Lung cancer 2

Challenges in molecular testing in non-small-cell lung cancer patients with advanced disease

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Summary

Lung cancer diagnostics have progressed greatly in the previous decade. Development of molecular testing to identify an increasing number of potentially clinically actionable genetic variants, using smaller samples obtained via minimally invasive techniques, is a huge challenge. Tumour heterogeneity and cancer evolution in response to therapy means that repeat biopsies or circulating biomarkers are likely to be increasingly useful to adapt treatment as resistance develops. We highlight some of the current challenges faced in clinical practice for molecular testing of EGFR, ALK, and new biomarkers such as PDL1. Implementation of next generation sequencing platforms for molecular diagnostics in non-small-cell lung cancer is increasingly common, allowing testing of multiple genetic variants from a single sample. The use of next generation sequencing to recruit for molecularly stratified clinical trials is discussed in the context of the UK Stratified Medicine Programme and The UK National Lung Matrix Trial.

Introduction

Lung cancer is the most common cause of cancer mortality in the UK, accounting for one in five of all cancer deaths,[1] and global incidence of around 1·83 million cases in 2012. However, a century ago, lung cancer diagnosis was a rare event. In 1912 Isaac Adler's collection of 374 case reports in his publication, *Primary Malignant Growths of the Lungs and Bronchi*,[2] represented the entire known global incidence at the time. A century later, the WHO histological classification of malignant epithelial tumours of the lung recognises different histologies with many variants for each subtype, and analyses from next generation sequencing (NGS) studies have divided this disease into molecular subtypes defined by distinct somatic alterations.[3–5] This Series paper focuses on key challenges faced in current clinical practice for molecular testing in non-small-cell lung cancer (NSCLC). In broad terms, the challenges are technical, logistical, and related to tumour biology. We highlight some of the pertinent issues (figure 1).

Identification of tumour histology

Historically, the treatment focus for patients with advanced NSCLC was selection of an appropriate cytotoxic chemotherapy regimen, irrespective of histological subtype. Several large studies[6–8] were published that showed that the efficacy of various platinum doublet combinations were comparable, but with differing drug specific toxicities. However, accurate classification of NSCLC subtype has become fundamental in the management of advanced NSCLC following the results of phase 3 clinical trials[9–12] that showed improved progression-free survival in *EGFR* mutation-positive adenocarcinoma treated with EGFR tyrosine kinase inhibitors (TKI),[9,10] and improved overall survival with pemetrexed in the first line and maintenance setting for patients with non-squamous histology.[11,12]

The proportion of tumours that cannot be given an accurate histological diagnosis (ie, adenocarcinoma *vs* squamous cell carcinoma) has reduced greatly with the use of immunohistochemical markers.[13,14] The use of markers for p63, p40, and cytokeratin CK 5/6 help to identify squamous cell carcinomas, while thyroid transcription factor 1 (TTF1), Napsin A and CK7, and mucin stains, are indicative of adenocarcinomas.[15] Interpretation of immunohistochemistry panels still requires the expertise of an experienced histopathologist, as markers are not reliable in isolation.[16] *TTF1*, a marker synonymous with adenocarcinoma, is expressed in only 80–90% of cases but is also usually expressed in neuroendocrine tumours.[15,17] Immunohistochemistry can only be meaningfully interpreted in a detailed morphological context.

Sampling challenges in advanced NSCLC

The analysis of lung cancer tissue is particularly challenging, as primary lung tumours often show much lower tumour cellularity than other tumour types. Even with macroscopic selection of areas of frank carcinoma the tumour purity (the fraction of a given region containing tumour cells) can often be less than 20% because of the high proportion of stromal cells, lymphocytic infiltration, and necrosis (unpublished observations from the UK Lung TRACERx longitudinal cohort study).[18]

The challenge of lung cancer tissue analysis is compounded by the nature of the specimen types routinely received by histopathology and molecular diagnostics laboratories. Presentation with metastatic disease is common, and only a small proportion of patients with NSCLC undergo curative surgical resection.[19] The large tissue samples obtained via open thoracotomy (wedge resection, lobectomy, and pneumonectomy) are usually of sufficient quantity and

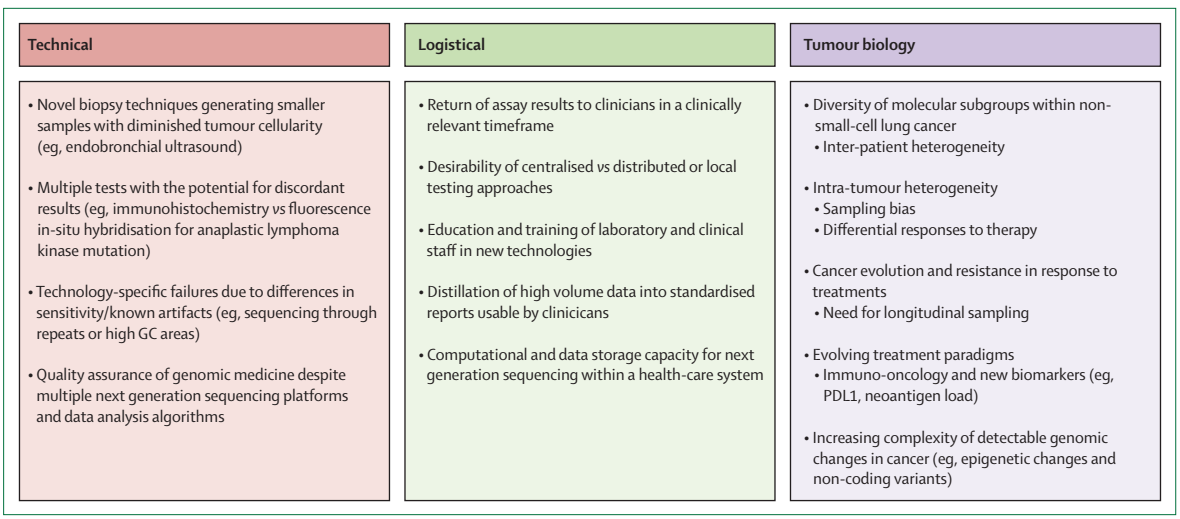

***Figure 1*: Summary of the key technical, logistical, and biological challenges for molecular testing in NSCLC**

quality for a number of histological and molecular assays, if handled appropriately. However, patients with advanced disease are predominately diagnosed with computerised tomography-guided percutaneous biopsy or ultrasonography-guided endoscopic biopsy, with 18 gauge needles or with fine needle aspiration. These patients are the cohort in which molecular diagnostics are most important for determining the standard of care and enabling participation in clinical trials, yet the sample quality and quantity from such needle biopsies is the most limiting for histological and molecular testing.

Obtaining adequate tissue for diagnosis, tissue subtyping, molecular profiling, and treatment planning are therefore key to patient management. The target tumour is not always easily accessible in patients presenting with a probable lung cancer. The development of endobronchial ultrasound transbronchial needle aspiration (EBUS-TBNA) is proving increasingly important in the investigation and management of thoracic malignancies as it offers a minimally invasive approach to sampling of mediastinal lymph nodes and masses. EBUS-TBNA is now increasingly embedded in routine clinical practice with wider use beyond high volume tertiary centres in the UK and USA. EBUS-TBNA, alone or in combination with endoscopic ultrasound fine needle aspiration (EUS-FNA), is now generally accepted as potentially sparing surgical mediastinoscopy or thoracotomy in the staging of NSCLC.[20,21] Importantly, EBUS-TBNA also offers the possibility of combining diagnosis and staging as a single procedure in patients with suspected lung cancer. In contrast to tissue biopsies or surgical samples that allow subtyping (adenocarcinoma *vs* squamous cell carcinoma) on morphological criteria alone in the majority of cases, investigation of cytological specimens obtained by EBUS-TBNA poses additional challenges that can be partly overcome with wider use of immunohistochemistry.[22]

Identifying driver mutations, such as EGFR and ALK, in these small samples is central to management of patients with advanced disease. Whether molecular analysis is successfully performed depends on the absolute number of tumour cells, the proportion of tumour cells compared with total nucleated cells present, and the method used for molecular analysis. In case of EBUS-derived samples, there is evidence to conclude that simple mutation analysis (EGFR, KRAS, or ALK) can be successfully performed in most cases.[23–25] The use of multi-gene targeted NGS panels, using only nanograms of DNA, to sequence fine needle aspiration samples is achievable and is becoming more commonly used in clinical practice.[26,27] Whole exome sequencing (WES) analysis and whole genome sequencing (WGS) analysis, which require greater amounts of DNA, micrograms in the case of WGS, will be more challenging from EBUS-TBNA samples.

Current challenges in molecular diagnostics for *EGFR* mutation analysis in clinical practice

The first randomised phase 2 studies of gefitinib demonstrated clinical activity,[28,29] and phase 3 studies, although negative for the primary outcome measure, suggested a benefit in patients with adenocarcinoma, people of Asian origin, and never-smokers.[30,31] During this period of testing, a number of seminal case series identified *EGFR* mutations as a marker of sensitivity to *EGFR* TKIs,[32–34] and analysis of samples from these early trials supported this conclusion.[35] Subsequent phase 3 trials incorporated *EGFR* mutation status and showed higher response rates and progression-free survival in patients with *EGFR*-activating somatic mutations treated with *EGFR* TKIs compared with chemotherapy.[9,10,36]

Development of diverse mechanisms of resistance and selection of resistant clones in response to treatment

The common *EGFR* mutations are located in the tyrosine kinase domain (exon 18–21) of the *EGFR* gene, with detection of Leu858Arg and deletions in exon 19 the clinical priority as these determine sensitivity to first and second generation TKIs.[37] The Thr790Met mutation in exon 20 results in resistance to these therapies.[38] Sensitive assays suggest that tumour clones harbouring the Thr790Met mutation are often detectable prior to initiation of a first generation TKI, but can also occur by genetic evolution in drug tolerant cells without Thr790Met mutation, in response to treatment.[39–41] Identification of this resistance mutation is more critical following the development of the third generation EGFR TKIs active against Thr790Met-mutation-positive NSCLCs.[42,43] But whether these Thr790Met-resistant clones pre-exist or evolve in response to treatment could have clinical implications with differing sensitivities to third generation TKIs.[41] The capacity for tumours to evolve in response to first generation TKIs results in an additional diverse array of mechanisms of resistance such as amplification of *MET*, selection for *PIK3CA*, or *BRAF* mutations and transformation to a small-cell phenotype.[44] Clearly, cancer evolution and selection of resistant subclones is not restricted to first generation TKIs. This is highlighted by recent reports of the emergence of Thr790Met-mutation-negative disease and the development of novel secondary EGFR resistance mutations (C797S) after treatment of Thr790Met-mutation-positive patients with third generation TKIs.[45,46]

EGFR mutation testing

The nature of *EGFR*-sensitising mutations, being single nucleotide variants (SNVs) or short deletions, lends them to molecular analysis of formalin-fixed small samples which contain fragmented DNA.[47] Various methods exist to detect *EGFR* mutations, including conventional Sanger sequencing, amplification refractory mutations systems, restriction fragment length polymorphisms and, more recently, as part of targeted NGS panels.[48,49] Reporting the limitations of an assay along with the result is critical for clinical interpretation. Bidirectional Sanger sequencing without a mutation enrichment step has a lower limit of detection of 10–25% of total DNA, meaning that the use of samples with low tumour cellularity can result in false negative mutation calls. Consequently, the use of methods that can detect mutations in low tumour cellularity (<10%) samples is recommended. PCR-based ultrasensitive and NGS methods can generate artifact mutations leading to false positive results. Techniques such as duplex sequencing are being developed to overcome the inherent error rate in sequencing technologies[50] Formalin-fixed samples are particularly prone to DNA damage and display disproportionate levels of C to T and G to A changes in the 1–10% allele frequency range, which can result in false positive mutation calls.[51] Publication of clinical trial results of response to *EGFR* TKI in patients with real but less common *EGFR* mutations can help guide clinical decision making.[52] Detection of EGFR mutations as part of a WES or WGS analysis allows various driver mutations to be queried simultaneously but the performance of bioinformatics tools to call mutations from NGS data varies. Such complexities need to be considered as these technologies are increasingly adopted into mainstream clinical practice.[53–55]

EGFR mutations, resistance, and tumour heterogeneity

There are very few reports of discordance of *EGFR* mutation status between primary disease and metastatic sites, and these reports might be due to technical limitations of the assays used.[56] Loss of the *EGFR* mutation was not a mechanism seen in seminal studies.[44] Studies looking at the extent of intra-tumour heterogeneity (ITH) in early lung cancer have shown *EGFR* to be exclusively a clonal event prevalent throughout the tumour.[57,58] As resistance to EGFR TKI is usually due to acquisition of secondary mutations in *EGFR* or other driver genes, the key challenge at the time of disease progression is to obtain a contemporaneous sample to inform selection of second line therapy. In general, the most easily accessible lesion is used but in patients with a poor performance status this might not be a trivial task. Due to tumour heterogeneity it is possible that a single sample might be insufficient to accurately represent all the resistance mechanisms present or the breadth of subclonal driver events present across multiple disease sites following progression on therapy.

Current challenges in molecular diagnostics for *ALK* testing in clinical practice

The discovery of an oncogenic anaplastic lymphoma kinase fusion gene (*EML4-ALK*) in 2007 identified another important molecular cohort in NSCLC.[59] Present in 2–7% of NSCLC ALK fusion genes are restricted to adenocarcinoma subtypes and are more common in younger patients and never-smokers.[60–62] Identification of this cohort is crucial, given the high response rates (57–74%) to ALK inhibition both as a first line therapy and after platinum-based chemotherapy.[60,63,64] Subsequently, other rare fusion genes have been identified involving *ROS1*, with similar exquisite sensitivity to kinase inhibition,[65] but also *RET* and *NTRK* where objective response rates were lower.[66,67]

ALK fusion gene detection

Testing for *ALK* fusion genes brings its own particular set of challenges. *ALK* is activated by genomic rearrangement, leading to the expression of a chimeric protein that contains the effector part of the *ALK* tyrosine kinase fused to the proximal portion of another protein.

In NSCLC cancer, this is typically a balanced translocation with the ubiquitously highly expressed *EML4* gene,[59] although other partner genes can be involved.[68,69] Expression of the chimeric protein leads to upregulation of mitogenic signalling through the RAS/RAF pathway and interruption of this pathway by *ALK* inhibitors causes cancer cell death and tumour regression.[63] ALK-mutated tumours often show unusual features on conventional microscopy, such as cribriform growth patterns and so-called signet ring cells with large vacuoles,[70] but this is not sufficiently sensitive or specific to guide treatment.

The first widely-adopted test for ALK-driven tumours was fluorescence in-situ hybridisation (FISH), approved by the US Food and Drug Administration (FDA) in 2011.[71] FISH is a technically demanding method, requiring specialised equipment and experienced practitioners. Tissue sections or cytology specimens are subjected to a protocol that labels either side of the *ALK* breakpoint locus with red and green fluorescent DNA probes. In non-transformed cell nuclei the coloured dots overlap and look yellow, while in translocated cells isolated red or green signals are seen. For a reliable FISH assay, the tissue must be adequate in quantity and quality. This can be more challenging with small biopsy samples, which might contain few cells or which show crushing artifacts that can impair interpretation.

In 2015, an immunohistochemistry method was approved by the FDA. This approach is simpler in principle, using an antibody stain to detect abnormal *ALK* antigen expression. However, currently available antibodies do not give a strong signal and an additional signal amplification step needs to be employed. This places the test beyond the capacity of many small labs. Nonetheless, the modified test is cheaper than FISH, easier to interpret, and has the theoretical advantage of additionally detecting *ALK* expression following rare atypical rearrangements. After much investigation, recent studies suggest immunohistochemistry can be an adequate stand-alone diagnostic, showing extremely high concordance with FISH.[72] UK guidelines do not dictate which test should be applied, and practices vary regionally, though FISH is still often regarded as the gold standard and is considered the definitive test in the USA.[49] As our understanding of tumour taxonomy and genotypes advances, it seems inevitable that some form of NGS platform will become the clinical standard for gene fusion detection.[73] These methods have the potential to detect ALK and other rearrangements in either a targeted panel or a WES or WGS approach.[73,74,75]

ALK fusion genes, resistance, and tumour heterogeneity

ALK fusion genes are considered to be clonal events with minimal discordance between primary and metastatic lesions.[76] They were previously thought to be mutually exclusive with *EGFR* mutations, but recent reports suggest a small minority of tumours can contain both *ALK* and *EGFR* positive clones.[77–79] The mechanisms of resistance seen following *ALK* inhibitor therapy again demonstrate tumour evolution with secondary mutations in *ALK*, *ALK* copy number gain, secondary driver mutations in other genes and outgrowth of *ALK* fusion gene-negative clones reported.[80–83] Consequently, contemporaneous sampling of progressive disease, by needle biopsy or analysis of cfDNA, might allow real time analysis of tumour evolution and guide therapy.

Integration of multi-gene NGS testing in clinical practice

Routine molecular profiling can be performed at scale on a national level. Large cooperative efforts in France and the USA used combinations of mutation specific PCRs, Sanger sequencing, and FISH analysis to assay 6–10 oncogenic drivers in thousands of patients with NSCLC, and survival was improved for those treated with gene directed targeted therapies.[84,85] The use of NGS to assay multiple oncogenic drivers simultaneously is attractive, because less DNA is required compared to multiple individual assays, there is a reduction in hands-on laboratory time, and complex FISH analysis for detection of fusion genes might be avoided. A recent NGS approach used an amplicon-based approach to assay 14 genes with only 50 ng of DNA from formalin-fixed, paraffin-embedded (FFPE) samples.[86] This study provided a comprehensive assessment of the spectrum of potentially clinically actionable or important prognostic mutations, and co-occurrence of mutations, in adenocarcinoma and squamous cell carcinomas with detection turnaround times of less than 2 weeks. These studies and those of The Cancer Genome Atlas highlight the inter-patient molecular heterogeneity of NSCLC (figure 2). Even within these molecular cohorts, intra-tumour heterogeneity could have significant effects on outcome as exemplified by a recent study showing that the clonality of *FGFR* amplification is an important predictor of response to *FGFR* inhibition.[90] A deeper understanding of the clonal or subclonal nature of driver events in NSCLC from sufficiently powered studies, is still awaited. Recruitment of patients with rare mutations to molecularly stratified trials is challenging,[91] and some clinical and opinion leaders advocate that modifications to existing paradigms in drug development are required in the era of genomic studies and precision medicine.[92] Multi-gene or WES NGS assays are likely to become standard practice in the future, and the ultimately automated provision of readable, applicable reports of complex genomic data is another important challenge.

Current challenges in molecular diagnostics for PDL1 testing in clinical practice

Activation of inhibitory T-cell checkpoint interactions in established tumours has been demonstrated in a number of solid tumours, including NSCLC, and this suppresses the anti-tumour immune response.[93,94] The

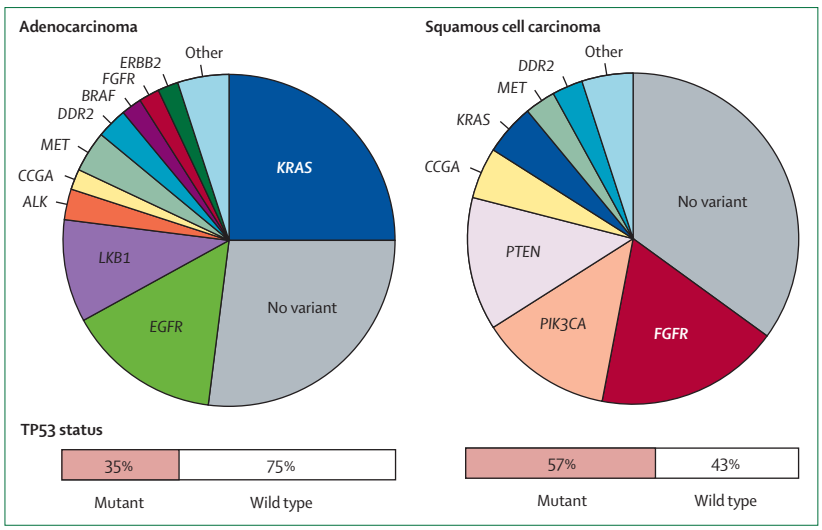

***Figure 2*: Approximate distribution of clinically relevant driver mutations in individuals with NSCLC**
The genomic variants shown are potentially clinically actionable variants.[87] The proportions presented are based on estimates from the referenced studies and data sources, including the Stratified Medicine Programme 2 (unpublished data).[3,4,86,88] CCGA=cell cycle genomic aberration.[89] EGFR=epidermal growth factor receptor. LBK1=liver kinase B1. ALK=anaplastic lymphoma kinase. MET=MET proto-oncogene receptor tyrosine kinase. FGFR=fibroblastic growth factor receptor. MRAS=neuroblastoma RAS viral (v-ras) oncogene homolog. DDR2=discoidin domain receptor tyrosine kinase 2. ATK1=v-akt murine thymoma viral oncogene homolog 1. PTEN=phosphatase and tensin homolog. PIK3CA=phosphoinositide-3- inase, catalytic, α polypeptide. BRAF=v-raf murine sarcoma viral oncogene homolog B1. ERBB2=human epidermal growth factor receptor 2. KRAS=v-Ki-ras2 kirsten rat sarcoma viral oncogene homolog. TP53=tumor protein P53.

aim of immunotherapy using antagonists of these inhibitory T-cell checkpoint interactions is to reactivate anti-tumour immunity. PDL1 (B7-H1) is a ligand present on antigen-presenting cells (APCs), including tumour cells, that interacts with its receptor (PD1) on T cells and inhibits T-cell effector functions. PD1-positive and CD8-positive effector T-cell populations are thought to be the tumour reactive subset responsible for anti-tumour immunity.[95] There is limited knowledge of the spatial or functional heterogeneity of tumour-infiltrating lymphocyte populations and the T-cell checkpoint ligand-receptor interactions within solid tumours.

Recent randomised trials[96–100] have shown activity of PD1 and PDL1 targeting antibodies in squamous and non-squamous NSCLC. In most instances, these agents have shown greater activity in patients whose tumour expresses PDL1 when tested using immunohistochemistry. However, durable responses are seen in patients without PDL1 expression. This is unsurprising given the technical and spatial heterogeneity of PDL1 expression in NSCLC, which hampers its use as a predictive biomarker.[101–103] Studies of the expression of PDL1 on APCs in NSCLC are also contradictory with respect to any correlation with tumour infiltration of the effector CD8-positive T cells.[93,101,102]

Regulation of PDL1 expression is complex and controlled by both cell-intrinsic and cell-extrinsic factors.[104] This means that oncogene driven expression of PDL1 can result in increased expression in the absence of significant underlying immunogenicity.[105] This underlying immunogenicity is thought to be a result of non-synonymous SNVs which generate neoantigens, mutated proteins that are recognised by populations with tumour-infiltrating lymphocytes[106,107] The number of neoantigens harboured by a tumour could act as a potential biomarker for immunotherapy although there are technical challenges inherent with such complex assessments. Recent data also suggest that neoantigen intratumour heterogeneity might also be associated with altered checkpoint inhibitor response, which could further complicate the use of such assays in a clinical setting.[108]

The advent of immunotherapy presents additional challenges for molecular diagnostics in NSCLC. Although immunohistochemistry for PDL1 can be performed on the small samples often used in lung cancer diagnostics there is the risk of significant sampling bias because of ITH. The dynamic nature of PDL1 gene expression,[109,110] means that a contemporaneous sample obtained by repeat biopsy might be the most accurate adding additional burden and expense to current clinical pathways. Characterisation of neoantigens as a potential biomarker would require sufficient tumour DNA for WES and carries significant expense but given the cost of these therapies would be justified if the assay were sufficiently predictive. However, neoantigen prediction algorithms are still in their infancy and evidence suggests that there are a proportion of patients who derive no clinical benefit from checkpoint inhibitor therapy, yet have tumours with a neoantigen burden above thresholds associated with sensitivity, and conversely patients with low neoantigen burden who benefit.

Molecular diagnostics in practice: The UK National Lung Matrix Trial

The Cancer Research UK Stratified Medicine Programme 2 (SMP2) screens samples from advanced NSCLC patients using NGS for known drivers that are considered clinically actionable. The aim of SMP2 is to establish high-throughput and quality genomic screening at a national level in the UK. Based on these results, patients are recruited to The National Lung Matrix Trial (NLMT); (NCT02664935) a phase 2 umbrella study with both targeted therapy and immunotherapy arms for patients who have progressed on first-line therapy.[89] By comparison, the Lung-MAP (NCT02154490) and SAFIR02 Lung trial (NCT02117167) are umbrella studies, outside of the UK, for patients with NSCLC where recruitment is preceded by molecular stratification (table).

SMP2 molecular pathology workflow uses DNA from excess diagnostic biopsy tissue. Sections are sent from the referring clinical site and extracted by one of three central technology hubs. Samples with sufficient amounts of DNA (>50 ng) are then analysed using a custom 28-gene targeted NGS panel. Having successfully screened over 1000 patients, patterns of mutation and prevalence are emerging across the genomic and clinical data. Preliminary analysis indicates prevalence and distribution of SNVs consistent with published reports,

including 31·6% *KRAS* mutations (of which 19·7% show concomitant *STK11* mutation) and 15·1% *EGFR* mutations in patients with adenocarcinoma. Over the past year, SMP2 has led to the recruitment of over 60 patients to the NLMT. A number of detailed audits have identified areas of improvement along the SMP2 pathway; from patient recruitment, to sample preparation, and result analysis.

Whilst using excess DNA from the FFPE diagnostic biopsy has significant advantage for patients and clinical workload (as repeat biopsies are not required), only 70% of samples sent have sufficient DNA to enter the sequencing pipeline. This is in part due to FFPE blocks being exhausted during the diagnostic process and a general reduction in the size of diagnostic cores over time. Consequently, the minimum number of sections has since been increased to ensure enough DNA is obtained upfront. Some recruiting centres quantify DNA upfront, which allows a faster feedback loop if insufficient DNA is present. Sites can then obtain additional samples from the diagnostic block or through re-biopsy, if appropriate. However, differences in quantification methodology between local clinical centres and central technology hubs have led to samples being sent with less than the required 50 ng, resulting in some of these samples failing quality control metrics prior to sequencing. Changes in extraction methods and a standardised DNA concentration have been introduced.

Unique to NLMT is the need to determine wild type status of some genes for eligibility to certain arms. Patients recruited to the CDK4/6 inhibitor palbociclib arm must have wild type retinoblastoma 1 protein (RB1) in addition to deficiencies in addition to cell cycle genomic aberrations (CCGA). CCGAs in NSCLC include loss of *CDKN2A* or amplification of *CDK4* or cyclin D1. The determination of wild type status requires a pre-sequencing assessment of tumour cellularity to determine appropriate sequencing depth. However, there can be significant discordance between pathologist assessments of this.[111] Clearer guidance and online training should ensure more concordance for visual assessment, and digital solutions might provide a useful alternative. A number of computational methods exist to assess tumour purity and control for both stromal cell admixture and cancer cell ploidy in DNA samples from NGS data.[112,113]

Extremes of GC nucleotide content in certain genes (*RB1* and *FGFR3*) can result in an increased number of sequencing failures. Additional probe coverage in the targeted panel and correction for GC content in the data processing stage will improve results for these difficult-to-sequence regions. By following these incremental improvements at each step of the molecular pathology workflow, we have shown that the number of successfully sequenced samples that would allow recruitment to the NLMT has increased, and there has also been an increase in identification of potentially actionable mutations that would permit recruitment to trials other than the NLMT.

	Line	Phase	Personal outcome measures	Molecular subgroups	Location
National Lung Matrix Trial	Second or later	Non-randomised phase 2	Progression-free survival; objective response rate	*AKT*, *PIK3CA*/*PTEN*, *TSC*, *LKB1*, *KRAS*, *NRAS*, *NF1*, *MET*, *ROS1*, *EGFR* (Thr790Met), CCGA, immunotherapy	UK
SAFIR_02 Lung Study	First-line maintenance	Randomised phase 2	Progression-free survival	*mTOR*, *AKT*, *FGFR*, *HER2*, *EGFR*, *MEK*, immunotherapy	France
Lung MAP	Second or later (squamous cell carcinoma)	Non-randomised phase 2	Progression-free survival	*PIK3CA*, *FGFR*, CCGA, immunotherapy	USA
Darwin I/II	First or later	Non-randomised phase 2	Progression-free survival	*EGFR*, *HER2*, *ALK*, *RET*, *BRAF*, immunotherapy	UK

CCGA=cell cycle genomic aberration.

Table: **Comparison of molecularly stratified umbrella studies in NSCLC**

Future solutions

The technical limitation of the small, and potentially low tumour cellularity NSCLC samples obtained from bronchoscopy and EBUS-TBNA means that the main challenge facing clinicians and pathologists is the need for ever greater amounts of information from diminishing amounts of tissue. It is therefore imperative that the quality of diagnostic samples in the advanced NSCLC setting is of the highest order. How best to achieve this represents a challenge for health service providers that has received very little attention thus far. The spectre of ITH and cancer evolution means that sampling bias and the presence of subclonal driver mutations, causing resistance to therapy, are likely to hinder clinical benefit of targeted therapeutics.[114,115] The UK Lung TRAcking Cancer Evolution through Therapy trial (NCT01888601) is currently characterising the extent of ITH in early surgically resected NSCLC and with longitudinal follow-up aims to determine the origins of tumour subclones contributing to relapse.[18] Evidence from other tumour types of parallel evolution, acquisition of mutations in the same gene or signalling pathway in distinct subclones, could highlight an evolutionary bottleneck that could be an Achilles heel for subsequent cancer therapy.[116–118] Clonal analyses of a drug target and putative resistance events, whether they are present in all tumour cells or only a proportion, might affect the response rate and progression-free survival times on targeted therapy and this is being addressed in clinical trials including the DARWIN studies (NCT02314481, NCT02183883). Ultimately it might be that only through warm autopsy studies—where subclonal phylogenetic structures can be determined through sampling various sites of disease—that effective strategies to forestall cancer evolution can be elucidated.[119]

The use of minimally invasive methods to detect mutations in circulating cell-free DNA (cfDNA) or liquid

biopsies offers the potential to obtain a mutation call in a patient where an invasive biopsy might not be feasible. As tumour DNA from all sites of disease has the potential to enter the bloodstream it might also be a better reflection of tumour heterogeneity than a single biopsy.[120,121] cfDNA has been used to detect resistance mechanisms in patients treated with EGFR TKIs, often prior to radiographic progression.[46,122,123] This has resulted in the development and approval of a commercially available assay of cfDNA in plasma that can detect a spectrum of *EGFR* mutations, including the Thr790Met mutation, amenable to being targeted with third generation TKIs.

Circulating tumour cells (CTCs) are tumour cells that can be isolated from the peripheral blood and are a complementary circulating biomarker to cfDNA. CTCs are a versatile tool, as cell enumeration can be prognostic, immunohistochemistry permits further characterisation, single cell DNA or RNA sequencing is possible, and tumour xenografts can be generated to assess drug response.[124–128] At present, the complexity of separation from other cells in the peripheral circulation and the need to process samples promptly for functional or genomic studies results in greater expense in comparison to cfDNA analysis. Circulating biomarkers will have a substantial impact on cancer management in the near future, and readers are directed to more extensive reviews focusing on CTCs, cfDNA, and other circulating nucleotides.[124,129–132]

Conclusion

The challenges for molecular diagnostics in NSCLC are largely similar to other tumour types. Resolving these issues will require technological improvements in addition to a greater understanding of tumour biology. The logistical challenges of implementing the next generation of molecular diagnostics into clinical practice are equally challenging. Clinical governance; information technology infrastructure; data storage; pathways in sample processing and training; and professional developments in histopathology, respiratory medicine, and oncology will need investment. With these great challenges comes opportunity to improve the success rate and efficiency of drug development in NSCLC and subsequent patient outcomes.

Contributors

All authors assisted in manuscript writing and critical reading. CTH was responsible for most of the manuscript writing and generating figures. CS was responsible for final decision making regarding manuscript content.

Declaration of interests

CS declares advisory board or speaker fees on laboratory research over the last 3 years for Roche, Pfizer, Celgene, Boehringer Ingelheim, Novartis, Glaxo Smithkline, and Eli Lilly. CS sits on the scientific advisory board and holds stock options for Epic Biosciences and Grail and is a founder of Achilles Therapeutics. CS is Royal Society Napier Research Professor. This work was supported by the Francis Crick Institute which receives its core funding from Cancer Research UK (FC001169), the UK Medical Research Council (FC001169), and the Wellcome Trust (FC001169); by the UK Medical Research Council (grant reference MR/FC001169/1); CS is funded by Cancer Research UK (TRACERx), the CRUK Lung Cancer Centre of Excellence, Stand Up 2 Cancer (SU2C), the Rosetrees Trust, NovoNordisk Foundation (ID 16584), the Prostate Cancer Foundation, the Breast Cancer Research Foundation, the European Research Council (THESEUS) and Support was provided to CS by the National Institute for Health Research, the University College London Hospitals Biomedical Research Centre, and the Cancer Research UK University College London Experimental Cancer Medicine Centre.

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