Preanalytics in Lung Cancer

Arne Warth, Thomas Muley, Michael Meister and Wilko Weichert

Abstract

Preanalytic sampling techniques and preparation of tissue specimens strongly influence analytical results in lung tissue diagnostics both on the morphological but also on the molecular level. However, in contrast to analytics where tremendous achievements in the last decade have led to a whole new portfolio of test methods, developments in preanalytics have been minimal. This is specifically unfortunate in lung cancer, where usually only small amounts of tissue are at hand and optimization in all processing steps is mandatory in order to increase the diagnostic yield. In the following, we provide a comprehensive overview on some aspects of preanalytics in lung cancer from the method of sampling over tissue processing to its impact on analytical test results. We specifically discuss the role of preanalytics in novel technologies like next-generation sequencing and in the state-of the-art cytology preparations. In addition, we point out specific problems in preanalytics which hamper further developments in the field of lung tissue diagnostics.

Contents

A. Warth · W. Weichert (✉)
Institute of Pathology, Heidelberg University, Heidelberg, Germany
e-mail: wilko.weichert@med.uni-heidelberg.de

T. Muley · M. Meister
Translational Research Unit, Thoraxklinik at Heidelberg University, Heidelberg, Germany

1   Introduction

1.1   Preanalytics

Preanalytic processing of tissue probes and cytology specimens comprises the choice of the appropriate method for tissue retrieval, optimal fixation algorithms, and subsequent careful tissue workup for morphologic and molecular analysis. Without an optimized preanalytic workflow, results of any morphological and/or molecular analytic procedure become unreliable. Today, the main focus of research and almost all our developmental efforts are centered on the analytic steps; whereas some processes and procedures in preanalytics hardly match the requirements of modern medicine. Several important aspects of lung preanalytics will be reviewed in the following chapters.

1.2   Lung Cancer

Lung cancer is the leading cause of cancer-related mortality with nonsmall cell lung cancer (NSCLC) accounting for about 80 % of all cases [1]. Since clinical symptoms are usually mild during the early course of the disease, most diagnoses (about 75 %) are made at a time point where the tumor is not resectable anymore. Therefore, in the diagnostic context pulmonary pathologists usually receive only small biopsy specimens or even only cytological specimens.

About a decade ago, the separation between small cell lung cancer (SCLC) and NSCLC was sufficient for clinical decision making in terms of therapy selection. SCLC patients received cisplatin/etoposide and NSCLC patients were either resected in cases where surgery was still possible or received platinum-based chemotherapeutic regimens and/or radiation therapy. This has changed since today a refined morphological, immunohistochemical, and molecular classification specifically of NSCLC is required for a stratification of patients for different therapeutic approaches. Due to these novel requirements, a rational usage of the usually sparse tumor tissue is essential.

2   Aspects of Tissue Retrieval

For the increasingly complex sequence of morphological diagnosis, tumor subtyping by immunohistochemistry and molecular analysis, the amount and quality of tissue at hand is of utmost importance. One of the most important variables influencing these factors is the mode of tissue retrieval in the clinical diagnostic context.

In those cases where a resection of the tumor is performed and usually ample tumor tissue is at hand, issues of fixation, and workup become the limiting factors for the extraction of clinically meaningful analytical results (see below). However, only the minority of lung cancer patients is actually treated by tumor resection and dissection of corresponding lymph nodes, since in most of the palliative cases a surgical approach has no impact on overall survival times. Therefore, preanalytical and analytical methods primarily must be optimized for the biopsy setting.

Several methods for bioptic tissue retrieval are at hand (Table 1). The selection of the appropriate method and the subsequently applied tissue workup procedures are essential to guarantee high-quality results. The choice of the biopsy method, however, usually depends on the state of the patient, tumor localization, techniques at hand, and experience of the clinician applying the respective procedures.

Bronchial as well as transbronchial biopsies have a high diagnostic yield as far as a sufficiently large biopsy forceps is used (open forceps diameter ~ 2 mm) and at least four tissue fragments are being taken. If the forceps is too small this will lead to artifacts, which hamper the morphological assessment and diagnosis. With a larger forceps, naturally, complication rates increase [2, 3], however, the respective

Table 1 Tissue retrieval methods in lung cancer

Tumor location	Method	Material
Central tumor with bronchial contact	Bronchoscopy	Conventional bronchial biopsy
		Bronchial cryobiopsy
		Brush cytology
		Bronchoalveolar lavage
Peripheral tumor	Bronchoscopy	Transbronchial biopsy
		Transbronchial cryobiopsy
		Transbronchial needle aspiration
		Brush cytology
		Bronchoalveolar lavage
	CT-guided percutaneous puncture	Transthoracic needle biopsy
		Transthoracic needle aspiration
Peribronchial/mediastinal tumor	Bronchoscopy	Endobronchial ultrasound with TBNA
		Classic TBNA

TBNA Transbronchial needle aspiration

complications, if they occur, are usually manageable if the appropriate clinical experience and equipment is at hand. Especially, the novel method of taking cryobiopsies, in which the tissue is frozen by a cryodevice at the tip of the endoscope in vivo and subsequently chunked out, is very effective and a reasonably secure method for the retrieval of large tissue fragments by means of endoscopy [4]. However, when applied in the transbronchial setting, this method is not very widely distributed and only available in specialized centers.

Percutaneous transthoracic needle biopsies, usually guided by computed tomography, complement the endobronchial methods for the diagnostic workup of peripheral lung lesions. The success rate of this method has been reported to range between 80–95 % [5, 6]. The negative predictive value of this diagnostic procedure is given with 84–96 %, false negative results can be expected in 2–4 % of the cases [7]. For this method, however, complication rates are somewhat higher than for endobronchial biopsy approaches and reach approximately 20–50 % [8–10]. However, complications (in most cases the occurrence of a pneumothorax) are usually manageable.

Apart from these methods, which usually guarantee that enough tissue material is at hand even when complex molecular analysis are required, techniques for obtaining cytology specimens also have their established place in the diagnostic workup in patients with lung neoplasms. Although cytology specimens are somewhat restricted with respect to tumor cell content and cellularity, optimized cytology workup procedures (see below) nowadays also allow for a broad array of diagnostic procedures (including morphology, immunohistochemistry, and molecular methods).

For those lesions which could not be reached by a direct biopsy approach (e.g., intrapulmonary tumors without bronchial contact, peribronchial nodal metastases) transbronchial needle aspiration techniques (TBNA) can be used either with or without ultrasound guidance [11, 12]. Larger needle diameters (e.g., 19G, inner diameter 0.69 mm) produce better results since more cellular cytology specimens and even small tissue fragments might be obtained. Endobronchial ultrasound (EBUS) guidance further improves the diagnostic yield, specifically when hilar and upper mediastinal lymph nodes are targeted (stations 2L, 2R, 10–12) [13]. The combination of EBUS with endosonographically-guided transesophageal fine-needle aspiration (EUS-FNA) increases the diagnostic accuracy even stronger in tumor patients, with both methods combined reaching a sensitivity of up to 96 % and a specificity of up to 100 % [14].

Bronchial brush cytology specimens can be taken prior or after the retrieval of bronchial biopsy specimens, the admixture of blood in the latter scenario does not compromise the quality of the probe [15, 16]. This method is specifically useful for tumors visually detectable by bronchoscopy. Bronchial lavage fluid can also be obtained prior or after biopsy retrieval, the use of 10–20 ml isotonic saline fluid has been recommended [15–17]. The obtained material is processed to produce smears and/or a cell block (see below), however, the diagnostic yield is worse than for brush cytology specimens. Bronchoalveolar lavage (BAL) techniques in which 3×50 ml isotonic saline fluid is administered and recovered from the peripheral airways are mainly used for the diagnosis of infectious and interstitial lung disease [18], in rare cases, however, adenocarcinomas can be diagnosed with this

technique, as well. Finally, sputum specimens can, in principal, be screened for the presence of tumor cells. The likelihood for a positive tumor diagnosis and the yield of tumor cells, however, is lowest for sputum material, followed by BAL, brush cytology specimens, and fine-needle material [19]. Sputum as diagnostic specimen, therefore, cannot be recommended [20].

For an optimal yield of material it is recommendable to combine different technical approaches, e.g., brush cytology and biopsy for centrally located tumors [21, 22]. In addition, several needle passages (at least 3–4 per lesion) do increase the likelihood to obtain diagnostically adequate material [23].

Taken together, clearly the type of method applied for specimen retrieval not only influences the ability to render a precise morphological diagnosis, but also impacts on the ability to perform immunohistochemistry and in situ hybridizations (FISH, CISH) on the material and also severely impacts on the yield of RNA and DNA from the respective specimens (see below).

3   Aspects of Tissue Fixation

Regardless whether the specimens submitted to a pathology lab consist of cytology, biopsy, or resection material, one must be aware that besides histomorphology, which requires an immediate and thorough fixation of the specimens, application of additional diagnostic methods is potentially required to obtain a final diagnosis. Since almost all methodological approaches established in routine diagnostics can be performed using formalin-fixed paraffin-embedded tissue (FFPE), separate bio-banking of fresh or cryopreserved tissue is usually not required in the majority of cases, but must be considered in specific clinical constellations (e.g., when bacterial cultures are needed) as well as in rare cases where an exploratory scientific approach (e.g., exome sequencing) is intended. Such approaches, however, might become increasingly popular especially when established treatment methods fail and exploratory targeted therapies are a last option.

FFPE tissue is suitable for immunohistochemistry (IHC), DNA extraction, RNA extraction, and FISH/CISH [24]; furthermore, it might be used for electron micros-copy after re-embedding. However, when electron microscopy is required in the first place, initial fixation of parts of the specimens with glutaraldehyde is recommended.

Pathological specimens are usually transferred directly into 4 % neutrally buf-fered formalin and stored until further processing. Time to fixation is critical for the preservation of architecture, antigenicity, and specifically for the integrity of DNA and RNA. Since most of the cases in lung tumor diagnostics consist of biopsy specimens, this is usually not critical, because tiny tissue fragments can directly be transferred to formalin and the penetration of formalin into the probe is almost immediate. However, with larger resection specimens these issues become increasingly critical; this is discussed in more detail in other chapters of this book. Another critical issue might be the time the probe is retained in the fixative solution, before further processing is possible. However, again this applies mainly for

resection specimens, since biopsy probes are usually processed more rapidly. Subsequent dehydration and paraffin embedding is usually done with fully automated systems in a highly standardized manner.

Apart from the conventional way of formalin fixation and paraffin embedding, several other ways of tissue preservation do exist. However, none of these approaches has found its way into a broad application in routine diagnostics yet. These methods include cryopreservation by immediate shock freezing of probes in liquid nitrogen but also other novel fixation techniques like HOPE [25], PAXgene® tissue system [26, 27], or RNA*later* [28, 29] fixation. All these procedures, however, require either specialized sampling equipment (liquid nitrogen) or tissue handling techniques which are currently not automated and therefore hardly introducible into a routine workflow. Furthermore, all of these methods are more expensive compared to standard FFPE processing.

4   Impact of Preanalytical Variables on Diagnostic Results in Biopsy Specimens

As outlined above, several aspects of preanalytics may strongly influence the outcome of a broad variety of diagnostically necessary analytical methods. This will be discussed in the following in a structured manner for the specific analytical methods applied in the diagnostic setting.

4.1   Morphology

Conventional histomorphology is still the backbone of pathological diagnoses. First and foremost, it is used to confirm the presence of a neoplastic process. In addition, recent data indicate that especially for pulmonary adenocarcinomas (ADC) a precise histomorphological subtyping is of high prognostic and maybe even predictive relevance [30]. Furthermore, it is of utmost importance to (at least) separate tumors with a squamous and a non-squamous phenotype [31], since major druggable driver mutations and amplifications involving, among others, *KRAS*, *EGFR*, *ALK*, *BRAF*, *ROS1*, *FGFR1*, *MET*, and thus the selection of targeted therapies, are significantly affected by these features and are currently only tested in the specific subentities. Morphology is easily assessed in FFPE material, this is the gold standard preanalytical method in this regard. However, novel fixation techniques like PAXgene® tissue system and HOPE also achieve high morphological standards comparable to that of FFPE procedures (Fig. 1). Cryosections from shock frozen tissue as well as RNA*later* fixed tissue show considerably lower quality and require a specialized cutting technique; routine diagnostics cannot be done on these specimens. Therefore, if molecular methods which require frozen tissue are necessary, extra
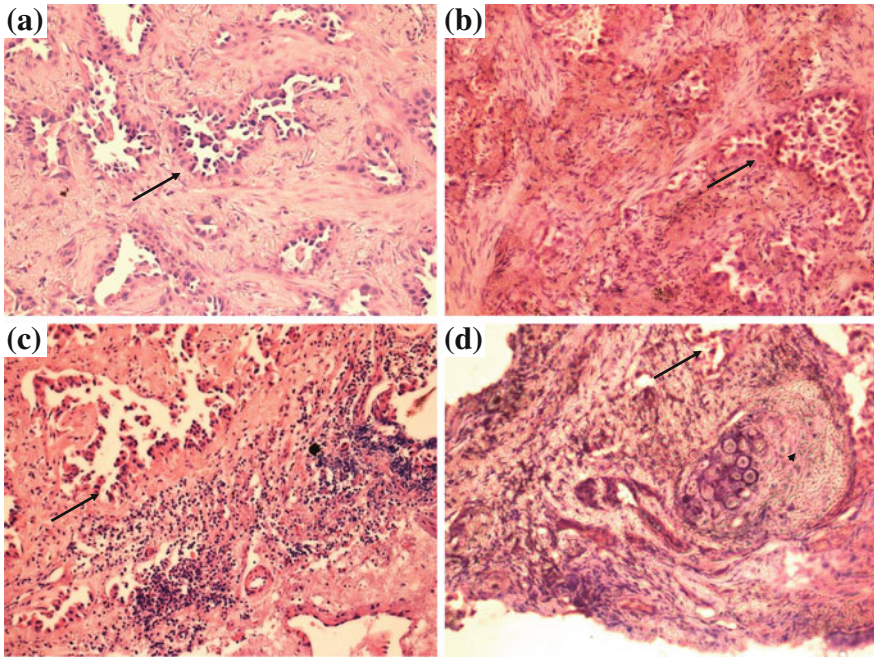
Fig. 1 Morphologic tissue quality in dependence of fixation procedure, **a** FFPE tissue with well-preserved morphology. **b** In cryosections morphology is slightly worse; however, the respective structures are still discernable. **c** HOPE fixation preserves morphology comparable to FFPE procedures. **d** Tissue fixed in RNA*later* shows a somewhat compromised morphology. *Arrows* neoplastic glands, *Arrowhead* cartilage

cryoconserved material must be taken in addition to the material necessary for conventional diagnostic purposes. In Fig. 1 morphology examples for differing tissue preserving methods are shown.

4.2   Immunohistochemistry and FISH

Since in up to 30 % of the NSCLC biopsies a precise subtyping requires additional IHC using specific markers for a squamous (p40, p63, CK5/6), an adenocarcinomatous (TTF-1, napsin, CK7) or even a neuroendocrine differentiation (chromogranin A, synaptophysin, CD56, Ki67) immunohistochemistry is critical for reliable diagnoses [31]. In addition, several IHC-based biomarkers which predict response to targeted agents are currently under development, this specifically includes MET and ALK [32]. For almost all diagnostically relevant antibodies, FFPE tissue is suitable. Since IHC staining is always interpreted in the context of morphology, optimal tissue fixation with high preservation of structural details is

important. Poor tissue fixation further results in a loss of immunoreactivity. Tissue fixation depends on time to fixation and several other variables which are not that critical in the lung biopsy setting (see above) but play an important role in the context of resection specimens in general (see other chapters in this book). Other tissue embedding methods including PAXgene® tissue system and HOPE do preserve both morphology and antigenicity; therefore, IHC is easily possible in this context. However, protocols and antibodies must be adjusted and cannot be transferred directly from the FFPE situation.

FISH analytics are necessary for amplification or translocation detection of predictive biomarkers (*ROS1*, *ALK*, *RET*). For FISH analyses, the requirements are comparable to IHC. Besides careful processing of the tissue, the most important thing is proper fixation to maintain the cellular structure and to avoid DNA degradation. From the technical viewpoint, FISH can be theoretically done with most types of fixatives (frozen, FFPE, PAXgene® tissue system, HOPE); however, data on this for some procedures are very sparse and protocols vary considerably.

4.3 Nucleic Acid Extraction

Quality and quantity of nucleic acid extracts are of utmost importance for all subsequent nucleic acid based molecular methods applied in routine diagnostics and translational research. First and foremost, tumor tissue must be marked on stained tissue slides and macro- or microdissected from the very same slide or subsequent unstained slides. Tumor cell content of the microdissected area should be documented, since it influences many factors of the analytics results like, e.g., allele frequencies in sequencing. Prior to tumor dissection the specimens need to be analyzed for a potential tumor heterogeneity including the amount of vital tumor, stroma, necrosis, or potentially contaminating normal lung or inflammatory cells in order to obtain tumor-specific, high-quality nucleic acids. Tumor cell content may vary considerably between biopsies.

Yield and quality of RNA and DNA strongly depends on the type of material used, fixation method, and extraction technology. RNA quality is frequently measured as RNA integrity number (RIN) by using capillary electrophoresis techniques (Agilent bioanalyzer) [33]. Results usually range between 2 (low quality) and 10 (optimum). RIN numbers better than 8 are widely considered as good quality. This RNA quality might serve as a template for most applications including microarray analyses.

The influence of biopsy techniques on RNA/DNA quality is low, when tissue specimens are processed/fixed quickly (1 min. range). Best results are obtained with cryobiopsies followed by forceps biopsies and core needle biopsies. DNA/RNA quality is excellent in most cases of cryobiopsies and suitable for all kinds of molecular analyses including next-generation sequencing.

Nucleic acid preservation is best for shock frozen tissue and RNA*later* material (Fig. 2). The quality of RNA isolates from RNA*later* stabilized tissue is equal to
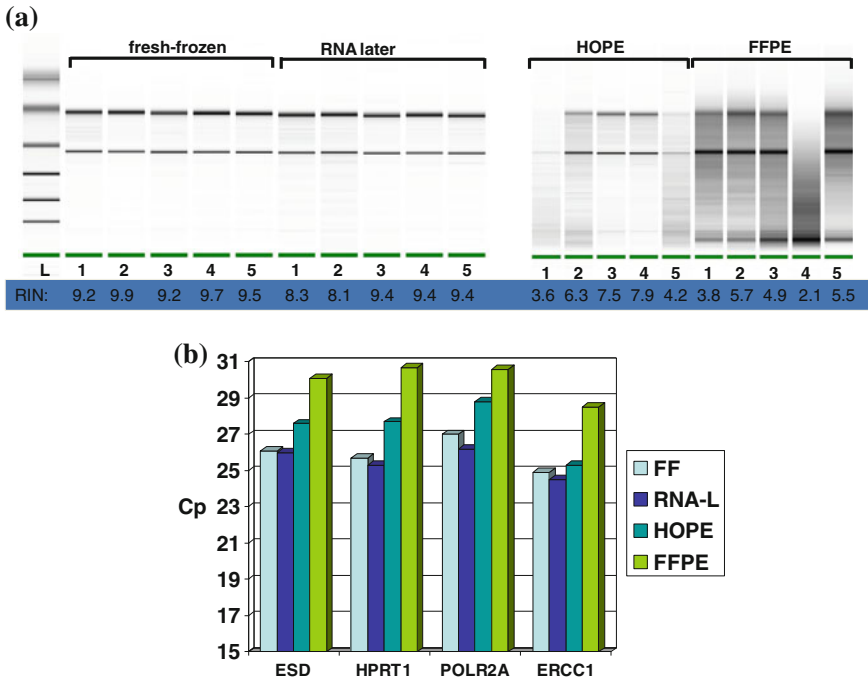
Fig. 2 RNA quality in dependence of tissue fixation method. **a** Gel electrophoresis and RIN numbers are given for representative tumor tissue samples fixed with different methods. **b** Reverse transcribed (RT) total RNA amplifiability of representative genes (*ESD* esterase D, *HPRT1* hypoxanthine phosphoribosyltransferase 1, *POLR2A* polymerase (RNA) II (DNA directed) polypeptide A, *ERCC1* excision repair cross-complementing rodent repair deficiency, complementation group 1) from samples with different fixation procedures (*FF* fresh frozen, *RNA-L* RNA*later*, *HOPE* HOPE fixans, *FFPE* formalin-fixed paraffin embedded) as measured by RT qPCR. Mean Cp values of 5 samples are shown

cryopreservation as measured by the RIN number [33]. From our experience, there is no major difference in DNA/RNA quality for nucleic acids isolated from FFPE and PAXgene® tissue system fixed probes [34]. This is exemplarily shown for RNA in Fig. 2, where the template quality for RT-PCR is compared.

High-quality DNA and RNA are usually isolated from cryopreserved or RNA-*later* stabilized tissues by hand using commercially available kits (e.g., Qiagen, Ambion, other), the respective workflow for frozen material is exemplarily shown in Fig. 3. DNA and RNA isolation from FFPE tissue might be a bit more challenging, isolation can be done by commercially available manual kits (e.g., Qiagen, others) but also by automated systems (Siemens, Qiagen, Promega). Time and method (bead mill, rotor-stator homogenizer) of tissue homogenization of small biopsies may have a significant influence on the size of isolated genomic DNA. This is important if the DNA is to be used for advanced molecular approaches such as next-generation sequencing techniques.
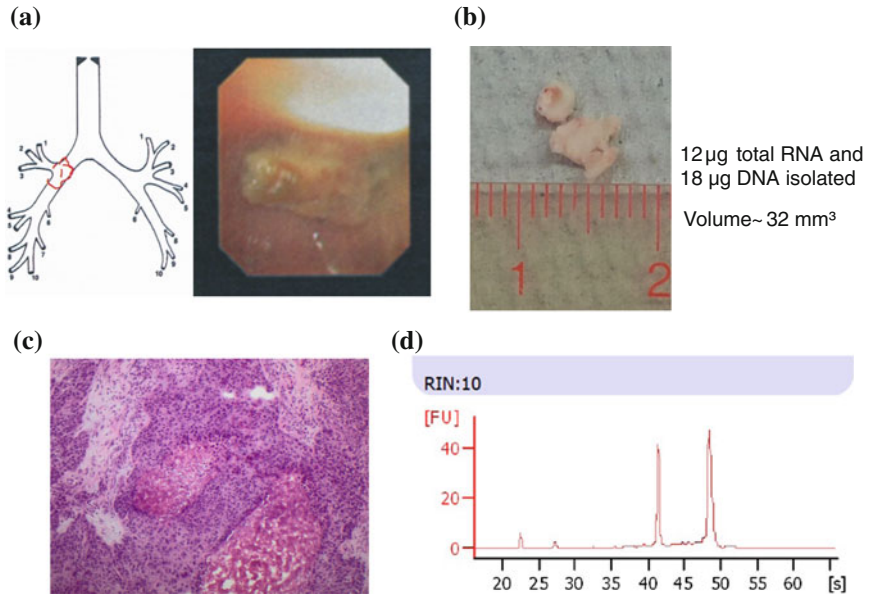
Fig. 3 Workflow of frozen cryobiopsy processing. **a** Localization of endobronchial tumor; **b** cryobiopsy; **c** frozen tissue section evaluation; **d** result of RNA analysis using an Agilent bioanalyzer

4.4 Sequencing

For conventional Sanger sequencing, a tumor cell content of about 30 % has to be considered as a lower limit for the reliable detection of point mutations. However, complex mutations like the typical exon 19 deletions can reliably be detected even in samples with lower tumor cell content [35]. DNA quality is not that critical for this method, however, major degradation may lead to compromised amplification by the presequencing PCR. For next-generation panel sequencing approaches 50 ng of DNA are usually sufficient [36]. Here, DNA quality is somewhat more important than for Sanger sequencing and must be rigorously controlled prior to sequencing.

5 Cytology Aspects

In general, morphological and molecular analyses of cytological specimens can be reliably performed using smears, cytospins, or cell blocks; there is no ultimate need for histological specimens. However, the sparseness of cellular material in some instances might hamper the sole use of cytology material. The diagnostic workup of cytological probes should be done as carefully as possible to spare material for subsequent molecular analyses. Nowadays, minimally invasive techniques are often used in a first attempt to obtain a definite diagnosis in lung cancer [37]. Therefore,

EBUS- and EUS-guided transbronchial needle aspiration (TBNA), and especially the combination of both, become increasingly popular as the initially applied diagnostic methods [11, 12, 14, 38]. As a consequence, the available material in lung pathology is increasingly shifting from histological to cytological specimens.

In order to increase the diagnostic yield of cytology specimens, the preparation of cell blocks is highly recommended. A cell block consists of fixed and embedded cell preparations that can be processed and cut like tissue material [39, 40]. The cell block method is a reliable, complimentary approach to conventional cytological or biopsy procedures.

Major advantages of conventional cytological smear or cytospin specimens over cell blocks are the intactness of the cell nuclei and the high DNA quality, usually allowing for good morphological and especially molecular analyses. However, in cell smears the amount of tumor cells is usually limited. Furthermore, destruction (from the morphological viewpoint) of the original specimen by, e.g., workup for molecular analyses may pose a problem considering the archiving requirements for original slides on which a malignant diagnosis was based. In those cases, image documentation of the slides, preferably by whole slide scanning, is mandatory prior to the dissection of tumor cells.

The advantages of cell blocks are the availability of established and validated protocols [39, 41] and the opportunity to have serial sections from the same specimens. Thus, changes to the original slides used for morphology review for molecular analyses can be avoided and molecular analyses can even be repeated or complemented by other methods using the available serial sections.

Preconditions for reliable molecular analyses using cytological specimens are experience, high expertise in cytology, sufficient technical equipment as well as a direct communication/close cooperation between histopathologists and cytologists involved in the case and those who perform the molecular analyses in order to ultimately correlate all respective results. It must be emphasized that cell blocks do not replace conventional cytological procedures (smears, cytospins) but are especially helpful as a complementary approach to fulfill the requirements for reliable and high-quality biomarker analysis [39, 41]. After sampling, cytological probes should be kept within a temperature of 2–8 °C (not frozen) or suitable fixatives (e.g., neutrally buffered formalin). The following points need to be considered for molecular analyses using cell block material:

- At least 50–100 tumor cells are usually required for molecular analysis; contamination with non-neoplastic cells should be as low as possible.
- Specimens should be fixed with buffered formalin (defined pH and buffer capacity). Bouin's solution should be avoided because the use of picric acid results in DNA damage.

Based on the various aspects outlined above in detail, the following points are recommended in order to improve the quality of molecular analyses using cytological specimens:

- Independently from the method used as much tumor material as possible should be obtained.
- Sputum and bronchoalveolar lavage specimens should always be combined with another method to gain sufficient amounts of tumor cells (TBNA or biopsy approaches).
- If a transport of the specimens is required this should be done in liquid form using suitable fixatives or at 2–8 °C. Freezing of the specimens should be avoided.
- Besides smears or cytospins, a preparation of a cell block from each cytological probe should be considered in order to optimize subsequent molecular analyses.

We recommend preparation of cell blocks immediately after a malignant diagnosis was made using the sediment of the original probe stored at 4 °C. For reasons of efficiency, we prepare cell blocks from EBUS-TBNA material and all specimens suspicious for malignancy immediately at the beginning of the diagnostic procedures.

6   Concluding Remarks

Regardless of whether cytology specimens, lung biopsies, or resection specimens have to be analyzed, preanalytic tissue processing is of utmost importance to optimize the results of the subsequent morphological, immunohistochemical, and molecular analytic process. Until today, only few efforts have been made to obtain reliable data on influences of preanalytics on diagnostic results. This is the reason why developments in preanalytics optimization are considerably lagging behind those in analytics. To change this, more efforts have to focus on this interesting field of research and development.

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