Pitfalls in Lung Cancer Molecular Pathology: How to Limit them in Routine Practice?

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Abstract: New treatment options in advanced non-small cell lung carcinoma (NSCLC) targeting activating epidermal growth factor receptor (EGFR) gene mutations and other genetic alterations demonstrated the clinical significance of the molecular features of specific subsets of tumors. Therefore, the development of personalized medicine has stimulated the routine integration into pathology departments of somatic mutation testing. However, clinical mutation testing must be optimized and standardized with regard to histological profile, type of samples, pre-analytical steps, methodology and result reporting. Routine molecular testing in NSCLC is currently moving beyond EGFR mutational analysis. Recent progress of targeted therapies will require molecular testing for a wide panel of mutations for a personalized molecular diagnosis. As a consequence, efficient testing of multiple molecular abnormalities is an urgent requirement in thoracic oncology. Moreover, increasingly limited tumor sample becomes a major challenge for molecular pathology. Continuous efforts should be made for safe, effective and specific molecular analyses. This must be based on close collaboration between the departments involved in the management of lung cancer. In this review we explored the practical issues and pitfalls surrounding the routine implementation of molecular testing in NSCLC in a pathology laboratory.

Keywords: Molecular testing, non small cell lung carcinoma, pathology, pitfalls, EGFR, KRAS, BRAF,ALK, targeted therapy, quality assurance.

1. INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide, with “classical” treatment options lacking adequate specificity and efficacy [1,2]. Despite these therapies, the 5-year survival rate is about 15% for patients with lung cancer across all stages of the disease [3]. However, in recent years, the treatment options have changed from cytotoxic chemotherapies alone to single-agent and combination targeted therapies [4]. Recent advances in the understanding of cancer biology have allowed the development of molecularly targeted therapies that block oncogenic signaling pathways that characterize lung cancer cells (Fig. 1). Therapeutic decisions are based on the molecular analysis of lung cancer tissue specimens. The development of personalized medicine has challenged the routine integration into pathology laboratories of somatic genetic testing, with molecular assessment being regarded as a powerful supplement to the histopathological diagnosis [5,6].

Epidermal growth factor receptor (EGFR) gene mutations are commonly found among molecular abnormalities analyzed in non-small-cell lung cancers (NSCLCs), particularly in lung adenocarcinomas [7,8]. The epidermal growth factor receptor (EGFR, HER-1/ErbB1) is a receptor tyrosine kinase (TK) of the ErbB family, which consists of four closely related receptors: HER-1/ErbB1, HER-2/neu/ErbB2, HER-3/ErbB3 and HER-4/ErbB4. Upon ligand binding, receptor homo- or hetero-dimerization and phosphorylation activate the EGFR signal downstream of the PI3K/AKT pathway, which is involved in cell survival, or activate the RAS/RAF/MAPK pathway leading to cell proliferation [9,10]. Patients harboring activating EGFR mutations demonstrate response rates higher than 70%, 14-month progression-free survival and 27-month median overall survival when treated with EGFR-tyrosine kinase inhibitors (EGFR-TKIs; gefitinib, erlotinib) (Fig. 1) [11-13].

Since 2009, gefitinib (Iressa™, AstraZeneca, Macclesfield, Cheshire, UK) was the only licensed oral preparation for use in adult patients with locally advanced or metastatic NSCLC with activating EGFR mutations in all lines of therapy [11]. Recently, erlotinib (Tarceva™, Roche Group, Basel, Switzerland) has been granted European and US approval for the use as a first-line monotherapy in patients with locally-advanced or metastatic NSCLC with EGFR activating mutations. This molecule was already FDA and EMEA approved for use in maintenance and second-line treatment of NSCLC [14].

The short in-frame deletions in exon 19 and the exon 21 L858R point mutation account for approximately 90% of all EGFR mutations and are the most predictive of EGFR-TKIs efficacy in advanced lung adenocarcinomas [15-17]. However, several EGFR mutations contribute to primary or acquired resistance to EGFR-TKIs treatment [18]. The most conserved (~49% NSCLC cases) mechanism of resistance to TKIs is associated with the emergence of a single recurrent missense mutation T790M within the EGFR kinase domain [19]. Other secondary EGFR gene mutations such as D761Y, L747S, and T834A mutations have been associated to TKIs resistance, but with extremely low frequencies [18].

Intense research has led to a more detailed understanding of mechanisms of resistance in these tumors [20]. There is emerging evidence that mutations in other genes of the EGFR family (HER2) or related tyrosin-kinase receptors (cMET) as well as their downstream genes (in particular, KRAS, BRAF, PIK3CA, AKT1, MEK1) are present in NSCLC [21]. The presence of these mutations can be associated with a lack of response to the first-generation EGFR-TKIs in the treatment of NSCLC [22-24]. The amplification or mutation of cMET (20%) and PIK3CA gene mutations (5%) are among the most frequent “bypass mechanisms” which may determine resistance to TKIs, with continue activation of critical intracellular signaling pathways, despite continued EGFR inhibition [23]. In addition, with the greater understanding of tumor biology, agents that specifically target these oncopgenes are currently under development and are being evaluated into clinical trials (Fig. 1).

Recent studies have suggested that resistance to EGFR-TKIs may be mediated through cMET amplification or point mutations [25]. About 20% of patients with an EGFR mutation who initially respond to an oral EGFR inhibitor and finally progress are found to have a cMET amplification or somatic mutation [26]. Several agents targeting cMET are currently under clinical investigation as single agents as well as in combination regimens (Fig. 1) [27]. The selective, non-ATP competitive orally administered MET inhibitor, tivantinib (ARQ-197), has recently completed a phase II clinical trial and demonstrated an important progression-free survival.
improvement when combined with erlotinib, particularly among patients with non-squamous histology, EGFR wild-type status and KRAS mutations [28]. Cabozantinib (XL184), a multikinase inhibitor that targets cMET, VEGFR2, AXL, KIT, TIE2, FLT3, and RET, dramatically decreased tumor cell proliferation coupled with increased apoptosis and dose-dependent inhibition of tumor growth in breast, lung, and glioma tumor in vitro models [29]. Finally, the dual cMET and ALK inhibitor, crizotinib (PF-02341066), demonstrated tumor and metastasis inhibitory effects in both cMET and ALK-positive patients [30,31]. This compound is under investigation in a phase I study that combines this multikinase inhibitor with the irreversible pan-HER inhibitor PF-00299804 [32].

HER2 (Erbb2) gene mutations are found in approximately 2% of NSCLC [33]. The mutations are predominantly small, in-frame insertions in exon 20 and lead to constitutive activation of the mutant HER2 kinase. In NSCLC, activating mutations of EGFR and HER2 occur in a mutually exclusive manner [34]. Therefore, tumors harboring HER2 mutations do not respond to treatment with anti-EGFR inhibitors or to anti-HER2 antibody therapy alone (eg., Transtuzumab; Fig. (1)) [33]. Instead, HER2 insertions potentially predict sensitivity to treatment with pan-HER molecules that target EGFR and HER2 (eg., Lapatinib) [35]. Moreover, anti-tumor effects were observed when afatinib (BIBW 2992) that inhibits EGFR and HER2 was used in combination with mTOR inhibitors (eg., Sirolimus; Fig. (1)) [33]. The pan-HER inhibitor PF-00299804, with affinity for EGF, HER2, and HER4, has demonstrated activity in a phase II study, and seems to have activity in preclinical models of gefitinib resistance. However, it may not overcome resistance generated by cMET amplification [20]. In addition, several small-molecule TKIs that inhibit receptors such as VEGFR-2, EGFR, MET, PDGFR, and KIT simultaneously have demonstrated clinical value over agents with single targets [26].

Additionally, intense efforts to target mutant KRAS are under way, including dual inhibition of the critical downstream RAS effector pathways PI3K/AKT/mTOR and RAS/RAF/MEK (Fig. 1) [36].

The frequency of KRAS gene mutations varies according to tumor histology (15%–35% of adenocarcinomas), patient ethnicity (more frequent in Caucasians than Asian patients) and smoking history (more frequent in smokers than never smokers). In addition, KRAS mutations are non-overlapping with other oncogenic mutations found in NSCLC [37]. It seems that KRAS mutations work better as a negative predictor of response to EGFR-TKIs than EGFR mutations do as a positive predictor [36,38]. A number of agents targeting KRAS have been developed and are currently under clinical investigation. Farnesyl transferase inhibitors (FTIs; tipifarnib and lonafarnib), antisense molecules (e.g. ISIS 2503), and peptide vaccines are being tested in combination with cytotoxic therapy in clinical trials in NSCLC [26].

Somatic BRAF mutations are associated with increased kinase activity and as part of the MAP kinase pathway, are involved in cell proliferation, differentiation, and transcriptional regulation (Fig. 1). BRAF mutations have been initially identified in melanomas [39]. The BRAF mutations prevalence in NSCLC is approximately 1–3%, most of which are adenocarcinomas [33]. In contrast to melanoma where the punctual V600E mutation in BRAF kinase domain is the most prevalent (~90%) somatic alteration, NSCLCs can harbor mutations at other positions V600E (50%), G469A (39%), and D594G (11%) [40]. BRAF mutations are non-overlapping with other oncogenic mutations found in NSCLC (e.g. EGFR or KRAS mutations, ALK rearrangements) [33]. In lung cancer in vitro models BRAF mutations predicted decreased sensitivity to the EGFR-TKIs [22]. Most of the current clinical data concerning the BRAF inhibitors comes from promising studies conducted with these molecules in melanoma. Improved rates of overall survival
and progression-free survival have been reported in a phase III trial comparing vemurafenib to dacarbazine in previously untreated, metastatic melanoma harboring BRAF V600E mutation [41]. The activity of sorafenib, the multikinase inhibitor of BRAF, VEGFR-1, -2, -3, and PDGF was evaluated in NSCLC, but showed no significant difference in survival of patients [33]. However, the potential impact of BRAF mutations as predicting biomarkers of response to selective BRAF or MEK inhibitors is currently under investigation in NSCLC.

MEK1 (also known as MAP2K1) is a serine-threonine protein kinase downstream of BRAF and is a central mediator in the MAP kinase signaling pathway involved in cellular growth and proliferation (Fig. 1). The frequency of somatic MEK1 mutations is low (~1%) in NSCLC and these are more common in adenocarcinoma than squamous cell carcinoma. MEK1 mutations are mutually exclusive to EGFR, KRAS, HER2, and BRAF mutations [33]. The presence of MEK1 mutations has been associated with in vitro resistance to EGFR-TKIs [24]. Inhibitors of MEK, which target further downstream along the RAS/RAF pathway, have recently been developed (CI-1040, PD-0325901, and AZD6244) (Fig. 1). Preclinical and clinical studies with these agents have shown promising antitumor activity in the treatment of NSCLC [24].

Phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases with a key role in the mediation between growth factor receptors and intracellular signaling downstream signaling pathways (Fig. 1). Preclinical data support the major role of the PI3K pathway in cell proliferation, growth, apoptosis, cytoskeletal rearrangement, disease progression and resistance to chemo- and radiotherapy in NSCLC cell lines [42,43]. The PI3K/AKT/mTOR signaling pathway may be activated in cancer through multiple mechanisms including mutations in PIK3CA, which encodes the catalytic subunit of PI3K, loss or mutation of phosphatase and tensin homolog (PTEN), AKT mutations and deregulation of mammalian target of rapamycin (mTOR) complexes [44,45]. Somatic mutations in PIK3CA have been identified in 1-4% of all NSCLC [46]. These mutations occur more frequently within two “hotspot” areas within exon 9 and exon 20 [47]. PIK3CA mutations appear to be increased in squamous cell carcinomas than in adenocarcinomas and occur in both never smokers and ever smokers [47]. PIK3CA mutations may occur concurrently with EGFR, KRAS, BRAF, and ALK abnormalities [48]. Pre-clinical data demonstrated that PIK3CA activating mutations are sensitive to the dual PIK3CA/mTOR inhibition (Fig. 1) [45]. In exchange, recent studies suggest that coexisting KRAS and PIK3CA mutations may be associated with resistance to PIK3CA/mTOR inhibitors [49]. Multiple PI3K inhibitors are in early clinical development, but thus far the response rates to single agents are low [33]. In addition, PIK3CA mutations have been detected in EGFR mutant lung cancers with acquired resistance to EGFR TKIs therapy [23,50].

AKT is a downstream effector of PI3Ks and is constitutively activated in NSCLCs (Fig. 1) [51]. The prevalence of AKT1 mutations in NSCLC is about 1%, and they have only been identified in squamous-cell carcinoma [52]. Recently, pre-clinical data suggested that the combination treatment with selective MEK (AZD6244) and AKT inhibitors (MK2206) had a significant synergistic effect on tumor growth in vitro and in vivo leading to increased survival rates in mice bearing advanced human lung tumors [53].

PTEN is a tumor suppressor gene by negatively regulating the PI3K/AKT signaling (Fig. 1). PTEN may be down regulated through several mechanisms, including mutations, loss of heterozygosity, methylation, and protein instability, which contributes to lung carcinogenesis [26]. PTEN somatic mutations were identified in 4.5% of all NSCLCs. PTEN mutations were found in ever-smokers and were significantly more frequent in squamous cell carcinoma than in adenocarcinoma. In pre-clinical studies, PTEN loss in EGFR mutant lung tumors is associated with decreased sensitivity to EGFR-TKIs [54].

mTOR plays a critical role in transducing proliferative signaling mediated through the PI3K and AKT signaling pathways, that is essential for cancer cell growth and proliferation (Fig. 1) [20]. It’s inappropriate activation is involved in the pathogenesis of numerous tumor types, including NSCLC [26]. Numerous molecules interfere with the PI3K/AKT/mTOR pathway at multiple levels. Some of them, such as the mTOR inhibitors temsirolimus and everolimus, are already approved by the FDA and EMEA for other indications, such as renal-cell carcinoma based on previously published phase III randomized trials. mTOR inhibition demonstrated promising results in KRAS-mutated cell lines [49].

Frequently mutated tumor suppressor genes in NSCLC include TP53 (50%), CDKN2A (p16) (17%), and LKB1 (STK11) (11%) [55,56]. TP53 mutations are more common in squamous cell carcinoma (62%) than in adenocarcinoma (39%), with the most frequent mutations occurring in exons 5–8 [55]. TP53 mutations are more commonly found in the presence of EGFR mutations in never-smokers patients [56]. LKB1 is more frequently mutated in adenocarcinoma than in squamous cell carcinoma (15% vs. 5%), in Caucasian vs. Asian patients (17% vs. 5%), and rather limited to male smokers. LKB1 mutations may co-exist with KRAS or BRAF mutations [57,58]. Notably, cell line studies have shown that NSCLC tumors with concurrent mutations in LKB1 and KRAS demonstrate sensitivity to mTOR and MAPK inhibition that is not apparent with either mutation alone [59]. Moreover, studies using gene therapy by replacement of tumor suppressors in preclinical, and in some early-phase clinical trials for NSCLC, have been performed. In this regard, the most evaluated strategy was that of restoring wild-type p53 expression in lung tumor cells [60]. In general, these trials have demonstrated safety, with low efficacy. Although, some phase I studies of p53 replacement with adenoviral vectors suggested clinical responses with a few partial responses, phase II studies failed to demonstrate difference in response rates for Ad.p53/chemotherapy-treated lesions chemotherapy alone [61]. There are no current trials ongoing in the United States or Europe using this approach in lung cancer. In the opinion of the researchers, the lack of strong bystander effects, along with the low transduction efficiency of adenoviral vectors, limited the potential application in lung cancer, unless more efficient vectors are developed [60]. In recent years, the field of gene therapy in NSCLC has shifted toward “immuno-gene therapy”. This strategy requires enough gene transcription to stimulate an endogenous immune response and to create a strong bystander effect. Although these strategies seem to be successful in initiating anti-tumor immune responses, it is generally recognized that there some limits remain (e.g. large tumor volumes; significant immuno-inhibitory networks created by the tumors involving cytokines such as TGF-β, interleukin-10, prostaglandin E2, and vascular-endothelial cell growth factor; and inhibitor cells such as T-regulatory cells and myeloid-derived suppressor cells) [62].

Recent data showed that fibroblast growth factor receptor 1 (FGFR1) may be a new promising molecular target for the treatment of smoking-associated lung cancer [63]. FGFR1 controls a wide range of biological functions in embryo genesis, development, wound healing, angiogenesis and metabolism, by regulating cellular proliferation, survival, migration and differentiation [64]. High-resolution genomic profiling demonstrated that the chromosomal region at 8p12 spanning the FGFR1 gene locus is amplified in up to ~20% of squamous cell lung carcinoma, and is a rare event (1-2%) in lung adenocarcinoma [65,66]. FGFR1 copy number aberration can be detected by several techniques, including fluorescent in situ hybridization (FISH) analysis [65]. The treatment of mice with FGFR1-amplified squamous cell lung cancer xenografts with a small anti-FGFR1
molecule (PD173074) resulted in significant tumor shrinkage in vivo [65]. In addition, this pathway may function as a mechanism of resistance to anti-EGFR and anti-VEGF treatment [67,68].

In 2007, the echinoderm microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK) fusion gene was identified in NSCLC [69]. EML4-ALK fusion results in protein oligomerization and constitutive switch on the RAS/RAF signaling pathway [49]. Transgenic mice expressing EML4-ALK under the control of a lung epithelial cell promoter develop multiple lung adenocarcinomas, demonstrating the oncogenic nature of this fusion gene [33]. EML4-ALK fusion is a rare abnormality detected in approximately 2-7% of unselected patients with NSCLC, a frequency that increases (13%) in a population of patients with at least two of the following characteristics: female sex, young adults, Asian ethnicity, never (<100 cigarettes in a life time) or light (<15 pack-year) smoking history, and adenocarcinoma histology [70]. EML4-ALK rearrangements are generally found in tumors with wild-type EGFR, KRAS and BRAF [71]. As for EGFR-TKIs, ALK inhibitors have been found to be highly effective in lung cancers that have this translocation [72]. The small molecule TKI crizotinib (PF02341066; Pfizer, New York, NY, USA) is an orally ALK inhibitor of phosphorylation and signal transduction. This inhibition is associated with G1-S phase cell cycle arrest and induction of apoptosis in positive cells in vitro and in vivo [26]. In a phase 1–2 trial, disease control was achieved in 47 (57%) of 82 patients and 27 (33%) patients with ALK-fusion-positive tumors had stable disease [73]. These dramatic findings led to two subsequent clinical trials of PF-02341066. The first is a randomized phase III trial of PF-02341066 compared with standard second line chemotherapy by pemetrexed or docetaxel in EML4-ALK rearranged NSCLC. The second is a phase II clinical trial of single agent PF-02341066 in EML4-ALK positive NSCLC designed for patients not eligible for the phase III trial or patients randomized to chemotherapy who subsequently developed progressive disease [26]. Moreover, patients who harbor this fusion gene do not benefit from EGFR-TKIs and should be directed to trials of ALK-targeted agents [70].

Finally, an area of increasing interest is the development of rationale combinations of conventional cytotoxic drugs with molecularly targeted therapies, or for combining molecular targeted alone to increase the therapeutic potential by blocking cancer cell survival mechanisms. However, at this point no general guidelines to deal with such combinations exist. Here, we reported several positive results with some combinations, although most of them were reported in pre-clinical studies. The drug interaction patterns observed in vitro may not be similar to those observed clinically. As previously noted, the synergy between cytotoxic and targeted therapies cannot always be reliably predicted from preclinical models and inevitably requires clinical validation [20]. Recently, in the INTACT-1, INTACT-2, TALENT, and TRIBUTE clinical trials, the addition of gefitinib or erlotinib to first-line chemotherapy failed to improve survival of NSCLC patients [75]. Some hypotheses were proposed to explain these disappointing results. First, further research efforts are necessary to select biomarkers that may predict response to targeted therapies. Second, all trials applied chemotherapy and targeted drugs simultaneously [76]. Recently, a pharmacodynamic separation model was proposed to bypass this issue: EGFR-TKIs primarily cause cell cycle arrest and accumulation of cells in G1; and thus, when administered concurrently with chemotherapy, may push tumor cells to the dormant phases of the mitotic cycle and render them resistant to classic cytotoxic agents [77]. Therefore, as no guidelines are available for the moment, the definition of the optimal schedule of administration of chemotherapy with molecularly targeted therapeutic agents largely remains a controversial clinical issue.

Taken together, these molecular events define molecular subsets in NSCLC that have been identified as potentially having clinical relevance to targeted therapies [33]. Therefore, additional molecular analyses of genes other than EGFR have become necessary to improve patient selection for EGFR or other driver mutation targeted therapies. Before the implementation of EGFR molecular testing as a clinical practice, EGFR genotyping was performed within the context of experimental settings or clinical trials. In this context, tissue samples, tissue processing, and storage conditions were homogeneous, whereas in a routine setting, these different elements are more heterogeneous [78]. Currently, molecular testing for predictive biomarkers in NSCLC is neither standardized nor validated and is yet to be implemented as routine practice in a pathology laboratory. Considering the medical consequences of EGFR genotyping and the need to ensure high quality and reproducible analyses, technical guidelines and recommendations for EGFR testing in NSCLC were proposed [2,79]. These recommendations emphasize the key role of the pathologist in the selection and preparation of tissue samples, EGFR assay selection, and standardized reporting of results.

This review will address the need for standardization and set out the main pitfalls of molecular testing in routine practice in the management of NSCLC patients.

2. RELEVANCE OF MORPHOLOGICAL SAMPLE ASSESSMENT FOR MOLECULAR TESTING

Targeted therapies directed against specific molecular alterations require precise histological sub-classification of NSCLC [80]. Data regarding EGFR mutations predicting responsiveness to EGFR-TKIs have established the importance of histology in treatment outcome with EGFR inhibitors. Medical oncologists place high demands on pathologists to distinguish squamous cell carcinoma from adenocarcinoma and NSCLC not otherwise specified (NOS) in patients with advanced lung cancer [81]. However, it is noteworthy that approximately 70% of lung cancers are well differentiated and staged by small biopsies or cytology rather than by surgical resection specimens. As expected, more limited samples will result in a less specific diagnosis, in particular with the recent increase in the use of transbronchial needle aspiration (TBNA), endobronchial ultrasound-guided TBNA and esophageal ultrasound-guided needle aspiration [82]. The latest classification of lung adenocarcinoma emphasizes the use and integration of immunohistochemical (i.e., thyroid transcription factor [TTF-1]/p63 staining), and histochemical (i.e., mucin staining) studies for more accurate diagnosis [81]. As well as helping subtype NSCLC, immunohistochemistry (IHC) is sometimes required to discriminate between primary and metastatic disease. However, the IHC profile does not always confirm diagnosis of squamous cell carcinoma or adenocarcinoma. These cases should be reported as ‘NSCLC’, but with the added caveat ‘probably squamous cell’ or ‘probably adenocarcinoma’ (Table 1) [83]. If there is doubt about the histological subtype then molecular testing should be performed. Performing unnecessary IHC tests wastes time, tissue, money and laboratory capacity. This is of great importance, especially when the reference pathology laboratory performs molecular testing on outsourced specimens without knowledge of the percentage of tumor cells left on the paraffin embedded tissue block after the immunohistochemical study. To evaluate tumor content, it is recommended that the pathologist assess a hematoxylin and eosin stained section of the tissue area of the paraffin block designated for mutation analysis before DNA extraction (Table 1).

Small tissue specimens have to be well managed not only for morphological diagnosis but also to maximize the amount of tissue
available for molecular studies. As 10 to 30% of tissue specimens continue to be diagnosed as NSCLC-NOS, the International Association for the Study of Lung Cancer (IASLC) has published recommendations for strategic use of the minimum amount of specimen necessary for accurate diagnosis to preserve as much tissue as possible for potential molecular studies made in a second intention [81].

3. REQUESTING MUTATIONAL STATUS

The patient EGFR mutational status remains the best predictor of benefit from EGFR-TKIs therapy [36]. EGFR somatic mutation testing is certainly the first molecular test prescribed in advanced stage lung adenocarcinomas [2]. KRAS and EGFR mutations are mutually exclusive molecular events [84]. Given that the EGFR-

KRAS signaling cascade is considered to function as unidirectional linear outside-in signaling, tumors harboring mutant KRAS are independent of EGFR activation, and are resistant to EGFR-targeted therapy [36]. One question surrounding KRAS testing is whether routine screening of KRAS mutations before EGFR testing to identify patients who do not benefit from EGFR-directed therapy, is worthwhile in terms of improving outcome in clinical practice, without negative consequences from delaying treatment until results are available [17,85].

In our pathology molecular laboratory (LPCE, Pasteur Hospital, Nice, France), KRAS testing is performed in conjunction with EGFR analysis. Detection of a KRAS mutation is a useful finding in a small-volume or poor-quality test samples, removing what may otherwise be an understandable concern regarding a false negative

<table>
<thead>
<tr>
<th>Step</th>
<th>Pitfalls</th>
<th>Recommendation</th>
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<tbody>
<tr>
<td><strong>Morphological sample assessment</strong></td>
<td>Distinguish squamous cell carcinoma from adenocarcinoma and NSCLC NOS</td>
<td>Immunohistochemical (TTF1/p63 staining) and histochemical (mucin) studies. May report “probably adenocarcinoma”</td>
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<tr>
<td><strong>Material for molecular analysis</strong></td>
<td>No knowledge of the percentage of tumor cells</td>
<td>Perform HE stain before molecular test</td>
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<tr>
<td><strong>Time point</strong></td>
<td>Extended turnaround time</td>
<td>At diagnosis or at disease progression if possible</td>
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<tr>
<td><strong>Who may order?</strong></td>
<td>Extended turnaround time</td>
<td>Treating physicians or pathologists</td>
</tr>
<tr>
<td><strong>Sample quality</strong></td>
<td>Warm ischemia time</td>
<td>Rapid transfer to the pathology laboratory (e.g., pneumatic air tube transport system)</td>
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<tr>
<td><strong>Fixation</strong></td>
<td>Type of fixative</td>
<td>Avoid prolonged fixation</td>
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<tr>
<td><strong>Delay of fixation</strong></td>
<td>10% NBF should be used. Bouin’s or fixative substitutive fixatives should be avoided. Cryopreservation should be the standard method for tissue fixation and preservation.</td>
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</tr>
<tr>
<td><strong>Type of specimen</strong></td>
<td>Limited size or available sample</td>
<td>Biopsy preferred to cytology specimens</td>
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<tr>
<td><strong>Tumor content</strong></td>
<td>Low percentage of tumor cells</td>
<td>Enrichment for tumor cells (macro- or microdissection)</td>
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<td></td>
<td>Minimum percentage of tumor cells required for molecular analysis</td>
<td>Adaptation according to the estimated analytical sensitivity of each method. Refusal if to low or absent.</td>
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<tr>
<td><strong>Sample preparation</strong></td>
<td>DNA extraction</td>
<td>Between 1 and 10 FFPE sections of 5- to 10-µm thickness. Avoid contamination (e.g., separate dedicated lab areas, changing blades, sample-to-sample traceability, regular cleaning and decontamination, dedicated sterile scalpel for dissection). Fresh-frozen material should be the standard. Interfering substances removal (e.g., melanin). Test DNA quality (Control PCR amplification). Optimized and controlled reagents.</td>
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<td><strong>Analysis success rate</strong></td>
<td>Analysis success rate</td>
<td>95% of samples with successful DNA extraction.</td>
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<tr>
<td><strong>Screening vs. targeted methods</strong></td>
<td>Validated methods vs. “in-house” tests</td>
<td>Should validate and verify each method</td>
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<tr>
<td><strong>Sensitivity</strong></td>
<td>Analysis methods</td>
<td>According to clinical needs in agreement with physicians</td>
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<tr>
<td><strong>Specificity</strong></td>
<td>Reduced performance of mutation immunohistochemistry</td>
<td>Needs further validation</td>
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<td></td>
<td>Interpretation of positive ALK rearrangement by FISH analysis</td>
<td>At least two experienced pathologists should perform the reading. Use positive and negative controls.</td>
</tr>
<tr>
<td><strong>Analysis success rate</strong></td>
<td>Analysis success rate</td>
<td>97% of samples with correct mutation test results.</td>
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<tr>
<td><strong>Quality assurance</strong></td>
<td>Quality assurance</td>
<td>Needs accreditation / Guidelines / External Quality Assessment Programs</td>
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<tr>
<td><strong>Final report</strong></td>
<td>Final report</td>
<td>Should be reported, in conjunction with the identification of patient and health care professional, the pathology diagnosis, details on the tissue block tested, sample source, sample size and quality, estimation of the proportion of tumor cells in the sample extracted for DNA amplification, the method used, estimated test sensitivity and specificity, test results (mutant or wild-type allele) and interpretation of results in the context of the indication for testing</td>
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EGFR mutation test [83]. Although KRAS mutation analysis is not recommended to exclude EGFR mutations and therefore to make EGFR-directed treatment decisions in clinical practice, there may be a place in the near future for this molecular test in making other treatment decisions. In this regard, the BATTLE program is assessing potential biomarkers for a variety of NSCLC treatment strategies, including the potential utility of KRAS for predicting treatment success with antiangiogenic agents (i.e.; sorafenib) [86].

Genetic mutations in the genes encoding proteins involved in EGFR signaling pathways (KRAS, BRAF, PIK3CA, AKTI, MAP2K1, and MET) are mutually exclusive somatic events with exception for PIK3CA gene mutations [87]. Development of new therapies targeting the downstream RAS effector pathways PI3K/AKT/mTOR and RAS/RAF/MEK will probably require knowledge about the mutational status for these latter molecules [4]. The future value of testing for the KRAS or BRAF mutational status may be to exclude the possibility of an EGFR mutation or ALK translocation or to identify a molecular subset of patients with NSCLC in whom a drug development strategy that targets the KRAS pathway is pursued [36].

There is a need to define a standardized stepwise algorithm for molecular testing in the management of NSCLC patients in relationship to available targeted therapies. Fig. (2) shows our current workflow for routine molecular testing of advanced lung adenocarcinoma.

This algorithm has already been used by several large academic centers in the United States and Europe [4]. Moreover, pathologists may order mutational testing at the time of the diagnostic workup in order to reduce the turnaround time for molecular testing and also to provide a complete histological/ molecular diagnosis (Table 1).

Considering the multiple genetic alterations occurring in NSCLC, how pathology departments that do not have adequate molecular laboratory support will manage NSCLC specimens is a strong issue. Testing by outside laboratories brings up another challenge, such as integration of all available data about the tumor (e.g., histology, stage, and mutational profile) in the final pathology molecular report and in the patient’s medical records, which is much easier if testing is performed in a single pathology laboratory [4]. Close collaboration, communication flow, and coordination between the departments involved in the management of lung cancer are essential to implement mutation testing in routine practice. Moreover, the development of the concept of integrative pathology associating a morphological pathology laboratory, a molecular pathology laboratory and a biobank in close proximity is probably a good system for optimal patient care.

4. PRE-ANALYTICAL ISSUES

4.1. Specimen Collection

As therapy for NSCLC evolves into more individualized approaches, it becomes increasingly important to optimize the collection, handling, and storage of tissues to improve the consistency and accuracy of molecular analysis. Furthermore, DNA integrity is strongly impacted by the choice of tissue sample and the method of preservation [80]. The influence of pre-analytical steps on biospecimen quality is well-recognized by tumor biobanks. Several markers of sample quality have been identified [88]. One pre-analytical variable of particular interest is the time of ischemia, which may affect the DNA integrity. This includes the time of warm ischemia (from the time of blood vessel ligation to surgical excision) and the time of cold ischemia (from excision to freezing...
or fixation). Ideally, the length of both times should be kept to a minimum and both should be recorded [89]. Clearly this is more difficult for the time of warm ischemia, which depends on the surgical procedure being of absolute priority [89]. Pneumatic air tube transport systems can provide safe, efficient and rapid means to optimize the time of cold ischemia [90]. This should be implemented in pathology services, notably in those performing molecular testing.

4.2. Fixation

Another key point in the pre-analytical steps is the fixed tissue on which molecular analyses are performed in the molecular pathology laboratory. Molecular testing is generally performed on formalin-fixed paraffin-embedded (FFPE) specimens. To date, formaldehyde as 10% neutral buffered formalin (NFB) is the most widely used universal fixative as it preserves a wide range of tissues and tissue components. The acid environment of the Bouin’s fixative damages both DNA and RNA, and should be definitively avoided for molecular testing [91]. The use of formalin substitute fixatives eliminates exposure of laboratory staff to formaldehyde. However, the DNA quality is somewhat variable when formalin substitute fixatives are used. In addition, the cost of formalin substitute fixatives must also be taken into consideration, and they still contain components that are potentially toxic for humans and are also inflammable [92]. Studies conducted on the preservation status of nucleic acids in FFPE tissues generally agree on the alteration of DNA [92,93]. Considerable evidence suggests that the DNA quality and PCR productivity from FFPE tissues is inferior to frozen tissue, although it may yield adequate results in many cases depending on the type of fixative solution and time of fixation before embedding. We are aware that the integration of tissue cryopreservation could be quite challenging in many pathology services due to the high cost or to the extra storage space required. Moreover, morphological control of a frozen section performed before DNA extraction is always necessary. However, immediate freezing of tumor samples in liquid nitrogen and storage at −80°C or in liquid nitrogen vapor represents the best method for tissue fixation and preservation and can be challenging in routine practice for future multiple molecular analyses and deep sequencing performed from small samples [94]. Finally, the two general rules to prevent DNA damage during storage are “low temperature” and “as dry as possible” [78].

Prolonged fixation in NFB rarely yields good quality DNA [93,95]. As a general rule, short fixation times, 6-12 hours for biopsy specimens and 8-24 hours for larger resection specimens in 10% NFB should be optimal for DNA-based tests, as well as for FISH assays [96].

5. TUMOR TISSUE SAMPLE

Many issues regarding tumor samples dedicated to molecular testing remain to be defined, notably in routine practice, such as the type of specimen, the tumor content, the area of necrosis, the biopsy site, and the reliability of molecular analyses on other specimens, such as cytology or plasma samples. To optimize molecular testing in NSCLC patients, there is a need to maximize tumor tissue acquisition, as described above. Currently, most of the NSCLCs are diagnosed at an advanced stage, and the current trends favor minimally invasive diagnostic procedures, which are challenging since it is difficult to get enough tumor tissue for molecular assessment. Cytological specimens are frequently available for diagnosis. Different methods using cell blocks, scraped cells from archival slides, and fresh cells have been used for molecular testing [97]. However, cytological specimens have not been widely used for molecular testing due to the heterogeneity within samples, and to the sparse cellularity [98-100]. Even though cytological samples may be suitable for analysis, this method needs validation for routine molecular testing, and further research is needed to fully understand the clinical reliability of mutational data obtained from these samples. Collection and handling of cytological samples still have to be optimized for molecular testing in routine practice. Until then, clinicians should be encouraged to provide tissue biopsy samples whenever possible. In this regard, the NSCLC Working Group published recommendations indicating that FFPE tissue blocks are the preferred sample type for EGFR molecular assays (Table 1) [99]. Finally, imaging-guided needle biopsy seems to be satisfactory for mutation analysis. An average of 1.8 needle passes with small (18–20 gauge) core needles yields sufficient and reliable samples for mutation analysis [101].

For mutational analysis, the proportion of malignant cells within the sample is critical for the detection of gene mutations [79]. Enrichment for tumor cells may be required to improve the reliability and accuracy of the detection of tumor-specific somatic mutations, particularly for direct sequencing [79,99]. Selection of material is based upon histological examination of HE sections, and, most often, macrodissection of glass-mounted unstained sections to select for suitable tissue. Laser capture microdissection is a more elegant and accurate approach, but time-consuming, cost effective and less often available or practical and usually not required [83,102]. The minimum number of tumor cells required for adequate mutational analysis is not defined. The European EGFR Working Group has recommended that a sample should contain at least 200 tumor cells, with no abundant necrotic tissue, with at least 50% of tumor cells in order to reduce false negative results from sequencing [79]. Reliable results can be obtained with a tumor percentage as low as 10 to 20% when sensitive technical procedures are used. Several methods with higher analytical sensitivity than direct sequencing can detect mutations present at very low levels (1–5% gene copies mutated), allowing the possibility of detection of mutations when the tumor cell proportion is less than 10% of the test sample [103]. In any case, the analytical sensitivity of the method used must be referred to in the final molecular report, as well as the percentage of tumor cells observed in a mirror tissue sample (Fig. 3).

Although pathologists are aware that they should strive to increase the proportion of tumor cell in material for analysis to well above the threshold of the method used, they should not rely upon the sensitivity of the test to compensate for poor specimens [83]. Moreover, one question surrounding the size and the minimum tumor content is whether to exclude clinical specimens for molecular testing. We strongly believe that the refusal to do molecular analyses should be based on specific validated planned exclusion in close collaboration between the physicians and the departments involved in the management of lung cancer. Recently, the European EGFR Working Group recommended that rebiopsy at the time of recurrence or disease progression, or even during initial patient work-up, must be considered specifically for mutation testing if the initial samples are inadequate for mutation analysis [79].

The biopsy site, such as primary versus different metastatic tumors, may also influence the final molecular results. Several studies have demonstrated a substantial rate of discordance in results of mutational and FISH assays between primary and metastatic lesions, suggesting tumor heterogeneity at the molecular level during the process of metastasis [104-108]. This heterogeneity may depend on the gene. Usually, clinicians sample only the most easily accessible tumor tissue [79]. However, it seems more relevant to perform molecular testing on the metastatic sites and on tissue from recurrent tumors.

6. TISSUE PROCESSING FOR MOLECULAR TESTING

FFPE processing impairs the extraction efficacy and quality of DNA, thus preventing the possibility of conducting high-quality
molecular analyses and potentially affecting the results of molecular analyses. Fresh-frozen tissue represents an ideal source of archival material for molecular investigations but is not usually possible in routine practice [109].

In most pathology laboratories, DNA-extracts from FFPE samples are the most common source of template for PCR amplification. Although commercial kits for molecular pathology testing are now available, most of the current tests use in-house methods since they are much cheaper. Thus, there is an urgent need for standardization of procedures in molecular pathology, starting from nucleic acid extraction [110]. The choice of a method of nucleic acid extraction depends on several factors, including assay targets (RNA/DNA-based), specimen type, sample throughput, laboratory workflow, cost, and the performance of the extraction system. Thus, we have to keep in mind that the sample preparation processes of DNA/RNA extraction and purification are time-consuming and labor-intensive. Several steps are required, including lysis, nucleic acid extraction, impurity washes, and DNA/RNA elution.

In regard to the type of tissue preservation, the extraction methods are different when using either FFPE or fresh-frozen samples [103]. FFPE sections require a supplementary time of deparaffinization. Moreover, the xylene used to remove paraffin is toxic. It can cause serious health problems and must be handled under a safety hood.

Sections cut from the FFPE tissue blocks are the standard resource for DNA extraction. Between one and ten sections of 5- to 10-µm thickness should be used (Table 1) [2]. Laboratories that use laser capture microdissection will require thinner sections [2]. To avoid contamination or sample-to-sample carry-over, some precautions are required, such as performing extraction and amplification in separate dedicated laboratory areas (preferably a self-contained area or laminar flow hood) using dedicated material, changing blades between each paraffin block, ensuring sample-to-sample traceability by appropriate labeling of the container at each transfer step, regular cleaning and decontamination of all material and instruments. If the percentage of tumor cells is too low, or if the section contains necrotic tissue, fatty tissue, hemorrhagic tissue or a melanin rich area (e.g. lung metastasis of malignant melanoma), then the section must be dissected. It is highly recommended to use a dedicated sterile scalpel to select the tissue part in order to enrich the sample in tumor cells.

The DNA-extraction protocols range from homemade to commercial kits and can be divided into several groups, such as DNA extraction with or without precipitation or purification, and silica-based adsorption columns. There is no consensus as to whether a single protocol is highly superior to others [110]. There is also general agreement that for molecular testing performance the quality of amplifiable DNA is more important than its quantity [79]. Most testing laboratories report a technical failure rate of 3–8%, which is often due to PCR failure as a result of poor quality DNA, most probably due to overfixation, or insufficient DNA [83]. It is highly recommended to perform a control PCR amplification to test the DNA quality and also to give the quality results to the clinician in the molecular pathology report (Fig. 3). If a sample that does not meet quality criteria has a wild-type genotype, it should be reported that a mutation was not found but that the presence of a mutation
cannot be excluded due to the poor quality of the sample [79]. We consider that no matter the method used for nucleic acid isolation, it is recommended to employ only optimized and controlled reagents in order to avoid variability in results. Some commercial nucleic acid extraction kits with automated instruments have been developed for molecular testing [111]. The automation of DNA extraction has the advantage of standardized sample treatment and avoidance of error during routine sample handling and contamination due to intermediate processes [111,112]. However, there is some debate as to the efficiency of DNA extraction by automated methods in comparison with manual extraction methods when a limited sample volume is used [113].

7. METHODS FOR MUTATION TESTING

There is a wide range of methods available for mutation detection in the oncology field. Many of these methods use laboratory-based assays and are not commercially available for use in routine molecular testing. Other methods are available as commercial test kits [114]. It is noteworthy that only a few of these tests have met the requirements of the European Directives (CE-Mark) for molecular diagnostic use.

All these methods have certain advantages and disadvantages, and there is no consensus agreement on which is the preferred method [79]. The technologies can be divided into two subgroups, screening and targeted mutation detection technologies [99]. Many of the tests available for EGFR molecular testing are also available for Kras and BRAF mutation analysis, and vice versa.

EGFR gene mutations have been commonly detected by direct sequencing, this method is available in many molecular pathology laboratories. This technique allows the detection of all variants, including novel variants. The detection of unreported mutations should always be independently confirmed to avoid false-positive results due mainly to fixation and long storage conditions [78]. With sequencing there is no need for batching of samples and it provides better control of contamination as the exact, specific mutation in the sample can be determined. However, the sensitivity of direct sequencing is suboptimal in comparison with targeted methods (10-30% mutant DNA in a normal DNA background of heterogeneous tumors) [83]. In the setting of NSCLC, in which diagnosis is often based on small sized samples with a low percentage of tumor cells, detection of a mutation by direct sequencing may frequently lead to false-negative results [115]. Moreover, this method requires experienced operators and tends to be more labor-intensive, frequently resulting in an extended turnaround time for reporting results [116].

Beside this method, there are a variety of “in-house” developed screening tests. These latter methods are subject to higher inter- and intra-laboratory variability and are not always prone to adequate quality assurance, which ensures the reproducibility of the results [114]. Furthermore, highly sensitive methods were used, with the caveat that only targeted mutations are identified. The assessment of the EGFR mutational status has mainly focused on the detection of the most common EGFR-sensitizing mutations: exon 19 in-frame deletions and point mutations in exon 21 (L858R and L861Q) [117]. The technologies used may detect only the mutations assayed and therefore are less time consuming. Sensitivity is increased compared to screening tests (1 to 10% mutant DNA in a normal DNA background of heterogeneous tumors) [115]. Moreover, some of these techniques do not require a large amount of tissue and have quite equivalent performance rates for either fresh tissue or FFPE samples [116,118,119]. However, despite its low sensitivity direct sequencing is still considered the gold standard for gene mutation analysis, as some of the targeted assays require sequencing for confirmation of results. Furthermore, the significance of low-abundance mutations detected in heterogeneous samples is uncertain and these mutations need further determination of their clinical or predictive significance [4]. Highly sensitive methods may also increase the risk of false-positive results [83]. Because of the need for mutation-specific primers, comprehensive detection of in-frame deletions in EGFR exon 19 may not always be possible [115]. The advantage of commercially available tests is the validation process that they have gone through, but cost factors could hamper the adoption of these kits [114]. As new targeted therapies against oncogenic targets other than EGFR are developed, there is an increasing need for multiple mutations testing in NSCLC. Multiplexed PCR are being developed to screen mutations in several clinically relevant genes within a single reaction. These methods seem robust and reliable in FFPE-derived DNA samples, although they are currently under evaluation [120].

Another assay that needs further validation, as it may provide a rapid and cost-effective molecular test, is IHC. IHC has the advantage of being widely available in a pathology laboratory, relatively easy to perform and retains morphological informative data [83]. Interestingly, two mutant-specific monoclonal antibodies directed against the most common mutant forms of EGFR have been recently developed for IHC use: the 15-bp deletion (E746_A750del) in exon 19 and the L858R point mutation in exon 21 [121]. So far, both antibodies demonstrated clinically performance with sensitivities ranged between 23% and 99% for exon 19 deletions and 75%-100% for the L858R mutation [122,123]. Therefore, EGFR IHC is still challenging for EGFR testing in NSCLC in routine practice [79].

Although, until now, routine testing for ALK rearrangements is not currently recommended outside clinical trials, several molecular tests are available, such as FISH, reverse transcriptase-PCR (RT-PCR), multiplex PCR and IHC [124-126]. At present, the FISH assay, using commercial break-apart ALK probes has been the method of choice for selection of patients in the current clinical trials. However, some limitations exist. Although, the recommended type of fixative is the 10% NBF, prolonged tissue fixation may lead to progressive degradation of signal intensity. The acid fixatives damage DNA and should be definitively avoided for FISH analysis [91]. The interpretation of a positive rearrangement through the introduction of a gap between the red and green probes could be challenging. Moreover, morphological indicators of tumor cells versus non neoplastic cells are almost totally lost under fluorescence [116]. In this regard, analyses must be performed by experienced pathologists with a recommended two-person scoring approach when the percentage of positive cells is close to the cut-off (between 5% and 25%) (Table 1). It is critical to use adequate positive and negative control specimens in each assay. By FISH analysis, the actual gene rearrangement is not known, although the clinical significance between isoforms is uncertain. RT-PCR as a screening method seems to be less adapted to clinical use. Moreover, the success rate of RT-PCR on FFPE tissues is unsatisfactory, with low efficiency of RNA extraction and impaired reverse transcriptase reaction by formalin-induced degradation [4]. The IHC seems to be a relatively specific technique for identification of ALK rearrangements, although commercially available antibodies have demonstrated poor sensitivity [127]. Molecular pathology laboratories should test the sensitivity of immunohistochemistry in their local conditions to be made aware of its limitations in routine practice [127].

Overall, the choice of a particular molecular methodology will depend upon the available technology or what is most appropriate for individual pathology departments or cancer networks to develop [83]. This has to consider the balance between efficacy, robustness, sensitivity and specificity, method validation, analysis success rate, and costs. Testing may be developed “in-house” or in conjunction with existing local molecular pathology/genetics laboratories with the necessary expertise and technology.
8. QUALITY ASSURANCE

All molecular pathology testing must be provided and practiced under a quality assurance framework, which is subject to adaptation and interpretation by regulatory and professional organizations. Accreditation has been recognized as an effective procedure to assure the analytical and diagnostic quality for optimal patient care (Table 1) [128]. To address the need for standardized molecular mutation testing and if no legislative requirements are in place, professional societies for pathology may play an important role in forming settings for adaptation of existing accreditation systems to the needs of molecular pathology testing. This should also be the basis for international recognition of providers of external quality assessment programs. While internal quality procedures begin to be implemented in every laboratory, External Quality Assessment Programs (EQAP) are important tools to increase the analytical or diagnostic proficiency of the molecular pathology laboratory. Although there is general recognition of the necessity of EQAPs, they are usually not mandatory in EU countries. The establishment of reference laboratories in Europe may also be helpful: such laboratories exist for KRAS mutation testing in colorectal cancer [129,130]. Finally to prevent poor-quality mutation testing, professional organizations have developed guidelines, recommendations and checklists to which molecular pathology laboratories must comply (http://www.oecd.org) [2,79].

9. MUTATIONAL ANALYSIS FINAL REPORT

Accurate characterization of molecular features is crucial with the expanding role of targeted therapy in advanced NSCLC patients. The mutational analysis should be reported in conjunction with the identification of patient and health care professionals, the pathological diagnosis, details on the tissue block tested, sample source, sample size and quality, the estimation of the proportion of tumor cells in the sample extracted for DNA amplification, the method used, estimated test sensitivity and specificity, test results (mutant or wild-type allele) and interpretation of results in the context of the indication for testing (Table 1) (Fig. 3).

A turnaround time of 2 weeks from the diagnostic procedure to the final report has been suggested, but organizing a system that would include multiple departments seems to be challenging [4]. However, such an engagement cannot be completely respected especially when molecular testing is ordered on samples from external laboratories. Furthermore, in our institution, pathologists order mutational profiling at the time of diagnosis as described above, to reduce the turnaround time for molecular testing.

CONCLUSION

The emerging biomarkers may potentially modulate the sensitivity of tumors to targeted inhibitors and could therefore contribute to the improvement of predictive models and the optimization of therapeutic options. The role of pathologists in guiding treatment decisions is increasing because the molecular profiling, together with the morphological analysis, represents the future of personalizing medicine for patients with NSCLC [131].

As new therapies targeting the growing list of mutant or amplified oncogenes (e.g., EGFR, KRAS, EML4-ALK, PIK3CA, HER2, BRAF) in NSCLC are developed, it is likely that we will have to move toward a more global approach of molecular testing, such as testing for a panel of mutations or possibly using gene expression profiling to identify molecular subtypes of NSCLC [36,49]. There is no doubt, that such global gene expression screening methods will be used in the future in an attempt to identify predictive signatures. Establishing new methods of mutational analyses is an active area of research that aims to reduce time and expense with acceptable sensitivity and to use specimens other than resected tissue [3].

Routine clinical testing for molecular abnormalities in NSCLC still needs to be optimized and standardized in regard to mutational methods, specimens and tumor types, and result reporting.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AKT</td>
<td>Protein Kinase B</td>
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<tr>
<td>ALK</td>
<td>Anaplastic lymphoma kinase</td>
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<tr>
<td>AXL</td>
<td>AXL receptor tyrosine kinase</td>
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<tr>
<td>BRAF</td>
<td>v-raf murine sarcoma viral oncogene homolog B1</td>
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<tr>
<td>CDKN2A/p16Ink4A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
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<tr>
<td>EGFR/HER-1/ErbB1</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EGFR-TKIs</td>
<td>EGFR tyrosine kinase inhibitors</td>
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<tr>
<td>EMEA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EML4</td>
<td>Echinoderm microtubule-associated protein-like 4</td>
</tr>
<tr>
<td>EQAP</td>
<td>External Quality Assessment Programs</td>
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<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
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<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
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<tr>
<td>FGFR1</td>
<td>Fibroblast growth factor receptor 1</td>
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<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
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<td>FLT3</td>
<td>FMS-like tyrosine kinase receptor-3</td>
</tr>
<tr>
<td>FTIs</td>
<td>Farnesyl transferase inhibitors</td>
</tr>
<tr>
<td>HER2/neu/ErbB2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
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<td>HER3/ErbB3</td>
<td>Human Epidermal Growth Factor Receptor 3</td>
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<td>HER4/ErbB4</td>
<td>Human Epidermal Growth Factor Receptor 4</td>
</tr>
<tr>
<td>IASLC</td>
<td>International Association for the Study of Lung Cancer</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>KIT</td>
<td>Tyrosine-protein kinase Kit or Mast/stem cell growth factor receptor</td>
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<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>LKB1/STK11</td>
<td>Liver kinase B1 or Serine/threonine kinase 11</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK/MAP2K1</td>
<td>Mitogen-activated protein kinase kinase 1</td>
</tr>
<tr>
<td>MET</td>
<td>Met proto-oncogene or hepatocyte growth factor receptor</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>NFB</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>NOS</td>
<td>Not otherwise specified</td>
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<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
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</table>
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**PDGFR** = Platelet-derived growth factor receptor

**PIK3CA** = Phosphoinositide-3-kinase, catalytic, alpha polypeptide

**PTEN** = Phosphatase and tensin homolog

**RET** = Ret proto-oncogene/Rearranged during transfection

**RT-PCR** = reverse transcriptase-PCR

**TBN** = Transbronchial needle aspiration

**TIE2** = Tyrosine kinase with immunoglobulin and EGF homology domains

**TK** = Tyrosine kinase

**TP53** = Tumor protein 53

**TTF-1** = Thyroid transcription factor 1

**VEGFR2** = Vascular endothelial growth factor receptor 2

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rarrangements potentially suitable for ALK inhibitor treatment. Clin Cancer Res. 2010, 16(22), 5581-5590.


