Molecular biomarkers for lung adenocarcinoma

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ABSTRACT The identification of oncogenic driver alterations that underlie sensitivity to small inhibitors has led to growing interest in identifying additional targetable oncogenes in nonsmall cell lung cancer. Although the therapeutic impact of the discovery of these alterations has now been widely demonstrated, the epidemiological data associated with each of these biomarkers remain insufficiently studied. In this review, we discuss the techniques used to discover each of these candidate oncogenes, their prevalence in nonsmall cell lung cancer, and briefly outline the epidemiological features of the major oncogenes and ways in which their identification can determine therapeutic strategies.

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Introduction

Mechanisms of oncogenesis in lung cancer have been largely deciphered over the past 20 years. The concept of "oncogene addiction" refers to tumour-cell dependence on the specific activity of an activated or overexpressed oncogene. The main oncogenic drivers in the field of thoracic oncology are mutations of *EGFR*, *KRAS* and *ALK* rearrangements. They are most often reported in adenocarcinomas. However, new molecular targets have been highlighted recently: *i.e. BRAF* mutations, *HER2* and *PIK3CA*, and new translocations, such as *ROS1* and *RET*. Therapeutic strategies have been designed to inhibit these signalling pathways, among which are monoclonal antibodies and tyrosine-kinase inhibitors.

Tumours are now classified according to their molecular profile, which is itself associated with new demographic data. Lung adenocarcinoma can now be considered as a cluster of discrete molecular subtypes, the majority of which are defined by a single alteration to the oncogenic driver. Multiplex genotyping and high-throughput genomic profiling by next-generation sequencing (NGS) has been increasingly refining molecular diagnoses.

In this review, we discuss the techniques used to discover each of these candidate oncogenes, their prevalence in nonsmall cell lung cancer (NSCLC), their associated epidemiological features, and ways in which their identification can inform therapeutic strategies.

Methods of tumour sampling

There is a current paradox between the need to obtain significant samples for multiple analyses for a growing number of molecular biomarkers and the development of minimally invasive or noninvasive techniques, resulting in small tissue samples with very small amounts of DNA. Various techniques (described below) are used for the initial molecular profiling of DNA extracted from formalin-fixed and paraffin-embedded (FFPE) tumour samples.

Cytological samples are often considered insufficient for exhaustive molecular examination. Yet, recent data have demonstrated that very limited amounts of tissue can be sufficient for this type of analysis. Only 10 ng of DNA is sufficient to analyse 22 genes using NGS [1]. Likewise, LASER microdissection on 50 cells can facilitate the analysis of *EGFR* and *KRAS* using pyrosequencing [2]. Combining new sampling procedures with higher sensitivity tests helps the pulmonologist to obtain a molecular profile from various samples. The main advantages and pitfalls of each technique are reported in table 1.

Endobronchial ultrasound and transbronchial needle aspiration

Multiple studies have demonstrated the usefulness of cytological samples obtained by ultrasound-guided cytopuncture of lymph nodes through echobronchoscopy and molecular testing in NSCLC. In the study by Boulanger *et al.* [3], screens for *KRAS* and *EGFR* mutations were possible in 95.1% and 97.6% of cases, respectively (n=82). More recently, Casadio *et al.* [4] tested 306 samples of NSCLC for *EGFR* and *KRAS*

TABLE 1 Advantages and pitfalls of the different methods of cytological sampling for molecular profiling of nonsmall cell lung cancer

Sample	Diagnostic accuracy	Advantages	Pitfalls
EBUS-TBNA	Sensitivity >95%	Histologic diagnostic, staging and molecular profile at the same time	Relatively invasive
cfDNA	Good sensitivities >95% (*EGFR, KRAS, BRAF)*	Noninvasive Rapid, simple monitoring Early detection or acquired resistance (*EGFR* T790M)	Low number of mutated alleles among wild-type alleles
CTCs	Sensitivity 78% (*KRAS*) Sensitivity 92% (*EGFR*)	Noninvasive Possibility of: - Cytomorphological analysis - FISH (*ALK*) - ICC (*ALK*)	Expensive and laborious Lack of standardisation Multiplicity of methods
Pleural fluid	88% sensitivity	Possibility of multiplexed molecular testing if previously centrifuged	Contamination by haematopoietic cell DNA
Bronchoalveolar lavage	Sensitivity 16% (Sanger) to 81% (NGS)	Good sensitivity with NGS	Low number of tumour cells Poor sensitivity with conventional sequencing methods

EBUS-TBNA: endobronchial ultrasound transbronchial needle aspiration; cfDNA: circulating free DNA; CTCs: circulating tumour cells; FISH: fluorescence *in situ* hybridisation; ICC: immunocytochemistry; NGS: next-generation sequencing.

(Sanger and RT-PCR) and *ALK* rearrangements (FISH), with very good diagnostic accuracy (96.9% of samples were suitable for molecular profiling, with very similar results compared to tissue data). In this context, pyrosequencing and high-resolution melting produce equally excellent results: 97% and 93% of cases, respectively [5].

Transthoracic fine-needle aspiration

Scarpa *et al.* [1] demonstrated the feasibility of obtaining the mutational status of the main genes potentially involved in thoracic oncogenesis using just a small amount of DNA (10 ng) and NGS. In this study, 22 genes were examined in cytological samples obtained by transthoracic fine-needle aspiration. A mutation of at least one of these genes was found in 67% of patients, which included 28% *KRAS* mutations and 16% *EGFR* mutations.

Biological fluids

Bronchoalveolar lavage and bronchial-smear rinse fluid

Few studies have assessed the feasibility of molecular analysis on alveolar-lavage fluid. The PCR-denaturing gradient gel-electrophoresis (DGGE) technique offers low sensitivity (43%) and only 33% consistency with the primary tumour (n=36) for *KRAS* mutations [6]. In a recent study, sequencing cells collected by bronchoscopic brushing enabled mutations to be found in only one of the 77 patients sampled [7]. Among 36 positive bronchoalveolar lavages (0.3–9% tumour cells) from patients with a known *EGFR* mutation, the same mutation was detected in only 16% of cases using Sanger sequencing, but in 81% of cases using NGS. NGS could even detect an *EGFR* mutation in 42% of cytological samples without evidence of a tumour [8]. *EGFR, KRAS* and *ALK* statuses can also be established from endobronchial ultrasound (EBUS)-guided brushing of peripheral nodules with good diagnostic accuracy (95.2%) and concordance with other samples [9].

Pleural fluids

Testing for mutations in pleural fluid is also possible [2], but is complicated by the presence of DNA of haematopoietic cells. Prior centrifugation could facilitate this procedure, and would concentrate and isolate the tumour cells from leukocytes [10]. Multiplexed molecular testing (*EGFR, KRAS, BRAF, PIK3CA, NRAS, MEK1, AKT1, PTEN* and *HER2* mutations, and *ALK, ROS1* and *RET* fusion genes, by pyrosequencing and RT-PCR) was shown to be feasible in 102 pleural fluid samples from 84 Japanese patients with NSCLC. *EGFR* mutations were detected in 29% of patients and *ALK* rearrangement in 4%, with a concordance rate of 88% with FFPE samples [11].

Circulating tumour cells

Various methods allow the isolation of circulating tumour cells, among which Cellsearch, an indirect method based on cytokeratin staining, and ISET (isolation by size of epithelial tumour cells), a direct method based on cell size, are the most widely used. Beyond its prognostic value and the possibility of cytomorphological analysis, ISET can detect some molecular alterations in NSCLC, such as *EGFR* mutations by PCR [12], or *ALK* rearrangement by FISH and immunocytochemistry, with good concordance to primitive tissue [13].

One study reported the feasibility of detection of *KRAS* mutations using PCR in circulating tumour cells: 100 patients carrying a *KRAS* mutation on the primary tumour were included (32 colorectal cancers, 51 breast cancers and 17 lung cancers). The study reported 90.2% sensitivity and 94.9% specificity [14]. More recently, sensitivity of 78% for *KRAS* analysis was reported with COLD-PCR (co-amplification at lower denaturation temperature-PCR)/high-resolution melting for DNA extracted from circulating tumour cells isolated in a size-selected assay (ScreenCell) [15].

Circulating free DNA in plasma

Circulating tumour DNA (ctDNA) corresponds to the fragmented DNA released by tumour cells either actively or passively, during apoptosis or necrosis [16]. Cell-free DNA is increased in lung cancer patients [17]. A high level is correlated with a poor prognosis [18] and its variations during treatment could help monitor tumour burden [19]. Nevertheless, cell-free DNA has some limitations because of its low specificity, as normal cells or multiple inflammatory of infectious benign disorders can increase its level [20]. This problem can be avoided by targeting mutated tumour-specific DNA. Thus, detection of several genomic alterations, including those of *EGFR*, *BRAF* and *KRAS* in circulating DNA is possible with good diagnostic accuracy and excellent specificity and sensitivity, especially when using digital droplet PCR (ddPCR) [21–23].

We have recently shown that monitoring mutated ctDNA with ddPCR is a noninvasive way to follow-up the mutant clone in mutated NSCLC, as is evident with *BRAF*-mutated lung adenocarcinoma [24].

Another very exciting application is the early detection of the mechanism of resistance during targeted therapy. The detection of *EGFR*–T790M-mutated ctDNA, for example, precedes the radiological progression of *EGFR*-mutated lung adenocarcinoma [21]. NGS allows the detection of very small amounts (0.4%) of mutated DNA (*KRAS, EGFR, BRAF, etc.*), as well as other genomic alterations (*HER2* insertions, *cMET* amplifications and *ALK*, *ROS1* and *RET* rearrangements) in plasma with 100% specificity and sensitivity, similar to ddPCR (77%) [25].

Decision tree algorithm for tumour sampling technique

For initial genotyping, tissue should still be the first choice for molecular profiling of the tumour. A biopsy is indeed required for the histological diagnosis and could be used in the first instance. However, less invasive techniques are should be preferred when possible. Conventional bronchoscopic biopsies are preferred in cases of proximal endobronchial tumours and linear EBUS-TBNA is preferred for peribronchial tumours or mediastinal lymph nodes [3–5].

New tools have been developed to access distal tumours and avoid the complications associated with computed tomography (CT)-guided transthoracic biopsies (haemorrhage and pneumothorax) [26]. A radial EBUS miniprobe (rEBUS-MP) can be introduced through the involved bronchus after careful analysis of the CT scan. When the miniprobe reaches the tumour, the normal lung "snowstorm" appearance is replaced by focal circumferential hypo-echogenicity, and the probe is then replaced by biopsy forceps. A meta-analysis has reported a pooled sensitivity of 78% for nodules >20 mm and 56% for nodules <20 mm in diameter [27]. However, this procedure must be reserved for patients showing the bronchus sign on a CT scan [28].

Virtual bronchoscopic navigation uses a magnetic field, a magnetic sensor probe and three-dimensional integration to enable CT scan reconstruction and indicate bronchoscope position. The diagnostic yield of this technology is good (73.8%), even for lesions <20 mm in diameter (67.4%). This procedure is expansive and seems to be particularly suited for the upper lobes and peripheral third of the lung field [29].

However, if molecular characterisation cannot be assessed on these samples, a liquid biopsy is preferred to avoid a second invasive sampling procedure. Sequencing of ctDNA is the only technique approved for the analysis of *EGFR* mutational status and should be used as a first-line method in such circumstances. Its specificity is strong, facilitating the prescription of epidermal growth-factor receptor tyrosine kinase inhibitors (EGFR-TKIs) based on the results. However, the sensitivity of this method is imperfect [30] and a negative result must be confirmed by a tissue biopsy, which will enable wider genotyping including rearrangements (ALK, ROS1, *etc.*) [31].

At progression, a liquid biopsy constitutes the best choice to analyse mechanisms of resistance. In cases of EGFR-mutated tumours, ctDNA constitutes a very useful tool for detection of the T790M mutation, which occurs in more than half of all patients. The specificity of this technique is high. Furthermore, because of tumour heterogeneity and the improved detection sensitivity of the techniques used, the T790M mutation can even be detected in patients considered T790M-negative based on tissue analysis, making ctDNA a complementary tool. The US Food and Drug Administration (FDA) proposed a new paradigm for detection of the T790M mutation at progression. Plasma genotyping should be proposed as a first-line technique, as its high specificity facilitates the prescription of a third-generation EGFR-TKI. Nevertheless, its sensitivity is imperfect and a negative result must be confirmed by tissue analysis, which enables the detection of other mechanisms of resistance (small-cell carcinoma transformation, MET or HER2 amplification, PIK3CA mutations, *etc.*) [32].

The decision tree algorithm is presented in figure 1.

Methods used to detect molecular alterations

Different methods are available to identify the diverse somatic genomic aberrations that can be encountered in NSCLC (point mutations, insertions or deletions and gene rearrangement) obtained from FFPE samples or other available sources of material, such as cytology specimens or circulating DNA. Some technologies allow mutations to be screened and sequenced in the region of interest, whereas others analyse specific and known mutations.

Owing to the cellular heterogeneity of solid cancers, the primary technical challenge is the detection of somatic variants in tumour biopsies. Somatic mutations can be present in low numbers within an elevated background of wild type sequences, and more sensitive assays are therefore needed than those used for germline variants. Moreover, the challenge has been to develop more sensitive techniques for small biopsies and liquid biopsies. In particular, circulating mutant DNA represents only a very small fraction of the total circulating DNA [33]. Following the introduction of the first-generation capillary Sanger

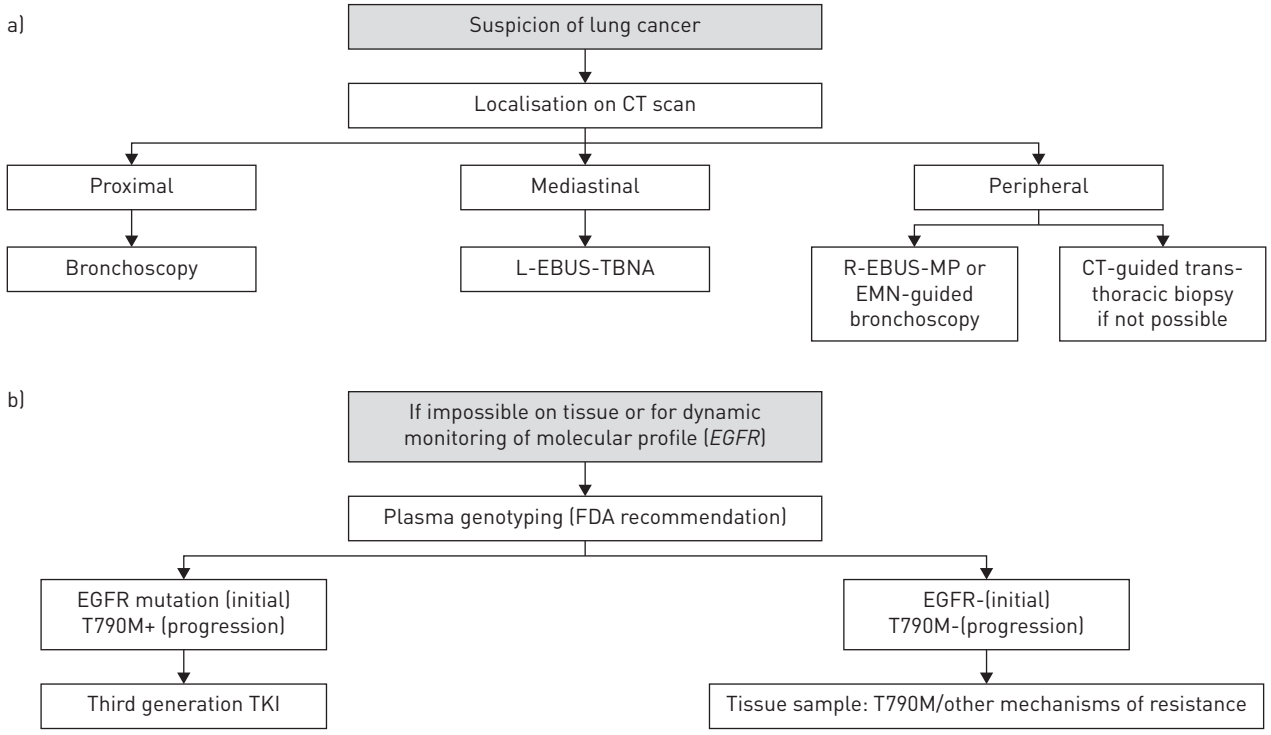

FIGURE 1 Decision tree algorithm for tumour sampling and molecular profiling at initial diagnosis (a) and during progression (b). CT: computed tomography; L-EBUS-TBNA: linear endobronchial ultrasound transbronchial needle aspiration; R-EBUS-MP: radial endobronchial ultrasound miniprobe; EMN: electromagnetic navigation; FDA: US Food and Drug Administration; EGFR: epidermal growth factor receptor; TKI: tyrosine kinase inhibitor.

sequencer (in 1995), increasing numbers of technologies have now been made available, including real-time PCR platforms, digital PCR and NGS.

Screening assays

Sanger sequencing

Until recently, Sanger sequencing was considered the gold standard for identification of all mutations. This technique uses sequencing according to termination performed on PCR products, which generates fluorescently labelled DNA fragments that are separated by size using capillary electrophoresis [34]. However, Sanger sequencing has very low sensitivity. The mutant variant should represent at least 15–20% of the total tumour DNA to be detected [35].

Pyrosequencing

Pyrosequencing is sequencing by synthesis, and it relies on the detection of pyrophosphate release during the incorporation of nucleotides into the DNA chain using bioluminescence through a series of enzymatic reactions. Pyrosequencing can identify individual bases or short stretches of nucleic-acid sequences at predetermined positions. The limit of detection of an allele is 5% [36].

High-resolution melting

High-resolution melting is used to screen for mutations in genes of interest prior to sequencing analysis. It is a post-PCR method based on the detection of small differences in PCR melting curve patterns, generated by the transition from a double to single strand in the presence of fluorescent dyes that intercalate with the double-stranded DNA and differentiate wild type sequences from homozygote or heterozygote variants. To improve the detection limit of this assay from a range of 2–10% to 0.1–1% of mutants in mixtures with wild type DNA co-amplification at lower denaturation temperatures, PCR (COLD-PCR) that allows preferential amplification of minority alleles could be combined with high-resolution melting [36].

Next-generation sequencing

NGS is a high-throughput sequencing method that detects somatic mutations in tumour samples at levels as low as 5%. Several chemical and analytical methods are available. The Illumina platform is based on the

preparation of DNA fragments using bridge PCR on a solid support to form clusters, which are then sequenced through repeated cycles on a single-base extension using a mixture of four fluorescently labelled nucleotides, and then imaged. In contrast, the ion-torrent platform relies on template preparation using emulsion PCR. The incorporation of a particular nucleotide is detected following the release of hydrogen ions.

The number of theranostic biomarkers and associated targeted therapies is rapidly increasing, and tissue samples can often be limited to very small amounts of DNA. NGS can rapidly evaluate the entire genomic landscape of the tumour simultaneously. At initial diagnosis NGS on tissue samples facilitates identification of the genotype of the tumour. Targeted panels including genes involved in the oncogenesis of lung cancer are preferred. A whole exome approach would significantly increase the turnaround, bioinformatic analysis and cost, as well as the risk of non-targetable genomic aberrations. When genotyping is not possible on tissue, plasma targeted NGS could be of great benefit [25] even if this approach has not yet been validated for routine use. The technique of circulating free DNA genotyping can overcome the dual problem of intra-tumour heterogeneity that exists between different regions of the same tumour (spatial heterogeneity), and between the primary tumour and local or distant recurrences in the same patient (temporal heterogeneity). NGS on plasma samples could also be an appealing technique for follow-up analysis of tumour biology under targeted therapy. If a targeted assay like ddPCR is the preferred method in EGFR-TKI resistant patients, in whom T790M resistance mutation is the main mechanism of resistance to monitor, a multiplex assay should be of great benefit in other TKI failure situations. For example, resistance in anaplastic lymphoma kinase (ALK) patients being treated with crizotinib tends to be more heterogeneous. Numerous ALK resistance mutations have been reported and the spectra of activity associated with different second and third-generation ALK tyrosine kinase inhibitors (ALK-TKIs) are not similar [37]. A plasma multiplexed NGS assay would thus be very useful in such circumstances.

Targeted assays

Most of the methods used to detect a specific mutation are based on multiplex real-time PCR using allele-specific PCR probes; however, they can only detect known mutations. In cases of lung adenocarcinoma, this targeted approach seems particularly suitable for detection of frequent and targetable alterations, like EGFR del19, L858R, KRAS G12X or EGFR T790M mutations.

Competitive allele-specific TaqMan® PCR

Competitive Allele-Specific TaqMan® PCR (castPCR™) technology (Life Technologies) combines allele-specific TaqMan® qPCR with allele-specific minor groove binder blockers to suppress nonspecific amplification from wild type alleles. CastPCR™ technology provides high sensitivity and specificity in the detection of rare mutant alleles among large quantities of wild type DNA (0.1%).

Therascreen® mutation kits

Therascreen® mutation kits (Qiagen) are real-time PCR assays that combine the amplification refractory mutation system (ARMS), which is an allele-specific amplification method, with Scorpion PCR primers, which are linked to a probe (a fluorophore and a quencher).

Cobas® kit

The Cobas® kit (Roche Molecular Systems) uses specific TaqMan probes (each with a different fluorescence intensity, generated by a fluorophore quencher pair) in real-time PCR to amplify and analyse mutations.

Peptide nucleic-acid clamp technology

Peptide nucleic-acid clamp technology consists of selective amplification of mutant alleles using specific peptide nucleic acids that suppress wild type sequence amplification.

BEAMing

BEAMing (beads, emulsion, amplification and magnetics) is a process based on emulsion digital PCR that binds DNA to magnetic beads, to facilitate separation and detection using flow cytometry [38].

Digital PCR

Digital PCR can detect and quantify very small amounts of mutant DNA, by partitioning a DNA sample into several thousand replicates. This partitioning facilitates the amplification and detection of single spots, which substantially increases sensitivity (0.005–0.01%) [39]. This method is thus particularly suitable for plasma genotyping and is now validated for the detection of common *EGFR* (deletion 19, L858R) and *KRAS* alterations (G12X, G13X) and for early detection of T790M in TKI-resistant patients [40]. Iterative

ddPCR quantitative analysis of the ctDNA could also be a useful tool to monitor the response to targeted or conventional treatments [21–24].

Molecular methods used to detect gene rearrangements

Fluorescence in situ *hybridisation*

Fluorescence *in situ* hybridisation (FISH) is the gold standard for detection of ALK or ROS1 rearrangements, but does not identify the fusion partner. This cytogenetic technique uses two small DNA strands coupled to fluorescent molecules that are complementary to the 5′ and 3′ ends of the gene. As observed under fluorescence microscopy, the two probes in wild type cells are very close together and their fluorescence can fuse; whereas in mutant cells, the two colours are well separated, or only one is detected. Several certified ALK and ROS1 break-apart fluorescence *in-situ* hybridisation assays are available [41]. FISH analysis is considered the gold standard for ALK NSCLC mutation testing. In 2011, the FDA approved the Abbot Vysis ALK Break Apart FISH Probe Kit for molecular diagnostic testing.

Immunohistochemistry

Immunohistochemistry (IHC) of ALK or ROS 1 assumes that the normal protein is absent; whereas the fusion proteins are overexpressed, as previously observed in anaplastic large-cell lymphoma [42]. In NSCLC, the ALK protein can be easily detected and some studies report comparable results between IHC and FISH for ALK rearrangements [42]. Moreover, IHC has been recommended for the detection of RET and ROS rearrangements [43].

Reverse transcriptase PCR

Multiplex reverse transcriptase PCR (RT-PCR) can discriminate between different fusion transcripts, but requires prior knowledge of the possible partners in order to design specific primer sets for each translocation [44, 45].

Biomarkers for targeted therapies

The incidence and characteristics of the main biomarkers of NSCLC are summarised in table 2. Their geographic distribution is presented in figure 2.

EGFR

Epidemiology

Epidermal growth-factor receptor (EGFR; erbB-1; HER1) belongs to the ERBB family of tyrosine-kinase receptors [53]. The *EGFR* gene is located on chromosome 7 (7p11.2) and encodes a 170 kDa transmembrane glycoprotein with tyrosine-kinase activity. Upon binding to a specific ligand, EGFR undergoes conformational

TABLE 2 Incidence and characteristics of the main biomarkers for nonsmall cell lung cancer

Target	Biology	Caucasian patients %	Approved treatments	Clinical trials
EGFR	Mutation	10–15	Gefitinib, erlotinib, afatinib osimertinib (second-line if T790M)	Rociletinib (second-line if T790M)
ALK	Translocation	3–5	Crizotinib	Ceritinib, alectinib, brigatinib, lorlatinib
BRAF	Mutation	2	NA	Vemurafenib, dabrafenib, dabrafenib+trametinib
ROS1	Translocation	1	Crizotinib	Ceritinib, Lorlatinib
HER2	Mutation	1	NA	Trastuzumab, afatinib, neratinib
KRAS	Mutation	20–25	NA	Trametinib, selumetinib, abemaciclib
PI3K	Mutation	2	NA	PI3K inhibitors, mTOR inhibitors
MET	Amplification mutation	2–5	NA	Crizotinib, INC280, tepotinib
RET	Translocation	1–2	NA	Cabozantinib, sorafenib, vandetanib

NA: not applicable.

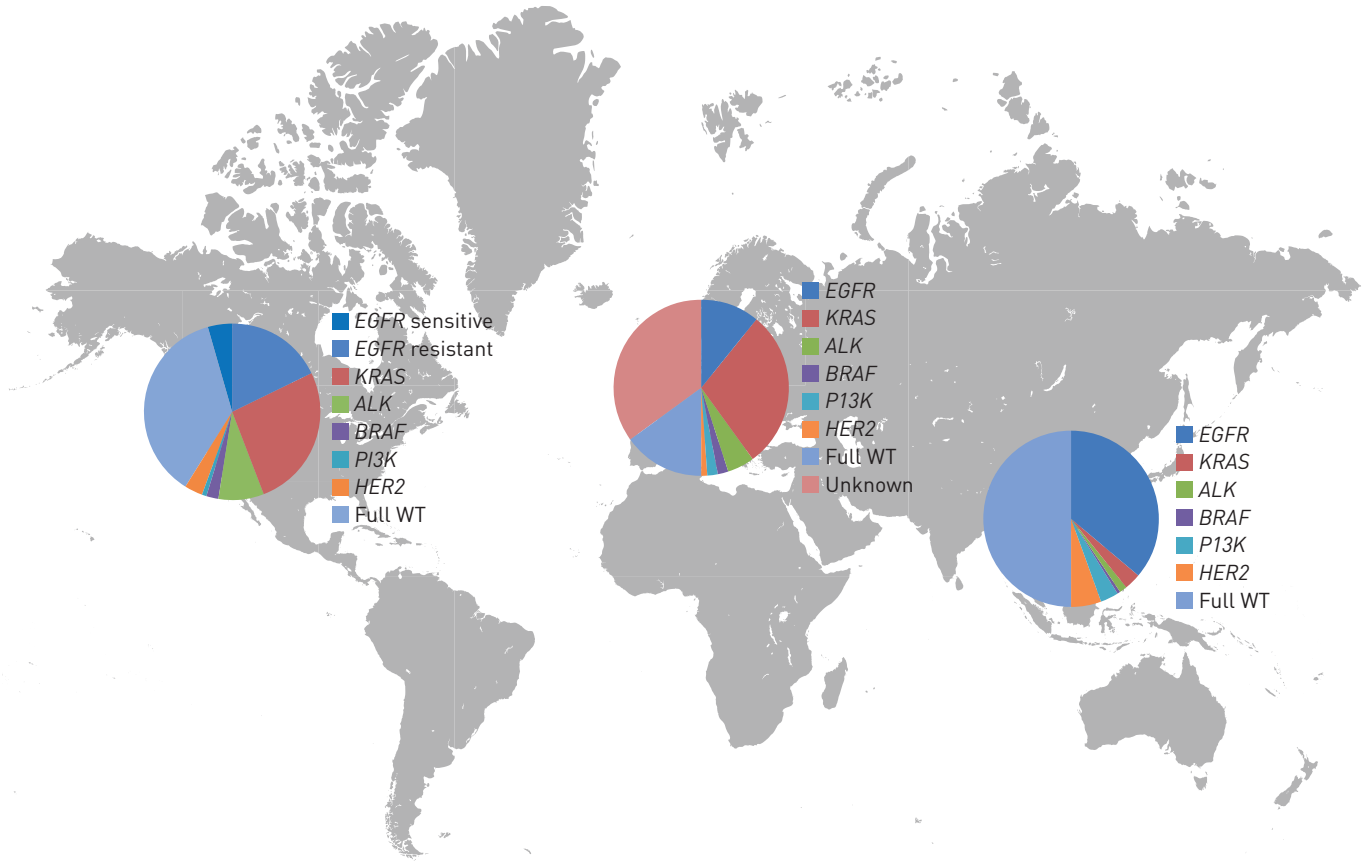

FIGURE 2 Molecular epidemiology of nonsmall cell lung cancer according to geographic origin. Asia: from Li *et al.* [46], Shan *et al.* [47] and Zhao *et al.* [48]. Europe: from Barlesi *et al.* [49], Mazieres *et al.* [50] and Mazieres *et al.* [51]. America: from Kris *et al.* [52].

change and phosphorylation, leading to activation of several pathways involved in cell survival and proliferation, such as PI3K/AKT, RAS/ERK and JAK/STAT, among others [54]. Since the 1980s, several clinical studies have reported increased levels of EGFR expression or increased gene-copy numbers in lung cancer, particularly in squamous-cell carcinoma, adenocarcinoma and large-cell carcinoma [55–58]. Although the prognostic association of EGFR is still discussed because of inconsistences in clinical reports [57–61], the development of selective inhibitors to EGFR tyrosine-kinase activity (*e.g.* gefitinib and erlotinib) has allowed the identification (in 2004) of specific mutations in the *EGFR* gene that are harboured in a subgroup of patients who present with a dramatic clinical response to these inhibitors [61–63]. Since 2004, much effort has been made to identify and characterise these mutations, and the large quantity of data thus generated now permits a relatively accurate overview of the prevalence of *EGFR* mutations among this population.

In NSCLC, the oncogenic mutations of *EGFR* are found within exons 18 to 21, which encode for part of the tyrosine-kinase domain located around the ATP-binding pocket of the enzyme, which is also the binding site of *EGFR*-TKi. The most common *EGFR* mutations are the in-frame deletions of exon 19 (mostly delE746-A750), which account for ~45% of all *EGFR* mutations, followed by L858R substitution in exon 21, found in 40–45% of all *EGFR* mutations [64]. For the remaining 10%, other rare *EGFR* mutations have been reported that include insertions in exons 19 and 20 (1% and 4%, respectively) and point mutations in exon 21 (L861, mostly L861Q: 1–2%) and exon 18 (mostly G719X: 3%) [65–68]. The choice of treatment might differ for patients harbouring common and rare mutations, as not all *EGFR* mutations confer the same degree of sensitivity to EGFR-TKIs (see below).

EGFR mutations are almost exclusively associated with adenocarcinoma or a bronchioloalveolar histological subtype, and are largely mutually exclusive with mutations in *KRAS* or *ALK* rearrangements (described below) [69]. They are more frequently observed in nonsmokers and women. The frequency of *EGFR* mutations is much higher in individuals of Asian origin (45–50%) than in individuals from Western Europe (10–15%) or North America (15–20%). It is noteworthy that high variability has been reported in the prevalence of *EGFR* mutations within the Asian-Pacific population, with a higher frequency in Vietnam (64.2%), Thailand (53.8%) and China (50.2%) than in India (22.2%) or Bangladesh (23%) [70, 71]. This variability has also been observed in South America, with a higher proportion of *EGFR* mutations among the

Amerindian subpopulation (principally Peru, 67%, but also Mexico, 31.2%, and Colombia, 24.8%) compared to Caucasian populations (Argentina, 19.3%) [72]. A world map representing *EGFR* mutation frequency in patients with NSCLC by country has been recently constructed from 151 studies worldwide [73].

Treatment

Targeted therapy remains the best strategy to treat lung cancer patients who harbour *EGFR*-activating mutations. Three drugs are currently available in the US and Europe (gefitinib, erlotinib and afatinib). Sensitivity to EGFR-TKIs seems to be highly dependent on the type of EGFR mutation. The best response rates (RR) are observed in patients harbouring the two main *EGFR* alterations (exon 19 deletions and the L858R mutation), with a higher susceptibility to treatment observed in patients with an exon 19 deletion (RR of 70–100% *versus* 20–67% for those with an L858R mutation) [65, 66, 74, 75]. Although rare *EGFR* mutations are often associated with poorer efficiency of EGFR-TKI as compared to common mutations, patients with L861Q or G719X mutations could benefit from this type of treatment, whereas insertions in exon 20 are more likely to confer resistance to TKI therapy [61, 74]. This was confirmed in a French multicentre study that analysed 10 117 samples from which 1047 (10%) were EGFR-mutated (102 with rare mutations) and exon 20 insertions were associated with EGFR-TKI resistance, whereas exon 18 mutations showed better sensitivity to treatment [66]. The choice of EGFR-TKI to treat patients with uncommon mutations could also be important; for instance, afatinib should be favoured in patients with G719X and L861Q mutations [64, 76]. For patients with very rare (other than G719X and L861Q) or complex mutations (with more than one mutation) in the *EGFR* gene, the efficiency of EGFR-TKI therapy has not been clearly demonstrated because of the small number of patients. However, the proportion of *EGFR* mutations that confers sensitivity to EGFR-TKI therapy seems to be greater than that which confers resistance [74, 77]. We believe that first-line EGFR-TKI therapy should be the most suitable strategy to treat patients with a specific *EGFR* mutation, unless the data indicate a clear disadvantage for targeted therapy in this particular context.

Despite a high global response rate (~60–70%), all patients usually relapse within a median delay of 12 months. In the majority of cases, this is due to the existence of the *T790M* gatekeeper mutation in 50–60% of patients, and much less frequently caused by amplification of *HER2* and the *MET* proto-oncogene, the epithelial to mesenchymal transition, or more rarely a small cell histologic transformation [78–81]. Several third-generation EGFR inhibitors have been recently developed (*e.g.* osimertinib and rociletinib) that show clinical efficacy in patients harbouring the *T790M* mutation [82, 83].

Osimertinib (AZD9291, Tagrisso) has recently received accelerated approval by the FDA for the treatment of patients with a confirmed *T790M* mutation. However, adaptive resistance mechanisms have already been described in a small subset of patients who relapsed after third-generation EGFR inhibitor treatment that included those with the *EGFR* C797S mutation [84]. Although the primary EGFR-activating mutation (mostly exon-19 deletion and a L858R point mutation) is usually conserved in resistant clones arising from erlotinib or gefitinib treatment, resistant clones derived from osimertinib- or rociletinib-treated *T790M*-positive tumours are rarely positive for the *T790M* mutation. This is because *T790M*-resistant tumours are often accompanied by T790M-negative resistant clones, which are likely to confer resistance to third-generation EGFR inhibitors [85]. This intra-tumoural heterogeneity is thus considered the Achilles heel of targeted therapy, and studying the origin of such clonal diversity could provide clues to promote more durable remissions.

ALK

ALK, a member of the insulin-receptor tyrosine-kinase family [86], is encoded by the *ALK* gene on chromosome 2. *ALK* was first identified as part of the NPM-ALK (nucleophosmin-anaplastic lymphoma kinase) oncogenic fusion protein that results from translocation between chromosomes 2 and 5 (t[2;5][p23;q35]) and is associated with anaplastic large-cell lymphoma [87]. The same translocation has also been described in Hodgkin's lymphoma [88]. Subsequently, a small inversion within chromosome 2p results in the formation of a fusion gene comprising portions of the echinoderm microtubule-associated protein-like 4 (*EML4*) gene and the *ALK* gene, which were identified in a resected adenocarcinoma specimen from a 62-year-old male smoker [89]. Several variants of *ALK-EML4* have been identified [90] and other fusion partners have been reported in NSCLC, including *KIF5B* [91].

ALK rearrangements occur in 3–5% of patients with NSCLC, and are more common among younger patients with a light smoking history, adenocarcinoma histology, and in tumours that are wild type *EGFR* and *KRAS* [92, 93]. These factors could help clinicians to identify high-risk populations that can undergo *ALK* testing, even if these criteria are not strong enough to select patients based on epidemiological characteristics. Consistent with these findings, the International Association for the Study of Lung Cancer

and the European Society for Medical Oncology guidelines both propose screening all patients with advanced-stage lung adenocarcinoma, irrespective of the clinical characteristics.

Crizotinib was the first drug to be approved for ALK+ NSCLC. It is an oral, small-molecule inhibitor that targets ALK, ROS1 and MET tyrosine kinases [94–96]. It has shown significant (~60%) overall response rates in a single-arm phase-I study [95], leading to its approval in the USA. The results of recent phase-III trials have confirmed the superiority of crizotinib compared to chemotherapy in second-line [97] and first-line [98] settings for *ALK*+ NSCLC. Unfortunately, the majority of patients also develop resistance within the first 12 months of therapy [99].

Developments in the understanding of the molecular biology and resistance mechanisms of *ALK*+ NSCLC have been made over the past few years. Alectinib and ceritinib, both second-generation ALK inhibitors, with higher intracranial activity than crizotinib, and used to treat leptomeningeal carcinomatosis, have recently gained approval as a form of therapy in patients who are refractory, or intolerant to crizotinib. Sequential use of ALK inhibitors has led to increased overall survival, with some *ALK*+ patients reaching an overall survival of 5 years.

Lorlatinib (PF06463922), a third-generation ALK/ROS1 inhibitor that can overcome certain resistant *ALK* mutations (with the exception of ALK L1198F), is now in a phase-II clinical trial that includes *ALK*+ and *ROS1*+ NSCLC patients. ALK I1171X and ALK F1174X resistance mutations have differential sensitivities to alectinib and ceritinib, which might determine which of the two inhibitors should be selected. Other resistant *ALK* mutations have been reported. In one case report, Shaw *et al.* [100] recently reported that C1156Y–L1198F mutations could confer resistance to lorlatinib, ceritinib, alectinib and brigatinib, but re-sensitise cells to crizotinib.

KRAS

Mutations in the *KRAS* gene (v-Ki-ras2 Kirsten rat sarcoma viral-oncogene homologue) are characterised by high frequency and specific epidemiology. Alterations to the *KRAS* gene are most frequently localised on codon 12 and more rarely, on codons 13 and 61 [101]. Alterations to the *KRAS* gene cause a loss of GTPase activity, and thus affect its feedback regulation. KRAS then continuously promotes cell proliferation *via* the MAP-kinase (BRAF/MEK/ERK) and phosphoinositide-3-kinase (PI3K) pathways [102]. G12C substitution is the most frequent alteration, representing 52% of all *KRAS* mutations. G12V, G12D, G12A and G12S mutations can be found in 11.6%, 7.2%, 2.9% and 1.5% of patients, respectively. In never-smokers who have adenocarcinoma, the G12D substitution is found in 50% of all cases [103, 104]. *KRAS* gene mutations can be found in 18–32% of adenocarcinomas, 12.8% of large-cell carcinomas, 10% of adenosquamous carcinomas and 1.6–7.1% of squamous-cell carcinomas in Caucasian patients [49, 105–108]. The association between *KRAS* mutations and tobacco is undeniable [108–110], and has been clearly identified in two meta-analyses [110, 111]. Nevertheless, *KRAS* mutations can also be found in 5–15% of nonsmokers [112–114]. *KRAS* mutations are more frequent in African-American patients compared to Caucasians (OR 2.4; p=0.048) [115], and are far less frequent in Asian patients, with an incidence of 11.2% [110].

KRAS gene mutations are usually described as an unfavourable biomarker in patients with resected pulmonary adenocarcinoma [116, 117], with a hazard ratio of 1.40 (n=5216, p=0.01) in a meta-analysis by Mascaux *et al.* [105]. Nevertheless, the prognostic implications of *KRAS* mutations are still under debate. In a study by Villaruz *et al.* [106], which focused on 318/988 cases of adenocarcinoma harbouring a *KRAS* mutation, the multivariate analysis (with adjustment for other prognostic factors, particularly smoking status) showed no prognostic impact of *KRAS*. In another recent study involving a cohort of 1935 patients, *KRAS* mutations appeared to correlate with a shorter overall survival, but did not influence progression-free survival (PFS) [111].

Contradictory findings are reported regarding the impact of different subtypes of substitutions; Ihle *et al.* [104] reported the adverse effects of G12V, whereas Cserepes *et al.* [109] observed better responses to chemotherapy and longer PFS in similar patients. No specific subtype of *KRAS* mutations showed any prognostic value in another study that evaluated adjuvant chemotherapy [118].

Previous retrospective studies also report detrimental effects [119–121], which seem to occur primarily in patients with a mutation located on codon 13 [118, 122]. Detection of a *KRAS* mutation in ctDNA is associated with a poor response to platinum-based chemotherapy [23]. Nevertheless, these results have not been consistently observed [122–124]. Because *KRAS* mutations cause constant activation of previously mentioned signalling pathways, independently of EGFR activation, the inefficiency of EGFR-TKI seems a logical inference. Most retrospective analyses are consistent with these results, with response rates usually <3% [125–130]. Nevertheless, a few clinical trials were unable to show any difference in survival [131, 132]. Unlike the case of colorectal cancer, *KRAS* mutational status does not affect the response to monoclonal anti-EGFR antibodies in pulmonary adenocarcinoma [133].

BRAF

After activation by the RAS protein, *BRAF* induces phosphorylation of two other kinase proteins, MEK and ERK. The activated form of ERK can translocate into the nucleus and phosphorylate transcription factors to regulate differentiation, proliferation, angiogenesis and apoptosis [134]. The majority of *BRAF* mutations localise to the kinase domain and increase kinase activity of *BRAF* toward MEK. *BRAF* mutations are found in 1.6% and 1.8% of cases of NSCLC in smokers and nonsmokers, respectively [49].

A thymidine to adenosine transversion at nucleotide T1799A at exon 15, which results in a valine to glutamate substitution at codon 600 (V600E), is the most common mutation, representing half of all cases. The other two most frequently observed mutations are the G469A mutation (39% of cases) and the D594G mutation (11% of cases) [135]. However, many of the non-V600E mutations show only intermediate and low kinase activity; thus, their roles as driver oncogenes remain unclear [136]. Among 916 NSCLC patients, *BRAF* mutations were identified in 1.9%, which comprised mostly adenocarcinomas (88%), female patients (64.7%) and smokers (70.6%) [137]. Retrospective analysis of 1046 patients revealed a prevalence of 4.9% and 0.3% in adenocarcinomas and squamous-cell carcinomas, respectively, with V600E representing 56.8% [138].

The prognostic impact of the *BRAF* mutation remains controversial in NSCLC. Paik *et al.* [135] and Cardarella *et al.* [139] demonstrated no significant impact of *BRAF* mutations on survival in advanced-stage patients. Nevertheless, the *BRAF* V600E subtype is associated with aggressive histology (micropapillary features in 80% of patients), and decreased disease-free survival and overall survival [139, 140]. Of the 35 patients receiving *BRAF*-targeted therapy (vemurafenib, dabrafenib or sorafenib) for *BRAF*-mutated lung adenocarcinoma in the European cohort, 83% had a V600E mutation, which appeared to be associated with a better prognosis than other subtypes of mutations (median survival 25.3 months *versus* 11.8 months, respectively) [141]. Another study on a broad series of 63 treated patients found a favourable prognosis of the V600 mutation compared to non-V600 mutations (3-year survival of 24% *versus* 0%, respectively). Smoking appeared to be more strongly associated with non-V600 mutations (11% of nonsmokers *versus* 42% of smokers with a V600 mutation) [142].

PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR signalling pathway has a diverse array of functions, including those of regulation of cellular survival, differentiation and display of stem-cell-like properties, growth, proliferation, metabolism, migration and angiogenesis [143, 144]. It plays a key role in downstream signalling by phosphorylation and activation of several effectors, including the serine/threonine kinase, AKT and mTOR (mammalian target of rapamycin), a master regulator of cell growth and metabolism. Signalling through the PI3K/AKT pathway is negatively regulated by the tumour-suppressor gene *PTEN* (phosphatase and tensin homologue) [145].

Although aberrations in the PI3K pathway are particularly prevalent in some cancers (including invasive breast cancer, head and neck cancer, colon cancer, cervical and ovarian cancer), they are rare in lung cancer. The most frequent alterations are mutations of *PIK3CA* and the loss of *PTEN* function (mutations or deletions). These alterations are more common in squamous-cell carcinoma (10–16% *PI3KCA* mutations, 15% loss of *PTEN*) than in adenocarcinoma (3–4% *PI3KCA* mutations, 3% loss of *PTEN*, frequently associated with *KRAS* or *EGFR* mutations) of the lung [146–149]. PI3K aberrations are associated with an unfavourable prognosis, higher burden of metastatic disease and higher incidence of brain metastases [150].

The effects of *PIK3CA* mutations on prognosis and response to standard therapies remain unknown. A retrospective study reported that a concomitant *PIK3CA* mutation resulted in decreased overall survival in *EGFR*-mutant lung adenocarcinoma (18 *versus* 33 months, p=0.006), although the authors found no evidence that a concurrent *PIK3CA* mutation affects the outcome of EGFR-TKI therapy [151–155].

HER2

Human epidermal growth factor 2 (HER2, erbB-2/neu) is a member of the erbB receptor tyrosine-kinase family. The *ERBB2* gene, which encodes HER2, is a major proliferative driver that activates downstream signalling through the PI3K-AKT and MEK-ERK pathways. No ligand has been described for this receptor, which is activated by homo-dimerisation or hetero-dimerisation with other members of the erbB family. *HER2* mutations consist of in-frame insertions into exon 20, leading to constitutive activation of the receptor and downstream AKT and MEK pathways. Inducible expression of a *HER2* mutant (HER2YVMA) in the lung epithelium of mice, resulted in the emergence of invasive adenosquamous carcinomas, with tumour maintenance requiring continuous expression of the driver, as evidenced in *EGFR*-driven cancer [156]. *HER2* mutations have been identified in about 2–4% of NSCLC patients [50, 157], but in less than 1% of the French national database [85].

This mutation is predominantly observed in females, nonsmokers and in the adenocarcinoma subtype, which is similar to *EGFR*-mutated NSCLC [50, 157]. *HER2* mutations could be more relevant to lung carcinogenesis than *HER2* amplification or overexpression, which reflects conditions that are opposite to those of breast cancer. Recent studies suggest that *HER2* mutations might be predictive for HER2-targeted therapies in lung cancer, such as HER2–TKI [158] and HER2-antibodies [159].

ROS1

The c-ros oncogene 1 (*ROS1*) is a relatively recent target for lung cancer. It encodes a tyrosine-kinase receptor from the insulin-receptor family. Chromosomal rearrangements involving the *ROS1* gene were originally described in glioblastomas, where *ROS1* (chromosome 6q22) was fused to the *FIG* gene and results have shown transformation in transgenic mice [160, 161]. In NSCLC cell lines and in primary tumours, *ROS1* fusion has been identified as a driver mutation [162]. *ROS1* fusion partners include *SLC34A2*–, *CD74*–, *TPM3*–, *SDC4*–, *EZR*–, *LRIG3*, *KDELR2* and *CCDC6* [163]. *ROS1* rearrangement occurs in 1–2% of NSCLCs [94]. The kinase domain is always fully retained on the ROS1 fusion protein, and the junction point at the mRNA level always occurs at the 5′ end of exons 32–36 [164]. The ROS1-kinase domain has significant homology with the ALK-kinase domain. *ROS1*-positive patients share similar characteristics with *ALK*-positive patients, such as adenocarcinoma histology, histomorphology, younger age and a high prevalence of nonsmokers [94]. Four new fusion partners (CLTC, LIMA1, MSN and TMEM106B) of ROS1 were identified in 2014 and 2015, and the incidence and clinicopathologic characteristics of *ROS1*+ NSCLC patients have been described in a comprehensive meta-analysis [165]. Crizotinib is associated with a very good response rate and PFS [51, 166]. Other drugs are currently being evaluated.

Emerging molecular targets

The *RET* gene is a proto-oncogene [167, 168]. Oncogenic activation can occur *via* its mutation or rearrangement. In nonsmall cell lung cancers, *RET* rearrangements occur in 1–2% of unselected cases. These are commonly found in adenocarcinomas of never-smokers [169]. In contrast to thyroid cancer, in which *CCDC6* and *NCOA4* are more common upstream partner genes, *KIF5B* is the most common upstream fusion partner of *RET* in NSCLC [170]. Cabozantinib and vandetanib showed efficiency in patients with *RET*-rearranged lung cancers in three phase II trials [171–173]. Other RET inhibitors, including cabozantinib, vandetanib, sorafenib and lenvatinib that are approved for the treatment of advanced thyroid cancers, and ponatinib, alectinib and sunitinib that are approved for other indications, have not been yet tested in clinical trials.

MET exon 14 mutations represent other new target molecular alterations in 3% of non-squamous NSCLC. These mutations are more likely detected in nonsmokers, and the response to MET inhibitors must be assessed in clinical trials, as a few case reports and limited series have demonstrated favourable outcomes [174, 175].

Conclusion

In conclusion, the discovery of many theranostic molecular biomarkers in NSCLC has greatly changed the classification of lung cancer, and thus its management. The epidemiology, impacts on prognosis and responses to conventional and targeted treatments are very varied, depending on genomic alteration.

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