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Review

Novel approaches against epidermal growth factor receptor tyrosine kinase inhibitor resistance

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ABSTRACT

Background. The identification and characterization of molecular biomarkers has helped to revolutionize non-small-cell lung cancer (NSCLC) management, as it transitions from target-focused to patient-based treatment, centered on the evolving genomic profile of the individual. Determination of epidermal growth factor receptor (*EGFR*) mutation status represents a critical step in the diagnostic process. The recent emergence of acquired resistance to "third-generation" EGFR tyrosine kinase inhibitors (TKIs) via multiple mechanisms serves to illustrate the important influence of tumor heterogeneity on prognostic outcomes in patients with NSCLC.

Design. This literature review examines the emergence of TKI resistance and the course of disease progression and, consequently, the clinical decision-making process in NSCLC.

Results. Molecular markers of acquired resistance, of which T790M and *HER2* or *MET* amplifications are the most common, help to guide ongoing treatment past the point of progression. Although tissue biopsy techniques remain the gold standard, the emergence of liquid biopsies and advances in analytical techniques may eventually allow "real-time" monitoring of tumor evolution and, in this way, help to optimize targeted treatment approaches.

Conclusions. The influence of inter- and intra-tumor heterogeneity on resistance mechanisms should be considered when treating patients using resistance-specific therapies. New tools are necessary to analyze changes in heterogeneity and clonal composition during drug treatment. The refinement and standardization of diagnostic procedures and increased accessibility to technology will ultimately help in personalizing the management of NSCLC.

INTRODUCTION

Overview of EGFR TKI resistance

Non-small-cell lung cancer (NSCLC) accounts for 85–90% of primary lung cancers [1, 2]. The identification of specific molecular targets against which therapies for NSCLC can be directed has prompted a shift towards personalized treatment [3, 4] and, with this, improved survival rates [4, 5]. In the tumors of patients with NSCLC of adenocarcinoma histology, three out of four of the known driver gene mutations are targetable with regulatory approved, specifically targeted treatments; these are: activating mutations in the epidermal growth factor receptor (*EGFR*); activating translocations of anaplastic lymphoma kinase (*ALK*); rearrangements of ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*); and the kinase activating mutation V600E in the *BRAF* oncogene [5–7]. The most common *EGFR* mutations are present in the tumors of approximately 13–20% of Western and 40–48% of Asian patients with NSCLC of adenocarcinoma histology (corresponding data for non-adenocarcinoma: 3–5% and 8%, respectively) and whilst *EGFR* mutations may occur in any patient, they show a clear association with Asian ethnicity, female gender, and never-smoker status [6, 8–15]. For patients with *EGFR* mutation-positive NSCLC, first-line treatment with EGFR tyrosine kinase inhibitors (TKIs; specifically gefitinib [IRESSA™], erlotinib [TARCEVA®], and afatinib [GIOTRIF®]) has been associated with superior objective response rates and progression-free survival compared with chemotherapy [5, 16–18].

The presence of *EGFR* mutations is the fundamental driver of response to EGFR TKIs [19–26]. However, most patients will acquire resistance to first-line EGFR TKIs and disease progression usually occurs within 6–24 months of treatment initiation [20, 22–24]. Unfortunately, the inevitability of acquired resistance keeps apace with new drug development, and resistance to second-line "third-generation" EGFR TKIs (e.g. osimertinib [TAGRISSO™] and rociletinib) has also been reported [27–31]. The molecular mechanisms underlying resistance to first- and second-line EGFR TKI therapy are becoming increasingly clear. Molecular alterations triggering resistance may alter the drug target itself (e.g. the T790M resistance mutation in the kinase binding domain of EGFR, the most common mechanism of acquired resistance to first-line EGFR TKIs) or activate alternate signal transduction pathways (e.g. *MET* amplification).

Tumor heterogeneity and development of resistance

Tumor heterogeneity — the presence of subclones of cells with distinct genotypes and divergent biologic behaviors — represents a key driver of cancer progression. This can include different cell subclones within a primary tumor (intratumor heterogeneity), between or within associated metastases (inter-/intra-metastatic heterogeneity), and between multiple tumors within an individual (intertumor heterogeneity) [32–37]. Tumor heterogeneity can be fostered by genomic instability [32] and genetically unstable cell subclones accumulate genetic alterations due to various kinds of selection pressure, including anti-neoplastic treatments and changes within the fluctuating microenvironment — a concept termed "tumor Darwinism" [32–34, 38]. Tumor stem cells may represent an important source of heterogeneity, as they have sufficient lifespan, and the proven capacity to self-renew and differentiate, which allows them to accumulate the genetic alterations necessary for treatment resistance [39]. Treatment resistance may also be due to failure of drug delivery; however, the mechanisms responsible for this are beyond the scope of this review.

Drug treatment (e.g. EGFR TKIs, cytotoxic chemotherapy) can promote the selection of resistant clones and subclones with genetic aberrations that eventually drive treatment resistance and disease progression [33, 40–42] (Figure 1). For example, the increased prevalence of the T790M resistance mutation detected over time during first-line gefitinib treatment for NSCLC provides evidence for clonal expansion during EGFR TKI treatment [43].

Tumor heterogeneity, therefore, has the potential to vastly complicate the treatment process, given that it is difficult to anticipate and then selectively target multiple molecular changes in rapidly evolving disease [33, 44, 45]. This can confound the predictive accuracy of prognostic biomarkers, particularly when relying on historic tissue samples. However, the emergence of liquid biopsy, and advances in "real-time" analysis methodology, may eventually help to accurately track the evolution of the tumor and, in this way, optimize targeted treatment approaches. Understanding tumor heterogeneity in terms of disease progression and relating observations in the patient to changes happening at the molecular level is the key to effective disease management. In NSCLC, treatment decisions are currently made on the basis of "clinical" radiologic indicators of disease progression, denoting a worsening of tumor burden with the emergence of clinical symptoms. Acquired resistance leading to disease progression is often driven by the development of secondary mutations that can be verified following detection of genetic biomarkers. This review examines the emergence of TKI resistance and the impact of tumor heterogeneity on the clinical decision-making process in NSCLC.

BIOMARKERS

The ongoing characterization of the key drivers of response and resistance to TKI therapies has allowed the identification of molecular biomarkers that may form the

basis of diagnosis and personalized treatment for patients with NSCLC [3]. In this section we review some of the key biomarkers and consider their relevance at either the point of initial diagnosis or at clinical or molecular disease progression.

Diagnostic biomarkers which can be used to guide treatment options

EGFR mutations (Figure 2) are important predictive biomarkers at diagnosis for the efficacy of first-line EGFR TKI treatment [46]. Determination of *EGFR* mutation status is, therefore, mandatory in the diagnosis of NSCLC, and should also be performed in squamous-cell lung carcinoma in never-smokers [2, 47–50]. For patients with *EGFR* mutation-positive NSCLC, EGFR TKI treatment is advocated, whereas chemotherapy or immunotherapy may be beneficial for patients with *EGFR* mutation-negative NSCLC [5, 19, 22].

Biomarkers which may indicate progression and may be used to guide subsequent treatment options

The nature of post-progression treatment should be tailored according to identified resistance mechanisms, as well as sites and the pace of disease progression [51]. Continued treatment beyond progression with concurrent local treatment in oligoprogressive disease when local treatment is feasible has been widely adopted in NSCLC [50]. The most recent National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines advocate the continued use of erlotinib, gefitinib, or afatinib in patients with asymptomatic progression, given that discontinuation of these EGFR TKIs has been associated with accelerated disease progression in terms of symptoms and tumor size [2, 50]. The basis for this post-progression prolongation of survival comes from the continued application of selective pressure on EGFR TKI-sensitive tumor subclones, thereby preventing regrowth and reducing the risk of rapid progressive disease once treatment is withdrawn [52]. The recommendation of treatment beyond progression may be based on prospective and retrospective analyses. Small retrospective studies of treatment beyond progression combined with local ablative therapy in patients with *EGFR* mutations or *ALK* translocations that experience oligoprogressive disease to TKI treatment have shown benefit in terms of progression-free survival and overall survival [53, 54]. The ASPIRATION study, to date the only prospective study to investigate the continuation of erlotinib beyond progression, shows benefit associated with continued

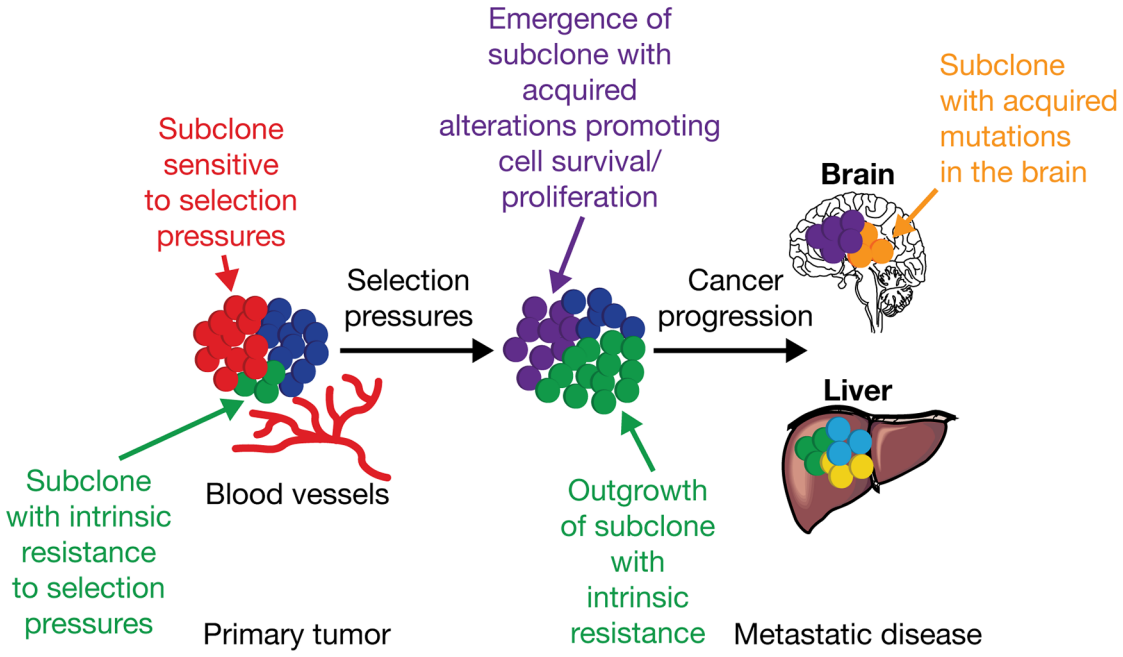

Figure 1: Intratumor heterogeneity and clonal evolution. Adapted from Jamal-Hanjani M, Quezada SA, Larkin J, Swanton C. Translational implications of tumor heterogeneity. Clin Cancer Res 2015; 21: 1258-1266, with permission from AACR [34]. Primary tumors consisting of different subclones may be subjected to various selection pressures (e.g. chemotherapy, and micro-environmental factors such as hypoxia, and infiltrating stromal and immune cells). Under the influence of selection pressures, subclones with intrinsic resistance (*green*) can outgrow a tumor mass, potentially leading to disease progression, and/or can acquire somatic alterations (*purple*) promoting cell survival, proliferation, and metastatic tumor formation. The outgrowth of some subclones (*red*) may be constrained by selection pressures that they are sensitive to; for example, targeted therapy against a tumor subclone with a somatic alteration sensitive to therapy.

treatment in select patients gaining a median 3.1 months of progression-free survival [55].

Whilst acquired resistance to EGFR TKIs may arise from multiple, complex mechanisms, several treatment strategies have been developed that specifically target the most frequent routes: *EGFR* T790M mutations, *MET* amplifications, and human epidermal growth factor receptor 2 (*HER2*) amplifications [56].

EGFR T790M

T790M mutations are secondary mutations in *EGFR* that are associated with acquired resistance to early-generation EGFR TKIs [57, 58]. Being the most common mechanism of acquired resistance, T790M mutations occur in approximately 50–60% of cases [56, 59] and are associated with impaired binding of the EGFR TKI to the tyrosine kinase domain of the EGFR [60, 61]. The T790M mutation increases the affinity of the binding pocket for ATP, thus interfering with the binding of EGFR TKIs and affecting specificity [61, 62]. The substitution is at a key site in the catalytic cleft of the EGFR TKI domain, located in the back of the ATP binding cleft. The amino acid substitution (threonine to methionine) leads to a bulkier side chain, resulting in steric hindrance which prevents the binding of reversible first-generation TKI molecules [62]. However, the T790M mutation itself does not interfere with ATP-binding and activation of EGFR, thus the tumor remains dependent on the EGFR pathway. This fact is important for further EGFR TKI treatment, as the T790M mutation remains sensitive to irreversible inhibitors. In contrast to the reversible inhibitors, the irreversible inhibitors overcome the resistance mechanism by covalent binding with Cys-797 in the ATP-binding cleft [61, 62].

As a collective drug class, the irreversible inhibitors are called "third-generation" EGFR TKIs and have shown potent and highly specific activity against T790M-mediated EGFR TKI resistance, with Phase I/II/III data in circulation (see Table 1 for a summary of efficacy outcomes only) [63–73].

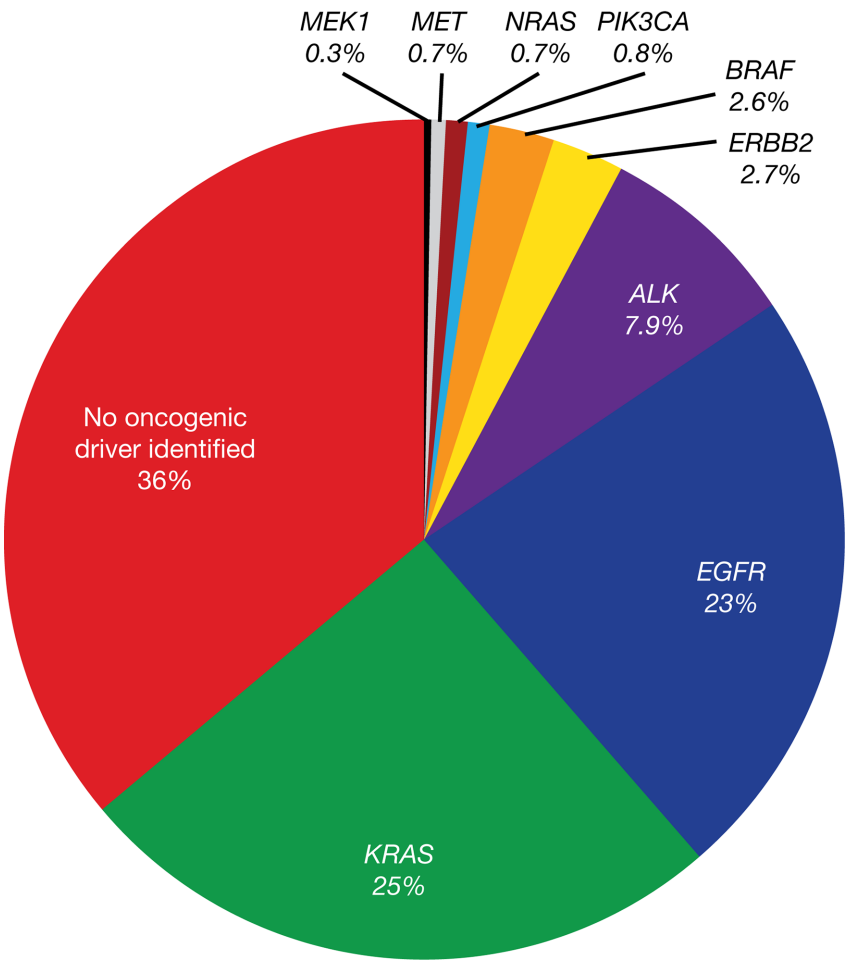

Figure 2: *EGFR* driver mutations identified in the Lung Cancer Mutation Consortium cohort (lung adenocarcinoma). Reprinted from Sholl LM, Aisner DL, Varella-Garcia M et al. Multi-institutional oncogenic driver mutation analysis in lung adenocarcinoma: the Lung Cancer Mutation Consortium experience. J Thorac Oncol 2015; 10: 768-777, with permission from Elsevier) [46].

Table 1: Summary of "third-generation" EGFR TKIs showing activity against acquired resistance mediated by T790M

"Third-generation" EGFR TKI	Study outcomes	Comment
Jänne et al 2015 [63] (AURA study)		
Osimertinib	ORR (61% vs. 21%) and PFS (median 9.6 months vs. 2.8 months) improved in patients with *EGFR* T790M mutation-positive NSCLC following progression during prior EGFR TKI therapy, vs. patients with non-T790M-mediated resistance	Granted FDA accelerated approval for treatment of T790M mutation-positive NSCLC regardless of line of therapy (November 2015)
Goss et al 2016 [64] (AURA 2 study)		
Osimertinib	Patients with *EGFR* T790M mutation-positive NSCLC showed early and durable objective response to osimertinib	Data support a potential change in clinical practice to evaluate tumors for the presence of *EGFR* T790M after progression
Mok et al 2017 [65] (AURA 3 study)		
Osimertinib	In patients with *EGFR* T790M mutation-positive NSCLC, prolonged PFS (median 10.1 months vs. 4.4 months; HR 0.30; $P<0.001$) and higher ORR (71% vs. 31%; OR 5.39; $P<0.001$) observed with osimertinib vs. platinum plus pemetrexed therapy. PFS also prolonged with osimertinib vs. platinum plus pemetrexed therapy in patients with CNS metastases	Benefits of osimertinib observed in Phase II trial and confirmed in Phase III trial
Sequist et al 2015 [66] (TIGER-X study)		
Rociletinib	Higher ORR (59%) in patients with *EGFR* T790M mutation-positive NSCLC vs. patients with T790M-negative disease (TIGER-X). Pooled TIGER-X/TIGER-2 data revealed lower rate of confirmed response (28–34%) [67]. Mature confirmed response rate (TIGER-X) 45% [68]	Clinical enrollment in all ongoing clinical studies terminated (2016)
Park et al 2015 [69]		
Olmutinib (HM61713)	Preliminary study reports ORR 58.8% (n=34) for HM61713 (dose >650 mg). Partial responses (unconfirmed; n=10) and disease stabilization (n=13) also observed	Granted Breakthrough Therapy designation by FDA (December 2015). Phase I/II studies ongoing (NCT01588145)
Tan et al 2015 [70]; Jia et al 2016 [71]		
EGF816	Potent inhibition of the most common *EGFR* mutations – L858R, exon 19 deletion and T790M – *in vitro* and in patient-derived xenograft models. Antitumor activity observed against T790M mutation-positive NSCLC across all dose levels examined	Phase I/II studies ongoing (NCT02108964)
Yu et al 2016 [72]		
ASP8273	Robust antitumor activity in patients with *EGFR* T790M mutation-positive NSCLC	Phase I, II, and III studies ongoing (NCT02113813; NCT02192697; NCT02588261)
Wang et al 2016 [73]		
PF-06747775	Under investigation in patients with advanced NSCLC with *EGFR* mutations (exon 19 deletion or L858R ± T790M)	Phase I/II studies ongoing (NCT02349633)

CNS, central nervous system; FDA, Food and Drug Administration; HR, hazard ratio; OR, odds ratio; ORR, objective response rate; PFS, progression-free survival.

MET

Although high-level amplifications of the proto-oncogene *MET* are uncommon in previously untreated NSCLC (~3% [74]), *MET* amplifications have been detected in 5–20% of tumor samples from patients with acquired resistance following first-line EGFR TKI therapy, and have been implicated in tumor cell proliferation and survival [35, 59, 75–82]. Co-occurrence of both MET and T790M resistance mechanisms may be found in between 7–39% of patients [83–85]; however, *MET* amplification may also occur independently of the T790M mutation, thereby representing a clinically distinct therapeutic target [76, 79, 80, 86]. In a pre-clinical setting, a combination of MET inhibition and EGFR inhibition has been shown to restore sensitivity to EGFR TKIs [87, 88] and preliminary clinical studies are ongoing (see later section; Table 2) [87–96]. *MET* amplification has also been implicated as a mechanism of resistance to the "third-generation" EGFR TKI osimertinib [94, 97, 98].

HER2

Amplification of *HER2* has been detected with a frequency of 12–13% in patients with progressive disease following first-line EGFR TKI treatment [78, 99]. Furthermore, it has been postulated that *HER2* amplification is involved in the development of resistance to "third-generation" EGFR TKIs, such as osimertinib — in a case report, a patient who had acquired a T790M mutation after progression with second-line gefitinib then went on to develop resistance to osimertinib, which was associated with *HER2* amplification in the absence of a C797S mutation in *EGFR* [100]. Targeted treatment of *HER2* amplification has been disappointing so far in NSCLC [101, 102], although HER2-directed antibodies and TKIs are under evaluation [103].

MEK/ERK pathway

Activation of the mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway has been observed in cell lines treated with EGFR TKIs, resulting in resistance to EGFR TKI monotherapy [104]. In patients treated with "third-generation" EGFR TKIs, MEK/ERK activation has also been described by different mechanisms [80]. Combinations of "third-generation" EGFR TKIs with MEK TKIs are being explored in Phase I trials – for example, osimertinib plus selumetinib (as *in vitro* data show reconstitution of EGFR dependency upon MEK inhibition) [91, 104].

Other rare resistance mechanisms

Many other genetic aberrations have been described in the setting of acquired resistance, either alone or in combination with other resistance mechanisms, such as EGFR TKI resistance or *MET* and *HER2* amplification. These genetic aberrations may also contribute to disease progression, including somatic phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutations [37, 105], seen in 1–5% of patients [56, 78, 106], and loss of phosphatase and tensin homolog (*PTEN*), which controls the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signal pathway [106, 107]. An additional mechanism of acquired resistance in *EGFR* mutation-positive NSCLC is transformation to the small-cell lung cancer (SCLC) phenotype, which has been reported in several patient cases [36, 108, 109]. Although relatively uncommon, the transition is detectable by standard pathologic examination of tissue biopsies and patients may respond well to SCLC-specific chemotherapy. It is also important to consider that other factors — such as the tumor microenvironment [106] — may be associated with resistance, further complicating full elucidation of resistance pathways. Indeed, the mechanism of resistance may be unknown in as many as 18–30% of patients [56, 59].

FOCUS ON *EGFR* MUTATION TESTING METHODS AT DIAGNOSIS AND PROGRESSION

The practicalities of *EGFR* mutation testing, either at diagnosis or at the point of progression, warrant careful consideration, particularly considering recent developments in liquid biopsy techniques. Diagnostic decisions regarding sample type and the timing of the test can have a critical influence on prognostic outcomes, and are discussed below; an author-drafted *EGFR* mutation testing algorithm is also presented in Figure 3.

Conventional biopsy

Historically, tumor-derived tissue has been instrumental in the elucidation of mechanisms of EGFR TKI action and secondary resistance [110]. Tissue biopsy represents the current gold standard sample type for *EGFR* mutation testing in NSCLC [110] and current guidelines (European Society for Medical Oncology [ESMO], American Society of Clinical Oncology [ASCO], and NCCN) advocate tumor subtype definition as a fundamental step in the diagnostic process [2, 49, 50]. Given its prominence, procedural techniques are highly standardized and widely accessible [111]. However, tissue biopsy has several associated limitations, one of which relates to limited availability of evaluable tissue samples, perhaps due to tumor location or perceived risk to the patient [11, 110, 112]. Such difficulties may also preclude the periodic monitoring of mutation status using sequential tissue samples. Furthermore, whilst there are advances in technologies facilitating the thorough analysis of mutations in archival formalin-fixed paraffin-embedded tissue samples [113] (noting that it is advisable to use strand-specific capture technologies), there are remaining

Table 2: Summary of combinatorial treatment approaches currently under pre-clinical and clinical investigation

Treatment combination	Outcome
Gibbons et al 2016 [89]	
Durvalumab plus gefitinib	Durvalumab plus gefitinib displayed encouraging activity in TKI-naïve NSCLC patients with sensitizing *EGFR* mutations and was generally well tolerated [Ongoing Phase I open-label study]
Oxnard et al 2015 [90]; Ahn et al 2016 [91]; Yang et al 2016 [92] (TATTON study)	
Osimertinib plus durvalumab (anti-PD-L1 monoclonal antibody), savolitinib (MET inhibitor), or selumetinib (MEK 1/2 inhibitor)	Encouraging clinical activity profile of osimertinib combinations in *EGFR*-mutation-positive NSCLC patients; emergence of interstitial lung disease in combination patients warrants further investigation (durvalumab arm now discontinued) [Ongoing Phase Ib study]
Janjigian et al 2014 [93]	
Afatinib plus cetuximab (antibody therapeutic)	Afatinib plus cetuximab displayed robust clinical activity and a manageable safety profile in resistant *EGFR*-mutant lung cancers with and without T790M mutations [Phase Ib study]
Ou et al 2016 [94]	
Osimertinib plus crizotinib (MET inhibitor)	High level of *MET* amplification post-progression on osimertinib, transient symptomatic benefit following osimertinib plus crizotinib (MET inhibitor) [Case report]
Nakagawa et al 2012 [87]	
WZ4002 (mutant-selective EGFR TKI) plus E7050 (mutant-selective MET TKI)	Suppression of growth of erlotinib-resistant tumors caused by gatekeeper T790M mutation, *MET* amplification, and *HGF* overexpression [Pre-clinical]
Smit et al 2016 [95]	
Erlotinib with/without INC280 (cMET inhibitor) vs. platinum chemotherapy plus pemetrexed	Erlotinib with/without INC280 compared with platinum plus pemetrexed, in patients with EGFR TKI-resistant NSCLC due to *cMET* amplification (*EGFR* T790M-negative) [Ongoing Phase Ib/II study]
Jia et al 2016 [96]	
EAI045 (allosteric inhibitor of drug-resistant EGFR mutants) plus cetuximab (antibody therapeutic)	EAI045 plus cetuximab effective in mouse models of lung cancer driven by EGFR(L858R/T790M) and by EGFR(L858R/T790M/C797S) [Pre-clinical]
Nanjo et al 2013 [88]	
Afatinib or WZ4002 plus crizotinib (MET inhibitor)	Crizotinib plus afatinib or WZ4002 potently inhibited the growth of mouse tumors induced by EGFR TKI-resistant cell lines. High-dose crizotinib plus afatinib associated with severe side effects [Pre-clinical]

HGF, hepatocyte growth factor; PD-L1, programmed death-ligand 1.

issues associated with the denaturation and fragmentation of DNA [111, 114–116]. Importantly, the usefulness of tissue biopsy techniques may also be confounded by inter-/intra-metastatic tumor heterogeneity, the clinical relevance of which has been discussed previously [33, 110, 115, 117].

Liquid biopsy

"Liquid biopsies" can provide access to a relative abundance of tumoral genetic material, including circulating free tumor-derived DNA (ctDNA), circulating tumor cells, and exosome vesicles, including exo-DNA [110, 117, 118]. Blood (plasma)-derived ctDNA in particular may represent an option for the identification and monitoring of *EGFR* mutations in patients with NSCLC [117], given the high rates of concordance with matched tumor samples when robust mutation testing methodologies are utilized in stringent research settings [11, 119–121], noting that this is not always the case in clinical practice [122]. Importantly, the presence of *EGFR*

mutations in ctDNA has been shown to predict response to EGFR TKIs [23, 119], with similar objective response rates and progression-free survival observed in patients *EGFR* mutation-positive by ctDNA sample versus tissue sample [119, 123].

Compared with conventional tissue biopsy, the liquid biopsy is minimally invasive, allowing for regular, repeated sampling, with a faster turnaround time compared with tissue biopsy [124]. Consequently, this allows for the possibility of early disease detection, along with real-time monitoring of disease progression, treatment response, or evolution of resistance — in some instances months before disease progression is clinically evident [110, 120, 125]. Crucially, liquid biopsy allows for the periodic assessment of tumor heterogeneity [86], provided that the chosen assay can detect somatic mutations, structural variants, and copy number changes. Despite these apparent benefits, the clinical application of ctDNA mutation testing methodologies has yet to be fully realized beyond use in settings with specific, approved, companion diagnostics, and lack of international standardization can limit the accuracy of outcomes [117]. The large, multicenter ASSESS and IGNITE diagnostic studies, which evaluated real-world *EGFR* mutation testing techniques across Europe, Asia, and Russia, observed great variation in testing methodologies, which subsequently impacted the mutation status concordance between ctDNA and matched tissue samples [11, 121]. It is further acknowledged that the robust and sensitive techniques specifically optimized for ctDNA mutation analysis may not be available in all

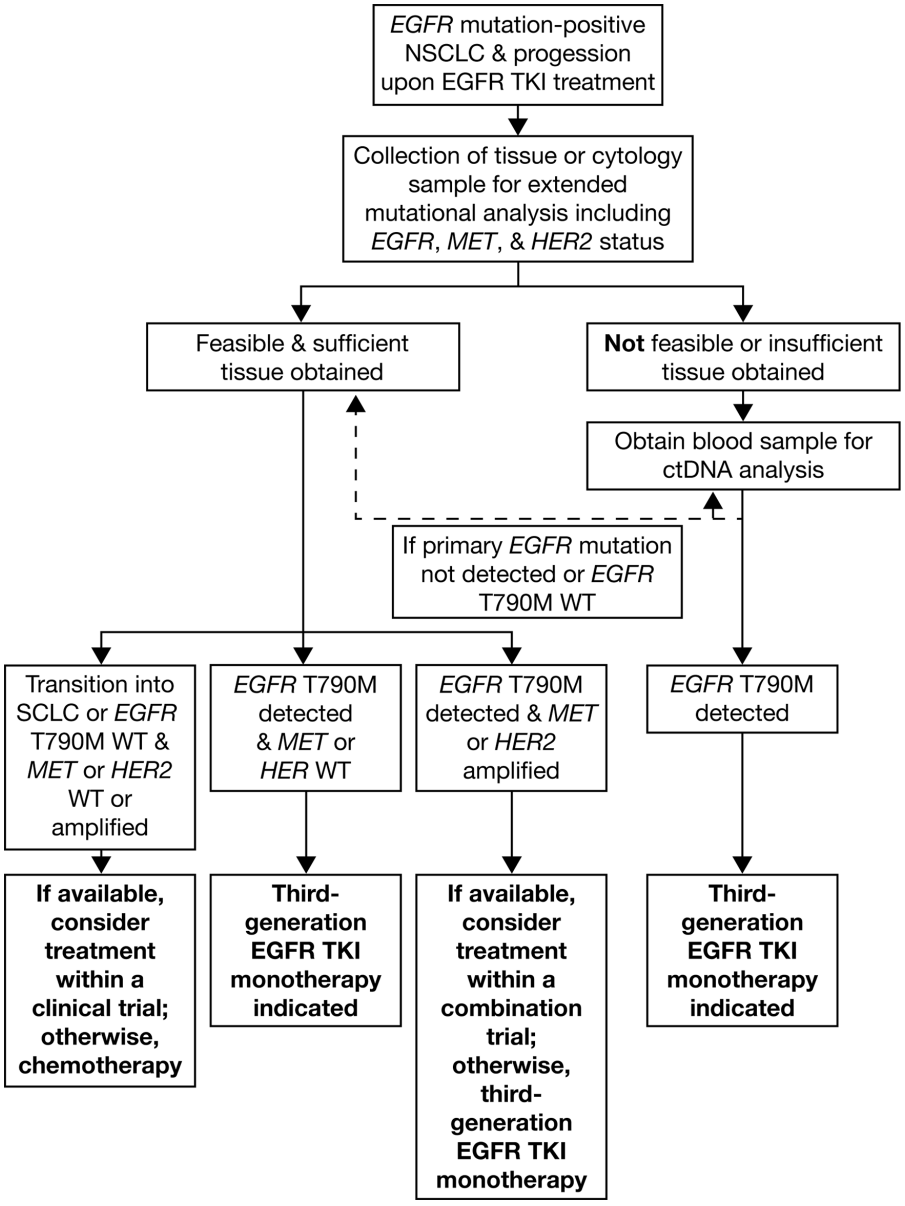

Figure 3: *EGFR* mutation testing algorithm. WT, wild-type.

laboratories, which is an important barrier to the adoption of these techniques into routine clinical practice [11, 110, 112]. Other disadvantages include the inability to detect morphologic changes within the tumor (i.e. transformation to another entity as a resistance mechanism) and the potential variability associated with pre-analytical preparation methodologies [126]. Furthermore, the amount of ctDNA available for analysis, when conducted, may not be sufficient to definitively rule out a specific mutation with, amongst other factors, the rate of tumor shedding impacting the fraction of mutant DNA in the bloodstream [126], and repeat testing may be necessary. Most importantly, single-gene diagnostic assays for liquid biopsy (for example, for *EGFR* mutations including T790M) do not provide information on other genetic mechanisms of resistance (e.g. amplification of *MET*). Thus, liquid biopsy molecular multiplex assays, such as next-generation sequencing, are needed to provide information comparable to that obtained from tissue biopsy. Technologies are under development (e.g. hybrid capture assays); however, sensitivity is not currently high enough to substitute tissue-based next-generation sequencing diagnostics.

Radiomics

Radiomics involves the post-processing and analysis of large amounts of quantitative imaging patient data for diagnostic, predictive, and prognostic modelling. Preliminary data in lung cancer have been reported [127]; however, findings were predominantly based on retrospective analysis. Given the very early stage of development, in depth discussion of radiomics is beyond the scope of this review.

EGFR mutation testing – what, when, and how?

What to test?

At diagnosis, the collection of a tumor sample is inevitable for histologic sub-classification, molecular analysis, and treatment choice. At disease progression, the preferred sample type for mutation analysis remains tumor tissue (or cytology) from a progressive lesion, where evaluable. However, ctDNA may be considered as an additional option to tumor tissue at progression, due to its less-invasive nature and the possibility of repeat testing allowing for assessment of disease heterogeneity that arises at progression. However, if a ctDNA sample yields an *EGFR* mutation-negative test result, contradictory to the initial biopsy, a new tissue sample should be obtained to confirm this [126]. This approach is limited, however, by the possibility that alternative mechanisms of resistance may not currently be detected in ctDNA analysis (i.e. protein-based biomarkers or SCLC transformation). In this context, it could be argued that tumor tissue testing is more appropriate at progression. As a consequence, guidelines [50], including the current German S3-Clinical Practice Guidelines (published summer 2017), call for tissue re-biopsy, wherever possible, with liquid biopsy an additional option.

When to test?

Mutation testing is advocated at diagnosis to confirm suitability for targeted therapies. As noted previously, progression can be defined in terms of a worsening of tumor burden or the emergence of secondary mutations that confer resistance to the ongoing therapy. Clinical progression is most commonly assessed in accordance with Response Evaluation Criteria in Solid Tumors (RECIST [128]). Whilst currently tumor re-testing is performed on the basis of suspected progression due to radiologic criteria, clinical indicators of progression often lag behind changes seen at the molecular level. Advances in clinical research that allow for the real-time analysis of molecular outcomes and the early detection of biomarkers associated with resistance may reconcile visible symptomatology with changes at the molecular level.

How to test?

EGFR mutation analysis methodologies for both tissue- and liquid-based testing include laboratory, in-house, and commercial technologies, and are discussed in detail elsewhere [129–131]. Optimal methodologies must be robust, sensitive, and tailored towards the relevant sample type. The sensitivity of the assay is particularly important for ctDNA, given that the amount of ctDNA that is present may be very low and highly fragmented and, consequently, methods used for tissue analysis may not be suitable or require adjustment. Sensitive methods include allele-specific polymerase chain reaction (PCR; e.g. RotorGene Kits [Qiagen], Cobas Kits [Roche], Amoy Kits [Zytomed]), droplet digital PCR (e.g. BioRad), next-generation sequencing, multiplex PCR, or hybridization-based methods (e.g. Qiagen, Illumina, Thermo Fisher, Multiplicom, Agilent) and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. Sanger sequencing or pyrosequencing are not suitable for ctDNA mutation analysis due to low sensitivity.

Experience with *de novo* T790M mutations (allele frequency tested in parallel)

Baseline T790M mutations have been detected in EGFR TKI-naïve patients, and in brain metastases, at low rates (<1%) using standard molecular analysis [31, 132]. Data obtained using more sensitive methodology (e.g. MALDI-TOF mass spectrometry, TaqMan quantitative PCR, amplification refractory mutation system) suggest that the prevalence of *de novo* mutations may be much higher (22–25%) [132, 133], although replication in larger patient samples is warranted before firm conclusions can be drawn. Regardless of prevalence, given that approved,

first-line EGFR TKI therapies have proven to be of limited value in these patients [31, 134], the possibility of such mutations should be considered in any diagnostic approach.

RESISTANCE TO "THIRD-GENERATION" TKIs

C797S as a mechanism of TKI resistance

As previously discussed, "third-generation" EGFR TKIs target the T790M mutation [63, 66, 135]. However, recent clinical findings have suggested the emergence of a tertiary acquired *EGFR* mutation *C797S* after treatment with a "third-generation" TKI [27, 30, 31, 80]. "Third-generation" TKIs rely in part on the formation of a covalent bond between the TKI and the 797-cysteine residue for their strong binding to the EGFR, but the mutation of the 797-cysteine residue to a serine (*C797S*) prevents such bond formation, compromising the TKI's efficacy and leading to subsequent resistance [96]. Combination strategies of first- and third-generation EGFR TKIs have been used to target C797S resistance to third-generation EGFR TKIs [136, 137]. Evidence supporting this approach comes from *in vitro* studies, which have shown that presence of T790M and C797S in trans (on different DNA strands), leads to resistance to third-generation EGFR TKI but a sensitivity to a combination therapy of first- and third-generation EGFR TKIs [138]. Recently, two case reports have shown that this combination EGFR TKI approach is effective in patients harbouring the T790M and C797S mutation in trans. However, these mutations can also occur in cis (on the same DNA strand), mediating treatment resistance prior to, or during, combination therapy. Thus, determining if the mutations are cis- or trans-allelic could be used to predict the outcome of this combinatorial therapy [136, 137].

Other mechanisms of resistance

It must be noted that patterns of resistance to "third-generation" TKIs may differ between settings, e.g. with or without the presence of a T790M mutation, and that the emergence of resistance to "third-generation" TKIs is not limited to the *EGFR* C797S mutation. It can occur via multiple mechanisms, including, but not limited to: *MAPK1* amplification, downregulation of negative regulators of ERK, *NRAS* mutation/amplification, and *KRAS* amplification [27]. Resistance to rociletinib and osimertinib, whilst not yet fully understood, is thought to recurrently involve MET, EGFR, PIK3CA, ERRB2, KRAS, and RB1 pathways, as well as the possibility of neuroendocrine transformation to SCLC [27, 86, 108].

Rationale for combination therapies

Given the prevalence of multiple resistance mechanisms, a single therapeutic agent may not be sufficient to treat a genetically heterogeneous and rapidly evolving tumor [35]. This has prompted combinatorial approaches to drug management that are tailored to the heterogeneity of the specific tumor [33] and which broadly fall into two categories: first-line combination therapies that delay the emergence of resistance, and later-line combinations for use after progression has occurred that directly target resistance. Treatment combinations currently under pre-clinical and clinical investigation are summarized in Table 2. Preliminary data appear promising but may be compromised by increased risk of toxicity [139].

FUTURE OUTLOOK

While the nature of acquired resistance mechanisms in NSCLC continues to evolve, we look to the future application of advances in our understanding in this area, in terms of clinical decision-making. Post-progression treatment should be tailored according to identified resistance mechanisms and clinical characteristics.

The identification of primary *EGFR*-sensitizing mutations at screening guides initial treatment approaches and is well established. However, the continued elucidation of the evolutionary mechanisms of acquired resistance in line with changes in individual tumor heterogeneity means that molecular targets are continuously changing, and therapeutic approaches must adapt accordingly. Whilst this remains a significant clinical challenge, advances in molecular profiling techniques, particularly the advent of liquid biopsy, may ultimately make possible real-time, holistic analysis of tumor behavior, thereby further informing appropriate treatment decisions.

As the range of molecularly targeted therapies broadens, it will be increasingly feasible to tailor treatment to the individual patient in response to changes in their genomic profile. Where possible, the immediate incorporation of emerging scientific approaches into current clinical practice will improve outcomes for patients with advanced NSCLC.

Author contributions

All authors made substantial contributions to the conception and development of this manuscript, including review of available literature, critical review of all drafts, and final approval of the version to be published. SM drafted the first version of the manuscript, and RB coordinated co-author review of all drafts and communication with the Journal.

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CONFLICTS OF INTEREST

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