From tissue to molecular phenotyping: pre-analytical requirements Heidelberg experience

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Abstract: Lung cancer is a leading cause of tumor-related death worldwide through years. Efforts to individualize lung cancer therapy to improve prognosis nowadays employ molecular analyses besides routine histopathological examination of tissue samples. In general, tissues are provided by bronchoscopy, CT-guided procedures or surgery. The sequence of tissue removal, storage, and processing has a considerable impact on the success and reliability of subsequent molecular biological analyses and will supposedly also influence therapeutic decisions. There is still an ongoing need for updated statements about the minimal requirements of tissue sampling for molecular diagnosis at international level and for certified/accredited quality control programs of the sampling procedures. Several of these issues may have to be adjusted to the individual local conditions. We will present several aspects of experiences gained in Thoraxklinik at the University Hospital of Heidelberg (TK-HD) with pre-analytical tissue requirements.

Key Words: Lung cancer; pre-analytical tissue; molecular phenotyping

Introduction

The prognostic situation in lung cancer has changed only marginally in the last 20-30 years (1,2). The poor 5-year survival rate of 15% is due to the fact that most of the patients are diagnosed in an advanced stage. Currently only about 30% of patients with manifested non-small cell lung cancer (NSCLC) can be treated curatively with surgery. Even in localized stage I which accounts for approximately 10% of the overall lung cancer population, the 5 year survival rate is suboptimal at 60-70%. The unsatisfactory overall survival rate leads to the quest of prognostic and predictive factors which might help to identify patients at risk, and can be further used as a surrogate for specific therapeutic options i.e. targeted individualized therapies.

With the advent of numerous new diagnostic techniques which help to identify driver mutations (for example EGFR, EM4-ALK) (3-5) and recent advances in understanding molecular biology of lung cancer more than 200 new agents are under investigation in preclinical and clinical studies (6). In addition, new algorithms for the sub classification of lung cancer have been developed in recent years (7).

As a consequence, there is a considerable increase in workload for pulmonary pathologists. Twenty years ago one of the major tasks was to differentiate between NSCLC and SCLC. Today, even sub classification within the major NSCLC subtypes adenocarcinoma, squamous cell carcinoma, large cell carcinoma is recommended since the prognosis might be considerably different between the subtypes (8).

New diagnostic "omics" methods analyzing genetic/epigenetic factors, gene expression, and protein analysis by high throughput technologies may help to establish and to monitor individualized approaches of therapy (9-12).

Table 1 Factors influencing the quality of molecular analyses

Tissue sampling techniques/sources	surgery, bronchoscopy, radiology
Tissue sampling conditions/preservation	amount, temperature, time, fresh frozen, fixation method
Tissue processing/evaluation	whole tissue, micro-; macro dissection, heterogeneity
Extraction of nucleic acids/treatment	extraction methods, digestion of samples, etc.
Data analysis	management of background, artifacts, and variations

Table 2 Number of biopsies necessary for accurate molecular diagnosis of the listed genes in respect to the various bronchoscopical biopsy techniques

	21 g -NA	19 g - NA	NA	14 g - NA	TBB	Cryo
			Cell block			
Number of biopsies	4	4	4	2-3	4-5	4-5
Kras mutation	+	+	+	++	+	++
EGFR mutation	+	+	++	++	++	++
TS (IHC/PCR)	+	(+)	+	++	++	++
ERCC1 (IHC/ PCR)	-	(+)	+	++	+	++
RRM1 PCR	-	(+)	w+	++	+	++
EML4-ALK	+	+	+	++	++	++

TBB, Transbronchial biopsy; NA, Needle aspiration; Cryo, Cryobiopsy; IHC, Immunhistochemistry; PCR, Polymerase chain reaction - not suitable; (+), Limited; +, Sufficient; ++, Good

The majority of these techniques require high quality tissue samples (*Table 1*).

Sources

Tissue samples might be obtained from various sources i.e. bronchoscopy, radiology (for example by CT-guided FNA) and surgery. The major limiting factor is the amount of acquired tissue (*Table 2*).

**Transbronchial needle aspiration cytology**

The diagnostic yield of conventional (non/ultrasound guided) transbronchial needle aspiration (TBNA) is high for endoscopically visible bronchial lesions (13). Conventional TBNA has also been used for staging the mediastinum in lung cancer since the early 80ties (14,15). The diameter of the needles is 19 or 21 G. The 19 G is preferred as tumor cells can be sampled. The availability of the cytopathologist to assess the quality of the sample is helpful (Rapid onsite evaluation ROSE), this increases the yield (16) (*Figure 1*).

Linear endobronchial ultrasound (EBUS)

In published meta-analysis EBUS-TBNA has been shown to have a sensitivity above 9o% and high specificity of 100% (17). Different publications have shown that, even in patients with lymph nodes smaller than 1cm a significant percentage could still be detected to have N2/N3 disease. Several studies have evaluated the feasibility of analyzing gene alterations in lung tumor tissue samples obtained by EBUS-TBNA. Determining the EGFR mutation status in adenocarcinoma (18,19).

Endoscopic ultrasound (EUS)

EUS is especially useful for sampling posterior mediastinal and paraoesophageal lymph nodes (stations 4 L, 7, 8 and 9). In addition, the left adrenal can be accessed (20). It has a so-called 'seagull' shape on ultrasound and is particularly well visualised in cases of metastatic enlargement. EUS is more accurate and has a higher predictive value than either PET or CT for posterior mediastinal lymph nodes (21). The reproducibility of cytological diagnoses on EBUS and EUS is good among experienced cytopathologists.
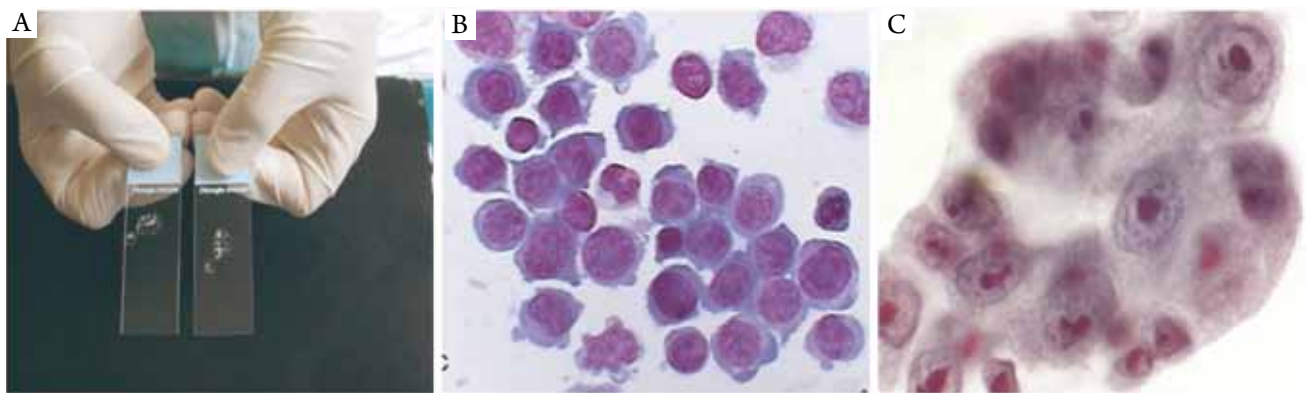
Figure 1 Results of aspiration cytology. A+B. celluar smear; C. cell block after centrifugation and paraffin embedding. The cell block can be handled similar like a FFPE tissue sample

Combining EBUS and EUS

EBUS-TBNA and EUS-FNA have a complementary reach for examining mediastinal nodes. EBUS has access to the paratracheal, subcarinal and hilar regions and EUS to the paraoesophageal lymph nodes. In combining these techniques, all mediastinal lymph node stations (apart from stations 5 and 6) as well as the left adrenal gland can be reached (22). For the EUS procedure the EBUS scope can be used (23).

Bronchial and transbronchial biopsy

Biopsies of endobronchial Tumortumors have a yield of 75-95% for diagnosis of malignancy (24). The larger the biopsy sample, the more accurate the diagnosis. A larger number of biopsy samples contributes to a more accurate diagnosis also (25,26). In malignant bronchial biopsy samples, between one third and one half of the biopsy fragments contain no tumor (27). Cryobiopsies are a very effective technique to produce large tumor biopsies with the potential to increase the diagnostic yield at least in endobronchial tumors (*Figures 2,3,4,5*) (28).

In patients with solitary peripheral pulmonary nodules, the endoscopic diagnostic procedure is usually performed as transbronchial lung biopsy (TBBx) under fluoroscopic guidance. This commonly performed procedure is associated with a low yield in SPNs not visible by fluoroscopy (29). Normally 4 to 5 biopsies are taken. For lesions smaller than 3 cm a navigation support based on a virtual bronchoscopy is recommended (30). Using these

systems herewith the yield for lesions around 2 cm can be improved to 80%.

### CT guided transthoracic needle biopsy (TTNBx)

For peripheral lesions transthoracic needle biopsy may be used. The main indications for these techniques is to determine the nature of a thoracic nodule or mass (31). TTNBx has an accuracy varying between 80-95% (32,33). The negative predictive value of pulmonary biopsy is 84-96% and false negative results are noted in 2-4% (34) CT-guided core needle biopsy and FNA allows acquisition of material for predictive analysis using either 18-gauge or 20-gauge tru-cut biopsy needles via 17-gauge or 19-gauge coaxial needles (35,36).

Heidelberg recommendation

The best strategy to increase the yield of the bronchoscopic samples is to combine several techniques. The optimal number of needle passes should be three ore more. Rapid On Site Examination (ROSE) is a quick cytological examination for the presence of tumor or lymphoid cells by pathologist or trained person (37). Initially, ROSE was set up for conventional (non-ultrasound guided) TBNA, for confirming the representativity of the sample. However, with the aim of obtaining as much tumor material as possible to allow more biomarker testing, the original goal is redundant, and additional needle passes may be required to obtain further tissue for molecular testing. Transbronchial biopsy could
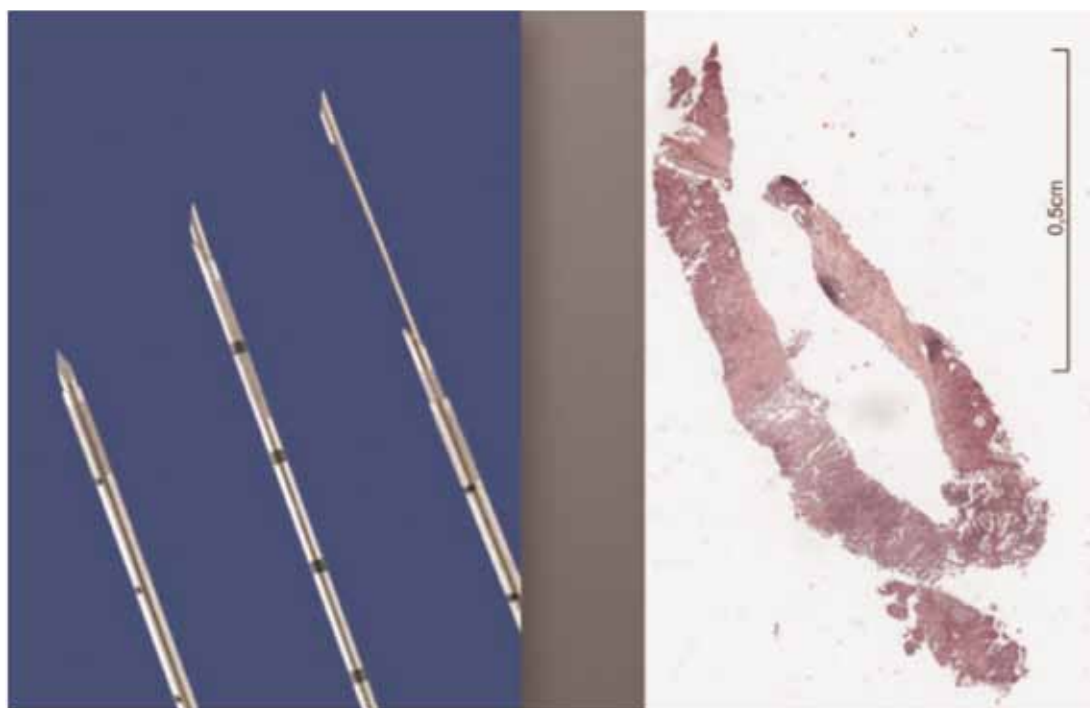
Figure 2 Endoscopic needles for aspiration cytology and punch cylinders. A major disadvantage of this technique is that the tumor content might change dramatically when the sample is sectioned longitudinally
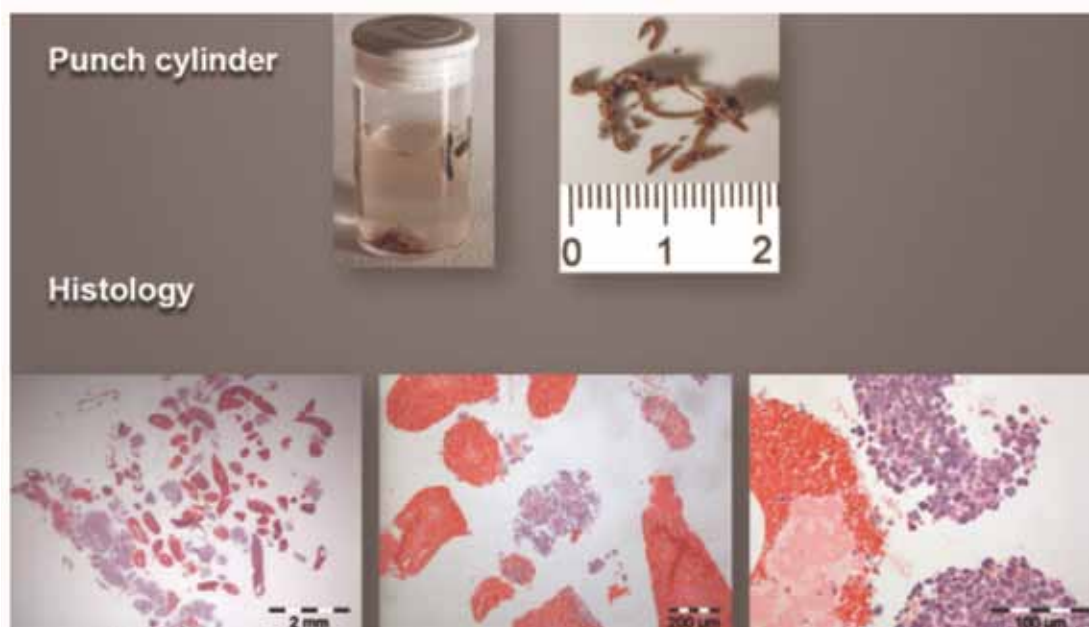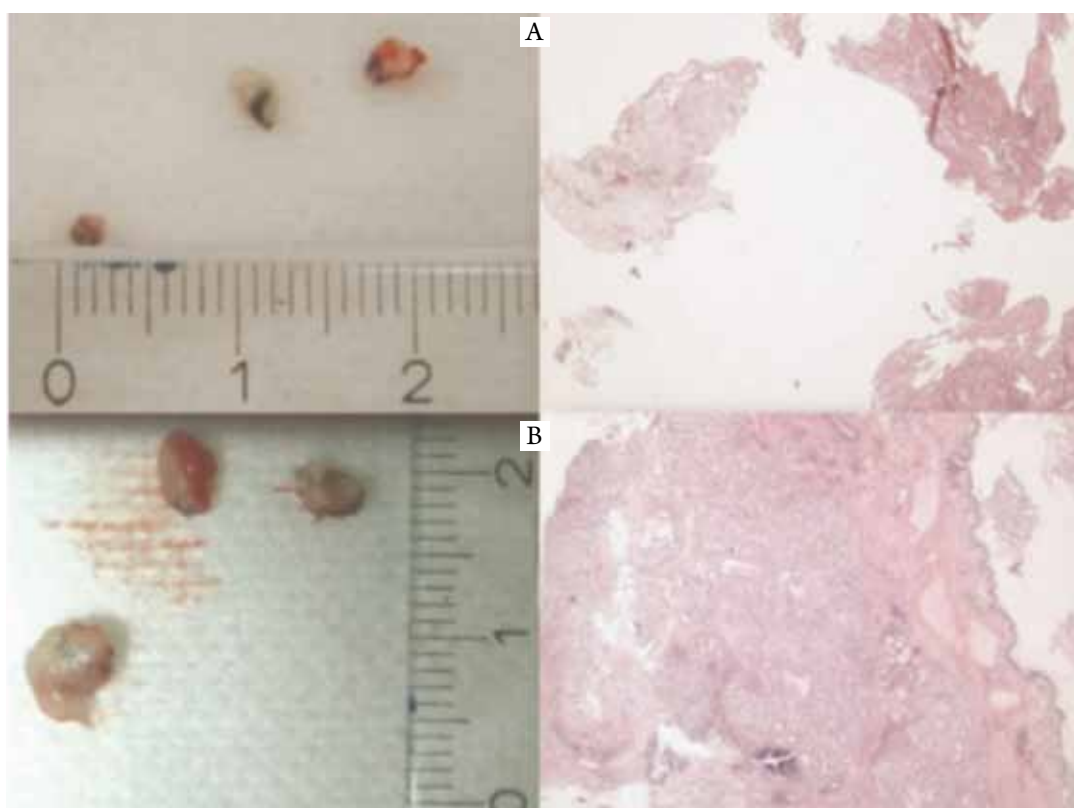

Figure 3 Endoscopic punch cylinders and histological samples after formalin fixation and paraffin embedding. Punch cylinders can be taken CT guided in the radiology department or alternatively acquired by bronchoscopy
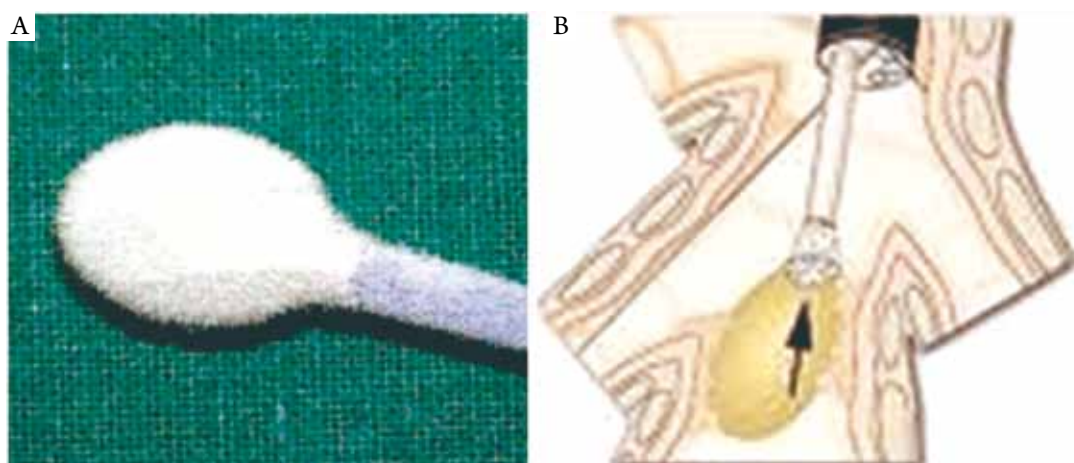
Figure 4 Cryobiopsy sample (A); schematic drawing of bronchoscopic cryobiopsy technique (B)

Figure 5 Conventional forceps biopsies (A) compared to cryobiopsies (B). Forceps biopsies are usually about 1-2 mm in diameter. The tissue might experience severe artifacts by squeezing of the sample, with an impact on the histopathological diagnosis. In addition, the amount of tissue is rather low requiring multiple samples for further analysis. Cryobiopsy has been proven as a save method in acquiring larger biopsies (>5 mm) at least in endobronchial tumors with an excellent preservation of the structure and superior diagnostic yield in comparison to conventional forceps biopsy. In addition, the sample might be fixed and paraffin embedded or stored fresh frozen in a condition which is as near to the original condition as possible. However, the technique has its limitations in small peripheral tumors. In our own experience this technique can be as well applied safely in transbronchial setting
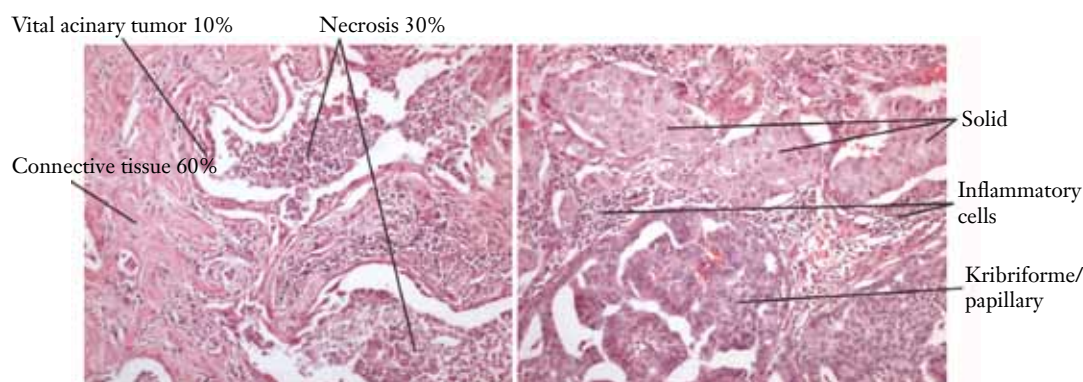
Figure 6 Heterogenity of tumor samples in respect to tumor cell content and sub types in resected adeno carcinoma of the lung. Samples of two patients are shown as an example for heterogeneity within a tissue sample and between patients

be done in peripheral lesions to improve the diagnostic yield of peripheral lesions. An upcoming alternative is the use of a navigation system.

Surgical options

A representative amount of tissue can be provided by surgery, e.g., open thoracotomy (wedge resection, lobectomy, pneumonectomy), thoracoscopy or mediastinoscopy. However, surgery is only possible in 30-40% of the patients. Several factors influencing the quality of the tissue have to be considered: (I) the processing time which is in general longer than for biopsies and (II) the influence of general anesthesia and the "warm ischemia" time on gene expression might be considerably high.

The interference of specific fixation methods with downstream analytical methodology should be considered as well (fresh frozen *vs*. FFPE or alternative fixation methods like HOPE, PAXgene® tissue, RNA later and others) (38-42). In addition, the determination of heterogeneity within the sample is of great importance since the content of viable tumor cells, stroma, necrosis and lung parenchyma may vary considerably between patients and within patient sub samples (*Figure 6*).

Recently, Freidin and coworkers presented a study at the WCLC 2011 in Amsterdam dealing with the effect of sampling time, fixation method and storage temperature on quality of extracted total RNA samples and the consecutive gene expression profiles (38). Tumor tissue samples were taken directly after chest opening (I) immediately after lung resection; (II) after transport to the

pathology department; (III) and after formalin fixation, paraffin embedding and long term storage; (IV) The quality of isolated total RNA, reported as RNA Integrity Number (RIN) (43) was fairly good for most fresh samples but considerably worse for FFPE material. In addition, the number of significantly expressed genes was comparable for most time points and storage conditions with the exception of FFPE tissue. In summary the best results were achieved when the tumor tissue samples were taken shortly after chest opening.

## Proceeding of the sampled material

Tissue banking

Tissue repositories are considered as an optimal source of fresh frozen tissue samples at least for research purposes (42). The TK-HD has built up a large lung tissue repository during the last 10 years (*Figure* 7), which got accredited in 2010 as part of the National Center for Tumor diseases (NCT)-tissue bank (44,45). Following these requirements SOP-guided quality-controlled tissue procurement can be guaranteed. Besides fresh frozen tissue, FFPE-tissue, multi tissue arrays and pathological platform technologies may be provided via the NCT tissue bank.

In addition, histological evaluations and quality controlled nucleic acid extraction services are routinely provided for scientists (46,47). The biorepository can be linked to an in house tumor documentation system, as a source of high quality supervised clinical data. A strong interdisciplinary cooperation is essential for successful tissue procurement (*Figure 8*). The whole process starts at the ward by the patient's informed consent, which is
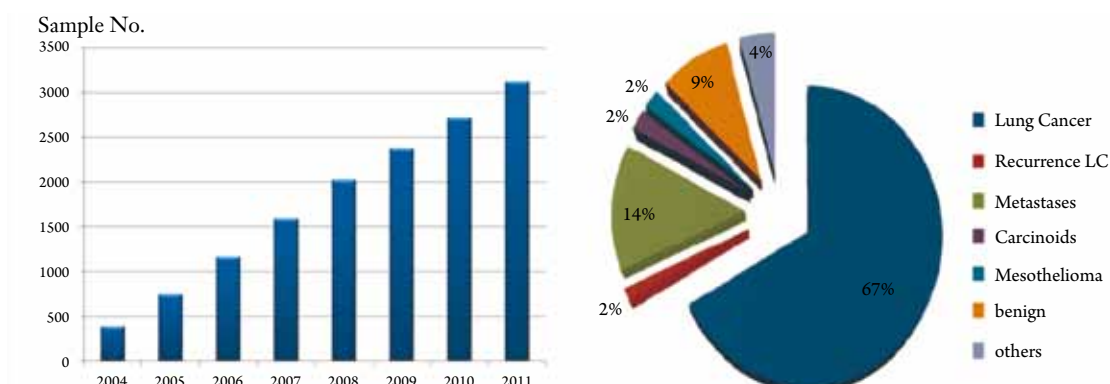
Figure 7 Progress in tissue sampling of TK-HD biorepository between 2004 and 2011 and distribution of diseases
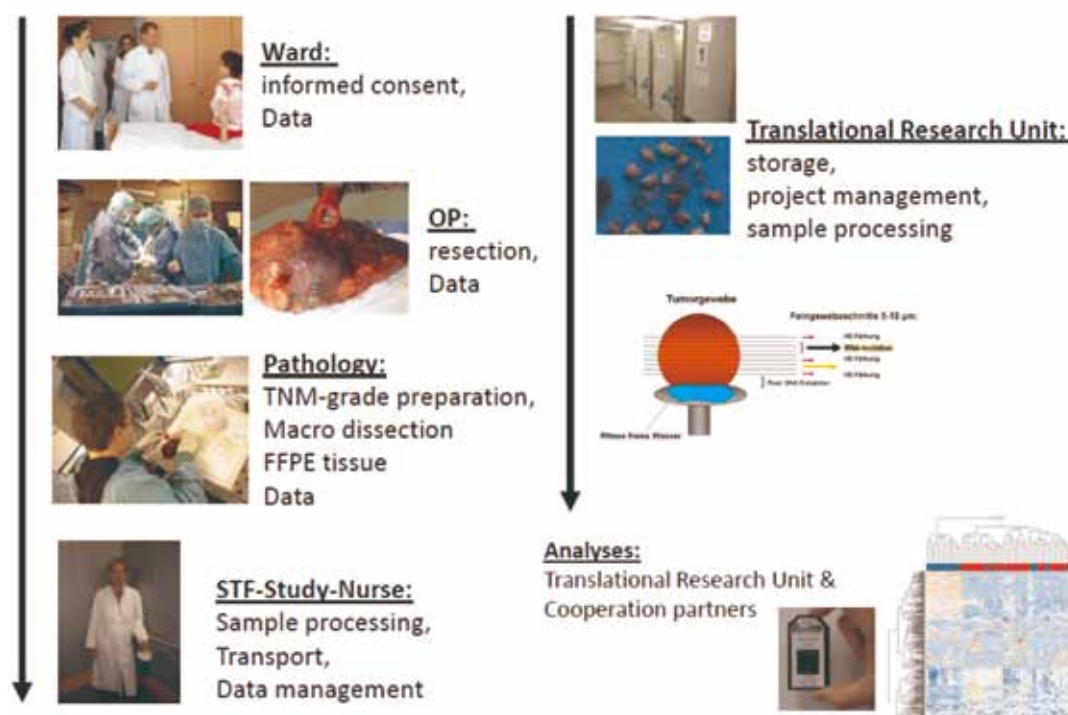

Figure 8 Workflow and interdisciplinary teamwork at the TK-HD biorepository from patient to molecular analyses

a prerequisite. Basic clinical data enter the clinical IT-system which employs a tumor documentation system as an integral part. The tumor documentation follows the recommendations of ADT ("working group of German tumor centers") (48).

During surgery all significant factors that may influence tissue quality are documented i.e. time of chest opening, interruption of blood supply, resection of the lung tissue, as well as transportation time to the pathology lab. In our hands, freezing of tissue samples can be accomplished
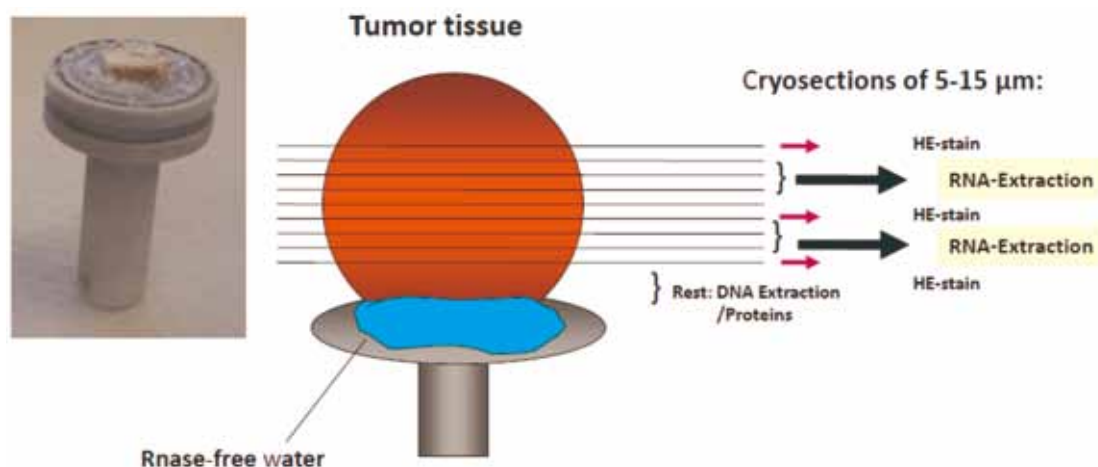
Figure 9 Tissue preparation for the evaluation of tissue heterogeneity in respect to content of vital tumor, necrosis, stroma and lung parenchyma. The first an intermediate and the last section of a series of cryo sections are H&E stained and evaluated by an experienced pathologist for tumor composition

within 15 to 30 minutes after resection in the majority of cases.

At the pathology lab a TNM grade sample processing is maintained and guaranteed even in small tumors (T1A). A high quality of the tissue and high tumor content can be achieved by macro-dissection of samples by an experienced pathologist. However, this needs a good interaction with your pathologist.

Tissue samples are routinely divided in pieces of about 5 mm × 5 mm × 5 mm, distributed in labeled cryovials, weighted, and immediately snap frozen in liquid nitrogen. Long term storage is performed at –80 °C in temperature monitored mechanical freezers.

Quality control of the banked samples

Before extraction of nucleic acids is performed, each individual sample is evaluated by a standardized protocol for tumor cell content as shown in *Figure 9*. One frozen piece of tumor tissue is removed from a vial, attached to a cryostat chuck using sterile RNAse-free water, and cut into 5-15 µm sections. The first, intermediate, and the last section of a series are Hematoxylin and Eosin (H&E) stained and reviewed by a dedicated lung pathologist to determine the proportion of viable tumor cell, stromal cell, normal lung cell content, infiltrating lymphocytes and necrotic areas. The sections in between the stained sections are transferred into pre-cooled micro vials and kept at –80 °C until nucleic acid extraction.

For DNA/RNA extraction we use those tissue samples with viable tumor content equal to or higher than 50%. Isolation of nucleic acids is performed with commercially available kits (AllPrep DNA/RNA kit, Qiagen, Hilden, Germany) and adopted protocols, which allow us to extract DNA and total RNA including miRNA in one session. The amount and quality of nucleic acids are routinely checked with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, MA, USA) and an Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Kit (Agilent Technologies, Boeblingen, Germany) (*Figure 10*).

On average the tumor content in a series of 447 selected NSCLC tumor samples was 57.8% (*Table 3*). The isolation of nucleic acids from these samples resulted in an RNA quality with a RNA-Integrity-Number (RIN) of approximately 9.0 (43). These samples are excellent templates for next generation sequencing, mutational analyses, methylation analyses, and microarray or qPCR based gene expression analyses (46,47). The average yield in DNA and RNA is fairly high and was not a limiting factor for above molecular biological analyses.

Conclusions

Various tissue sampling techniques are currently available and most of them will result in a high diagnostic yield in lung cancer. Nevertheless, the large variability in the number of cells and the heterogeneity within a tumor
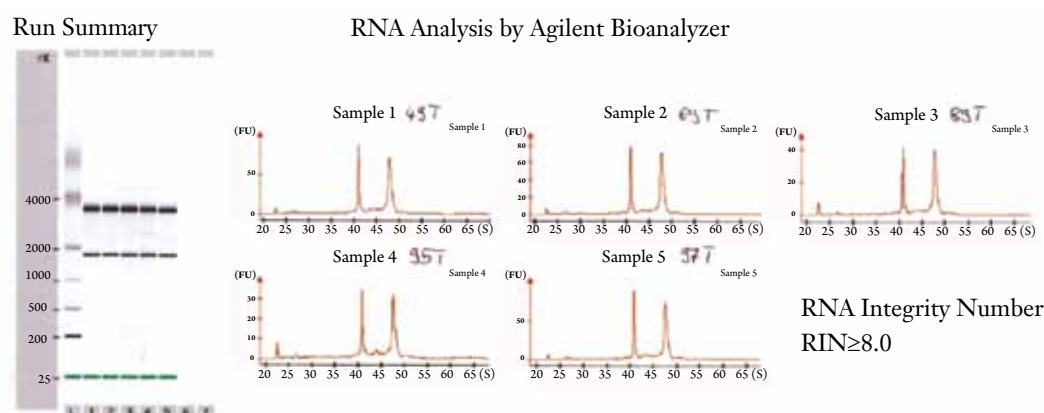
Figure 10 RNA quality assessment by micro chip-based capillary electrophoresis using Agilent Bioanalyzer. RNA quality is given as RNA integrity number (RIN) ranging for 0 (bad) up to 10 (excellent). Peaks indicate 28s and 18s ribosomal RNAs. Lane 1-5 electrophoresis run summary and densitometric read out for RNA of 5 individual patients' samples

Table 3 Results of DNA and RNA extraction from lung cancer tissue samples (n=447) with an vital tumor cell content equal to or larger than 50% using standardized nucleic acid extraction protocols

	Mean	SD	Median	Min	Max
DNA					
OD 260/280	1.9	0.02	1.9	1.85	2.07
Amount (μg)	105.6	19.12	106.9	44.6	203.3
Total RNA					
RIN	9.3	0.67	9.5	4.7	10
Amount (μg)	52.9	25.2	49.5	2.4	138.1
Tumor cell content (%)	57.8	7.8	55	50	87.5
RIN = RNA integrity number; OD = optical density					

sample itself represents a challenge for molecular analyses. Therefore, not every tissue sampling technique may be ideally suited for all kinds of marker analyses. There is clearly an ongoing need for updated statements about the minimal pre-analytical requirements of tissue sampling for molecular diagnosis on an international level. Additionally, there is also an existing need for local adaptations of the program and certificated or accredited quality control programs. It is anticipated that the combination of classical clinical, pathological, and molecular biology techniques will influence the diagnosis and improve the treatment options of patients suffering from lung cancer.

Acknowledgements

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