

# Molecular methods for somatic mutation testing in lung adenocarcinoma: *EGFR* and beyond

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**Abstract:** Somatic mutational profiling in cancer has revolutionized the practice of clinical oncology. The discovery of driver mutations in non-small cell lung cancer (NSCLC) is an example of this. Molecular testing of lung adenocarcinoma is now considered standard of care and part of the diagnostic algorithm. This article provides an overview of the workflow of molecular testing in a clinical diagnostic laboratory discussing in particular novel assays that are currently in use for somatic mutation detection in NSCLC focussing on epidermal growth factor receptor (*EGFR*) mutations and anaplastic lymphoma kinase (*ALK*), *ROS1* and *RET* rearrangements.

**Keywords:** Epidermal growth factor receptor (*EGFR*); anaplastic lymphoma kinase (*ALK*); *ROS1*; *RET*

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## Introduction

There has been a recent and significant paradigm shift in the diagnosis and management of lung cancer, with the discovery of driver mutations that can be targeted by specific therapeutic inhibitors (1). This translates into clinical outcomes for patients whose cancer harbour these mutations or rearrangements. Personalized treatment is driving the demand for somatic mutation testing in cancer not only in absolute patient numbers for which worldwide lung cancer affected approximately 1.8 million patients in 2012 and caused an estimated 1.6 million deaths (2), but also in the number of genes. Molecular testing of lung adenocarcinoma for the epidermal growth factor receptor epidermal growth factor receptor (*EGFR*) and anaplastic lymphoma kinase anaplastic lymphoma kinase (*ALK*) is now considered standard of care with other “driver mutations” in oncogenes such as *KRAS*, *ROS1*, *RET*, *HER2*, *BRAF*, *PIK3CA*, *NRAS*, *AKT1*, *MET* and *MEK* (3) also being part of the diagnostic algorithm and work-up of these patients. The results of the base biomarker findings are now incorporated into the standardized structured reporting by the College of

American Pathologist (CAP) (4) and the Royal College of Pathologists Australasia (RCPA) (5). Recently, the CAP, International Association for the Study of Lung Cancer (IASLC) and Association for Molecular Pathology (AMP) published a joint guideline communicating the recommendations for molecular testing in lung cancer (6). In these guidelines the pathologist plays a crucial role in this endeavour optimizing tissue handling and triaging of tumor material for appropriate testing downstream. This article provides a brief overview of the workflow of molecular testing in a clinical laboratory and also discusses the various assays that are currently in use for somatic mutation testing specifically focussing on *EGFR*, *ALK*, *ROS1* and *RET* mutations.

## Molecular genetics of non-small cell lung cancer

### Background

#### Adenocarcinoma

Recently The Cancer Genome Atlas (TCGA) Research Network published results from their work on the

comprehensive molecular profiling of lung adenocarcinoma (using messenger RNA, microRNA, DNA sequencing, copy number analysis, methylation and proteomic analyzes) (7). In this study, aberrations in eighteen genes were found to be statistically significant, with the genes identified being: *-TP53* (46%), *KRAS* (33%), *EGFR* (14%), *BRAF* (10%), *PIK3CA* (7%), *MET* (7%), *RIT1* (2%), *STK11* (17%), *KEAP1* (17%), *NF1* (11%), *RB1* (4%), *CDKN2A* (4%), *SETD2* (9%), *ARID1A* (7%), *MARCA4* (6%), *RBM10* (8%), *U2AF1* (3%) and *MGA* (8%). The key pathways affected in lung adenocarcinoma are the *RTK/RAS/RAF* pathway activation, the *PI(3)K-mTOR* pathway, p53 pathway, cell cycle regulator pathway, oxidative stress pathways and mutations in chromatin and RNA splicing factors. The analysis identified that amplification in *MET*, *ERBB2* and mutations in *NF1*, *RIT1*, *TP53*, *KEAP1* were enriched in oncogene negative tumors (i.e., tumors that lack receptor tyrosine kinase activation and that do not harbour *H/N/KRAS*, *EGFR*, *ERBB2*, *BRAF* mutations and *ALK*, *RET*, *ROS1* rearrangements) (7). The list of mutations are ever increasing, highlighting the drive to identify potential therapeutic targets. In the following discussion, we will be highlighting the recent updates pertaining to *EGFR*, *ALK*, *ROS1* and *RET*.

### Epidermal growth factor receptor (*EGFR*)

In 2004, the discovery of *EGFR* gene (also known as *HER1* or *ERBB1*) mutations linked to clinical response with *EGFR* tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib in patients harbouring mutations, transformed the management of lung cancer and fuelled the drive for the discovery of other oncogenic drivers (8-10). Subsequently second generation *EGFR* TKIs are being trialled to improve efficacy in first line treatment of *EGFR* mutated non-small cell lung cancer (NSCLC) and to provide an alternative strategy for treating cases of acquired resistance (10,11). The proposed mechanism by which these second generation TKIs circumvent the issue of acquired resistance is said to occur via three methods: (I) by intensifying *EGFR* inhibition (through binding with/inhibition of other members of the *ERBB* family); (II) by specific inhibition of the *EGFR* downstream signalling pathway; (III) by dual targeting of parallel signalling pathways combining *EGFR* with another pathway inhibitor (i.e., vascular endothelial growth factor *VEGF* pathway) (10). Second generation *EGFR* TKIs (neratinib, dacomitinib, afatinib) are pan *HER* inhibitors aiming to intensify *EGFR* inhibition by forming irreversible covalent binding to *EGFR* kinase domain and other members of the *ERBB* family (*HER2*, *HER4*) (10,11). The

most common form of acquired resistance is the T790M mutation, and specific *EGFR* T790M inhibitors (CO-1686) have been developed and investigated to address this issue (10,11). In preclinical studies, AP26113, a dual *EGFR/ALK* inhibitor has shown selective activity against mutated *EGFR* tumors including those harbouring the T790M mutation (10,11). Dual *EGFR/VEGF* inhibitors such as XL647 (vandetanib) and BMS-6905214 aim to inhibit the cross talk between the *VEGFR* and *EGFR* signalling pathway, as *VEGFR* expression is said to be associated with *EGFR* resistance (10,11).

*EGFR* gene mutations occur more commonly (but not exclusively) in light/never smokers, females and Asians as compared to other ethnic groups, however demographics alone should not be the sole criteria to exclude patients for mutational testing (6). *EGFR* mutations have been described in association with lepidic predominant adenocarcinoma, papillary, micropapillary adenocarcinoma subtypes and adenocarcinoma in situ (AIS) and are less common in adenocarcinoma with mucinous differentiation or with a solid growth pattern (12). *KRAS* mutations on the other hand, are commonly associated with invasive mucinous adenocarcinoma (formerly mucinous BAC pattern) and extracellular mucin (13).

*EGFR* mutations are present in approximately 15% of primary lung adenocarcinomas and are mutually exclusive of *KRAS* and *BRAF* mutations. *EGFR* is a member of the *ERBB* family of receptor tyrosine kinases and the gene is located at 7p12. It encodes a transmembrane receptor protein with cytoplasmic tyrosine kinase involved in downstream signalling transduction pathways. The most common activating somatic mutations in the cytoplasmic tyrosine kinase domain of *EGFR* occur in exons 18-24. Of these, the two most common mutations are the short inframe deletion in exon 19, clustered around amino acid residues 747-750 and the L858R missense mutation in exon 21, together accounting for approximately 80-90% of all *EGFR* mutations (14). Nevertheless, a significant number of mutations that may respond to TKIs have been identified outside of these "hot spots" and this has a bearing on the methodology of mutation detection (see below). Acquired secondary resistance to *EGFR* TKI can occur during the course of treatment, with the most common mechanism identified as the T790M mutation in exon 20 (10,11,15). This can sometimes be present below the assay limit of detection if retesting for this mutation is performed on the original biopsy, suggesting in some patients clonal outgrowth occurs under selective therapeutic pressure. Other pathways conferring resistance includes reactivation of

downstream signalling pathways (*MET* amplification, *HER2* amplification, mutation in *PI3K* gene), phenotypic alteration (transformation of original NSCLC histology to small cell histology) and epithelial mesenchymal transition (15).

### Anaplastic lymphoma kinase (*ALK*)

In 2007, a rearrangement in the *ALK* gene on 2p23 resulting in a fusion oncogene was discovered as an oncogenic driver mutation in a subset of lung adenocarcinomas (2-5%). It is commonly found in younger, light/never smokers (14). The histological features said to be associated with *ALK* rearranged tumors range from those with a solid growth pattern, signet ring cells with mucin production to those with well differentiated tubulopapillary and cribriform patterns (16). Treatment response in the early clinical trials in patients with such a rearrangement led to the accelerated U.S. Food and Drug Administration (FDA) approval of crizotinib in 2011. Crizotinib is an oral selective *ALK/MET* TKI for the treatment of NSCLC patients harbouring such an *ALK* rearrangement. In lung cancer, the most common *ALK* rearrangement is an inversion on chromosome 2, inv[2] (p21 p23) resulting in fusion of the 3' kinase domain of *ALK* with the (echinoderm microtubule-associated protein-like 4) *EML4* gene and its promoter region. The *EML4-ALK* gene fusion results in constitutive activation of the *ALK* kinase domain. This leads to activation of the three major downstream signalling pathways: *MAPK/MEK/ERK*, *PI3K/AKT*, and *RAS/STAT3*. The breakpoints in *EML4* are variable, whilst the *ALK* breakpoint is mostly in exon 20. This results in multiple variant of *EML4-ALK* due to the different truncations in *EML4* (16). There are at least 11 known *EML4-ALK* reported variants. The most common variants are variant 1 (E13, A20) with this nomenclature representing breakpoint in exon 13 of *EML4* juxtaposed to exon 20 of *ALK* (33%) and variant 3a/b (E6a/b, A20) representing breakpoint in exon 6 of *EML4* juxtaposed to exon 20 of *ALK* (29%). The other *EML4* variants are known as variant 2 (E20, A20) (9%), variant 7 (E14, A20) (3%), variant 5' (E18, A20) (2%), variant 4 (E15, A20) (2%), variant 5a/b (E2, A20) (2%) and E17, A20 (1%). Besides *EML4*, other less common translocation partners exist (*KIF5B-ALK*, *TFG-ALK*) (14). To date, further novel rearrangements have been identified including *HIP1-ALK* (17), *KLC1-ALK* (18) and *STRN-ALK* (19). A recently discovered variant *PTPN3-ALK* results from translocation of part of the *ALK* gene to the third intron of *PTPN3*, which does not result in a protein with enzymatic activity but instead results in a loss of one allele of *PTPN3* and is hypothesized to contribute

to tumorigenesis through loss of the tumor suppressive functions of the *PTPN3* gene. The *PTPN3-ALK* will not respond to crizotinib as the *ALK* kinase domain is absent (20). The significance of these diverse *ALK* fusion variants is unknown. As in *EGFR*, resistance to crizotinib may arise from secondary "gate keeper" mutations in the *ALK* tyrosine kinase domain, activation of alternative signalling pathway or outgrowth of clones that contain a different driver mutation (21). The most common "gatekeeper" mutation identified in the *ALK* tyrosine kinase domain is the L1196M which results in structural alteration of the adenosine triphosphate (ATP) binding pocket of the receptor, which in turn obstructs crizotinib from binding to its target (21). Other secondary mutations are distributed over *ALK* kinase domain. Activation of alternative downstream signalling pathways via the *PI3K/AKT/mTOR* pathways, heat shock protein 90 (HSP90) and activation of *EGFR* through increased phosphorylation and upregulation of *EGFR* ligands (rather than by *EGFR* gene mutations) have been shown to contribute to crizotinib resistance. Novel new generation *ALK* inhibitors (Ceritinib, Alectinib, AP26113) show activity against the L1196M gatekeeper mutation and other mutations (*ROS1* and *EGFR*). HSP-90 Inhibitors (retaspimycin, ganetespib) are also currently in clinical trial (21).

### *ROS1*

*ROS1* is a receptor tyrosine kinase of the insulin receptor family and is located on chromosome 6q22 (22). *ROS1* kinase alterations lead to activated downstream signalling of several oncogenic pathways controlling cell proliferation, survival and cell cycling (*STAT3*, *PI3K/AKT/mTOR*, *RAS-MAPK/ERK* pathways). As compared to *ALK* and *RET* rearrangements, whereby coiled-coil domains in the 5' fusion partners lead to ligand independent homodimerization, many of the *ROS1* fusion proteins do not have dimerization domains and the mechanism of constitutive activation of *ROS1* fusion proteins is unknown (22). *ROS1* rearrangements have been identified in 2% of lung adenocarcinoma, with patients sharing similar clinical profiles (younger age at diagnosis, non-smoking history) to those harbouring *ALK* rearrangements. The different *ROS1* fusion partners identified to date include *EZR*, *CD74*, *SLC34A2*, *LRIG3*, *SDC4*, *TPM3*, *FIG* or *GOPC*, *CCDC6*, *KDELRL2* (22-30). Two novel translocation partners *LIM1* and *MSN* were detected recently (31). With all different translocation partners, the breakpoint in *ROS1* occurs at the 5' end of exons 32, 34, 35 or 36 and the *ROS1* kinase domain is retained (22). Cell lines harbouring *ROS1* fusions

and case reports have shown that *ROS1* mutated lung adenocarcinoma show response to crizotinib therapy (25). The structural homology of crizotinib binding sites in the *ROS1* and the *ALK* tyrosine kinase domains is said to account for this (28). A phase 1 study using crizotinib in 50 patients with *ROS1* rearranged advanced NSCLC showed marked clinical response (in terms of duration of response and progression free survival, with no difference between type of *ROS1* translocation partners). In this study, the objective response rate was 72%, with 3 patients showing complete responses and 33 patients showing partial responses in their tumor with crizotinib treatment (31). This highlights the importance of including *ROS1* in the current testing algorithm.

### **RET**

*RET* (rearranged during transfection) is a receptor tyrosine kinase mapped to chromosome 10q11.2 (14). *RET* rearrangements have been identified in thyroid carcinoma whereby germline gain of function mutation leads to multiple endocrine neoplasia (MEN) type 2 and somatic gain of function mutation to sporadic medullary thyroid carcinoma. In lung adenocarcinoma, *RET* rearrangements were discovered in 2011, with the investigators using whole genome/transcriptome sequencing, multiplexed reverse transcriptase polymerase chain reaction (RT-PCR) and Sanger sequencing, immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) as identification and verification methods (1). *RET* rearrangements have been identified in 1-2% of lung adenocarcinomas (24,32) but the prevalence is higher (quoted up to 16%) when preselected and enriched for tumors which are pan negative for other known driver mutations (i.e., *EGFR*, *KRAS*, *NRAS*, *BRAF*, *HER2*, *PIK3CA*, *MEK1*, *AKT*, *ALK*, *ROS1*) (33). Patients with *RET* translocated NSCLC tend to be younger and never smokers (23). The most common fusion is the *RET-KIF5B*, formed from the intrachromosomal rearrangement/somatic inversion of chromosome 10 in the pericentric region, resulting in ligand independent dimerization and constitutive activation of the *RET* tyrosine kinase. Seven different *KIF5B-RET* variants have been recognized; each differs with respect to *KIF5B* (1). *CCDC6*, *NCOA4*, *TRIM33*, *CUX1* account for the remainder of fusion partners (23,24,32,34,35). The coiled-coil domain of the translocation partner functions to promote ligand independent dimerization, inducing homodimerization leading to constitutive activation of *RET* and downstream growth signalling. The oncogenic mechanism is similar to that seen *ALK* rearrangements (34). Histologic features

of lung adenocarcinoma with *RET* rearrangement include those with solid growth pattern containing signet ring cells, mucinous cribriform pattern with abundant extracellular mucin. Lung adenocarcinomas with *ALK*, *ROS1* and *RET* rearrangements share similar histological features (solid signet-ring cell pattern and mucinous cribriform patterns) and it has been proposed that these features could be a marker of an underlying rearrangement associated adenocarcinoma (23). Commercially available multikinase inhibitors such as vandetanib have been shown to inhibit the proliferation of cell lines with *KIF5B-RET* and *CCDC6-RET* fusion (24). Preliminary data from a phase II trial using multitargeted kinase inhibitor cabozantinib showed three *RET* positive patients experienced partial response and disease control (33). This data highlights that *RET* rearrangements are an oncogenic driver in a subset of lung adenocarcinoma and is a potential druggable target, hence the importance of incorporating this into diagnostic assays.

### **Case selection for testing**

The new IASLC/American Thoracic Society (ATS)/European Respiratory Society (ERS) international multidisciplinary lung adenocarcinoma classification guideline highlights the role of the pathologist in reporting lung cancer in resection specimens, small biopsies/cytology specimens and provides guidelines for the management of tumor tissue in patients with advanced lung cancer. The histologic distinction into NSCLC subtypes (adenocarcinoma versus squamous cell carcinoma) is still based on tumour morphology. The use of a limited panel of immunohistochemical markers (TTF1/Napsin A, p63 or p40) is employed when this distinction is not possible, i.e., when dealing with small biopsy/cytology samples (NSCLC-NOS) with the ultimate aim of conserving tissue for further molecular testing (12). Currently all lung adenocarcinoma, mixed tumors with an adenocarcinoma component or a small sample where an adenocarcinoma component cannot be excluded should be forwarded for molecular testing. Cytology specimens are suitable for molecular testing with cell block preparations preferred over smears (6). Samples for metastatic lesions to bone are an issue as acidic decalcifying solutions cause extensive DNA fragmentation but fixatives such as EDTA preserve DNA integrity to some extent. The choice of testing of the primary lesion versus metastatic lesion is dictated by the quality of the specimen (tumor content and preservation) (36), although the most recent site of metastatic disease should be tested in a case of

a previously treated TKI sensitive tumor which progresses on treatment. There are many potential algorithms for the sequence of molecular testing that are usually dictated by local requirements and availability of testing. It is suggested that *EGFR* and *ALK* should be reflexly tested at the time of diagnosis to ensure results are available at the time when therapy needs to be instituted as DNA degrades even with optimal storage and block retrieval can take significant time and can delay instigation of treatment. Nevertheless, the choice of reflex testing versus clinician requested testing may best be decided at a multidisciplinary team setting (6).

### *Workflow in a laboratory*

The routine work flow for analysis of somatic mutation starts with histologic assessment, review and confirmation of the diagnosis on a representative haematoxylin and eosin (H&E) stained slide of the tumor. The proportion of tumor content is documented and the area containing the highest proportion of tumor is demarcated on the slide. The aim of this initial step is to enrich and prepare a high concentration of tumor cells that can be isolated using tissue macrodissection. The assessment should also document the presence of mucinous material, necrotic tissue, pigment and haemoglobin as these can inhibit the polymerase chain reaction (PCR). Nevertheless, in the authors' experience, depending on the assay selected, a result can be obtained on as few as 50 well fixed cells. The Illumina Truseq Custom Amplicon Cancer Panel recommends 250 ng of input DNA, however results can be obtained with as little as 150 ng. Although limited tissue availability is one issue, preanalytical factors such as fixation, tissue processing, long term and poor storage conditions have a far greater impact on the nucleic acid integrity of the tumor. During tissue processing, inadequate fixation/low pH formalin can induce DNA degradation and fragmentation. 10% neutral buffered formalin is an important and widely used fixative in diagnostic pathology to preserve tissue architecture, prevent enzymatic degradation/tissue autolysis and to support high quality and consistent staining with H&E. The two common forms of DNA changes caused by formalin fixation is fragmentation of DNA and sequencing artefacts (37-39). Formalin by its nature of fixation via cross linking of DNA also causes fragmentation of DNA, resulting in template DNA of short and variable fragment lengths. Other factors affecting the quality of the template DNA is the type of fixative used, time in fixation and temperature during tissue storage which can significantly alter/modify

the DNA fragment. After DNA is extracted from formalin fixed paraffin embedded (FFPE) material, a quick quality control measure is introduced to quantify the amount of DNA/RNA material. The aim of this step is to identify and select samples that would be suitable for further sequencing. The DNA can be quantified by spectrophotometry (the NanoDrop Spectrophotometer is an example of this) or by fluorometry using dyes that bind to double stranded DNA (the Qubit<sup>®</sup> assay is an example of this). Whilst these methods determine the bulk or concentration of DNA, they do not provide information regarding the quality of the template DNA (in terms of the underlying potential molecular damage and fragmentation) (39). The information regarding the DNA quality and template fragment lengths from FFPE material may be determined by using a multiplex PCR assay. This quality control measure uses amplicons of known varying lengths (e.g., 100, 200, 300, 400 and 600 bp) to assess the tumor DNA template for fragment size and to ensure that there are enough templates of suitable lengths for further molecular processing/next generation sequencing (38). Formalin also causes chemical modification of DNA, and cytosine deamination resulting in C > T sequence artefact post PCR amplification, which is particularly evident when using very fragmented template DNA (37-39). These will need to be taken into account when interpreting downstream results. In general, pre-analytical factors are difficult to control, but play a vital role in the quality of the DNA material for further molecular testing. It is imperative that tumour tissue be managed properly to ensure accurate and reliable data output as molecular assays are highly dependent on the quality of input DNA.

### **Molecular method/assays used in lung adenocarcinoma**

There are a wide variety of commercially available molecular assays used to detect mutations in lung adenocarcinoma. An ideal assay should be sensitive and specific enough to comprehensively cover all clinically relevant targets using limited samples, while being cost effective and efficient. In NSCLC the main types of somatic mutations in cancer include single nucleotide variants (SNV)/point mutations, small duplications/insertions or deletions (indels), exon/gene copy number changes and structural variants (from translocations/inversions) (3).

The methods used will depend on the type of mutation that is being detected. The techniques used to identify

*EGFR* mutations can be divided into “screening (or scanning)” or “targeted” (or specific mutation) genotyping methods (40). “Screening” technologies such as Sanger sequencing, Next Generation Sequencing (NGS), High Resolution Melt Analysis (HRMA) and Pyrosequencing have the potential to detect all *EGFR* mutations in the region of interest including novel mutations. In contrast, “targeted” assays such as the Agena MassARRAY Oncocarta panel, the Cobas *EGFR* Mutation Test (Roche Molecular Systems), the Therascreen *EGFR* Kit (Qiagen) and SNaPSHOT (by Life Technologies/Applied Biosystem) are usually highly sensitive to detect a preselected/ specific known mutations or “hot spot” mutations but by their design are unable to identify novel mutations. The consensus opinion of the CAP/IASLC/AMP is that any routine *EGFR* assay used in clinical practice should be able to detect the common *EGFR* TKI sensitizing mutations (exon 19 deletions and L858R) and mutations that confer decreased sensitivity to *EGFR* TKI (T790M, exon 20 insertions). Assays used should also be able to detect the following common and less common mutations in the *EGFR* gene: - exon 19 (15-bp, 18-bp, 9-bp, 12-bp, 24-bp, 27-bp deletions and 15-bp, 18-bp insertions), exon 18 (E709, G719 mutations), exon 20 (S768, T790M, insertions), exon 21 (L858R, T854, L861Q mutations) (6).

The techniques used for clinical detection of the underlying gene rearrangement as occurs with *ALK*, *ROS1* and *RET* include FISH, reverse transcription-PCR (RT-PCR) and IHC to detect the overexpressed protein caused by the underlying fusion transcript. Target specific break-apart FISH probes can detect a rearrangement regardless of the fusion partner but this technique is highly technical and expensive, and not feasible for screening of large samples for rearrangements of *ALK*, *ROS1* and *RET* that occur at low frequency. IHC offers an alternative option for screening, and is widely available in diagnostic pathology laboratories. Fusion specific RT-PCR combined with Sanger or next generation sequencing of the PCR products allows specific identification of the fusion partners, however the predesigned fusion specific primer/probes used may miss novel or unknown translocation partners that may not be detected by the preselected probes. The results of RT-PCR are also affected by the often degraded and poor RNA quality obtained from the FFPE material. A novel multiplexed expression gene expression/ transcript based assay known as the Nanostring nCounter assay works on the premise that a rearrangement causes mRNA overexpression of the 3' end of the gene compared to the 5' end of the gene. Novel next generation sequencing assays based on either

the relative expression of 5' versus 3' amplicons derived from the cDNA of the oncogenic partner of known fusions, or specific fusion targeted amplicons, have recently become available. The Archer™ *ALK*, *RET*, *ROS1* Fusion Detection Kit is a targeted sequencing assay based on Anchored Multiplex PCR (AMP) to simultaneously detect and identify fusions of human *ALK*, *RET* and *ROS1* genes (41).

### ***Molecular methods/assays for EGFR mutations: screening assays and targeted assays***

#### **Screening assays**

##### **Sanger sequencing**

Traditional Sanger sequencing or direct DNA sequencing is considered the gold standard for characterizing all mutations. Sanger sequencing is performed on PCR products and requires sequencing primers spanning the region of interest, DNA polymerase for primer extension, labelled nucleotides/ bases and a low concentration of modified nucleotide/bases (also known as dideoxynTP). All four nucleotide bases (adenosine, thymine, guanine and cytosine) are each labelled with a different fluorophore. Sanger sequencing is also known as “sequencing by termination” or “chain terminator sequencing” as it uses the ddNTP (modified nucleotides/bases) to stop primer extension. This creates DNA fragments of different lengths, which are then separated out with capillary gel electrophoresis. Sanger sequencing is often the orthogonal method used to confirm results due to its ability to characterize a wide variety of mutations (SNVs, small insertions/duplications/deletions/indels), however it is limited in detecting gene copy number changes. It is not scalable (as compared to massively parallel sequencing/ next generation sequencing). Sanger sequencing works on a small amount of input DNA (5-10 ng) however has low sensitivity. It requires that the mutant variant, which may be a minor component of the mixture be present at least 20% of the total tumour DNA to be detected (3,42).

##### **High resolution melt analysis**

High-resolution melt (HRM) analysis is a cheap, rapid and sensitive mutation screening (or scanning) method. It is used to identify samples that contain mutations for further characterization by sequencing. The starting DNA material is amplified in a real-time PCR reaction and a melt analysis is subsequently performed in the presence of a DNA binding dye (the dye fluoresces brightly only when bound to double stranded DNA). The process of HRM begins with increments in temperature to a point (melting temperature,

$T_m$ ) where the double stranded DNA (with high fluorescence) will “melt apart” to become single stranded DNA fragments (low fluorescence). The DNA containing the mutation will “melt” at a different temperature compared to the wild type DNA. This difference in melt curve signature is used to detect the presence or absence of a mutation. As HRM is a screening tool, a more specific method like DNA sequencing is needed to identify the precise mutation (42).

### **Pyrosequencing**

Pyrosequencing is also known as “sequencing by synthesis” and uses chemiluminescent detection of inorganic pyrophosphate to detect specific base additions. This is a quick, sensitive method to detect mutant DNA that utilises the template containing the region of interest, primers, DNA polymerase and a set of enzymes/substrates (ATP sulfurylase, luciferase, apyrase, adenosine 5’phosphosulfate and luciferin). During primer extension, pyrophosphate is released each time a nucleotide is sequentially incorporated onto the 3’ end of a DNA which through an enzymatic reaction results in light emission. The resultant sequence is determined from the pyrogram generated. Compared with Sanger sequencing, pyrosequencing is a sensitive method that allows detection of mutations in tumor samples as low as 5% (as is often the case when tumor material is heterogeneous and admixed with adjacent normal tissue) compared with 10-20% tumor material needed for Sanger sequencing. Pyrosequencing is best used to detect SNVs and is limited in its ability to detect gene copy number changes/ structural chromosomal changes (3,42). Pyrosequencing, and the related next generation sequencing systems utilizing this technology (Roche 454, Ion Torrent Personal Genome Machine (PGM) (Life Technologies/Thermo Fisher Scientific) next suffer from insensitivity in homopolymer repeats greater than 7-8 nucleotides in length.

### **Next generation sequencing (NGS)**

Massively parallel sequencing or next generation sequencing (NGS) is a mutation screening method. NGS technology has the ability for high throughput sequencing of a large number (up to millions) of DNA templates in a single reaction with multiple patient samples. NGS platforms can detect somatic mutations as low as 5% of tumor material (43). The many applications of NGS include sequencing of the whole genome, exome (protein-coding regions of the genome), or transcriptome (all expressed sequences). There are many available NGS platforms available that differ in their sequencing chemistries and methods of sequence detection but all share the same fundamental principles

and steps (44,45). Firstly a library is constructed followed by PCR amplification and sequencing. The initial library preparation may be created via random fragmentation of the starting DNA of interest and ligation/annealing of the DNA fragments to an adapter sequence/linker to create a “library”. The library is then amplified by repeated cycles of PCR reaction (on a solid surface) and then sequenced. The presence of specific adapter/linker sequences allows selective amplification by PCR reaction. Amplicon libraries may also be generated directly from unfragmented target DNA. The clonal amplification of templates can be performed by emulsion PCR (e.g., Ion Torrent PGM, Ion Proton, Roche 454 platform and ABI SOLiD) or with bridge PCR amplification to form clusters on a flow cell surface (e.g., Illumina platform) (44,45).

In massively parallel sequencing, the repeated cycles of nucleotide addition and detection of the incorporated bases (i.e., sequencing and detection) occur simultaneously (44,45). The platforms utilize different sequencing chemistries (44,45). In the Illumina platform, sequencing is by synthesis with reversible dye terminators. The identity of the incorporated nucleotide is determined by the specific fluorescence it emits (each nucleotide carries a specific fluorescent label, hence emits a specific wavelength) and this signal is detected. After the detection step, the 3’OH group is deblocked such that the fragment continues to be extended in each cycle. The Ion PGM instruments use a chemistry related to pyrosequencing, however the base addition is detected by the release of hydrogen ions during native nucleotide incorporation rather than inorganic pyrophosphate. This is a variation of pyrosequencing which monitors the pH change rather than pyrophosphate/light to detect the incorporation of nucleotide. Pacific Biosciences uses single molecule real time (SMRT) DNA sequencing whereby the fluorescently labelled nucleotide is added to the growing strand by DNA polymerase. The fluorescence which is attached to the terminal phosphate end of the nucleotide is cleaved by the DNA polymerase and the diffusion of emitted light is detected by zero-mode-waveguide (ZMW) (44). The sequenced “reads” are then aligned to a reference genome and analyzed with bioinformatics software (45). While whole genome sequencing provides extensive data on SNV, indels, complex structural arrangements and copy number changes, it is relatively expensive and the huge amount of data generated requires complex bioinformatics analysis and storage. Due to its high sensitivity, often incidentally discovered novel variants may pose challenges in interpretation as these are

of unknown clinical significance.

Compared with whole genome sequencing, targeted NGS/exome sequencing offers a more affordable, efficient and clinically applicable method for somatic mutational profiling in cancer as it focuses on clinically relevant genes. Targeted NGS/exome sequencing enriches the target of interest and focusses higher coverage or read depths over genomic regions of interest (46). In this method, the target of interest is enriched (either by PCR amplicon method or hybridization capture) and the application of deep sequencing focuses a high number of reads targeted to a region known to contain variants of clinical significance. A variety of bench top sequencers are now being used in diagnostic laboratories for targeted mutational profiling, as these have the ability to generate clinically important data at a lower cost and with a faster turnaround time.

A significant advantage of NGS that is particularly valuable for NCSLC is its ability to test multiple targets/genes of interest (as compared to sequential testing) on limited material from small biopsies and cytological samples. It also, unlike targeted genotyping assays (discussed below), is able to detect any type of mutation in the region of interest as compared to an assay used to detect only the specific mutations. Nevertheless, NGS technology uses PCR for amplifying target DNA and as such, is susceptible to issues inherent to PCR enzymatic amplification such as preferential amplification of certain library fragments. False artefacts/false variants may also occur due to substitution errors by PCR polymerase. Due to its inherent sensitivity, application of NGS in the diagnostic setting raises issues pertaining to the discovery of low frequency variants and their clinical validation and how these should be reported and applied to patient care. There are currently no standardized model or guidelines for the application of NGS in clinical practice, highlighting the need for validation of NGS technologies mainly in terms of the NGS analytical process (minimum coverage/depth of coverage) and standardization of bioinformatics packages (47).

### Targeted assays

Commercially available targeted assays for *EGFR* mutations include those from Agena Bioscience MassARRAY, SNaPShot by Life Technologies/Applied Biosystems, cobas<sup>®</sup> (Roche Molecular Systems) and theascreen<sup>®</sup> Mutation Kits (Qiagen). Targeted assays are also available for *KRAS* and *BRAF* mutations. The theascreen<sup>®</sup> KRAS kit (Qiagen) covering 7 mutations in *codons 12, 13* was approved by the U.S. FDA in June 2012 as a companion diagnostic

device for cetuximab for patients with metastatic colorectal carcinoma. The cobas<sup>®</sup> KRAS (Roche Molecular Systems) is designed to detect 19 *KRAS* mutations in *codons 12, 13* and *61*. In 2011, the U.S. FDA approved the cobas<sup>®</sup> 4800 BRAF V600 Mutation Test (Roche Molecular Systems) as a companion diagnostic test in conjunction with the approval of vemurafenib for patients with metastatic melanoma with the BRAF V600E mutation. The theascreen<sup>®</sup> BRAF kit is also available. These targeted assays allow for multiplex genotyping of known validated, “hotspot mutations” or genetic alterations simultaneously within a single assay, although the Agena assay looks at multiple genes depending on the particular assay. These multiplex testing platforms detect specific alterations/mutations that are known to be present in specific genes however are limited in their abilities to detect new or additional mutations outside the targeted region. Targeted assays are highly sensitive and can be performed with a lower amount of starting DNA material (5-10%) depending on the mutation compared with traditional Sanger sequencing (48-52).

### Agena bioscience massarray<sup>®</sup> system

Agena MassARRAY<sup>®</sup> system utilizes PCR amplification and allele specific single-base primer extension. Each nucleotide/base added to the primer contains a defined molecular mass and the primer extension products are analyzed using the principle of MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight). The time of flight is proportional to the mass/charge which is translated into specific genotype calls (43,53). There are multiplexed somatic mutation panels (reagent sets) that allow detection of known oncogenes. These customised panels with selected candidate genes are selected and distilled from large scale sequencing studies, to target clinically actionable mutations. Currently there is a multi-gene panel OncoCarta<sup>™</sup> Panel v1.0. covering key “actionable” mutations in the *EGFR*, *BRAF*, *KRAS*, *NRAS*, *c-Kit* genes and a LungCarta panel which comprises 214 somatic mutations in 26 tumor suppressor and oncogenes (*EGFR*, *KRAS*, *NRAS*, *BRAF*, *ALK*, *AKT1*, *DDE2*, *EPHA3*, *EPHA5*, *ERBB2*, *FGFR4*, *JAK2*, *MAP2K1*, *STK11*, *MET*, *NOTCH1*, *NRF2*, *NTRK1*, *NTRK2*, *NTRK3*, *PIK3CA*, *PTCH1*, *PTEN*, *PTPRD* and *TP53* (48,49).

### Snapshot<sup>®</sup> multiplex kit (applied biosystems<sup>®</sup>)

The SNaPshot multiplex kit/platform from Applied Biosystems uses multiplex PCR and single base primer extension using fluorescent labelled probes. The fluorescently labelled primer extension products are then detected by conventional capillary electrophoresis.



The SNaPshot panel tests for a smaller panels of genes and mutations (8 to 14 genes, >50 hotspot mutations) compared to the Agena MassARRAY<sup>®</sup> system (43). It allows multiplexing and rapid identification of single nucleotide polymorphism (SNP)/point mutations at specific sites of the PCR generated templates. This can be then be combined with a further sizing assay to detect deletions (e.g., in exon 19) and insertions (e.g., in exon 20). Although this is a commercially available platform, it allows users the flexibility to customize the kit and design the assay to meet the needs of the individual laboratories as an in-house assay. The workflow is simple and easily incorporated into diagnostic laboratories. The capillary electrophoresis automated DNA sequencer is a familiar and available equipment present in most clinical laboratories, avoiding further overhead costs. SNaPshot assays require less input DNA compared to Sanger sequencing. The main disadvantage of the SNaPshot platform is the limit to the number of assays/reactions that can be multiplexed (optimally below 10). It is not designed to detect amplifications, insertions or deletions.

#### **cobas<sup>®</sup> EGFR mutation test**

The cobas<sup>®</sup> EGFR Mutation Test (Roche Molecular Systems) is another allele specific real time PCR assay. In 2013, the cobas EGFR Mutation Test was approved by the U.S. FDA as a companion diagnostic test to select patients with EGFR exon 19 deletions or L858R substitution in exon 21 for treatment with erlotinib, concurrently as it was approved for use as first line treatment of metastatic NSCLC (50). The pivotal trial leading to the approval of erlotinib as new first line treatment was based on the results of the phase 3 European Randomized Trial of Tarceva Versus Chemotherapy (EURTAC) trial assessing the safety and efficacy of erlotinib compared to standard platinum based chemotherapy (54). The Cobas EGFR mutation test was used in this study to determine the EGFR mutation status of the trial patients. This assay uses Taqman probes in a qPCR reaction to simultaneously amplify and detect the mutations using specific probes (each with their own fluorescence). TaqMan probe based assays use two target specific primers flanking the region of interest and a third sequence specific probe to hybridize with the area of interest. The sequence specific probe contains a reporter molecule at the 5' end and a quencher molecule on the 3' end of the probe. When these two molecules are in close proximity, the interaction between the quencher molecule and reporter molecule prevents emission of fluorescent signals. The TaqMan probe relies on the exonuclease activity of Taq polymerase to cleave the dual labelled

sequence specific probe upon encounter during the PCR amplification phase. The cleaving process separates the reporter molecule from the quencher, resulting in a signal that can be detected. For the EGFR gene, it is able to detect 41 mutations in Exons 18, 19, 20 and 21 of the EGFR gene. The mutations covered by the cobas<sup>®</sup> system includes G719X (G719S/G719A/G719C) in exon 18, 29 deletions and mutations in exon 19, T790M, S768I, 5 insertions in exon 20 and L858R in exon 21 (2 variants) (51).

#### **therascreen<sup>®</sup> EGFR kit (qiagen)**

The therascreen<sup>®</sup> EGFR kit (Qiagen) is also another allele specific real time PCR assay. In 2013, afatinib was approved by the FDA as first line treatment of patients with metastatic NSCLC with EGFR exon 19 deletions or L858R mutations. This approval was based on the results of the LUX-Lung 3 trial. The therascreen<sup>®</sup> EGFR kit, used in the study was approved as a companion diagnostic test at the same time (50,55). For the EGFR gene it has been designed to detect 29 mutations in exons 18, 19, 20 and 21 of the gene. The mutations detected include G719X (G719S/G719A/G719C) in exon 18, 19 deletions in exon 19, T790M in exon 20, S768I in exon 20, 3 insertions in exon 20, L858R in exon 21 and L861Q in exon 21. The therascreen<sup>®</sup> kit uses ARMS (amplification-refractory mutation system) and Scorpions for the detection of these mutations. ARMS is an allele specific amplification process using Taq DNA polymerase to selectively amplify specific mutated sequences. Scorpions are used to detect the ARMS amplicon, hence detect the presence of mutations. Scorpions are molecules that contain a PCR primer linked to a probe (which contain both a fluorophore and quencher). When the Scorpion primer binds to the ARMS amplicon, it starts primer extension resulting separation of the fluorophore and quencher, with release of fluorescence (52).

Currently there is no consensus regarding the best method to conduct EGFR mutational testing (6). The two early pivotal trials in 2004 that showed an association with EGFR activating mutations in the tyrosine kinase domain being strong predictors to response to EGFR TKIs used traditional direct Sanger sequencing (8,9). The Iressa Pan-Asia Study (IPASS), a phase III randomized study of gefitinib versus carboplatin/paclitaxel in previously untreated never/light smokers with advanced NSCLC tested the clinically enriched population for EGFR for mutation status (using PCR ARMS EGFR mutation detection kit), EGFR gene copy number (with FISH) and EGFR protein expression (with IHC). The presence of EGFR mutation, rather than gene copy number and protein expression correlated with

better outcome with gefitinib (56). There are a number of commercially available PCR based targeted *EGFR* mutation detection kits (as listed above) which have high analytical sensitivity but may not cover all possible spectrum/variables outside the scope of their detection. Diagnostic laboratories providing this service will need to report all findings and integrate the findings into a clinically usable report for the oncologist to aid therapeutic decision making. All findings should be reported, with a comment if the mutation is: (I) one of the commonest mutation known to show sensitivity to EGFR TKIs; (II) uncommon, but has been reported in the literature to confer EGFR TKI sensitivity; (III) uncommon with unknown clinical significance; (IV) known to confer EGFR TKI resistance; (V) uncommon mutation of unknown clinical significance but the mutation is occurring in an exon where mutations are usually related to EGFR TKI resistance.

#### ***Molecular methods/assays for ALK, ROS1 and RET mutations***

Rearrangements and inversions characterize the mutations within the *ALK*, *ROS1* and *RET* gene in lung adenocarcinoma. As opposed to the above methods which are geared towards detecting SNVs and indels, FISH is the technique used to identify exon/gene copy number changes and structural variations from rearrangements and inversions in clinical practice. An alternate approach to the detection of *ALK*, *ROS1* and *RET* rearrangement is IHC. In NSCLC, IHC can be used to either detect either mutant specific product (e.g., specific *EGFR* L858R, *EGFR* exon 21 deletion, BRAF V600E) or in the case of *ROS1*, *RET* and *ALK*, IHC can detect overexpression of protein (resulting from underlying translocation) that does not occur in non-rearranged tumours.

In general, FISH and IHC testing methods detects *ALK* rearrangements without prior knowledge of the translocation partner. In the Australian experience, testing for *ALK* rearrangements vary depending on the individual testing laboratory. In general, centralized laboratories perform *ALK* testing either in parallel with or in a sequential manner after a negative result from *EGFR/KRAS* mutational testing. Simultaneous testing reduces turnaround times (TAT) but sequential testing is more cost effective. Many laboratories perform *ALK* IHC as a rapid and cheap triage, with equivocal or positive results being sent for confirmatory FISH testing at a reference laboratory (57). However, this often uses more of the limited material available for

testing and it is recommended that the two are performed in parallel. The other issue with IHC is the relatively poor quality assurance that occurs in laboratories without an orthogonal method that ensures that the IHC is accurate and reproducible. *ROS1* testing has also been implemented in some laboratories using both FISH and IHC.

#### **Fluorescence in situ hybridization (FISH)**

FISH is the current gold standard for the detection of *ALK* rearrangements although it cannot identify the fusion partner. FISH technology utilizes dual probes containing specific sequences of DNA to bind specifically to the nucleotide sequence on the target DNA. The probes are conjugated to a fluorescent molecule allowing detection. In NSCLC, FISH testing using the Vysis *ALK* Break Apart probe Kit (Abbott Molecular) was approved as a companion diagnostic test concurrently with crizotinib based on the clinical response seen in patients with *ALK* rearranged tumors using this method (58). There are other *ALK* FISH probes that are commercially available but not yet FDA approved (59) (e.g., ZytoLight<sup>®</sup> SPEC *ALK/EML4* TriCheck<sup>™</sup> Probe, Cytocell *ALK* Breakapart probe, Cytocell Aquarius *EML4* breakapart probe). In *ALK* wild type, the close proximity of the probes result in closely opposed or a fused (yellow) signal. Additional copies of the fused signal indicate polysomy, which can occur in both wild type and *ALK* rearranged tumours. A tumor is considered to have a rearrangement when (I) there is separation of the red and green signal by more than 2 signal widths or (II) when there is a single red signal without a corresponding green signal in addition to fused (normal) signals although the translocation partner will be unknown. Interpretation of *ALK* break apart FISH differs from other FISH probes as the translocation and inversion occurs on the same chromosome arm. False positive break apart signals may be due to the slight separation of the probes in some wild type cells and truncation artefact which may result in artificial signal separation (59). FISH is relatively expensive compared with IHC, requires technical expertise for interpretation and is usually only available in larger reference centres.

FISH is also used to detect *RET* and *ROS1* rearrangements using *ROS1* and *RET* Dual Colour Break Apart Probes (23).

#### **Immunohistochemistry (IHC)**

The use of IHC for *ALK* protein expression is based on the premise that *ALK* protein is normally absent in the lung

and the overexpression of ALK protein infers an underlying rearrangement of the *ALK* gene leading to constitutive activation and subsequent overexpression of the protein (59). There have been many studies comparing IHC with gold standard FISH testing using a variety of different antibodies (60,61). A recent study used five different ALK antibody clones 5A4 (Novocastra), D5F3 (Cell Signaling), ALK1 (Dako), ALKO1 (Ventana) and SP8 (Abcam), and comparing the results to ALK FISH showed that the D5F3 and 5A4 ALK clones stained all ALK FISH rearranged cases with weak/moderate/strong intensity with some false positive cases (61). The 5A4 and D5F3 clones have generally been shown to have higher staining intensity compared with the ALK1 clone (61,62). In studies using ALK IHC, two scoring systems are used for evaluation. One of these is a four tiered scoring systems with 0 (negative), 1+ (weak intensity cytoplasmic staining), 2+ (moderate intensity cytoplasmic staining) and 3+ (strong intensity cytoplasmic staining). Samples have been evaluated by the presence or absence of staining, or using several semi-quantitative methods including a histoscore (H score) of 1+ to 3+ by assessing the percentage of cells showing expression together with the intensity of staining. Cases are considered positive if there is 1+, 2+ or 3+ staining. The other scoring algorithm is a binary system from Ventana. In 2011, Ventana/Roche collaborated with Pfizer Inc. and Cell Signaling Technology to develop an automated and standardized IHC companion diagnostic test for *ALK* rearrangements to identify patients who would be eligible for treatment with Pfizer's Xalkori® (crizotinib). As such, the binary scoring system can also be applied when using the Ventana anti-ALK (D5F3) rabbit monoclonal primary antibody, as the assay has been developed to maximize concordance with ALK status as determined by FISH. A positive ALK IHC is determined by the presence of strong granular cytoplasmic staining in tumor cells, regardless of the percentage of positive tumor cells. The specimen is considered negative for ALK when there is an absence of strong granular staining in tumour cells. Staining may be seen in non-tumour elements (alveolar macrophages, nerve and ganglion cells, normal mucosal glandular epithelium, scattered lymphocytes, mucin, and necrotic tumour areas) and this is not regarded as a positive result. Some 1-2% of ALK negative cases may demonstrate a weak, diffuse granular cytoplasmic staining but these cases are considered negative for ALK due to the lack of strong intensity staining (62).

It is critical that IHC for ALK testing in NSCLC is optimized and modified for this specific use in lung

tissue, as the ALK expression in NSCLC is lower than it is in anaplastic large cell lymphoma. In NSCLC, *ALK*-rearranged staining is noted to be less intense, more granular, with staining within the cytoplasmic compartment as compared to in lymphoma (whereby the staining is more intense and with nuclear and cytoplasmic expression) (60). Although the low prevalence of *ALK* rearrangements would support IHC as a feasible pre-screening triage test with selected cases to be confirmed using FISH, IHC is subject to pre-analytical factors (technical aspects pertaining to tissue fixation), analytical factors (type of antibody clone used, endogenous peroxidase activity, necrosis/crush artefact) and post analytical factors (interobserver variation in evaluating scoring, different cut offs used for a positive/negative result). The observation that even the presence, absence or semi-quantitative analysis of protein expression by IHC in general community laboratories that do not have an orthogonal method to ensure accuracy and reproducibility is poor suggests that IHC use should be performed only where FISH is available. The European Society of Pathology (ESP) provides an external quality assurance assessment (EQA) scheme for testing of biomarker mutations in NSCLC. In 2012, a pilot EQA programme was conducted for *ALK* testing (IHC or FISH) and a second pilot was conducted for *EGFR*, *KRAS*, *ALK* (IHC, FISH or RT-PCR). *ROS1* testing was included in the 2014 scheme. Participation in such a scheme provides laboratories with an opportunity to verify and standardize their current practices, and to also improve the reliability of their testing platforms (63).

IHC has also been used to detect *ROS1* and *RET* rearrangements in NSCLC, with comparable results to FISH and RT-PCR (23). In this study, the novel *ROS1* rabbit monoclonal antibody D4D6 from Cell Signaling Technology showed differences between *ROS1* rearranged tumors and those without a *ROS1* rearrangement. The optimal immunostaining interpretive criteria to predict underlying rearrangements is not yet clearly defined. In a study by Yoshida (29), adenocarcinomas containing the *ROS1* rearrangement showed a range of staining pattern from diffuse to focal cytoplasmic staining, with some tumors showing cytoplasmic membrane accentuation at the apical or lateral surfaces. They suggest that H-score of more than 150, diffuse staining extent of more than 75% and moderate-strong intensity staining was felt to discriminate between *ROS1* rearranged tumors and those without the rearrangement. In rare cases, there was occasional staining of non-neoplastic type II pneumocytes and macrophages (29). As these rearrangements are

rare, IHC can be used as a screening tool for further confirmatory test.

### EGFR IHC

In terms of using IHC for *EGFR* testing, three main types of EGFR IHC tests exist: (I) IHC for total *EGFR*; (II) IHC for phosphorylated *EGFR*; (III) mutant specific EGFR IHC. Experience with the former two IHC types are limited and currently not recommended as standalone tests for patient selection for *EGFR* TKI therapy (6). The mutation specific EGFR IHCs that are commercially available target the two most common *EGFR* mutations (the L858R mutation in exon 21 and the common 15 bp/5AA deletion (E746\_A750del) in exon 19. The L858R antibody has shown high sensitivity and specificity for detecting the specific mutation compared to the accepted orthogonal methods. The other *EGFR* E746\_A750 exon 19 deletion antibody is limited at identifying other rarer variant exon 19 deletions other than 15 bp (64,65). As such, mutant specific EGFR IHC testing should be used in conjunction with orthogonal molecular methods in cases negative for mutant specific EGFR IHC tests. Mutant specific antibodies may play an important role in situations whereby molecular testing is limited by the amount of available tumor tissue, however mutant specific IHC are limited in identifying other less common *EGFR* mutations that account for up to 10% of cases. They also suffer from the vagaries of ALK IHC and thus it is not recommended as a first line test.

### Reverse transcriptase polymerase reaction (RT-PCR) to detect translocations/gene fusions in *ALK*, *ROS1*, *RET*

Besides FISH and IHC, multiplex RT-PCR is another method used to detect the different translocation in *ALK*, *ROS1* and *RET*. This method of detection is popular with Japanese investigators as highlighted in their work (24). RT-PCR combined with DNA sequencing allows precise and specific variant detection of the translocation partner, however this requires prior knowledge of the possible fusions/translocation partner in order to design multiple primer sets to detect this. For example, in *EML4-ALK* rearrangements whereby there are many breakpoints for *EML4*, the RT-PCR method would require multiple primer sets to discriminate between all known variants (18,23,29,66,67). Other rare non *EML4* fusion partners for *ALK* also exist (KIF5B, TFG, KLC1, STRN and *HIP1* as mentioned earlier) and this limitation needs to be taken into account when using the RT-PCR method for clinical detection of *ALK* rearranged NSCLC. FISH

and IHC methods can detect all fusions regardless of the fusion partner, and are useful for screening but specific identification of the (potentially novel) translocation partner will require multiplex RT-PCR.

### The future

The ability to multiplex and simultaneously detect many mutations at once is advantageous and important especially when dealing with small tumor samples as with NSCLC that are often procured during advanced disease. The patient may have metastatic disease to sites hampering access to adequate tumor material. The clinical condition of the patient may also limit the options of an invasive procedure to obtain tumor material. Archival FFPE tumor tissue hold a wealth of material for research however FFPE material is often degraded and of poor quality. As such, the need to adapt to these conditions is highly important as there is an increasing demand for more information from the often small amount of material received.

A recently described automated digital multiplexed gene expression/transcript based assay to simultaneously test for *ALK*, *ROS1* and *RET* fusions in NSCLC holds exciting promise as a practical modality for high throughput detection of fusion transcripts (66,68). Known as the nCounter gene expression analysis system (by Nanostring Technologies), this platform combines the advantages of FISH and IHC methods to determine the mutational/expression status of many genes simultaneously in a single test. The novel Nanostring nCounter system is capable of multiplexing up to 800 genes in a single test using a small amount of tumor material (100 ng of total RNA). The technology can be used on RNA/DNA samples and is compatible with RNA of variable quality, in particular FFPE material. As the targets are directly quantified, the nCounter system does not require a polymerase reaction (no conversion step to cDNA by RT-PCR or an amplification PCR step, hence avoiding errors that may potentially be introduced when using short/fragmented DNA material from FFPE). The low yields of RNA/DNA extracted from FFPE material are often degraded or may contain modifications that can inhibit the polymerase reaction, hence this may introduce possible bias to the results. Lira et.al used the nCounter transcript based assay to simultaneously detect *ALK*, *ROS1* and *RET* fusions in NSCLC samples, showing concordance with FISH and IHC methods (68). The benefit of the nCounter system is its ability to directly detect and quantify many targets in a single reaction using a limited sample. Whilst it

can detect the presence/absence of a fusion/translocation, the 3' overexpression detection method depends on only the higher expression levels of probes distal to the known fusion junctions. As such, it is limited in its ability to discriminate between the specific variant types/translocation partners (68).

The coupling of NGS technologies in conjunction with detection of circulating tumor cells (CTCs) and cell-free circulating tumor DNA (ctDNA) from lysed CTCs in plasma or serum provides a non-invasive method to monitor treatment and track disease progression (69,70). CTCs are thought to shed into the blood stream from the primary or the metastatic tumor deposits, while ctDNA are fragments of DNA that have been released from cells during cell turnover, cell lysis or cell death. The relative levels of CTCs and ctDNA in a patient can be used as a marker of tumor burden and treatment response. Molecular genotyping of the CTCs and ctDNA can be a proxy of the underlying mutations in the tumor from which they derive. CTCs can be characterized by their morphology (the whole cell can be analyzed), by IHC or FISH and genotyped with DNA/ RNA based assays. ctDNA are easier to isolate and extract as compared to CTCs and can be genotyped (for point mutations point mutations, copy number variations, chromosomal rearrangements and structural variations and methylation patterns). These "liquid biopsies" provide a surrogate and additional method of sampling tumor material (compared to more invasive biopsies and resection specimen). CTCs are thought to be mechanism by which tumour cells spread to its distal sites, and this methodology enables real time study of tumor *in vivo* complementing traditional radiologic imaging which is used for follow-up of these patients, to monitor treatment response. It also has the potential for early diagnosis of malignancy and intervention. The application of NGS technology for mutational analysis of CTCs enables detection of treatment resistance and guide clinical decision making (69,70).

## Conclusions

Molecular testing to detect oncogenic drivers for targeted treatment is now part and parcel of oncology practice in the era of personalized medicine. There are a multitude of platforms available for somatic mutational testing and the selection of platform is based on the type of mutation to be detected and local clinical and laboratory circumstances. It highlights the importance in using the right test and to select the right patient for the right drug. Screening assays offer the ability to detect all *EGFR* mutations and have the

potential to detect novel mutations, while targeted assays offer higher specificity and sensitivity to detect specific known mutations that are clinically actionable. FISH is used to detect fusions characteristic of *ALK*, *ROS1* and *RET* in lung cancer. IHC for *ALK* can be used as an effective screening strategy to select out cases for FISH testing. Novel technologies with the ability to simultaneously detect *ALK*, *ROS1* and *RET* fusions in a single assay show promise for use in the clinical setting as do liquid biopsies. The challenges of genomic testing lie in the complexity of cancer pathways, their heterogeneous nature with an evolving tumor genome that has potential to develop resistance. Rather than sequential testing of specimens for single mutations at the time of treatment, there is an increasing demand for multiplexing and simultaneous detection of many targets at once at the time of diagnosis.

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