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The expanding capability and clinical relevance of molecular diagnostic technology to identify and evaluate *EGFR* mutations in advanced/metastatic NSCLC

Parth Shah [a], Jacob Sands [b,*], Nicola Normanno [c]

[a] Dartmouth-Hitchcock Medical Centre, 1 Medical Center Dr, Lebanon, NH 03766, USA
[b] Dana-Farber Cancer Institute, 450 Brookline Ave, Boston, MA 02215, USA
[c] Cell Biology and Biotherapy Unit, Istituto Nazionale Tumori-IRCCS-Fondazione G. Pascale, Via Mariano Semmola 52, 80131 Naples, Italy

A B S T R A C T

Epidermal growth factor receptor (*EGFR*) mutation testing in advanced non-small-cell lung cancer (NSCLC) has evolved rapidly over the past decade, largely triggered by the introduction of the targeted *EGFR* tyrosine kinase inhibitors (TKIs). Initially used to detect common *EGFR* mutations and determine the most appropriate first-line therapy at diagnosis, testing methodologies have expanded to test for multiple mutations at multiple time points throughout the disease course. Here we review the current mutation testing approaches, including types of biopsies, and the available assays commonly used in the clinic. Specific application of these approaches in advanced NSCLC, including current guideline recommendations, and potential future developments are discussed.

1. Introduction

Globally, non-small-cell lung cancer (NSCLC) accounts for 80–85% of all lung cancers [1]. Of these cases, about 30% are positive for mutations in the epidermal growth factor receptor (*EGFR*) gene [2]; however, regional differences have been reported, with *EGFR* mutations found in 10–25% of white patients but up to 30–50% of Asian patients with NSCLC (Table 1) [2-5]. Treatment of these patients has been revolutionized with the introduction of *EGFR* tyrosine kinase inhibitors (TKIs), which have been shown to be more effective and better tolerated than the previous standard of care, platinum-based chemotherapy [6-14]. Subsequently, several clinical trials have reported superior progression-free survival (PFS) and/or overall survival (OS) with the second- and third-generation *EGFR* TKIs, afatinib, dacomitinib and osimertinib, versus the first-generation agents, erlotinib and gefitinib [15-20]. Among these, osimertinib has demonstrated substantial improvements in both PFS and OS versus erlotinib or gefitinib, together with an additional improvement in side effect profile, when administered as first-line therapy [20].

The most common *EGFR* mutations are exon 19 deletions (Del19) and L858R on exon 21, which account for ~ 45% and ~ 30% of cases of *EGFR* mutation-positive NSCLC, respectively [21,22] (Fig. 1). However, up to 25% of *EGFR* mutation-positive NSCLC tumors harbor uncommon *EGFR* mutations [23]. These mutations represent a highly heterogeneous group, but the most prevalent include: exon 20 insertions, G719X, L861Q, S768I, and exon 19 insertions. All approved *EGFR* TKIs have demonstrated efficacy in patients with these common mutations [6-11]. However, for many uncommon mutations there is a paucity of clinical data as most randomised trials excluded patients with such mutations and preclinical data suggest that sensitivity of these uncommon mutations to TKIs may vary widely. The second-generation TKI afatinib appears to have broader activity than first-generation TKIs [24] and afatinib has demonstrated clinical efficacy against the major uncommon mutations G719X, L861Q and S768I [13], as reflected in the latest version of the United States Food and Drug Administration (US FDA)-approved label for afatinib. Further, there are increasing data to suggest afatinib may have activity against compound mutations and some exon 20 insertions [23]. The third-generation TKI osimertinib has also demonstrated some activity against the major uncommon mutations and exon 20 insertions [25-27], but evidence for the other *EGFR* TKIs is limited. The differing activities of the *EGFR* TKIs against the various uncommon mutations highlights the importance of accurate detection of *EGFR* mutations to enable selection of the most appropriate therapeutic option at diagnosis.

Mutation testing is also important at the time of disease progression. For example, the T790M mutation is the most common resistance mutation following treatment with afatinib, erlotinib, and gefitinib [28-30], and has particular sensitivity to osimertinib. Osimertinib, was initially

* Corresponding author.
E-mail addresses: parth.s.shah@hitchcock.org (P. Shah), Jacob_sands@dfci.harvard.edu (J. Sands), n.normanno@istitutotumori.na.it (N. Normanno).

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Table 1
Mutation testing in NSCLC: genes recommended for testing and their incidence in NSCLC patients [92,93].

Gene		Incidence in NSCLC patients
Category 1	*EGFR*	Overall: ~30% (10–25% in Caucasian and up to 50% in Asian patients) Del19 and L858R: 10–40% of patients at diagnosis, depending on ethnicity, sex and smoking status T790M: 50–70% of patients after progression on erlotinib, gefitinib or afatinib, higher prevalence in patients with Del19-positive disease Other 'uncommon' mutations seen at varying, lower incidences
	ALK	3–7%
	ROS1	1–2%
Category 2	BRAF	1–4%
	MET	MET amplification: 3–4% Exon 14 skipping mutations: 2–4%
	ERBB2 (HER2)	2–3%
	RET	1–2%
	KRAS	20–40%

EGFR, epidermal growth factor receptor; NSCLC, non-small-cell lung cancer.

developed as a second-line therapy option in patients with T790M-resistance to first- or second-generation TKIs and has demonstrated clear efficacy against T790M-positive tumors [31], providing a strong rationale for T790M mutation testing at disease progression.

Expert guidelines across the US, Europe and Asia recommend that all patients with NSCLC of adenocarcinoma or non-squamous, non-small-cell histology should undergo *EGFR* mutation testing that assesses mutations in *EGFR* exons 18–21 [32-36]. However, despite guideline recommendations, the *EGFR* testing rate is suboptimal in many countries; in the multinational PIvOTAL study, rates of 43–85% were reported across Italy, Spain, Germany, Australia, Japan, Korea, Taiwan, and Brazil [37]. Furthermore, a large proportion of *EGFR* mutation-positive patients still do not receive *EGFR* TKI therapy. In a large real-world study of 630 patients in the US, only 64% of patients with *EGFR* mutation-positive disease received *EGFR*-targeted therapy [38]. These data suggest that there is still scope to improve rates of *EGFR* mutation testing and first-line administration of TKI-targeted therapy.

The aim of this review is to provide a detailed summary of current molecular diagnostic tools used to detect *EGFR* mutations, including the limitations/benefits of each system, together with guidance for their use in clinical practice. We performed a literature review of studies and reports that discussed *EGFR* mutation testing in the clinic. We searched PubMed (up to 1 April 2020) with the following search terms: ('mutation' or '*EGFR*' or 'mutation testing') and ('NSCLC'). For this narrative review, articles identified from the database searches were selected based on their potential relevance to the topic of this review, focusing on *EGFR* mutation testing in patients with advanced NSCLC. Reference lists of the selected articles were also checked for additional potentially contributory articles.

2. Detecting and characterizing *EGFR* mutations

Biopsy type: tissue versus liquid biopsies

The current clinical gold standard approach for mutational analysis of lung cancers is genotyping of solid tumor biopsies [34,39,40]. Tissue samples are obtained from the tumor itself, and whole cells are used for molecular analysis. For patients with NSCLC, such samples can be obtained by a variety of methods, including transbronchial lung biopsy, endobronchial ultrasound-guided transbronchial needle aspiration, bronchial brushing or washing, computed tomography-guided biopsy, and pleural fluid sampling [41]. However, while tissue samples may be obtained routinely at diagnosis, obtaining additional biopsy samples (for example, at the time of disease progression) can be challenging. Repeat biopsies can be associated with high rates of clinical complications or be clinically unfeasible, or patients may simply refuse to undergo additional invasive procedures [42,43]. Further, the time needed to schedule a tissue biopsy can delay testing and clinical decision-making.

Liquid biopsies, in which tumor components are detected in bodily fluids, are an alternative to tissue biopsy and are finding increasing application in NSCLC [44]. The tumor components detected comprise the fraction of cell-free DNA (cfDNA) that is derived from tumor cells, known as circulating tumor DNA (ctDNA). Liquid specimens suitable for molecular analysis include blood (plasma), urine, saliva and liquid cytology specimens, with plasma being the most commonly used [44-47].The feasibility of liquid biopsies have been demonstrated by several studies. For example, a prospective study in the United States and Canada demonstrated that ctDNA testing could successfully identify guideline-recommended mutations in patients with newly diagnosed metastatic NSCLC at a rate at least as high as tissue testing (concordance rates of 98–99% were reported for Del19 and L858R *EGFR* mutations) and within a shorter time frame [48]. However, it should be noted that, while the use of liquid biopsy samples can be simpler, faster, less invasive, and more cost-efficient, the amount of tumor DNA in a liquid biopsy sample can be much lower than with solid biopsies [49], and negative results may require confirmation with a solid biopsy sample [35].

An important advantage of liquid biopsies over tumor biopsies is the increased convenience and avoidance of invasive procedures, potentially enabling mutation detection in patients unable to undergo tissue biopsies. In a recent analysis of 229 NSCLC patients in the United States, tissue biopsies alone detected targetable mutations for 47 patients (20.5%), whereas the addition of plasma sequencing increased the number of targetable mutations detected to 82 (36%) [50]. By sampling bodily fluids rather than a single tumor site, it has also been suggested that liquid biopsies can provide more representative samples of the entire tumor burden, potentially capturing molecular data across multiple metastatic sites. This may also minimize the risk of missing a mutation as a result of tumor heterogeneity or because a non-progressing lesion was sampled [40,51,52]. An example of this was seen in a study directly comparing cfDNA from liquid biopsies versus tumor biopsies in 42 patients with gastrointestinal cancers. In this study, single-lesion tumor biopsies frequently failed to identify the presence of multiple clinically relevant resistance mechanisms, with cfDNA identifying additional concurrent resistance mechanisms in 78% of cases [53]. Therefore, liquid biopsies could be of particular value following *EGFR* TKI therapy, owing to the heterogeneity of NSCLC [54] and evidence suggesting that more heterogeneity may be detected after treatment than at initial diagnosis [55].

3. Mutation detection approaches

A wide range of molecular diagnostic tests are currently available, many suitable for testing either solid or liquid biopsy samples. These diagnostic tests can be broadly categorized into three types: whole-exome sequencing, targeted sequencing (targeting specific exons), and single-allele approaches (targeting a single allele) (Tables 2 and 3).

Sequencing approaches

Non-allele-specific sequencing approaches can be used to detect both known and novel mutations [5,56]. Such approaches include targeted sequencing and whole-exome sequencing approaches. However, it should be noted that whole-exome approaches are mainly restricted to the research setting at present, with targeted approaches, focusing for example on *EGFR* exons, more commonly used in clinical practice. An early example of a non-allele-specific approach was Sanger sequencing (Table 2). Sanger sequencing is highly accurate but it has a low sensitivity and can only be used on samples with a tumor DNA content of at least 25–30%, limiting its use to solid tissue biopsy samples only [5].

While it is still used as a reference standard in comparative studies, Sanger sequencing has largely been replaced by massively parallel sequencing (MPS; also known as next-generation sequencing) approaches [5,56]. Indeed, analysis of data in Italy demonstrated that a switch from Sanger sequencing to more sensitive sequencing techniques or real-time PCR approaches was correlated with improved performance in external quality assessments [57].

For molecular oncology, including *EGFR* mutation testing, targeted

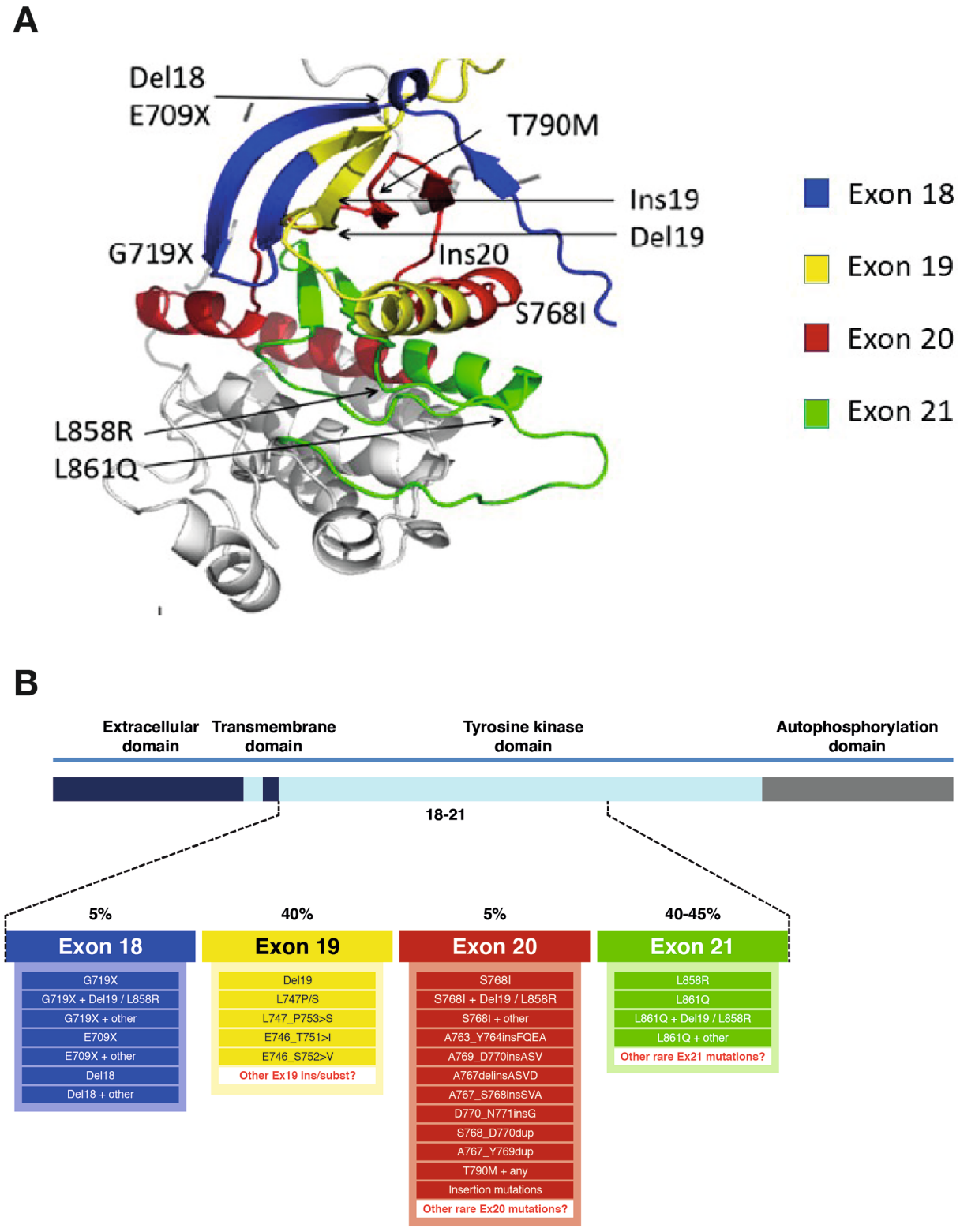

Fig. 1. (A) Tertiary structure of *EGFR* indicating the positions of exons 18–21 plus the positions of key *EGFR* mutations. (B) Schematic showing the key uncommon mutations in exons 18–21. Reprinted (with amendments) from Journal of Thoracic Oncology, Volume 16, Issue 5, Pages 764–773, Passaro A, et al., Recent Advances on the Role of EGFR Tyrosine Kinase Inhibitors in the Management of NSCLC With Uncommon, Non Exon 20 Insertions, EGFR Mutations, Copyright (2020), with permission from Elsevier.

Table 2
Key features of molecular platforms used for the detection of *EGFR* mutations: non-allele-specific assays.

Traditional sequencing (e.g. Sanger sequencing)	
Methodology	• Utilizes the original dideoxy sequencing technology pioneered by Frederick Sanger • Suitable samples: tissues only [5]
Advantages	• Accurate results [5] • Can enable discovery of new mutations
Limitations	• Can be costly • Can miss mutations that are present at a low VAF, or if tumor cellularity is <25–40% after enrichment [94,95] • Low throughput and limited scope for automation [5,96] • Low sensitivity and requirement for tumor DNA content of at least 25–30% in samples
Next-generation sequencing	
Methodology	• Involves the generation of a library of amplified DNA. Each individual DNA fragment is then separated and sequenced in parallel using an amplicon or hybrid-capture sequencing platform • Can sequence certain regions, the whole exome or the whole genome • Suitable samples: tissue biopsies, cytological samples, fine needle aspirates [97,98], plasma [29]
Advantages	• High sensitivity and high throughput [5,99] • Can detect multiple targets and multiple alteration types simultaneously even with small amounts of input nucleic acids [44] : PCR-based NGS: 5–10 ng total DNA (including malignant and normal DNA); hybrid capture-based NGS: 100–200 ng RNA or DNA; whole-genome NGS: micrograms of DNA [100] • New developments include approaches such as CAPP-Seq • Can be useful for detecting uncommon *EGFR* mutations
Limitations	• Whole-exome sequencing is not suitable for small samples due to the amount of DNA required [100] • Can be highly complex and robust bioinformatics tools are required [56,97] • Errors may originate in the wet laboratory process or the software used to evaluate the results [97] • Often slower, less sensitive and more labour-intensive than allele-specific methods

CAPP-Seq, CAncer Personalized Profiling by deep Sequencing; *EGFR*, epidermal growth factor receptor; NGS, next generation sequencing; PCR, polymerase chain reaction; VAF, variant allele frequency.

MPS approaches involve generation of a 'library' of amplified DNA, followed by separation of each individual DNA fragment and parallel sequencing [58]. This high-throughput approach makes it more rapid than Sanger sequencing. The most common MPS approaches are amplicon sequencing and hybrid capture sequencing (Table 2) [34,44]. The benefits of MPS include the breadth of mutations detected (multiple genes can be analyzed simultaneously and it can detect base substitutions/point mutations, insertions and deletions, gene fusions/rearrangement, and copy number alterations), the sensitivity of the assay, and the requirement for less DNA [34,44,56,59]. An example of an advanced MPS approach is Cancer Personalized Profiling by deep Sequencing (CAPP-Seq) [60,61], which can survey more loci than amplicon sequencing, giving it a higher sensitivity and specificity than previous MPS approaches.

Single-allele assays

Single-allele-specific assays utilize sequence-specific primers to selectively amplify mutated or non-mutated regions in a gene of interest and can be used to detect known mutations or copy number variants and are relatively low cost and rapid (Table 3). As tumor DNA is amplified using PCR, allele-specific tests are more sensitive than traditional sequencing assays and require less baseline tumor DNA; however, they cannot be used to detect novel mutations [59]. Several single-allele assays can be combined into multiplexed assays that can simultaneously detect multiple known substitutions, insertions, and deletions in one or more gene. Numerous single-allele systems are in routine clinical use,

Table 3
Key features of molecular platforms used for the detection of *EGFR* mutations: allele-specific assays.

ARMS, SARMS, ARMS-Plus, Super-ARMS	
Methodology	• Use sequence-specific PCR primers that allow amplification only if the target allele is present [69] • Suitable samples: tissue/cytologic samples [62,67,101], plasma [29,62,102]
Advantages	• Can detect as little as 1% mutant in normal DNA background [62] • Quick and easy to use [62] • Results can be analyzed in a real-time, closed-tube format by incorporating fluorescent probes or intercalating fluorescent dyes, which eliminates PCR product contamination and reduces the time to generate results [62,69] • Can detect multiple specific mutations, e.g. the Cobas® kit can detect 42 *EGFR* mutations across exons 18–21 [56], including key activating and resistance mutations [59]; Therascreen® can detect 29 mutations in exons 18–21 of *EGFR*, including T790M [52] • Can be used to quantify specific *EGFR* mutations (ARMS-Plus) [101]
Limitations	• Is allele-specific, i.e. only the specific alterations that are targeted by the assay are assessed • Although PCR can be highly sensitive, it can generate false-positive results; thus two independent PCR reactions from the original genomic DNA may be needed to eliminate false-positive errors [62,100] • Some assays (e.g., Therascreen® kit) have a low sensitivity for the T790M mutation [103]
Droplet digital PCR	
Methodology	• Separates template molecules into individual reaction vessels, facilitating independent amplification and fluorescence reading of thousands of individual droplets [5,69] • Suitable samples: tumor tissue [80], plasma [29,45,84], urine [45,46]
Advantages	• Extremely sensitive (0.04–0.1%) [5,40,41] • Rapid turnaround [5] • Quantitative PCR methods can be used to quantify specific *EGFR* mutations in longitudinal samples, and can allow for monitoring of disease/mutation evolution over time [41,52,63,69,101] • Particularly useful for detecting T790M mutations • Require less baseline DNA than sequencing methods [59]
Limitations	• Allele-specific, and can only detect previously-known mutations or targeted sites (cannot detect rearrangements) [5,41] • Each well can detect only one mutation site [5]
Peptide nucleic acid clamping [95]	
Methodology	• Uses peptide nucleic acids (artificially synthesized polymers that strongly bind to complementary DNA sequences) to suppress PCR amplification of wild-type sequences, facilitating greater amplification of mutant sequences [95] • Suitable samples: tissue/cytologic or plasma [67,69,104]
Advantages	• Can be used to detect mutant alleles, even when present at levels 100-fold lower than wild-type alleles [95] • Less technically complex than direct sequencing
Limitations	• Allele-specific
MassARRAY	
Methodology	• MassARRAY systems incorporate benchtop MALDI-TOF mass spectrometry customized for the detection of DNA molecules. The molecules are distinguished by their time-of-flight after being ionized in a vacuum chamber • Suitable samples: tissue and cytological samples [104], plasma [105]
Advantages	• Certain systems (e.g. iPLEX® HS panel for the MassARRAY® system) can detect 1% VAF [94] • Rapid turnaround time • Low per-sample cost [94]
Limitations	• Allele-specific
Cycleave PCR	
Methodology	• PCR-based assay utilizing chimeric DNA-RNA-DNA probes labelled with a fluorescent dye and a quencher at each end. The RNA sequence of separate probes corresponds to the wild-type sequence and specific target mutations. If PCR-amplified mutant sequences are present, the RNA portion of the matching probe hybridizes to the mutant DNA, and is subsequently cleaved by

(*continued on next page*)

Table 3 (*continued*)

ARMS, SARMS, ARMS-Plus, Super-ARMS	
	RNase. The separation of the fluorescent molecule from the quencher generates a fluorescent signal, which is captured and measured • Suitable samples: tissue [106], plasma [107]
Advantages	• The intensity of the wild-type probe serves as an internal control and permits calculation of the mutant allele fraction
Limitations	• Allele-specific

ARMS, amplification refractory mutation system; ddPCR, droplet digital PCR; *EGFR*, epidermal growth factor receptor; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; qPCR, quantitative PCR; PCR, polymerase chain reaction; SARMS, Scorpion ARMS; VAF, variant allele frequency

including amplification-refractory mutation system PCR (ARMS) and digital PCR (dPCR) (Table 3).

ARMS PCR is widely used for the detection of *EGFR*-sensitizing mutations, and can detect mutations in samples with as little as 1% tumor [62] (Table 3). ARMS uses sequence-specific PCR primers that allow amplification only if the target allele is present. The approach can also be used to detect multiple specific mutations (multiplexed assays); examples include the Cobas® *EGFR* Mutation Test (Roche, Basel, Switzerland), which can detect 41 *EGFR* mutations across exons 18–21, including key activating and resistance mutations [56,59], and the Therascreen® *EGFR* RGQ PCR Kit version 2 (Qiagen, Hilden, Germany), which can detect 29 mutations across these exons, including T790M (Table 4) [52]. ARMS-based multiplexed allele-specific assays are now widely used in routine clinical practice, owing to their ease of use, low cost, rapid turnaround time, and high sensitivity and specificity.

Digital PCR involves separating template molecules into individual reaction vessels prior to amplification. The most common application of digital PCR involves generating thousands to millions of water droplets in an oil emulsion and is termed droplet digital PCR (ddPCR). This approach has a rapid turnaround time and is highly sensitive. Digital PCR is a quantitative assay so it can be used to monitor treatment response and disease progression [40]. In one study, ddPCR-based serial quantification of plasma-derived cfDNA samples facilitated pretreatment detection of *EGFR* mutations, monitoring of plasma response to *EGFR* TKI treatment, and identification of increasing levels of the T790M mutation emerging up to 16 weeks prior to radiographic disease progression [63].

Other single-allele approaches include MassARRAY (Agena Bioscience, San Diego, US), cycleave PCR, and peptide nucleic acid clamping (Table 3). However, to date, these approaches have been of limited use in the clinical detection of *EGFR* mutations.

Table 4
Key FDA-approved tests for *EGFR* molecular testing in NSCLC [92].

Test	Mutations detected
Cobas *EGFR* Mutation Test V2 (Roche Diagnostics)	*EGFR* exon 19 deletions and *EGFR* exon 21 L858R alterations *EGFR* exon 20 T790M mutations
Cobas *EGFR* Mutation Test V2 – liquid biopsy (Roche Diagnostics)	*EGFR* exon 19 deletions and *EGFR* exon 21 L858R alterations *EGFR* exon 20 T790M mutations
Therascreen *EGFR* RGQ PCR kit (Qiagen)	*EGFR* exon 19 deletions and *EGFR* exon 21 L858R alterations
Oncomine Dx Target Test (Thermo Fisher Scientific)	*EGFR* L858R and exon 19 deletions BRAF V600 EROS1 fusions
FoundationOne Cdx (Foundation Medicine)	*EGFR* exon 19 deletions and *EGFR* exon 21 L858R alterations *EGFR* exon 20 T790M mutations BRAF V600E ALK rearrangements MET exon 14 skipping mutation

EGFR, epidermal growth factor receptor; NSCLC, non-small-cell lung cancer; PCR, polymerase chain reaction.

4. Mutation testing in the clinic

While *EGFR* is the most commonly mutated gene in patients with advanced NSCLC (Table 1), mutations have also been identified in many other genes, some of which could be therapeutically targeted. Hence, at diagnosis, initial molecular analysis for patients with advanced NSCLC should not be limited to detection of *EGFR* mutations, and the use of expanded, MPS panel testing is encouraged. National Comprehensive Cancer Network (NCCN) guidelines recommend that all laboratories that test lung cancer samples should assess *EGFR* mutations, ALK fusions, ROS1 fusions, BRAF mutations, MET exon 14 skipping mutations, and RET rearrangements [64,65]. In Europe, the European Society for Medical Oncology (ESMO) guidelines recommend testing for *EGFR* mutations, ALK fusions, and ROS1 fusions, with testing for BRAF and NTRK1 mutations increasingly common as targeted therapies become more widely approved. Indeed, MPS testing for all these mutations was recommended in a recent report from the ESMO Precision Medicine Working Group [66]. As in the United States, MET exon 14 skipping mutations, RET rearrangements, and HER2 are evolving targets/biomarkers and are likely to be included in expanded MPS panels [36]. Similarly, several agents targeting KRAS are in clinical trials and testing for mutations affecting KRAS is increasingly included in expanded panels.

For patients progressing after *EGFR* TKI therapy, it is recommended that comprehensive testing for targetable resistance alterations is performed. This should include testing for T790M when patients have received first- or second-generation TKIs as frontline therapy [34], but also testing for non-*EGFR*-based resistance mechanisms. This can be accomplished with plasma-based testing, but if no alteration is detected by plasma, tissue testing should be performed if feasible.

The choice of test to detect *EGFR* mutations is influenced by several factors, including the type of biopsy (solid or liquid), cost, desired sensitivity, breadth of genomic targets to be analyzed and timing of the test (for example, at diagnosis or disease progression). Whole-exome approaches are often associated with a relatively high cost and high DNA input requirement. Hence, while they can provide valuable data on novel genomic aberrations, their use is limited in clinical practice. In contrast, single-allele approaches offer standardized platforms, sensitive assays, a relative short turnaround time, and lower cost, and require less tumor material, but are extremely limited. Consequently, at sites where MPS is available, a panel testing approach often offers an appropriate compromise, allowing a range of targets to be assessed without the high cost and high DNA requirement of whole-exome sequencing.

Turnaround time can also be a factor when selecting a molecular assay. In our clinical experience, turnaround time is shortest for ARMS detection methods such as the Therascreen® *EGFR* RGQ PCR kit and the Cobas® *EGFR* mutation test v2 (3–4 days) (Table 4); as noted above, these have been FDA-approved and are widely available. Turnaround time can be longer for MPS approaches with different company/institutional assays, generally in the range of 1–3 weeks [40]. As a result, many centers use rapid assays for *EGFR* testing when the response is needed urgently, and follow-up with MPS panel testing if the result is negative.

Regardless of the molecular diagnostic chosen, it is important that testing of both plasma and tissue samples is conducted in specialized laboratories using robust and sensitive methods [67]. Further, if analysis of a liquid biopsy sample is negative it should be followed-up with a tissue-based test when possible.

Applying the results

The results from diagnostic *EGFR* mutational analyses have primarily been used at diagnosis to identify suitable patients for targeted *EGFR* TKI therapy. Of the three generations of *EGFR* TKIs available, the third-generation agent osimertinib will be most appropriate in patients with T790M. For patients with the major uncommon mutations G719X,

L861Q and S768I, there is an increasing body of evidence that that the second-generation TKI afatinib is effective, with some data also emerging to support the use of osimertinib [23,26]. Now, however, with the increased use of liquid biopsies and development of new, more sensitive assays, the role of molecular testing in clinical decision-making is expanding, enabling more frequent and broader analysis. Mutation analysis using non-targeted approaches like MPS may detect co-existing alterations, particularly at progression (e.g. additional *EGFR* mutations, *KRAS*, *BRAF*, *MET*) that can influence outcomes and lead to treatment resistance [68]; such information could potentially be used to guide subsequent or combined treatment approaches [51].

When used at the time of progression, molecular analysis can also assist in identifying and characterizing resistance mechanisms [69], and potentially, direct appropriate next-line therapy. For example, in patients who progress following treatment with a first- or second-generation *EGFR* TKI, molecular analysis can identify patients with T790M-mediated resistance who are candidates for subsequent treatment with osimertinib (up to 75% of cases) [28,30,70]. However, the relevance of T790M detection after first-line treatment has decreased with the increased use of first-line osimertinib in many countries. Compared to first- and second-generation *EGFR* TKIs, resistance mechanisms to osimertinib are more heterogeneous at the time of progression and there does not appear to be one predominant mutation [71,72]. To date, the most commonly identified osimertinib resistance mechanism is the tertiary *EGFR* mutation, C797S, which has been detected in ~ 11% and ~ 22–40% of cases following first- or later-line administration of osimertinib, respectively [72,73]. It is important to identify whether the C797S mutation is in a cis or trans state with the T790M mutation. If the mutations are in a cis state (i.e., on the same allele) no *EGFR* TKI inhibitors are available as therapeutic options. However, in cases where they are in a trans state, preclinical and individual case report data suggest that a combination of a first- and third-generation TKIs may have some efficacy [74,75]. Importantly, these data are generally not obtained from allele-specific PCRs and instead require the use of MPS assays. Putative osimertinib resistance mechanisms also include amplification or mutation of other genes (e.g., *MEK1*, *KRAS*, *PIK3CA*, *HER2*, *BRAF*), so-called *EGFR*-independent resistance mechanisms [68]. Such aberrations have generally been identified at low frequencies [72,73,76] but their detection highlights that, ideally, a broad panel of genes should be screened at the time of progression. Recently, oncogenic kinase fusions have been identified in approximately 4% of cases of acquired resistance to osimertinib as second-line therapy [77]. These fusions include *FGFR3–TACC3*, *RET–ERC1*, *CCDC6–RET*, *NTRK1–TPM3*, *NCOA4–RET*, *GOPC-ROS1*, *AGK–BRAF* and *ESYT2–BRAF*. In some cases, addition of a second drug to osimertinib treatment has maintained disease control; for example, the Ret inhibitor BLU-667 in a case of the *CCDC6–RET* fusion [78].

A further benefit of molecular testing at both diagnosis and disease progression is that results may have value in monitoring the success of *EGFR* TKI therapy [79,80]. For example, in the AURA3 studies, which investigated the use of osimertinib as second-line or subsequent therapy in patients with T790M-mediated acquired resistance, plasma samples from 40 patients were assessed after disease progression [81]. Patients without detectable plasma mutations at progression had numerically longer post-progression survival (median 10.8 months, 95% CI 7.2–not reached) than patients with detectable *EGFR* mutations. Of patients with detectable *EGFR* mutations, those who had lost the T790M mutation but had other *EGFR*-activating mutations had the shortest post-progression survival (median 2.6 months, 95% CI 1.3–not reached).

While still investigational, the convenience of cfDNA sampling may also enable regular mutation testing throughout the disease course and allow longitudinal assessment of tumor response [82]. For example, mutational analysis conducted during *EGFR* TKI treatment may be used to identify patients with molecular disease progression prior to clinical progression, and to monitor for the emergence of T790M [83-86]. In one study, ddPCR was used to dynamically monitor serial plasma samples for T790M in *EGFR* mutation-positive NSCLC patients receiving *EGFR* TKIs [83]. In almost half of patients who developed T790M, detection of the mutation preceded disease progression. Median time between detection and progression was 2.2. months. In a study of patients treated with intercalated chemotherapy and erlotinib or placebo, PFS and OS were significantly shorter in patients who had cfDNA that was *EGFR* mutation-positive after 3 cycles of treatment than in those without detectable mutant *EGFR* alleles [79]. Similar results have been reported in other studies of plasma *EGFR* mutation status after treatment with *EGFR* TKIs [84,86,87], suggesting that mutant allele clearance may be used as an early predictor of imminent disease progression. In another study, Reckamp et al. used short footprint mutation enrichment NGS to detect and monitor activating and T790M *EGFR* mutations in serial urine and plasma samples from previously-treated *EGFR*m+ patients receiving the investigational third-generation *EGFR* TKI, rociletinib [88]. In nine patients monitored while receiving rociletinib, a rapid decrease in urine T790M levels was observed by day 21. Further, the ratio of T790M alleles to activating *EGFR* mutations (T/A ratio) may predict response to osimertinib; in one small study, patients with a higher T/A ratio had longer PFS and treatment duration [89]. However, other research suggests that patients with a low T/A ratio (i.e. lower T790M relative to the activating mutation) also respond to osimertinib [90,91].

5. The future

The use of *EGFR* mutation testing in advanced NSCLC has expanded rapidly since the introduction of the *EGFR* TKIs with technological developments such as liquid biopsies and new assays increasing the clinical application. Looking to the future, we would expect the number of target mutations to continue to expand beyond *EGFR* mutations, making broad MPS testing increasingly common at diagnosis. This increased use of MPS testing will itself improve the identification of patients with *EGFR* mutation-positive disease and also aid the detection of rare genetic variants not detected by routine diagnostic methods. Indeed, in the evolving landscape of increasing genomic alterations with effective therapy, a MPS panel is becoming increasingly important for optimal patient care.

We also expect an increased use of liquid biopsies. At diagnosis, this will clearly be valuable in patients with no available tumor tissue, but also as a complementary analysis to a solid biopsy by providing additional information on tumor heterogeneity and prognostic information; for example, patients with a negative liquid biopsy often have a prolonged PFS. At the same time, an increased use of liquid biopsy analyses would be expected to lead to more mutation testing throughout the disease course, with regular monitoring at 3–6 weeks after initiation of therapy and then regularly throughout treatment to monitor early signs of disease progression and identify resistance mechanisms.

Finally, in terms of assay development, we would expect that the speed at which assays can be conducted will continue to increase and that additional targeted sequencing panels covering a range of actionable mutations will be developed, further facilitating precision medicine in NSCLC.

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Author contributions

PS: Writing – review & editing. JS: Conceptualization, supervision, writing – original draft and review & editing. NN: Conceptualization, investigation, supervision, validation, writing – original draft and review & editing. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

PS declares no potential conflict of interest. JS has received honoraria for scientific advisory board from AstraZeneca, Takeda, Medtronic, Pharma Mar, Jazz Pharmaceuticals, Daiichi Sankyo, Blueprint Medicines, Lilly, Foundation Medicine and Guardant; and consulting fees from Boehringer Ingelheim, AstraZeneca, Merck, Pharma Mar and Jazz Pharmaceuticals. NN has received honoraria from MSD, Qiagen, Bayer, Biocartis, Illumina, Incyte, Roche, BMS, Merck, Thermo Fisher Scientific, Boehringer Ingelheim, AstraZeneca, Sanofi, Eli Lilly; and disclose non-financial relationships being President of the International Quality Network for Pathology (IQN Path) and President of the Italian Cancer Society (SIC).

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