treatment and testing algorithm that combines existing and potential new diagnostic strategies is represented in Figure 2. Multiple clinical trials are ongoing or planned to investigate sequencing and combinations based on tailored therapy and resistance mechanisms, and results will hopefully lead to further survival improvements for patients with advanced NSCLC. In order for the results of these studies to be translated into impactful patient benefit in the real world, appropriate biopsy and diagnostic platforms and strategies will need to be implemented universally. By introducing treatment paradigms which shift the diagnostic emphasis toward both upfront pretreatment testing and timely monitoring for potential actionable resistance mutations, the continued use of inappropriate treatments may be minimized. At the same time, the clinical effectiveness of diverse targeted treatments as potent personalized treatment options for the patients who will derive the most benefit may be maximized.

8. Five-year view

The spectrum of currently available approved targeted therapies is still limited, and therefore, the justification for comprehensive evaluation of mutation status of vast numbers of oncogenes not just in clinical trials, but in routine clinical practice is still controversial. However, as the pace of research into new lung cancer driver and/or resistance mutations rapidly accelerates, this is unlikely to be the case in the near future. The key challenge will be in the implementation of highly complex techniques such as NGS in a uniform and preferentially centralized way that is accessible to all patients. This uniformity will need to include the software used for evaluation of results and recommended guidelines on cutoffs and minimum read numbers required per mutation tested, as well as the basic platforms used. NGS techniques as routine diagnostic tools in the real world are currently limited by specialist sample preparation requirements and costs associated with both these techniques and the widespread application of commercial platforms in routine community clinical practice. The optimization of the above factors will need to be carried out without compromising turnaround times. Nonetheless, the potential benefit to a large proportion of patients is exemplified by Figure 1, which shows that up to 30% of patients with adenocarcinoma are likely to have tumors bearing a known actionable genetic mutation, and even higher ratios in some resistance settings, with others more than likely to be discovered over the next 5 years. A further key future challenge will be in moving these diagnostic and treatment algorithms forward in the management timeline of patients with NSCLC, so that patients with earlier stage disease may also benefit from targeted personalized therapies.

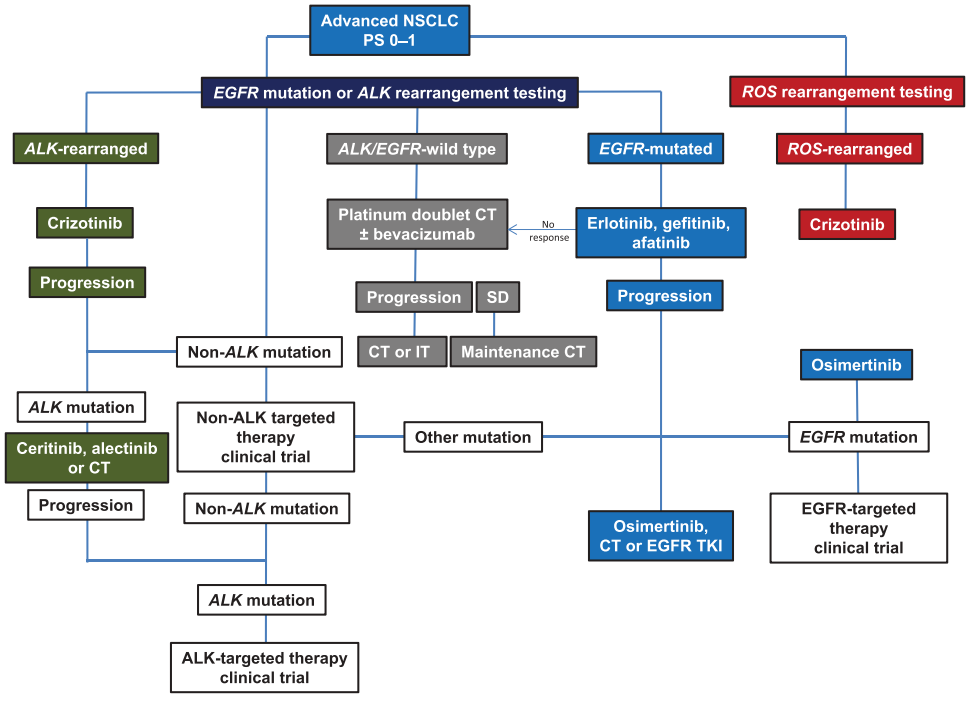

Figure 2. Flow chart recommending where mutational analysis can fit (white boxes) into current NSCLC treatment algorithms to direct therapy first line or in resistant disease; current recommended treatment flow is in shaded boxes and is based on current ASCO and NCCN Guidelines [8]. CT: chemotherapy; IT: immunotherapy; NGS: next-generation sequencing; PS: performance status; SD: stable disease; TKI: tyrosine kinase inhibitor.

This may lead to improved cure rates in NSCLC, in addition to already improved survival for patients with advanced disease. Ultimately, with all of the technological developments, we will have a strong impact on the cure for lung cancer.

Key issues

- In addition to *EGFR* and *ALK*, important oncogenic driver mutations/rearrangements that may be considered for diagnostic screening include *KRAS, MET, PIK3CA, HER2, BRAF, NTRK, ROS1, RET*, and *MEK*
- Acquired resistance to targeted agents occur by several mechanisms during treatment, and mutations that enable escape of dependence on the initial oncogenic driver present important second-line diagnostic targets
- Identification of resistance mutations or pathway activation upon progression is increasingly important as new and specific treatment options emerge
- In post treatment second tissue samples over half of NSCLC cases have additional genomic changes, including newly acquired alterations or losses; re-biopsy is thus important for prognosis, and to direct a change in therapy, but can be challenging due to limited tissue availability and quality
- Liquid biopsies are important as a non invasive means of performing dynamic genetic surveillance in patients receiving targeted therapy treatment where acquired resistance may be expected, and may overcome problems associated with tumor heterogeneity
- Circulating tumor DNA, circulating tumor cells, and microRNAs can be used to monitor treatment response non invasively, and track and reveal molecular mechanisms of resistance
- DNA mutational analysis via direct sequencing is considered the gold standard for characterizing mutations, and can be targeted to specific mutations including those associated with resistance, or aimed at screening or scanning larger regions
- Next-generation sequencing and pyrosequencing have the potential for simultaneous detection of mutations across different genes, including gene rearrangements, using material from small biopsies and cytological samples
- Patient-derived models of acquired resistance have provided valuable data that help identify effective drug combinations, and could help direct combination therapy strategies in individual patients that are not predicted by genetic analysis alone
- Combinations of targeted therapies based on resistance mechanisms are currently being evaluated in a variety of lung cancer settings

Declaration of interests

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. Editorial writing assistance was provided by Matthew Naylor, funded by Novartis Pharmaceuticals Corporation.

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• of interest
•• of considerable interest.

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