Tumour tissue sampling for lung cancer management in the era of personalised therapy: what is good enough for molecular testing?

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ABSTRACT In the era of personalised cancer therapy, the demand for molecular profiling of the patient’s tumour is steadily increasing. In advanced nonsmall cell lung cancer (NSCLC) patients, testing for epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) gene rearrangements has become an essential component of clinical practice to select patients who are most likely to benefit from EGFR and ALK tyrosine kinase inhibitors, respectively. Furthermore, obtaining tissue specimens from recurrent or metastatic tumours or from patients who develop resistance to initial effective therapies are essential for our understanding of the molecular basis of tumour progression and development of drug resistance. Therefore, the sampling of tumour tissue that is representative and is adequate in quantity and quality for pathological diagnosis and genomic profiling is crucial. In this review, we will discuss factors that should be considered in obtaining and processing biopsy specimens to enable routine molecular analysis in NSCLC patients.

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Introduction
During past decades, extensive efforts have been devoted to overcoming the lethality of cancers. However, the effectiveness of chemotherapeutic agents has reached a plateau [1]. Once cancers have metastasised, they are rarely curable as they almost invariably recur following initial chemotherapies. The recent discovery of driver mutations and their association with greater responsiveness to specific targeted agents led to a paradigm shift in cancer treatment from conventional chemotherapy to biomarker-driven targeted therapy. This new era of personalised therapy brings an increasing demand for characterisation of tumour genotypes and phenotypes. More than two-thirds of patients with nonsmall cell lung cancer (NSCLC) are in an advanced, inoperable stage at diagnosis [1]. Therefore, for most of them, small biopsy or cytology specimens will be the only available tissue for both diagnosis and molecular profiling. Furthermore, many investigators have recently focused on obtaining greater understanding of the molecular pathology of recurrent, metastatic or drug-resistant tumours. Therefore, repeat biopsy is assuming a greater role as the next generation of biological specimens. The latter can be defined as clinical specimens obtained from patients at distinct time-points in the course of disease or during the treatment period for tumour analysis to decide ongoing treatment options [2].

Mutations on the tyrosine kinase domain of epidermal growth factor receptor (EGFR) and rearrangement involving the anaplastic lymphoma kinase (ALK) gene are closely associated with high response rates to their respective targeted tyrosine kinase inhibitors (TKIs) [3–5] and, thus, are the major determinants for therapeutic use of these TKIs [6–10]. Testing of patient tumour samples for EGFR mutations and ALK gene rearrangement has become an essential component of clinical oncology and pathology practice, and is currently considered a standard of care for advanced stage lung adenocarcinoma patients.

The first step for molecular genotyping is the sampling of adequate tumour tissue. The accuracy of molecular tests depends on sample quality as much as the molecular analyses themselves, emphasising the importance of good sample collection and proper processing techniques [11].

The selection of biopsy site
Selection of the lesions for biopsy is usually decided by the clinicians, surgeons or interventional radiologists. The genetic analysis from a single biopsy specimen may not cover the entire tumour’s characteristics and may under-represent the heterogeneous genetic profile of the tumour [12]. Recent genomic studies have demonstrated the heterogeneity and genetic evolution in different regions of the same tumour, between primary and metastatic tumours, and between different metastatic sites. Despite this apparent heterogeneity, key driver mutations are well preserved in the metastatic as well as the primary tumours [13–15]. This suggests that genomic profiles of the primary tumour are likely to reflect the genomic spectrum of the metastasis, and the primary tumour tissue can be a surrogate for genomic profiling of all tumours [14]. A majority of EGFR mutations and ALK gene rearrangements, as driver mutations of NSCLC, appear to follow this pattern.

There has been controversy about the heterogeneity of EGFR mutations in primary tumours and between primary and metastatic tumours [16]. Some systematic studies using laser-captured microdissection reported that 24–50% of NSCLC showed a heterogeneous population of EGFR mutated and non-mutated tumour cells [17–21]. Furthermore, patients with intra-tumoural EGFR heterogeneity had a tendency to show lower response rates to EGFR-TKI treatment (table 1) [18, 20]. When applying highly sensitive detection methods such as next-generation sequencing, subpopulations of EGFR exon 19 deletion showing different deletion patterns from the main type were detected in 43% of NSCLCs, but only 4% of tumours showed substantial subpopulations of deletions representing >2% of genomic DNA [25]. However, other studies using manual microdissection failed to show intra-tumoural heterogeneity of EGFR mutations [22–24]. In addition, studies comparing EGFR mutation status of biopsies and resection specimens revealed no discrepancies (table 2) [26–28]. This suggests that, in some NSCLCs heterogeneous for EGFR mutation [17, 29, 30], the number of tumour cells showing different mutational patterns in the primary tumour is very small and, thus, easily obscured by the predominant population and not detectable with usual methods. However, this intra-tumoural heterogeneity of EGFR mutations might partially account for the discrepancies in clinical response to EGFR-TKIs in EGFR-mutated patients and for cases with unexplained primary and secondary resistance [28, 31, 32]. In the clinical setting, the overall EGFR status of the cancer tissue appears more important and, in most patients, intra-tumoural mutation heterogeneity may not significantly affect the efficacy of EGFR-targeted treatment [28]; however, further confirmation is needed. According to the recent College of American Pathologists (CAP)/International Association for the Study of Lung Cancer (IASLC)/Association for Molecular Pathology (AMP) guideline on molecular testing in NSCLC, EGFR testing of multiple different areas within a single tumour is not necessary [33]. By contrast, for patients with multiple lung adenocarcinomas that seem not to originate from a single tumour, each
<table>
<thead>
<tr>
<th>First author, year [ref.]</th>
<th>Population studied</th>
<th>Cases/sections or areas per case n/N</th>
<th>Detection methods</th>
<th>Tumour enrichment strategy</th>
<th>Pure mutated EGFR cases (group A)</th>
<th>Pure wild-type EGFR cases (group B)</th>
<th>EGFR mutant with heterogeneity (group C)</th>
<th>PFS response to EGFR-TKIs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yatabe, 2011</strong> [22]</td>
<td>Resected lung adenocarcinoma with EGFR mutation</td>
<td>50/3 5/100</td>
<td>Cycleave PCR/FA</td>
<td>Manual MD</td>
<td>100</td>
<td>NA</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Sun, 2012</strong> [23]</td>
<td>Early-stage mixed adenocarcinoma</td>
<td>10/2-3</td>
<td>DS</td>
<td>Manual MD</td>
<td>80</td>
<td>20</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Mattsson, 2012</strong> [24]</td>
<td>Resected NSCLC with EGFR mutation</td>
<td>6/3</td>
<td>DS</td>
<td>Manual MD</td>
<td>100</td>
<td>NA</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Tanguchi, 2008</strong> [18]</td>
<td>Resected NSCLC treated with gefitinib for postoperative recurrence</td>
<td>21/50-60</td>
<td>SNaP shot</td>
<td>Laser MD</td>
<td>71.4</td>
<td>NA</td>
<td>28.6</td>
<td>18 months for group A versus 7.5 months for group C</td>
</tr>
<tr>
<td><strong>Bai (2012)</strong> [19]</td>
<td>Stage III/IV NSCLC with palliative resection</td>
<td>79/30-40</td>
<td>DHPLC/ARMS</td>
<td>Laser MD</td>
<td>20.3</td>
<td>41.8</td>
<td>38</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Bai, 2013</strong> [20]</td>
<td>Stage III/IV NSCLC with palliative resection</td>
<td>85/total 2699 areas</td>
<td>DHPLC/ARMS</td>
<td>Laser MD</td>
<td>24.7 (DHPLC) 36.5 (ARMS)</td>
<td>42.4 (DHPLC) 35.3 (ARMS)</td>
<td>32.9 (DHPLC) 28.2 (ARMS)</td>
<td>16 months for group A versus 11 months for group C versus 3 months for group B</td>
</tr>
<tr>
<td><strong>Tomonaga, 2013</strong> [21]</td>
<td>Stage I resected mixed adenocarcinoma</td>
<td>38/NA</td>
<td>RFLP</td>
<td>Laser MD</td>
<td>26.3</td>
<td>50</td>
<td>24</td>
<td>NA</td>
</tr>
</tbody>
</table>

Data are presented as %, unless otherwise stated. EGFR: epidermal growth factor receptor; PFS: progression-free survival; TKI: tyrosine kinase inhibitor; FA: fragment analysis; MD: microdissection; NA: not available; DS: direct Sanger sequencing; NSCLC: non-small cell lung cancer; AR: acquired resistance; SNaP shot: single nucleotide primer extension reaction using the SNaPshot multiplex kit (Life Technologies, Carlsbad, CA, USA); DHPLC: denaturing high-performance liquid chromatography; ARMS: amplification refractory mutation system; RFLP: restriction fragment length polymorphism.
tumour may be tested separately [33]. If EGFR mutation is identified in one synchronous lung primary tumour, targeted therapy should be applied according to the therapeutic guidelines [34]. When considering heterogeneity between the primary tumour and corresponding metastases, the percentage of discordant results of EGFR mutation status varied from 0% to 38.8% (table 3) [23, 32, 35–39]. The genetic profiles of metastatic tumours might evolve or be clonally selected when metastasis occurs, and the time interval between primary tumour and metastasis is sufficiently long for outgrowth of heterogeneous subpopulations. In addition, chemotherapy or TKI therapy may affect EGFR mutation status by changing the overall frequency of EGFR mutant clones after drug treatment [19, 29]. Therefore, in a small subset of patients, primary tumours may not be representative of the EGFR mutation status of the metastatic tumours. The CAP/IASLC/AMP guideline suggested that EGFR mutation testing with cytological or small biopsy samples from the primary tumour or its metastases might be equally suitable for tumour genotyping [33]. In fact, in a population-based EGFR testing programme, S HIAU et al. [40] reported that close to 80% of submitted archival samples are from the primary tumour. However, for other genomic profiling studies, no guideline has been established as to whether the primary tumour or metastasis might be appropriate for molecular analysis. The clinical significance of genetic heterogeneity remains to be fully evaluated in future studies.

Among multiple candidate lesions, the selection of the biopsy site for molecular tests is usually based on tumour size and accessibility [2, 41]. Imaging may be used to guide biopsy and avoid necrotic areas. Bony metastases that require a decalcification process are not ideal for molecular analysis. For a better understanding of the genetic mechanisms of response and resistance to targeted therapies, and for procurement of biological specimens for further research, sequential tumour biopsies at different time-points during the course of disease or treatment are encouraged [2]. If additional samples need to be obtained, several clinical factors such as the patient’s overall clinical condition, accessibility of lesions, radiographic features, responsiveness to previous therapies and the most proper time-point should be considered [2, 33].

The type of sample for molecular testing
Pathological diagnosis and classification can be accurately made in most small biopsy and cytology specimens with few tumour cells, as they depend mainly on recognising the morphological (qualitative) features of the tumour cells. By contrast, molecular tests tend to require more tissue as assay sensitivity may require minimum amount of material (e.g. DNA, RNA or tumour cells). More tumour tissue also provides greater areas to enrich for the tumour cell contents by macro- or microdissection [33].

**TABLE 2** Comparison of EGFR mutational status of biopsy and resected non-small cell lung cancer specimens

<table>
<thead>
<tr>
<th>First author, year [ref.]</th>
<th>Population studied</th>
<th>Cases with paired biopsy and resection n</th>
<th>Detection methods</th>
<th>Tumour enrichment strategy</th>
<th>EGFR mutation in both biopsy and resection</th>
<th>EGFR WT in both biopsy and resection</th>
<th>EGFR mutation in biopsy and EGFR WT in resection</th>
<th>EGFR WT in biopsy and EGFR mutation in resection</th>
<th>Discordance rate between biopsy versus resection</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOLOMON, 2010 [26]</td>
<td>Stage I/II ADC</td>
<td>16</td>
<td>DS</td>
<td>NA</td>
<td>31.3</td>
<td>68.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MASAGO, 2008 [27]</td>
<td>Paired biopsy</td>
<td>19</td>
<td>DS</td>
<td>MD</td>
<td>47.4</td>
<td>47.4</td>
<td>0</td>
<td>5.3*</td>
<td>0#</td>
</tr>
<tr>
<td>HAN, 2012 [28]</td>
<td>Paired biopsy and resection specimens with &gt;20% tumour cellularity</td>
<td>23</td>
<td>DS</td>
<td>NA</td>
<td>52.2</td>
<td>47.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data are presented as %, unless otherwise stated. EGFR: epidermal growth factor receptor; WT: wild-type; ADC: adenocarcinoma; DS: direct Sanger sequencing; NA: not available; MD: microdissection; PNA: peptide–nucleic acid. *: the amount of DNA from one biopsy specimen was not sufficient and only one exon (exon 19) was sequenced and demonstrated to be wild type, and the corresponding resection specimen showed L858R mutation in exon 21; therefore, this case was not counted as discordant.
<table>
<thead>
<tr>
<th>First author, year [ref.]</th>
<th>Population studied</th>
<th>Cases n</th>
<th>Detection methods</th>
<th>Tumour enrichment strategy</th>
<th>Primary tumour/metastatic tumour</th>
<th>Rate showing discordance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mutant / mutant</td>
<td>WT/WT</td>
</tr>
<tr>
<td>SUN, 2011 [35]</td>
<td>Surgically resected NSCLC</td>
<td>80 pairs of PT/LNM</td>
<td>DS</td>
<td>Macrodissection</td>
<td>26.3</td>
<td>67.5</td>
</tr>
<tr>
<td>CHANG, 2011 [36]</td>
<td>Surgically resected NSCLC</td>
<td>56 pairs of PT/LNM</td>
<td>DS</td>
<td>Macrodissection</td>
<td>19.6</td>
<td>51.8</td>
</tr>
<tr>
<td>YATABE, 2011 [22]</td>
<td>Lung adenocarcinoma with LNM</td>
<td>77 pairs of EGFR PT/LNM</td>
<td>Cycleave PCR/FA</td>
<td>NA</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>CHEN, 2012 [32]</td>
<td>Patients with tumour samples available from &gt;2 disease sites</td>
<td>84</td>
<td>DS/HRMA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>KALIKAKI, 2008 [38]</td>
<td>NSCLC and corresponding metastasis</td>
<td>25 pairs of PT/DM</td>
<td>DS</td>
<td>Microdissection</td>
<td>4</td>
<td>72</td>
</tr>
<tr>
<td>GOW, 2009 [39]</td>
<td>67 pairs of PT/DM</td>
<td>DS/SARMS</td>
<td>NA</td>
<td>13.4</td>
<td>47.8</td>
<td>13.4</td>
</tr>
</tbody>
</table>

Data are presented as %, unless otherwise stated. EGFR: epidermal growth factor receptor; WT: wild-type; NSCLC: nonsmall cell lung cancer; PT: primary tumour; LNM: lymph node metastasis; DS: direct Sanger sequencing; HDA: heteroduplex analysis; FA: fragment analysis; NA: not available; HRMA: high resolution melting analysis; DM: distant metastasis; SARMS: scorpion amplified refractory mutation system. *: two cases among 80 pairs demonstrated different mutational profiles of EGFR.
Less than 30% of NSCLCs are resectable at the time of presentation [1]. Therefore, most clinical samples from patients with advanced-stage lung cancer available for molecular testing are small, formalin-fixed and paraffin-embedded (FFPE) biopsies or cytological specimens. Paradoxically, while the number of biomarkers for testing per specimen has been increasing, tumour sampling has moved towards favouring less invasive procedures, which usually yield a smaller amount of sample. Small specimens may contain limited amounts of tumour cells and a variable proportion of non-neoplastic cells, which can affect the accuracy of the results (fig. 1). Therefore, limiting the use of ancillary diagnostic immunohistochemistry markers and clinical prioritisation of molecular testing is important [42]. The requirement for larger and multiple biopsy samples needs to be balanced by the risk of procedural morbidity, such as pneumothorax or haemoptysis [43]. The data on the number of biopsies or cores that can be obtained from one lesion are limited [43–45], despite guidelines emphasising the benefits of obtaining as much tumour tissue as possible [41].

Although using tissue specimens is the gold standard for molecular testing, a considerable fraction of advanced stage NSCLC patients are diagnosed by cytology only [46]. Previous studies have demonstrated that, if FFPE samples are not available, a cytology sample can be appropriate for accurate diagnosis as well as for molecular testing [46, 47]. The detection rates of EGFR mutation with cytology specimens seem to be as high as those with tissue samples [40, 47]. However, more sensitive mutation testing methods should be applied when cytology samples have low tumour cell content [28]. Diagnostic fine needle aspiration (FNA) specimens often have an inherently high proportion of tumour cells [48], as tumour cells are less cohesive and, thus, are more easily aspirated out. FNA samples archived for a long time have been shown to yield abundant genomic DNA suitable for molecular profiling [49]. A recent study demonstrated that the tumour cells selectively scraped from smeared slides yielded high quality nucleic acid for molecular analysis [49], and showed significantly higher overall tumour cell numbers and tumour cellularity than cell block specimens [50]. However, cell block specimens are generally preferred over smear slides because tumour cellularity in cell block sections can be easily assessed, the original diagnostic slides can be archived and additional ancillary tests are possible when necessary [51].

In cases when biopsy or cytology samples cannot be obtained, the patient’s plasma might become an option. Plasma samples may contain circulating free DNA released from the tumour cells or circulating tumour cells. The concordance rates of the mutation status between plasma and tumour samples are variable from 33% to 87% according to the study design, the detection methods and the eligible patient groups [52–54]. Although technological advances have led to more accurate detection of cancer-associated alleles in the blood, many hurdles still need to be overcome before routine clinical application [52]. Nevertheless, plasma may provide information on the genetic landscape of tumours overcoming genetic heterogeneity and offering the opportunity to assess tumour dynamics, such as detection of minimal residual disease, early detection of recurrence and prediction of response to treatment in the advanced stage of lung cancer [12]. It shows promise in providing the least invasive and most convenient and cost-effective way of obtaining samples for molecular testing in the future.

The amount of tumour cells for molecular testing

In general, the amount of cells and the concentration of the extracted DNA is less of an issue in most mutational analysis using frozen samples [55]. The cytology specimen, as well as small biopsy samples that yield a very low amount of DNA, can detect mutations when using more sensitive detection methods
method [50]. In population-based EGFR demonstrated 100% concordance with the corresponding tissue samples when using a pyrosequencing abundant cell content. minimal cell content cases reporting significantly lower test-success rates than cases with small clusters of overall cell content of the cell block, but not tumour cellularity, was correlated with test-success rates, with macrodissection area, and a rate (among surgical biopsy specimens tested, core biopsy of the primary lung lesions had the highest test-failure rate (~10%). The highest test success-rate was associated with ≥30% tumour cellularity within the macrodissection area, and a ≥2 mm tumour area marked for macrodissection. For cytology samples, only overall cell content of the cell block, but not tumour cellularity, was correlated with test-success rates, with minimal cell content cases reporting significantly lower test-success rates than cases with small clusters of abundant cell content.

Specimen quality: tumour cellularity and tumour cell enrichment
Preservation and quality of the amplifiable DNA seemed to be more important than its quantity in the samples [41, 60]. If multiple specimens are available for molecular testing, the choice of which sample is the best for molecular assays should be based on the quality of the specimen, percentage tumour cellularity and the validated specimen type in each laboratory [34]. False-negative results may be problematic when only small samples are available for molecular testing, as the detection sensitivity may be further diminished by the pre-analytical histological or cytological factors.

The relative proportion of tumour cells versus non-neoplastic cells is one of the important parameters that determine the sensitivity of molecular testing [42, 60]. Yet, there is no definite consensus on optimal tumour cellularity or how to assess it. Some groups have set thresholds for >60% tumour cellularity and <20% necrosis for comprehensive genomic analysis [61–63]. For Sanger sequencing, if heterozygosity and disomic tumour cells are considered, the suggested threshold for reliable results is 40–50% when tumour cellularity is estimated and 30% when more accurate counting is performed [41, 56]. However, mutations may also be detected in samples with <40% tumour cellularity. The correlation between mutated allele frequency and tumour cellularity is highly influenced by gene copy number changes, as EGFR-mutated alleles are preferentially amplified [64–66].

As considerable variation in the tumour cellularity between samples may exist, a histological evaluation of each sample to confirm the diagnosis and assess the tumour cellularity is critical [2]. However, estimation of tumour cellularity can vary significantly among pathologists [56, 67–69], and visual assessment with estimation of tumour cellularity may have a tendency to overestimate tumour cellularity when compared with cell number counting [56, 68]. The training of pathologists with gold standard calibration specimens might be helpful for more consistent estimations of tumour cellularity [33, 67]. The development of computational image analysis systems for accurate counting of the cellular content may be an alternate unbiased option for estimating tumour cellularity [70].

An ideal specimen for molecular testing would have a high proportion of tumour cells and a minimal amount of mucin or necrotic cells. To obtain the best quality of specimens, techniques to enrich for tumour cells can be applied. Pathologists can designate the tumour cell rich areas on the slides during the histological review of the case and scrape off these areas from an unstained section, or alternatively core out the marked area from the paraffin block. Flow cytometric sorting or laser-captured microdissection techniques can be also used, but they are not practical or suitable in clinical practice.

With the rapid advance in detection methods with increasing sensitivity the analytical limitation of tumour cellularity lessens, and samples with lower tumour cellularity can be molecularly profiled. The CAP/IASLC/AMP guidelines recommended that, for EGFR-targeted therapy, sensitive molecular methods that can detect mutations in specimens with as little as 10% tumour cells should be available [33]. Each laboratory should also validate its sensitivity threshold for each assay and for each specimen type. However, the results of ultrasensitive molecular assays with a sensitivity of below 1% should be carefully interpreted due to an increased chance of artifactual mutations. The tumour cellularity of the samples and the detection limit of the molecular assay should always be considered together [28, 69]. In addition, the significance of very low frequency EGFR mutations detected with ultrasensitive methods should also be validated.

Pre-analytical processing of the specimens
The quality of a specimen is primarily influenced by pre-fixation time, the type of fixative and fixation time. Significant biochemical alterations start to occur in tissues within 10 min after sampling or resection. The pre-fixation time should be minimised to reduce the degradation of RNA and protein. The gross
examination of the resected specimen and sectioning into thin slices should be performed as soon as possible to facilitate the penetration of the fixatives [71].

Fresh, frozen, formalin-fixed or alcohol-fixed specimens can be used for molecular analysis. Fixation with 10% neutral-buffered formalin is routinely used to preserve clinical tissue specimens. However, formalin fixation is not optimal for molecular analyses as it causes chemical cross-linking of proteins, and fragmentation and chemical modification of nucleic acids [72, 73]. Random base alterations that generate artefactual mutations can occur, typically in FFPE samples with low DNA concentration [55, 74]. Nevertheless, formalin fixation preserves morphological details and allows long-term storage of samples at very low cost. Furthermore, most ancillary tests, including immunohistochemistry and molecular assays, have now been optimised and validated for use on FFPE samples, and many high-throughput techniques are being optimised for application to these samples [75, 76]. Clinical testing of *EGFR* mutations and *ALK* gene rearrangement can be reliably performed using FFPE samples.

Frozen specimen provides a high quality of nucleic acids and proteins, and has been considered the most optimal sample for most molecular analysis. However, freezing does not preserve the morphological details of tissues well and the handling and storing of frozen samples requires highly controlled conditions and costly infrastructure. Alcohol fixation generates a superior yield of nucleic acid compared with formalin, as it does not cause the chemical changes found in formalin-fixed specimens [71, 77]. As most fixatives for cytology preparation are alcohol bases, cytology specimens are appropriate for DNA-based molecular assays [42]. However, they may alter the morphological details of the nucleus, and are not recommended for tests that still depend on morphological evaluation, including fluorescent *in situ* hybridisation (FISH) [78]. Heavy metal fixatives are not suitable for most molecular analyses because they cause DNA fragmentation, and the metals in the fixative compete with magnesium and interact with other enzymes that are critical in molecular assays [79–81]. Bone is a common site for metastatic spread of lung cancer. Decalcification of the specimens obtained from bony metastases should be avoided and tissues treated with decalcifying solutions should not be used for molecular testing, as decalcifying solutions extensively degrade DNA [34]. This can be overcome by using non-acid EDTA-containing decalcification solutions.

Optimal fixation time for molecular analysis is 6–12 h for small biopsy samples and 8–19 h for larger surgical specimens [41]. The recent guidelines suggest that fixation times of 6–48 h are acceptable for clinical molecular tests [33, 82]. A fixation time <6 h is not recommended, since haematoxylin and eosin staining, immunohistochemistry and FISH analysis are adversely affected. If the fixation time exceeds 48 h, the quality of DNA deteriorates [72], and signals in FISH analysis become weaker [78].

**TABLE 4** Recommendations for pre-analytical preparation for molecular analysis in advanced lung cancer patients

| Selection of biopsy site | Primary tumour versus metastasis: equally suitable  
| Necrotic mass or bone metastasis: not suitable  
| Multiple synchronous primary tumours: each may be tested  
| Multiple-site biopsy in one tumour: not necessary  
| Sequential biopsies at different time point: optional |
| **Sampling** | Biopsy is preferred over cytology  
| Obtain as much tumour tissue as clinically feasible  
| If only cytology is available, cell block is preferred  
| Plasma: needs further evaluation |
| **Fixation** | Pre-fixation time should be minimised  
| Formalin fixation is recommended for biopsy specimen  
| Heavy metal fixatives or non-EDTA decalcifying solution should be avoided  
| Fixation time: ideally ≥6 h, but <48 h |
| **Pathologic diagnosis** | Limit the amount of immunohistochemistry for histological subtyping  
| Preserve maximum amount of specimen for molecular testing  
| ADC/NSCLC with ADC component and nonsmokers or light smokers: submit specimen for *EGFR* and *ALK* testing |
| **Tumour cell content** | ≥40% for Sanger sequencing  
| Lower tumour cell content acceptable with higher (1–10%) sensitivity techniques  
| Tumour cell enrichment with macro- or microdissection: recommended |

ADC: adenocarcinoma; NSCLC: nonsmall cell carcinoma; *EGFR*: epidermal growth factor receptor; *ALK*: anaplastic lymphoma kinase.
The role of the pathologists

In the era of personalised therapy, pathologists play an important role as a bridge between molecular testing and the clinical oncologists. Communication between the pathologist and the clinician is very important in determining the priority of the tests for individual patients. When the specimens are obtained, the clinical data such as tumour stage and treatment plan are often not readily available. Therefore, it is judicious to handle small diagnostic samples carefully, keeping in mind that they might be the only sample available for molecular testing. The pathologists should be involved in all steps from sampling of the specimen to ensure an adequate amount of specimen is obtained and to keep the specimens in good condition for molecular analysis (table 4).

After the diagnosis of malignancy is established, further histological subtyping of lung cancer is relevant for determining molecular testing strategies. Most tumours harbouring EGFR mutations as well as ALK gene rearrangement are adenocarcinomas or nonsmall cell lung carcinomas with an adenocarcinoma component [33]. A small biopsy or cytology sample showing histological subtypes other than adenocarcinoma does not exclude the presence of an adenocarcinoma component in the non-biopsied lesion. Additionally, the discrimination between adenocarcinoma and squamous cell carcinoma can be challenging in small biopsy samples. Therefore, the guideline suggested that if the histological subtype is uncertain or when squamous or small cell carcinoma patients have clinical features favouring EGFR mutation-positive subsets (e.g. nonsmokers), it is best to send the sample for molecular testing [33].

Because EGFR and ALK testing are the most important steps for therapeutic decision making after a diagnosis of adenocarcinoma is established, the CAP/IASLC/AMP guideline recommends preservation of a maximum amount of specimen for potential molecular testing and limitation on the amount of immunohistochemistry staining for histological subtyping [33]. For tumours with equivocal morphological features, e.g. NSCLC with not otherwise specified feature, it has been recommended that only one or two ancillary stains for each subtype be applied: thyroid transcription factor-1/napsin-A/mucin for adenocarcinoma and p63/p40 or cytokeratin 5/6 for squamous differentiation [83, 84]. If the immunohistochemistry study fails to result in a more specific diagnosis, the remaining tissue should be submitted for molecular testing, rather than further exhaustion of specimen for subtyping [34]. All specimens available from a patient including cytology samples should be considered for testing, and the choice of which specimen would be the most adequate for testing should be determined by a pathologist based on the specimen quality [33, 46, 47].

Reflex molecular testing ordered by the pathologist at the time of initial diagnosis would provide a more readily available molecular profile when the oncologist sees the patient, but the pathologist should communicate closely with institutional clinicians on which tests should be prioritised according to the individual patient’s need. Reflex preparation of the block, which involves cutting multiple additional unstained sections when the sample is processed initially in expectation of additional immunohistochemistry or molecular testing, may prevent waste of tissue by avoiding the re-cutting of blocks [34]. The essential information should be shared among clinical oncologists, pathologists and molecular pathologists. This can improve diagnosis accuracy and avoid waste of material that is intended for use in molecular analyses.

Conclusions

For the genomic profiling of lung cancer patients who will benefit from targeted therapy, sampling of tumour tissue that can be taken as representative of the malignancy, and that is adequate in quantity and quality is critical. Tumour specimens should be obtained as far as possible at the time of biopsy. A system of rapid on-site evaluation by a pathologist or well-trained technician to assure adequacy of the sample is desirable in cytological aspiration biopsies. Specimen quality such as tumour cellularity as well as preservation and fixation status is crucial for optimal molecular analysis. The significance of genetic heterogeneity of the tumour requires further investigation, and the significance and clinical application of multiplex mutational analysis or next-generation sequencing is currently being evaluated in many centres. The close communication between clinical oncologists, pathologists and molecular pathologists is critical to ensure the most optimal way of obtaining adequate tumour tissues for accurate histopathological diagnosis and molecular testing, and the proper guidance of treatment strategy for personalised cancer therapy.

References


