The evolving genomic classification of lung cancer

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Abstract

EGFR gene mutations and *ALK* gene fusions are well-characterized molecular targets in NSCLC. Activating alterations in a variety of potential oncogenic driver genes have also been identified in NSCLC, including *ROS1*, *RET*, *MET*, *HER2*, and *BRAF*. Together with *EGFR* and *ALK*, these mutations account for ∼20% of NSCLCs. The identification of these oncogenic drivers has led to the design of rationally targeted therapies that have produced superior clinical outcomes in tumours harbouring these mutations. Many patients, however, have *de novo* or acquired resistance to these therapies. In addition, most NSCLCs are genetically complex tumours harbouring multiple potential activating events. For these patients, disease subsets are likely to be defined by combination strategies involving a number of targeted agents. These targets include FGFR1, PTEN, MET, MEK, PD-1/PD-L1, and NaPi2b. In light of the myriad new biomarkers and targeted agents, multiplex testing strategies will be invaluable in identifying the appropriate patients for each therapy and enabling targeted agents to be channelled to the patients most likely to gain benefit. The challenge now is how best to interpret the results of these genomic tests, in the context of other clinical data, to optimize treatment choices in NSCLC.
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Introduction

Historically, lung cancers have been sub-divided by histology into small-cell and non-small-cell lung cancers (NSCLCs) [1], with NSCLC further classified into squamous cell carcinoma (SCC), large-cell carcinoma, and adenocarcinoma. More than half of all lung cancers are adenocarcinomas [2]. While treatment advances have been made with the use of platinum-based chemotherapy [3], lung cancer remains the most frequent cause of cancer-related mortality worldwide [4] and has a 5-year overall survival (OS) rate of just 16% for all stages [5].

Crucial to enhancing outcomes for patients with lung cancer is the ability to build a detailed profile of the disease, to guide treatment decisions and to enable the development of more effective therapeutic strategies. The last decade has seen a shift to a more molecular-based classification, in which information about genetic alterations and protein expression level is considered alongside histology in order to better understand the pathogenesis of the disease [6,7].

In NSCLC, multiple genetic alterations have already been identified as therapeutic targets, including mutations of the epidermal growth factor receptor (*EGFR*) gene and rearrangements of the anaplastic lymphoma kinase (*ALK*) gene. Drugs designed specifically as inhibitors of these molecular targets have significantly extended the survival times for patients with NSCLC whose tumours harbour these mutations [8–14].

As novel molecular targets are discovered, and ultimately new therapies developed, we may edge ever closer to a personalized treatment approach in NSCLC and further extend survival for patients. Within this article, we review recently identified molecular targets in NSCLC, new genetic techniques for classifying the disease, and the implications of these findings for clinical practice and future clinical trial design.

Novel molecular targets in NSCLC

Superior clinical outcomes have been achieved in NSCLC by treating molecularly selected groups of

patients with rationally targeted therapies (ie drugs directed against activated oncogenes, such as *EGFR* or *ALK* gene-fusion products) as opposed to the modest benefits achieved in unselected patients [15]. However, *de novo* or acquired resistance often develops, driving the search for novel targets and treatment mechanisms. In addition, *EGFR* and *ALK* alterations account for only a small minority of NSCLC cases, and both alterations occur predominantly in adenocarcinomas from non-smokers [11,16]. At present, the community does not have an answer for patients who have or will get lung cancer as a result of exposure to tobacco carcinogens.

Modern treatment strategies focus on the pathological classification of NSCLC, which includes assessment of protein expression by immunohistochemistry (IHC) to assess cell differentiation markers such as TTF1 and p63 (the splice variant p40), as well as the detection of molecular predictive markers, including validated driver mutations in genes involved in cell growth and survival. A variety of novel driver mutations or molecular targets have recently been identified in NSCLC (Figure 1 and Table 1). Here, we review some of these key targets (and interventions), including known oncogenic drivers (*EGFR*, *ALK*, *ROS1*, and *RET*), non-driver targets [MET, fibroblast growth factor receptor 1 (FGFR1), PTEN, and phosphatidylinositol 3-kinase (PI3K)], immunotherapies [programmed death ligand 1 (PD-L1/PD-1)], and antibody–drug conjugates (ADCs; NaPi2b). Mutations of a number of other important molecular targets identified in NSCLC, such as HER2, BRAF, and MEK1 (Table 1), have been described in detail elsewhere [2,15,17] and

will not therefore form the focus of this review. In addition, the *RAS* oncogenes (*KRAS* and *NRAS*) have been excluded from this review, as we are not aware of any molecules in clinical development that directly inhibit RAS.

Oncogenic drivers

For the purposes of this review, a target was considered an oncogenic driver if it is genetically activated in NSCLC and if there is an approved inhibitor (clinically validated target) or convincing proof-of-concept data (high response rates in a targeted population or a positive randomized phase II trial).

EGFR

Mutations of the *EGFR* gene are a well-established example of an oncogenic driver in NSCLC. *EGFR* activating mutations are present in ∼10% of NSCLCs in Caucasians and ∼40% in Asian patients, and are primarily seen in adenocarcinomas [18]. In prospective phase III trials, patients with previously untreated *EGFR* mutation-positive NSCLC achieved significantly longer progression-free survival (PFS) with the reversible EGFR tyrosine-kinase inhibitors (TKIs) erlotinib and gefitinib than with platinum-doublet chemotherapy [8,12,14]. Erlotinib has been approved by the FDA for the first-line treatment of patients with *EGFR* activating mutation-positive NSCLC detected by the approved cobas® *EGFR* Mutation Test. Several other platforms (mostly sequencing assays) are
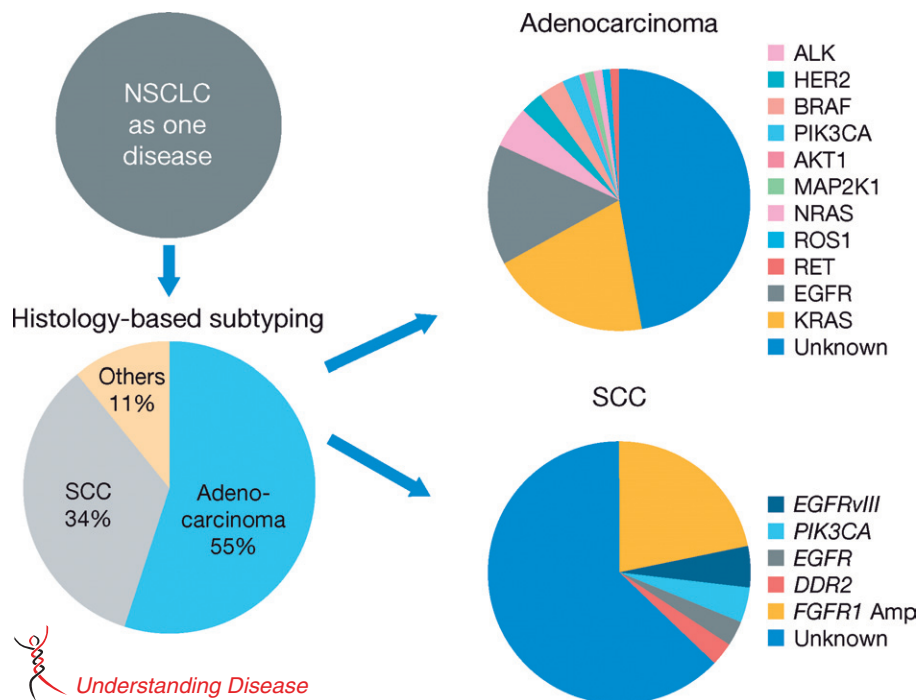

Figure 1. Evolving genomic classification of NSCLC. Li T *et al*: *J Clin Oncol* 2013; **31**: 1039–1049. Reprinted with permission. © 2013 by American Society of Clinical Oncology. All rights reserved [17].

Table 1. Current molecular targets in adenocarcinoma

Target	Prevalence (%)	Therapeutic agents
EGFR	Asians ~40 Caucasians ~10	Erlotinib, gefitinib, afatinib
ALK	< 5	Crizotinib
HER2	< 3	Afatinib, neratinib, dacomitinib
PIK3CA	< 5	GDC-0941, XL-147, BKM120
BRAF	< 5	Vemurafenib, GSK2118436
MEK	~1	AZD6244
ROS1	~2	Crizotinib
RET	~2	Sunitinib, sorafenib, vandetanib, cabozantinib
MET	1–11	Onartuzumab, rilotumumab, cabozantinib, tivantinib, crizotinib
FGFR1	~3	AZD4547, S49076, ponatinib, brivanib
PTEN	< 10	Vandetanib
PD-1/PD-L1	~30	Nivolumab, MPDL3280A
NaPi2b	~70	DNIB0600A (early development)

ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; FGFR1, fibroblast growth factor receptor 1; PD-L1, interaction of programmed death ligand 1; PIK3CA, phosphatidylinositol 3-kinase, catalytic subunit alpha.

used to study *EGFR* mutations in DNA extracted from tumour tissue specimens. Gefitinib is also approved as monotherapy for *EGFR* mutation-positive NSCLC following failure of platinum- and docetaxel-based chemotherapy.

The second-generation irreversible EGFR TKI afatinib recently gained FDA approval as first-line therapy for *EGFR* mutation-positive NSCLC in conjunction with Qiagen's *therascreen* RGQ polymerase chain reaction (PCR) diagnostic test. Another second-generation irreversible EGFR TKI, dacomitinib, demonstrated preclinical efficacy in NSCLC tumours harbouring the T790M gatekeeper mutation [19,20], which is present in ~50% of NSCLCs that have acquired resistance to erlotinib or gefitinib [21,22]. In a randomized phase II study, dacomitinib demonstrated significantly improved PFS versus erlotinib in patients with advanced NSCLC [23]. A phase III study of dacomitinib versus erlotinib as second-/third-line therapy for advanced NSCLC is currently underway (NCT01360554) [24].

ALK

Rearrangements of the *ALK* gene are another recent example of oncogenic drivers in NSCLC. ALK is a transmembrane tyrosine-kinase receptor expressed in the small intestine, testes, and brain, but not normally in the lung. In NSCLC, ALK signalling is activated by the creation of oncogenic fusions of the *ALK* gene with an upstream partner, *EML4* [25], although other fusion partners exist [26]. *EML4–ALK* rearrangements occur in 2–7% of NSCLC patients [11,27], usually in young never-smokers with adenocarcinoma [28–31]. *ALK*-rearranged tumours are resistant to the EGFR TKIs gefitinib and erlotinib [28].

The first-in-class ALK inhibitor crizotinib was approved by the FDA for the treatment of *ALK*-positive advanced NSCLC, with the concurrent approval of a companion fluorescence *in situ* hybridization (FISH) diagnostic test, based on impressive results in phase I/II trials. In the subsequent phase III trial, second-line crizotinib demonstrated superior PFS and response rates to chemotherapy alone in patients with locally advanced or metastatic *ALK*-positive NSCLC [13]. A first-line phase III trial of crizotinib in newly diagnosed *ALK*-positive NSCLC is currently recruiting patients (NCT01154140). Results of a recent study confirm that *ALK* rearrangements in lung adenocarcinoma can also be effectively detected using IHC for ALK expression in malignant cells [32].

ROS1

ROS1 is a tyrosine-kinase receptor of the insulin receptor family. *ROS1* gene rearrangements are known oncogenic drivers in NSCLC, and several fusion partners have been identified, including CD74, SLC34A2/NaPi2b, and FIG [33,34]. *ROS1* fusions are present in ~2% of NSCLC cases and are often seen in young never-smokers with adenocarcinoma, a population similar to those with *ALK*-rearranged NSCLC [33]. *ROS1* rearrangements rarely present simultaneously with *EGFR*, *ALK* or *KRAS* alterations [35].

Crizotinib has shown inhibitory growth effects on *ROS1*-positive cell lines, and a near-complete response was reported in a patient with advanced *ROS1*-positive NSCLC treated with crizotinib in a phase I clinical trial [33]. In an expansion cohort of the trial, 14 patients received crizotinib for *ROS1*-rearranged NSCLC (as tested by FISH) and nine (64%) had a confirmed response [36]. A further case of a complete metabolic response to crizotinib was reported in a patient with advanced *ROS1*-positive NSCLC [37]. A ROS1 monoclonal antibody (D4D6) has recently been developed and validated for use in IHC assays [34].

RET

The tyrosine-kinase receptor RET is involved in cell proliferation, migration, and differentiation. A novel fusion oncogene between the *RET* gene and *KIF5B* was recently described in a young never-smoker with adenocarcinoma and no family history of lung cancer [38,39]. Fusions between the *RET* gene and *CCDC6* have since been identified [40]. *RET* fusions are known to occur in ~2% of lung adenocarcinomas [38], are usually independent of other oncogenic drivers [35], and can be targeted with TKIs such as sunitinib, sorafenib, vandetanib, and cabozantinib [15,41]. Preliminary data have been published for the first three patients with *RET* fusion-positive NSCLC enrolled in a phase II trial of cabozantinib; confirmed partial responses occurred in two patients (one with a novel *TRIM33–RET* fusion), with prolonged disease stabilization (31 weeks) in the third patient [42].

Other targets

MET

Binding of the hepatocyte growth factor (HGF) to the transmembrane tyrosine-kinase receptor MET activates multiple signalling pathways involved in cell proliferation, survival, motility, and invasion [43]. Dysregulation of the MET/HGF pathway can occur via several mechanisms and is observed in many human malignancies, including NSCLC [43]. Mutations in *MET* are rare, but high *MET* gene copy number has been detected in 1–11% of NSCLC cases and is often associated with high MET protein expression and poor prognosis [44–46]. *MET* amplifications have also been linked with secondary resistance to EGFR TKIs in patients with *EGFR* mutation-positive NSCLC [47,48]; *MET* amplifications can be found in up to 20% of these patients [45].

A number of therapeutic agents targeting the MET/HGF pathway are in clinical development, including small molecule MET inhibitors (eg cabozantinib), specific MET TKIs (eg crizotinib), antagonistic antibodies against MET (eg onartuzumab), and neutralizing antibodies against HGF (eg rilotumumab). Although tivantinib was initially believed to be a MET inhibitor, several recent reports suggest that, at least preclinically, tivantinib does not appear to inhibit MET signalling [49,50].

Onartuzumab is a humanized monovalent (one-armed) monoclonal antibody that binds to the extracellular domain of MET to prevent HGF binding and activation [51,52]. In a randomized phase II trial, onartuzumab plus erlotinib improved PFS and OS versus placebo plus erlotinib in patients with tumours pre-defined as MET-positive by IHC ($\geq$ 50% of tumour cells expressing moderate-to-strong staining intensity) [53]. Clinical outcomes were worse in MET-negative patients treated with onartuzumab plus erlotinib, exemplifying the need for parallel diagnostic testing in drug development [54]. A randomized phase III study is investigating the combination of onartuzumab and erlotinib in patients with MET-positive advanced or metastatic NSCLC (NCT01456325). The MET IHC assay is being developed as a companion diagnostic for onartuzumab within this study, based on the 50% cut-off used in the phase II trial [54].

FGFR1

FGFR1 is a membrane-bound tyrosine-kinase receptor involved in the regulation of cell proliferation and angiogenesis [15]. *FGFR1* amplification occurs more frequently in SCC (21%) than in adenocarcinoma (3%) [55]. Activation of *FGFR2* and *FGFR3* has also been reported in NSCLC cell lines treated with EGFR inhibitors [56]. Recently, high *FGFR1* gene copy number was reported to be an independent favourable prognostic factor in NSCLC [57].

Several small molecule FGFR TKIs are currently under clinical investigation, including AZD4547, a selective inhibitor of FGFR1/2/3 [58,59], and S49076, an ATP-competitive TKI of MET, AXL, and FGFR1/2/3, which demonstrated marked anti-tumour activity in MET- and FGFR-dependent tumour xenografts [56]. In addition, the novel FGFR inhibitor ponatinib suppressed the growth of NSCLC cells overexpressing FGFR1, and significantly inhibited the growth of primary lung cancer cultures *in vitro*, suggesting that ponatinib may also be effective in patients whose tumours overexpress FGFR1 [60].

PTEN

Many cancers are associated with deletions or mutations of the *PTEN* tumour suppressor gene, which plays a significant role in cell cycle progression, apoptosis, growth, proliferation, and migration via negative control of the PI3K/Akt pathway [61]. *PTEN* mutations [62] and loss of PTEN protein expression are relatively common in SCC of the lung [63]. However, in many cases, the functional consequences of *PTEN* mutations remain to be elucidated. PTEN loss has also been linked with acquired resistance to EGFR TKIs in *EGFR* mutation-positive NSCLC [18]. In a meta-analysis of mutation incidence in NSCLC, *PTEN* mutations were influenced by ethnicity, with a higher frequency amongst Asian patients with SCC (9.8%) versus adenocarcinoma (1.6%), and in western patients with adenocarcinoma (6.0%) versus SCC (0%) [64]. The TKI vandetanib has shown efficacy against *EGFR* mutation-positive lung cancer cell lines showing loss of PTEN, suggesting that it may also be effective in patients with *EGFR* mutation-positive NSCLC whose tumours lack PTEN expression [65].

PI3K

PI3Ks are lipid kinases involved in the regulation of cell growth, proliferation, and survival. Mutations in the *PIK3CA* gene that encodes the catalytic subunit of PI3Kα have been identified in several cancers [66]. Furthermore, aberrant signalling through the PI3K/Akt/mTOR pathway has been observed in a number of human cancers, including NSCLC [67]. *PIK3CA* mutations occur in less than 5% of NSCLCs [15] and often co-exist with other oncogenic mutations, particularly *EGFR*, *KRAS* or *ALK* [68]. PTEN loss is thought to be a marker for PI3K dependency in some tumours [69].

Several PI3K inhibitors are in clinical development (eg GDC-0941, BKM120, BEZ235, XL-147, XL-765, perifosine), but the response rate to single agents has been low [2,70]. Ongoing phase II trials in lung cancer are examining combinations of PI3K inhibitors and chemotherapy (NCT01297491) or targeted agents (NCT01493843, NCT01487265). In human NSCLC lines, the novel PI3K inhibitor imidazopyridine demonstrated anti-proliferative effects, including the induction of apoptosis, in a dose-dependent manner [71].

Immunotherapies

PD-L1/PD-1

Interaction of PD-L1 with the PD-1 and B7.1 receptor on activated T cells plays a key role in tumour evasion of the host immune system [72–75]. Whether PD-L1 is overexpressed in solid tumours and associated with increased tumour aggressiveness remains unclear. However, high levels of PD-L1 were recently reported in patients with sarcomatoid lung cancer, a rare, high-grade, poorly differentiated form of NSCLC [76]. Furthermore, in a 5-year follow-up study in patients with NSCLC, PD-L1 was a significant independent poor prognostic factor, with PD-L1-positive patients having a shorter 5-year OS than PD-L1-negative patients [77].

Nivolumab (BMS-936558), an anti-PD-1 mono-clonal antibody, has shown anti-tumour activity in a phase I clinical trial in patients with advanced solid tumours, including NSCLC [78,79]. Of 129 patients with NSCLC treated with nivolumab, 22 (17.1%) achieved an objective response (13 with non-squamous cell histology and nine with squamous cell histology) and 13 patients (10.1%) had disease stabilization for at least 24 weeks [78]. The 24-week PFS rate was 33% [79]. Patients are currently being enrolled in a phase II study of nivolumab in SCC (NCT01721759) and a phase III study in non-SCC (NCT01673867).

MPDL3280A, an engineered anti-PD-L1 monoclonal antibody, has also demonstrated anti-tumour activity in a phase I NSCLC clinical trial. MPDL3280A treatment was associated with a 22% objective response rate in 41 heavily pretreated NSCLC patients, with 12% of patients having disease stabilization for at least 24 weeks [80]. A correlation between PD-L1 status and efficacy was reported: 4/5 patients (80%) with PD-L1-positive tumours achieved an objective response and 0/4 had disease progression, while 4/28 patients (14%) with PD-L1-negative tumours had an objective response and 15/26 experienced disease progression [80]. The 24-week PFS rate was 46% [80]. Clinical studies of MPDL3280A in PD-L1-selected or unselected locally advanced or metastatic NSCLC are currently open for recruitment (NCT01846416, NCT01903993, NCT01375842).

MK-3475, a humanized monoclonal antibody against PD-1, has not presented any clinical data in the setting of NSCLC at the time of this publication. Clinical studies of MK-3475 are currently recruiting patients with NSCLC (NCT01295827, NCT01840579, NCT01905657).

ADCs

ADCs are a new class of targeted therapy being investigated for use against a number of cancers. They consist of a monoclonal antibody, or antibody fragment, against a known molecular target that is stably linked to an active cytotoxic drug. Due to their targeted nature, ADCs offer the potential for fewer side effects than standard chemotherapies.

NaPi2b

The human sodium-dependent phosphate transporter NaPi2b is encoded by the *SLC34A2* gene and belongs to the type II family of sodium-dependent phosphate transporters [81]. In normal tissue, NaPi2b is expressed at the intestinal brush border membrane and plays a role in the synthesis of surfactant in lung alveoli. Immunohistochemical analysis of NaPi2b using the monoclonal antibody MX35 revealed heterogeneous NaPi2b expression in lung cancer tissues, ranging from no immunoreactivity to 100% positively stained cells [82]. An ADC targeting NaPi2b (DNIB0600A) is currently in clinical development, with preliminary evidence of anti-tumour activity in NSCLC; of the 16 patients with NSCLC enrolled in the phase I trial, 70% expressed high levels of NaPi2b by IHC [83].

The impact of molecular testing on lung cancer management

The identification and characterization of molecular targets are having a growing impact on the management of patients with lung cancer. Several clinical practice guidelines, including those published by the National Comprehensive Cancer Network (NCCN), the American Society of Clinical Oncology (ASCO), and the International Association for the Study of Lung Cancer (IASLC)/College of American Pathologists (CAP)/Association of Molecular Pathology (AMP), now recommend that all patients with NSCLC containing an adenocarcinoma component undergo biomarker testing for *EGFR* mutations and *ALK* rearrangements [6,84,85]. The French National Cancer Institute, in collaboration with the French Ministry of Health, recently introduced a programme of free molecular diagnostic testing for all patients with solid tumours, which includes testing for *EGFR* mutations and *EML4–ALK* rearrangements in lung cancer [86]. Not only has this mandate granted equal access to molecular tests and appropriate targeted therapies for all patients across France, but it has also reduced the unnecessary treatment of unselected patients.

Across the USA, the Lung Cancer Mutation Consortium (LCMC), a collaborative approach between 16 academic centres, is actively promoting molecular mutation testing in lung cancer in order to match patients to optimal treatment strategies [87]. The LCMC is conducting an observational study with the aim of genotyping ten driver mutations in tumour specimens from 1000 patients with advanced lung adenocarcinoma (NCT01014286); results of *EGFR* testing are passed to treating physicians, while patients with other driver mutations are offered enrolment into LCMC-linked clinical trials of various targeted agents

[88]. Preliminary reports from the first LCMC study confirm the presence of several key target genes, including *KRAS* mutations (24%), *EGFR* mutations (20%), and *ALK* rearrangements (8%) [88–90].

New techniques in the genomic classification of lung cancer

The landmark studies that led to the approval of the first targeted agents in NSCLC used gene-based molecular tests that were focused on single biomarkers. However, with the advent of many more potential molecular targets, and the challenges associated with obtaining tissue from patients with late-stage NSCLC, there is a growing need to develop and utilize molecular technologies that can determine the expression or mutation status of several genes simultaneously, so-called multiplex testing, in order to obtain the maximum diagnostic information from the limited tumour tissue available (Figure 2).

Predictive and prognostic gene signatures

A number of research groups have developed predictive and prognostic gene signatures in surgically resected lung cancer [91–93]. However, the use of these signatures in clinical practice is often hampered by issues such as reproducibility, cost, and limited availability, as well as lack of validation [92]. Using samples from the JBR.10 clinical trial, Zhu *et al* developed a 15-gene expression signature that demonstrated the potential to select patients with stage IB/II NSCLC most likely to benefit from adjuvant chemotherapy with cisplatin/vinorelbine [91]. Kratz *et al* developed a prognostic gene signature that was able to identify patients with early-stage, non-squamous NSCLC at high risk for mortality after surgical resection [92]. The 14-gene mRNA expression assay was based on quantitative PCR using formalin-fixed, paraffin-embedded (FFPE) tissue samples and improved prognostic accuracy beyond NCCN criteria for stage I high-risk tumours ($p < 0.0001$). Blinded and independent validation of the assay was confirmed in a cohort of 433 patients from the USA and in a larger sample of 1006 patients from China [92].

More recently, Tang *et al* developed an 18-gene prognostic signature in resectable NSCLC, which was then integrated with genome-wide functional data and genetic aberration data to derive a 12-gene predictive signature for survival benefits with adjuvant chemotherapy [93]. The prognostic signature predicted the prognosis of patients with adenocarcinoma in all validation datasets across four microarray platforms, including Illumina® (Illumina Inc, San Diego, CA, USA), Affymetrix® (Affymetrix, Santa Clara, CA, USA), and Agilent® (Agilent Technologies Inc, Santa Clara, CA, USA). The predictive signature was successfully validated in two independent datasets in 266 patients. Prospective clinical trials are needed to further
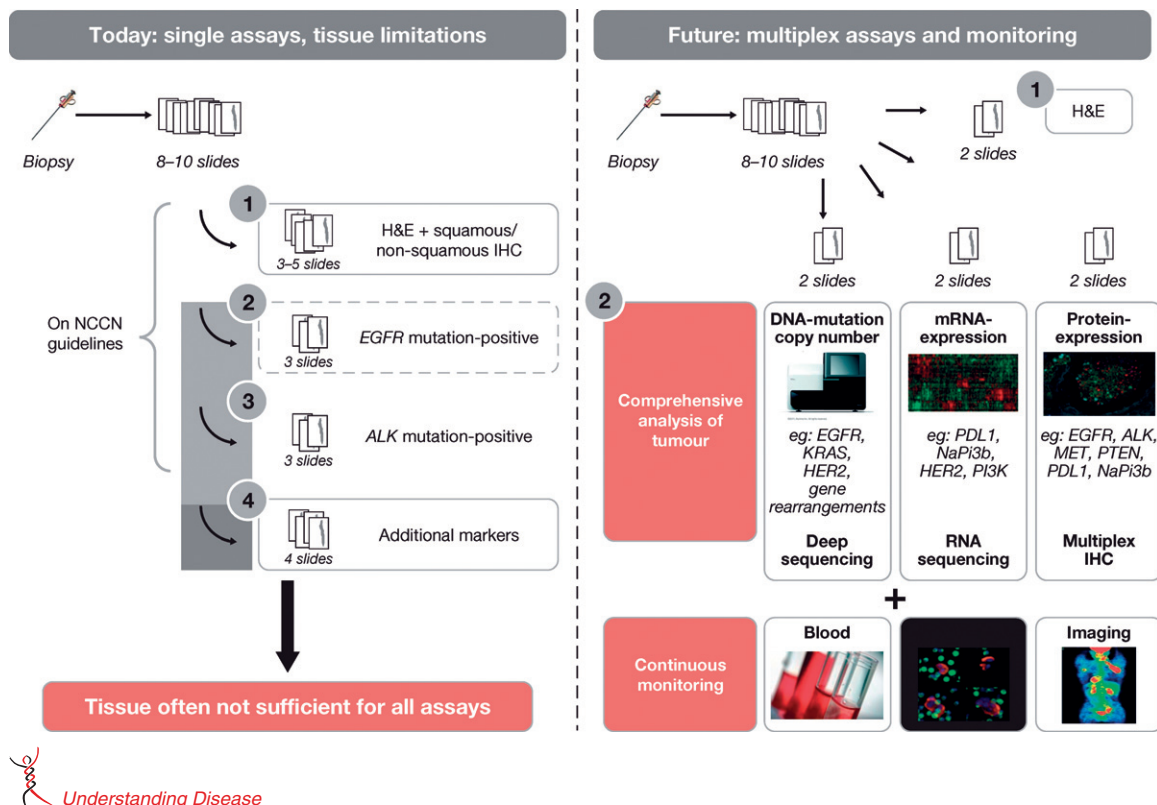

Figure 2. Lung diagnostic testing today and in the future.

validate the use of prognostic and predictive signatures in lung cancer [93,94].

Multiplex PCR (mutation detection and gene expression)

Multiplex PCR involves the simultaneous amplification of two or more cDNA/DNA targets in a single reaction vessel with uniquely labelled probes for each target [95]. A number of multiplexed PCR-based assays are available, including SNaPshot® (Applied Biosystems, Foster City, CA, USA), which detects hotspot mutation sites in key cancer genes using fluorescently-labelled primer extension products [96], and Sequenom MassARRAY® (Sequenom Inc, San Diego, CA, USA), which analyses primer extension products using mass spectrometry [97]. A high-throughput microfluidics method (Fluidigm, South San Francisco, CA, USA) has been developed for mutation detection [mutation multi-analyte panel (MUT-MAP)] based on quantitative PCR, which includes ~120 hotspot mutations and works effectively with less than 100 ng of FFPE tissue [98].

Similar assays and formats are widely used in the cancer research community and are starting to be applied in clinical trials. For example, the LCMC is predominantly utilizing the SNaPshot® and Sequenom MassARRAY® platforms, together with the FDA-approved FISH test for *ALK* gene rearrangement in their ongoing genotyping trial. Multiplex PCR has the advantage of needing only a small sample of tumour compared with conventional tests, but it is restricted to codons previously determined as mutation hotspots, and is unable to detect chromosomal rearrangements or determine gene copy number [70].

Next–generation sequencing (NGS)

High-throughput NGS technology has been commercially available since 2004 and offers the ability to analyse DNA, mRNA, transcription factor regions, and DNA methylation patterns throughout the entire genome [99]. Several NGS platforms are available, including Illumina® HiSeq 2500 (Illumina Inc, San Diego, CA, USA), SOLID™4 System (Applied Biosystems, Foster City, CA, USA), and Ion Torrent™ (Applied Biosystems, Foster City, CA, USA) [17,99]. NGS has been applied to clinical settings in almost all tumour types and is being used as a research tool, as well as to screen patients for clinical trial enrolment. NGS can detect chromosomal rearrangements and gene copy number alterations at a very high resolution [70]. Indeed, identification of the *KIF5B–RET* fusion was made possible through the application of NGS [38].

NGS has huge potential over traditional sequencing techniques; however, currently each platform requires a specific investment in computational analysis and bioinformatic support to produce and interpret the data, and the assays are expensive and often cumbersome [99]. Over the next few years, the cost and complexity of NGS-based testing will continue to decrease rapidly and testing is likely to become even more widespread. In the USA, NGS-based clinical assays are already being offered as laboratory-developed tests (LDTs) in several Clinical Laboratory Improvement Amendments (CLIA)-approved laboratories (eg Foundation Medicine and laboratories in academic institutions). However, there are currently no NGS-based FDA-approved companion diagnostic tests. Given the high-resolution data that NGS can provide, traditional prospective clinical validation can be challenging, particularly for rare genomic alterations. Overcoming this challenge, and/or refining the definition of prospective clinical validation, will require the cooperation of clinical scientists, regulatory authorities, and payers. Collaboration between institutions and patient referral centres are necessary to identify these rare patients, as are innovative clinical trial designs, as described later. NGS-based companion diagnostics will also require the flexibility to be updated as additional clinical information emerges. As the cost of NGS-based tests continues to drop to the point where whole genome sequencing becomes routine clinical practice, the test update required may simply be a software update that changes the clinical report to include, for example, detection of a newly validated rare *EGFR* mutation or *ROS1* fusion partner.

Clearly, other important platforms for identifying molecular targets, such as FISH (as exemplified with crizotinib and *ALK* rearrangements) and IHC (as exemplified with onartuzumab and MET expression), should be considered alongside these newer techniques. The key for clinicians and pathologists, therefore, will be to determine the optimal method for molecularly classifying lung cancers moving forward.

Challenges of widespread genetic testing

The merits of molecular testing in lung cancer are clear; however, there are a number of challenges to overcome in the widespread use of these tests. Firstly, the community will need to come to some consensus as to what an actionable test result might be. A valid test result can mean very different things depending on the technology, bioinformatics pipeline, and what the investigator or treating physician perceives to be clinically relevant, and there are obvious potential dangers in this. Additionally, the quantity, quality, and type of tumour tissue available for testing vary extensively between different centres and countries. One of the greatest challenges is obtaining adequate tumour samples for all genomic tests, while avoiding contamination with normal and necrotic cells, in a minimally invasive manner [70]. Substrates derived

from peripheral tissues, such as circulating tumour cells and circulating tumour DNA, are less invasive alternatives to surgical or biopsy specimens and have yielded comparable results in molecular tests [100], although further research in this field is required [70]. Intratumour heterogeneity can result in a mixed response to a molecularly targeted agent in different tumour sites, and throughout the course of successive treatment lines, due to alterations in the genetic make-up of the tumour as the disease progresses or in response to therapy [17]. Such heterogeneity may represent a major treatment challenge if the therapy choice is based on genomic analysis of a single tumour biopsy sample at a specific time point [101]. Thus, serial biopsy or cytology sampling during the course of the disease may provide a more accurate genomic analysis of the tumour (Figure 3).

Another challenge with genomic testing will be for clinicians to decide which of the genomic data is of relevance to an individual patient's treatment choice [102]. Importantly, in cases where a patient has more than one activating alteration, the physician will need to decide which lesion to treat first. An additional consideration will be the time it takes to perform the tests – should physicians start treating a patient with the current standard of care for unselected patients while testing is taking place, and then switch the patient once a positive result is identified? What decisions should be made in the event that a patient has a mutation in a gene for which there is no currently approved therapy in NSCLC, but where a targeted treatment is approved in other indications? At a minimum, a multidisciplinary team approach will be required to accurately interpret the results of the tests, and central to this will be engaging patients to help them realize the importance of molecular testing in the first instance [17]. Several institutions have implemented multidisciplinary molecular tumour boards to discuss the management of patients whose lung tumours harbour rare genetic abnormalities with no validated targeted therapy available.

The costs of widespread genetic testing will also come into question, in terms of the cost–benefit ratio of the newer platforms versus the more conventional tests, and the challenges faced by diagnostic laboratories in keeping up with the costs of buying new equipment and validating new assays as the sequencing platforms continually evolve [103]. Finally, as the current regulatory environment does not allow for the rapid adoption of new technology, we may face unavoidable delays in the implementation of genomic testing and, ultimately, the optimization of treatment for patients with lung cancer.

Implications for clinical trial design

Lung cancer is a competitive landscape with many new drugs in development and several failed phase III clinical trials. To reduce the risk of further clinical trial

failure, genomic testing of NSCLCs must be included alongside histological testing, such that candidate patients can be identified and treatment choices optimized. For example, non-mucinous bronchoalveolar carcinoma (BAC) is now classified as lepidic predominant adenocarcinoma (100% TTF1, ~45% *EGFR* mutation-positive, 5% *BRAF* mutation-positive), while mucinous BAC is classified as mucinous invasive carcinoma (15% TTF1, ~80–100% *KRAS* mutation-positive, 0% *EGFR* mutation-positive) [7,104]. The potential impact of targeting different molecular targets or histologies within the context of historical 'all-comer' studies is illustrated in Figure 4; not only do the specific targeted subsets need to be considered in terms of their particular prognostic behaviour (*EGFR* mutation-positive patients do much better on both chemotherapy and EGFR TKIs than *EGFR* wild-type patients), but the impact of removing these patients from the remaining pool should also be considered if spurious interpretations of trial data are to be avoided.

Furthermore, it is likely that most patients with NSCLC will test positive for at least two potential molecular targets when IHC and genetic tests are considered together. Thus, there is a real need to understand how these molecular targets fit into current and future treatment algorithms, especially for patients with multiple biomarkers.

Earlier clinical trials were not designed adequately for the testing of multiple molecular targets, but rather restricted enrolment to patients with a known single mutation. Evaluating biomarker combinations or overlap (ie between mutations, gene expression, and IHC) could inform rational drug combinations or sequencing trials in the future. Ongoing programmes, such as the LCMC study and the MD Anderson BATTLE trials, are already utilizing novel designs to evaluate multiple targeted therapies in NSCLC [70,106]. Careful ethical consideration must also be given to the design of control arms in clinical trials of biomarker-selected patients. From the patients' and treating physicians' perspective, strong arguments can be made to permit crossover in biomarker enabled trials, such that patients whose tumours have the relevant biomarker can gain benefit from the targeted agent at some point; however, from regulatory and payer perspectives, similarly strong arguments are made to prevent crossover, to demonstrate differences in OS. In addition, recently obtained tumour samples should be used, rather than archival tissue from surgical resection, as the tumour profile can change considerably over time, and trial enrolment should be based on the current disease profile, rather than that at the initial diagnosis.

Conclusions

A deeper understanding of the molecular classification of lung cancer may ultimately lead to personalized treatment strategies, which will improve care for
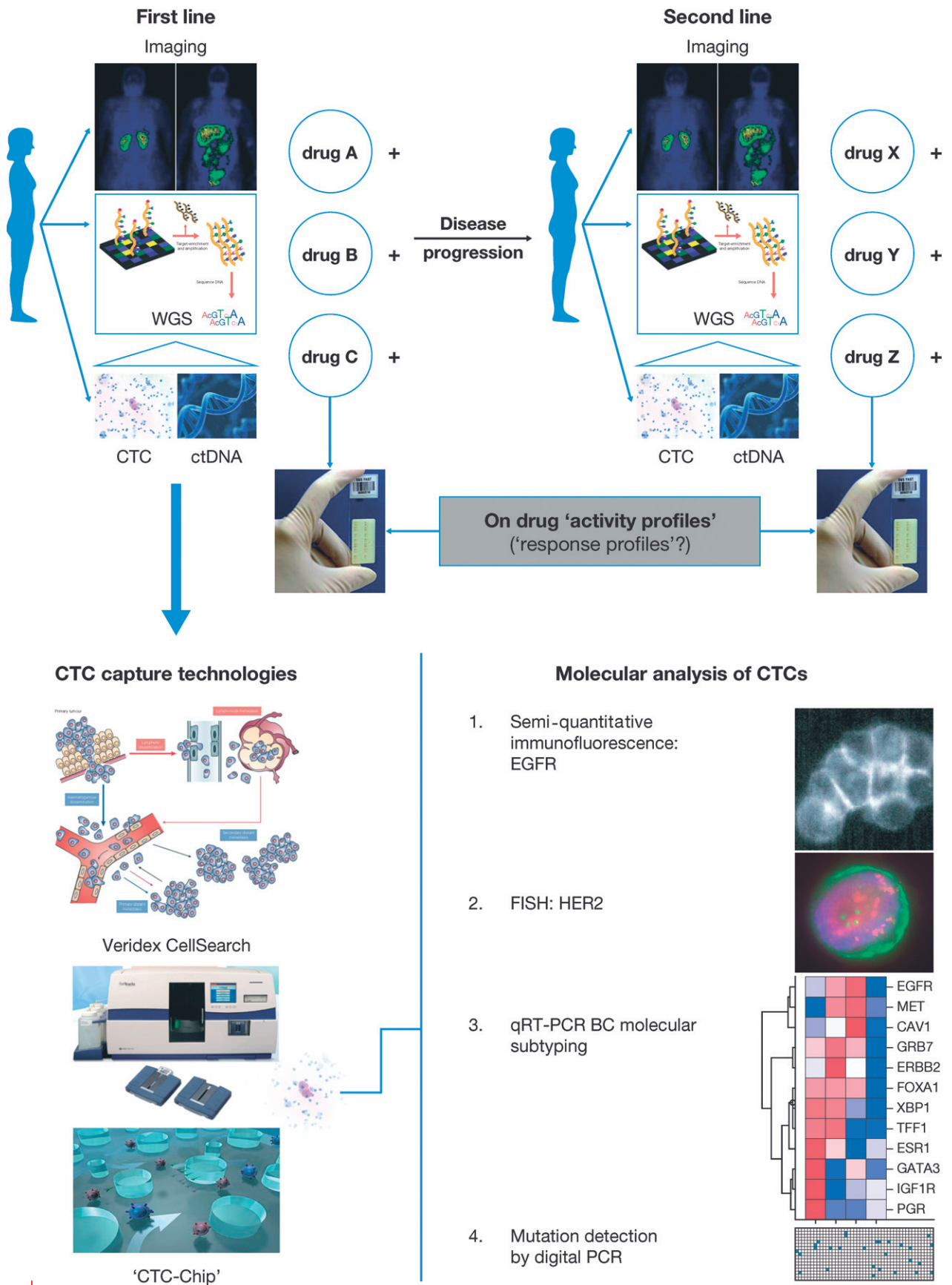
Figure 3. The future of oncology testing in NSCLC. BC, breast cancer; CTCs, circulating tumour cells; qRT, real-time reverse transcription; WGS, whole genome sequencing.
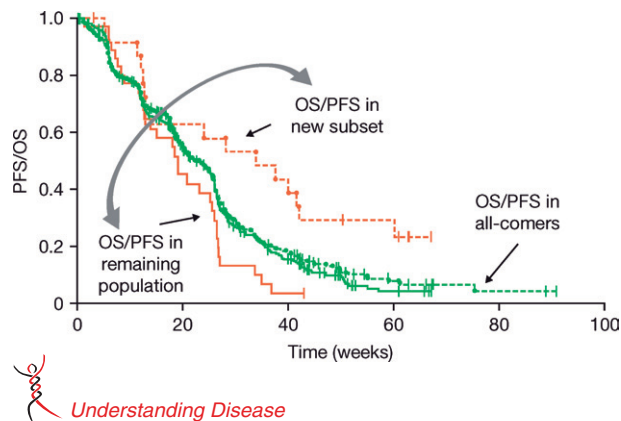
Figure 4. Hypothetical effect on OS/PFS in prognostic/predictive subsets and remaining 'all-comer' patients. Modified from ref 105.

those patients most likely to benefit, and spare the cost and morbidity associated with failed treatment interventions. Multiplex PCR assays, high-throughput technologies such as NGS, and hopefully some form of multiplex protein-based platform will play an important role in lung carcinoma management and rational therapy selection, but there are many challenges ahead. Careful design of clinical trials will help to evaluate molecularly targeted agents in the context of those populations most likely to benefit, but clinicians will be faced with difficult decisions, such as how to include an ethically fair control arm, what treatment to choose when a new patient subset is no longer part of the first-line population, and what the preferential order of treatment should be where multiple molecular targets are present. Only through a better understanding of the disease can treatment choices be enhanced and the outlook for patients with lung cancer improved.

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Author contribution statement

DSS and IIW conceived and wrote this manuscript.

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