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Molecular Diagnostics of Lung Cancer in Serous Effusion Samples

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Abstract: For molecular diagnostics of lung cancer samples, often only a small amount of material is available. The ever-increasing number of biomarker testing is in contrast to the amount of material obtained. In that case, cytological specimens, such as serous effusion samples, are one possible option. Effusion samples were prepared as sediment smears or cytospins or as a cell block if needed. Suitable tumor cells areas were marked by a cytopathologist and used for molecular diagnostics, including fast track analysis, parallel sequencing, and/or fluorescence in situ hybridization. In 62 cases of malignant effusion with cells of pulmonary adenocarcinoma, molecular diagnostics were carried out. A fast-track result with the high-resolution melting method for hotspot mutation of *KRAS* Exon 2 and *EGFR* exon 21 and fragment length analysis of *EGFR* exon 19 was available for 43 out of 47 samples (92%). Parallel sequencing was successful for 56 out of 60 samples (93.3%). In the same period, 108 FISH analyses were performed for *MET* amplification, followed by *ROS1*, *RET*, and *ALK* translocation analysis. If only a limited amount of tissue/biopsy is available, a malignant effusion is advisable to perform on the molecular diagnostics with a high success rate.

Keywords: lung cancer; pulmonary adenocarcinoma; serous effusion samples; molecular diagnostics; fast-track analysis; parallel sequencing; fluorescence in situ hybridization

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1. Introduction

Lung cancer is one of the most frequent malignancies and also one of the leading causes of death from a malignant disease worldwide [1]. In the course of the disease, serous effusions occur quite frequently, with pleural effusions being prevalent [2]. If cells of a pulmonary adenocarcinoma are found in a serous effusion, an advanced stage of the disease is diagnosed [3]. In these cases, rapid molecular diagnostics should be performed to search for driver mutations which might provide an option for targeted therapy. Approved drugs are available for the epidermal growth factor receptor (*EGFR*), proto-oncogene B-Raf (*BRAF*), mesenchymal–epithelial transition factor (*MET*) or Kirsten rat sarcoma viral oncogene homolog (*KRAS*) G12C mutations, anaplastic lymphoma kinase (*ALK*), *RET* proto-oncogene (rearranged during transfection; *RET*) and c-ros oncogene 1 (*ROS1*) translocations [4–10]. The molecular diagnostics were carried out with panel-based parallel sequencing (NGS) and covered all kinds of mutations, e.g., point mutations, deletions, and insertions [11–13]. Translocations, i.e., rearrangements of larger chromosome segments, and amplifications were very well identified by fluorescence in situ hybridization (FISH) analysis. Typical examples are the *EML4*::*ALK* translocation, *ROS1* translocation and *MET* amplifications [14].

In this study, we performed a retrospective analysis of all cases of malignant effusion due to pulmonary adenocarcinoma, which were processed in our cytopathology lab in 2018 and 2019 and have undergone molecular diagnostics in our institution [15].

Far more molecular analyses of effusion preparations in cases of pulmonary adenocarcinoma have been performed than analyzed in this study. We have restricted this study to

effusion samples that had been processed in-house to minimize variation in preanalytical steps as far as possible [16,17].

2. Materials and Methods

Effusion samples were sent to the lab in containers of various forms and sizes depending on the sample volume and the preferences of the clinical department. The samples were expected to be fresh and without any additives. After centrifugation, the technician decided whether sediment smears or cytospins were prepared. Routinely, four slides were prepared; one was immersed immediately into 96% ethanol for fixation, and the other three slides were left air-drying. The alcohol-fixed slide was used for the Papanicolaou stain, two of the air-dried slides were used for Hematoxylin and Eosin (H&E) stain and May-Gruenwald-Giemsa (MGG) stain, and the fourth slide was left for additional stains if needed. If immunochemistry was requested, a cell block was prepared following an in-house protocol based on "Gautinger Protokoll" [18].

If the final diagnosis of standard cytomorphology was malignant effusion in advanced pulmonary adenocarcinoma, comprehensive molecular diagnostics according to the national Network Genomic Medicine (nNGM) Lung Cancer were suggested to the attending clinician and offered to the patient. After obtaining written informed consent, molecular diagnostics were performed. Screening for *ALK* and for *ROS1* rearrangement by immunochemistry was performed on sections of a cell block, as this is part of the fast-track analysis and our lung cancer routine diagnostics (results are not listed here). If positive, a confirming test by fluorescence in situ hybridization (FISH) was performed. If no cell block was available or cellularity was too low, *ALK* and *ROS1* were analyzed by FISH using smears or cytospins (Supplementary Table S4).

Since the result of the molecular diagnostics is of immediate clinical relevance for the choice of therapy, we perform a rapid analysis for *EGFR* exon 19 and 21 and *KRAS* exon 2 (also called "fast-track" analysis) with a very fast turnaround time. Analyses were carried out with high resolution melting or fragment length analysis, which detects a positive or negative result but does not provide the exact mutation description (Supplementary Method Description 1; Supplementary Table S1). Next-generation sequencing-based analysis of several genetic markers was performed using a panel approach. Validated gene panels with 14 genes using AmpliSeq gene panels for poor DNA quality or low DNA content samples (Thermo Fisher Scientific, Waltham, MA, USA; LUN3, 14 genes) or GeneRead gene panels for good DNA quality samples (Qiagen, Hilden, Germany; LUN5 panel) including 19 genes were used (Supplementary Method Description 2; Supplementary Tables S2 and S3 [19,20]). In many cases, a cell block of sufficient cellularity was available. In cases in which no cell block was available or the cellularity of the block was too low, stained sediment smears or cytospin preparations were used either for FISH analyses or for sequencing or for both assays. One of the cytopathologists reviewed the slides and decided which slides were used for the requested assays and marked the relevant areas on the glass slides (Figure 1).

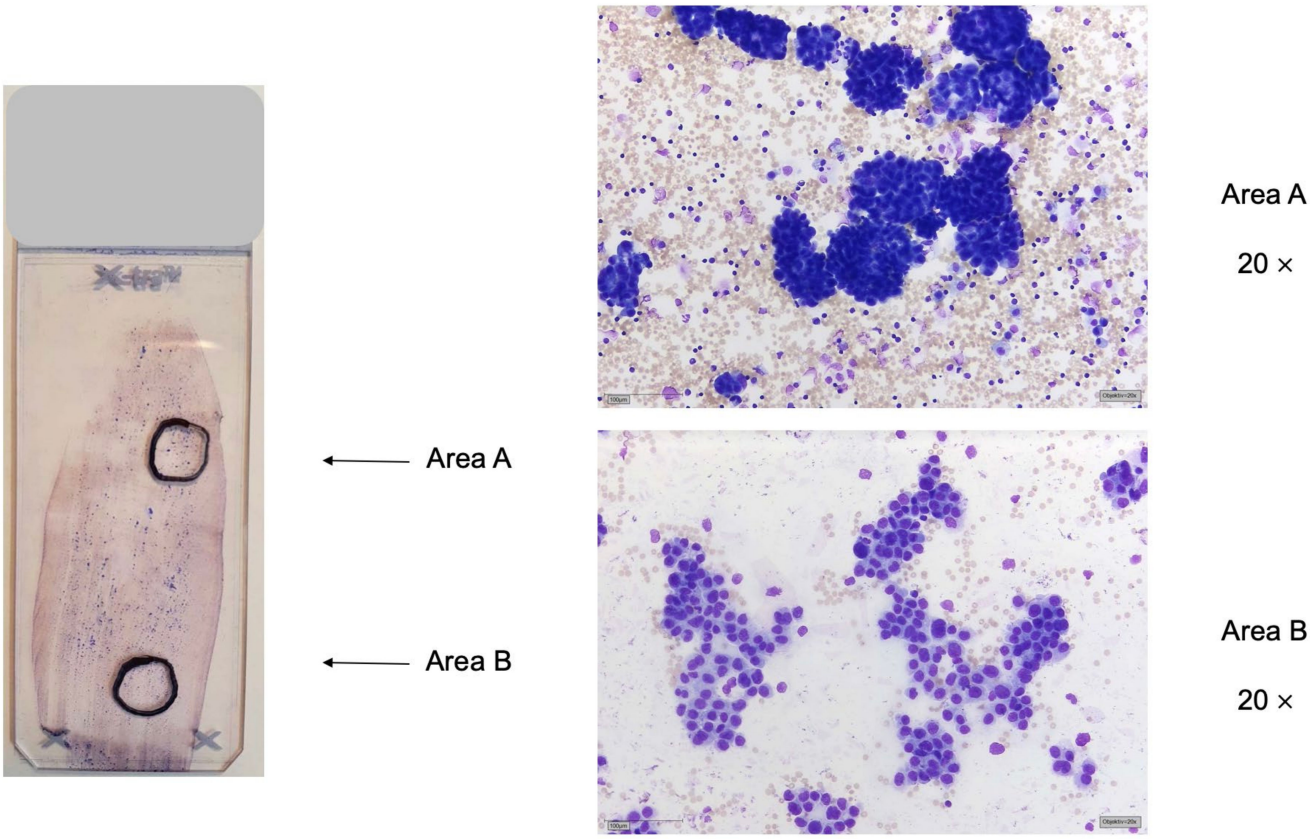


Figure 1. Marking of suitable areas for DNA extraction (area (**A**)) or FISH analysis (area (**B**) by a cytopathologist. Left: sediment smear of pleural effusion of pulmonary adenocarcinoma, MGG stain. Two areas are marked up, area (**A**) for DNA extraction and parallel sequencing and area (**B**) for FISH analysis. Right: area (**A**): dense tumor cell clusters, suitable for DNA extraction and parallel sequencing; area (**B**): flat sheets of tumor cells, suitable for FISH analysis.

2.1. Preanalytics of Cytological Preparations

Cytological preparations can be sent in air-dried (not covered) or covered (coverslip or coverslip film) and already stained for analysis. In both cases, appropriate areas to be used for further analytical tests must be marked by an experienced cytopathologist (Figure 2A). For the uncovered specimen, the areas were marked on the back of the slide with a waterproof pen. Depending on how the cells were distributed, there may be one or more areas on a slide. If the marking was on the front side (coverslip or coverslip film), the marking was transferred to the back side of the slide. For all cytological preparations, the mark on the back of the slide was scratched into the glass with a diamond stylus (Figure 2B). This mark was then traced again with a waterproof pen. The smallest color pigments, which were not washed away by the xylene, were caught in the grooves (Figure 2D). These color traces and the traces of the diamond pen make it easier to find the marked area again afterwards.

An air-dried smear or cytospin was placed in water for one minute for DNA extraction. Subsequently, the marked area can be scraped off by macrodissection with a scalpel. The dissected material was transferred with the scalpel tip into a reaction vessel containing lysis buffer. The mixture was shaken overnight at 70 °C and further processed according to the manufacturer's instructions. The extracted DNA was suitable for all subsequent PCR-based assays.

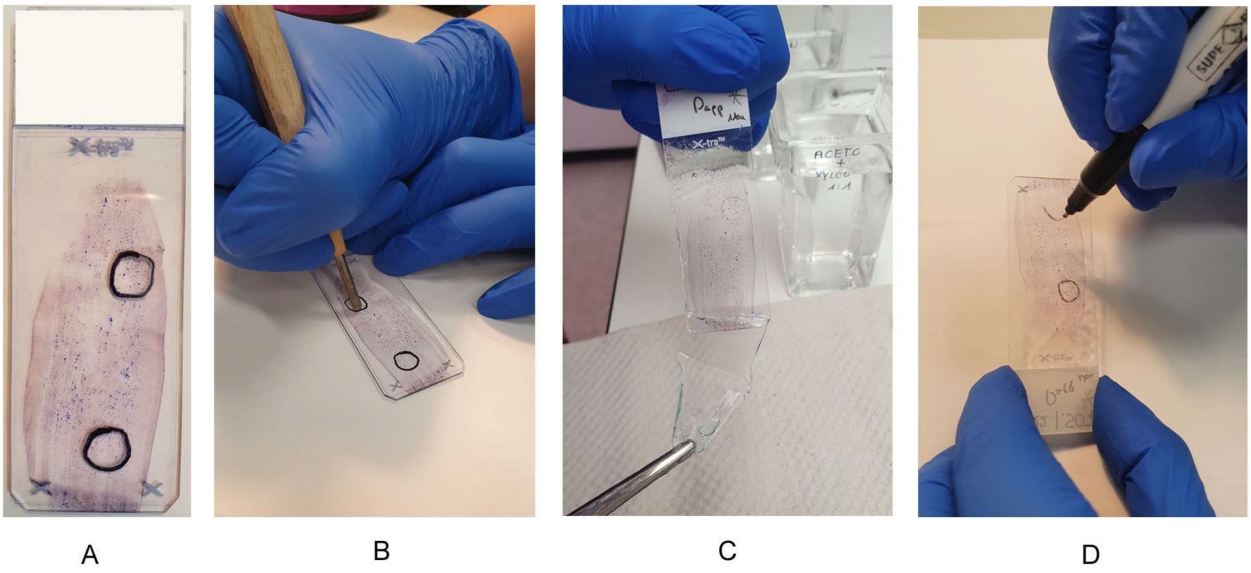


Figure 2. Preanalytical steps on cytological preparations. Preanalytical steps on cytological preparations. (**A**) Suitable smear with two areas, one area for DNA extraction and one area for FISH analysis; (**B**) use of a diamond stylus, scratching the marking on the back of a slide; (**C**) uncovering the slide in xylene; (**D**) trace the diamond scratches with a waterproof pen.

If the samples sent in were covered, the coverslip or coverslip film had to be removed first. A preparation that was covered with a coverslip was placed in xylene overnight. If the coverslip could not be removed the next morning, the smear or cytospin was placed in a −80 °C freezer for 30 min. The smear or cytospin was then placed back in xylene, and the coverslip should then be removable. If the submitted samples were covered with film, they were placed in acetone for 3.5 min, in an acetone/xylene mixture (1:2) for 1 min, and again in xylene for 1 min to completely remove the coverslip film (Figure 2C). It should be noted here that even markings made with xylene-resistant pens do not always adhere. The labeled area of the preparation was then "scraped off" with a scalpel and placed in a reaction tube filled with lysis buffer for DNA extraction.

2.2. Preparation for FISH Analysis

The cytological slides must be marked on the reverse side with a diamond pencil and then uncovered for FISH analysis. For FISH analysis, the smallest possible areas with at least 100 tumor cells that do not overlap were marked. If different FISH analyses have to be performed on one slide (smear or cytospin), the distance between the areas should be as large as possible. The pretreatment of the slides was automated using the VP2000 from Abbott and was identical to the pretreatment of formalin-fixed, paraffin-embedded (FFPE) sections. Slides that were already H&E, MGG, or Papanicolaou stained are well suited for FISH analysis. These preparations were decolorized by pretreating the slides for hybridization. After pretreatment, the 1st probe (3 µL) was applied and covered with a round coverslip (13 mm). The coverslip was sealed with Fixogum (Marabu). If the desired areas were close together, coverslips could be divided. After sealing the 1st area with Fixogum, the 2nd probe was applied to the 2nd area, covered, and sealed with Fixogum.

2.3. DNA Extraction from Cytological Preparations

For DNA extraction and subsequent PCR (polymerase chain reaction)-based analysis, at least one hundred malignant cells are required, which should be present "pure", i.e., as little as possible mixed with benign epithelia and/or inflammatory cells. The tumor cell content should be at least 10% of the total cells for parallel sequencing. For other PCR-based assays, such as Sanger sequencing, the tumor cell content must be 20%, as this

method is less sensitive. The percentage of tumor cells should be specified in order to be able to make a statement about an allelic fraction in case of a mutation detected by parallel sequencing. To increase the probability of mutation detection, it is useful not to extract all cells of a slide but to enrich the tumor cells in the number of cells that are extracted. Therefore, all available slides are stained (H&E) and reviewed microscopically. For DNA extraction, the cytopathologist marked a section of the preparation with a waterproof pen on the slide in which larger quantities of tumor cells are stored, if possible, without mixing with benign cells or inflammatory cells. Overlaying of the tumor cells themselves is not a problem (Figure 1).

Cytologic specimens may be air-dried or covered when labeled by the cytopathologist. Therefore, marking of the specimen may occur either on the coverslip or coverslip film or on the back of the slide. The mark was then transferred as described in the preanalytics section, see Figure 2. If a cell block was available from malignant effusions, molecular pathology studies were usually performed on the cell block. Marking of suitable areas was performed in the same way as on a biopsy. If necessary, additional sections, e.g., for FISH analysis, can be prepared. In our institution, the simultaneous sequencing of 14/19 genes and gene regions in a "lung cancer panel" using parallel sequencing is established as a routine method. The isolated DNA is measured for amplifiability and concentration by quantitative PCR (qPCR). If available, 10 ng of genomic DNA is used per primer pool. Since the "lung cancer panel" for parallel sequencing consists of four primer pools, a total of 40 ng of DNA must be used (Supplementary Table S1). According to the result of the parallel sequencing and depending on the request, different FISH analyses follow.

2.4. Fluorescence In Situ Hybridizations on Cytological Preparations

For FISH analysis, at least one hundred malignant cells are required in the preparation, which are well spread out (Figure 1). If possible, they should be without the overlay and well distinguishable from benign cells. An advantage of cytological preparations for FISH analysis is the intact cell nuclei, which are not partially incised as in a tissue preparation by cutting the FFPE blocks. However, the evaluation of FISH analyses can be more difficult because a whole-cell nucleus means that one has to look through the slide under fine focus. This diagnostic procedure is time-consuming but is worthwhile if a patient's material is low. One problem with cytology slides is the residual stain from prestained slides, which cannot be completely destained during pretreatment for hybridization. Residues can elicit strong background autofluorescence so that the FISH analyses cannot be evaluated. For each FISH analysis, such an area in the preparation with one hundred well-spread, well-delineated cells is necessary (Figure 1). If sufficient suitable slides are available, a separate slide was used for each FISH examination. In the case of cell-rich, high-quality smears, several FISH analyses can be performed on the same slide (Figure 1). Especially with sediment smears of effusion fluid, this is often possible (Supplementary Method Description 3, [21–25].

3. Results

By searching the archived files, we identified 5702 samples of pleural effusions, ascites, and pericardial effusions, which were processed in the cytopathology lab of the Institute of Pathology of the University Hospital Cologne from January 2018 to December 2019. Of these, 288 cases with a final diagnosis of pulmonary adenocarcinoma with malignant effusion were identified. Among these cases were 256 pleural effusions, 15 ascites, and 17 pericardial effusions. For further analysis, the location of the effusion was not taken into account. In 62 cases of malignant effusion with cells of pulmonary adenocarcinoma, molecular diagnostics were performed. Of these, 40 cases were primary diagnoses of pulmonary adenocarcinoma in advanced stage. Cell blocks were available for molecular diagnostics in 53 out of 62 cases (Table 1).

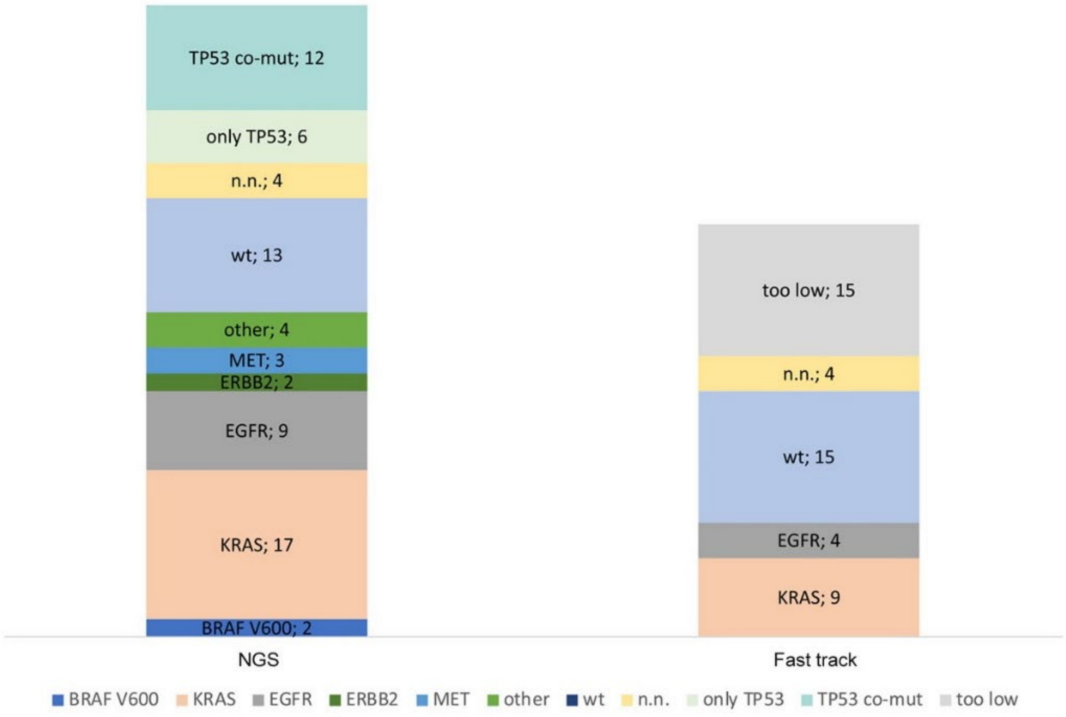
Table 1. Samples for molecular diagnostics.

	2018	2019	Total
Cell block only	25	26	51
Smears/cytospins only	5	4	9
Cell block and smears/cytospins	2	0	2
Total	32	30	62

Evaluation of Molecular Pathological Examinations of Cytological Specimens

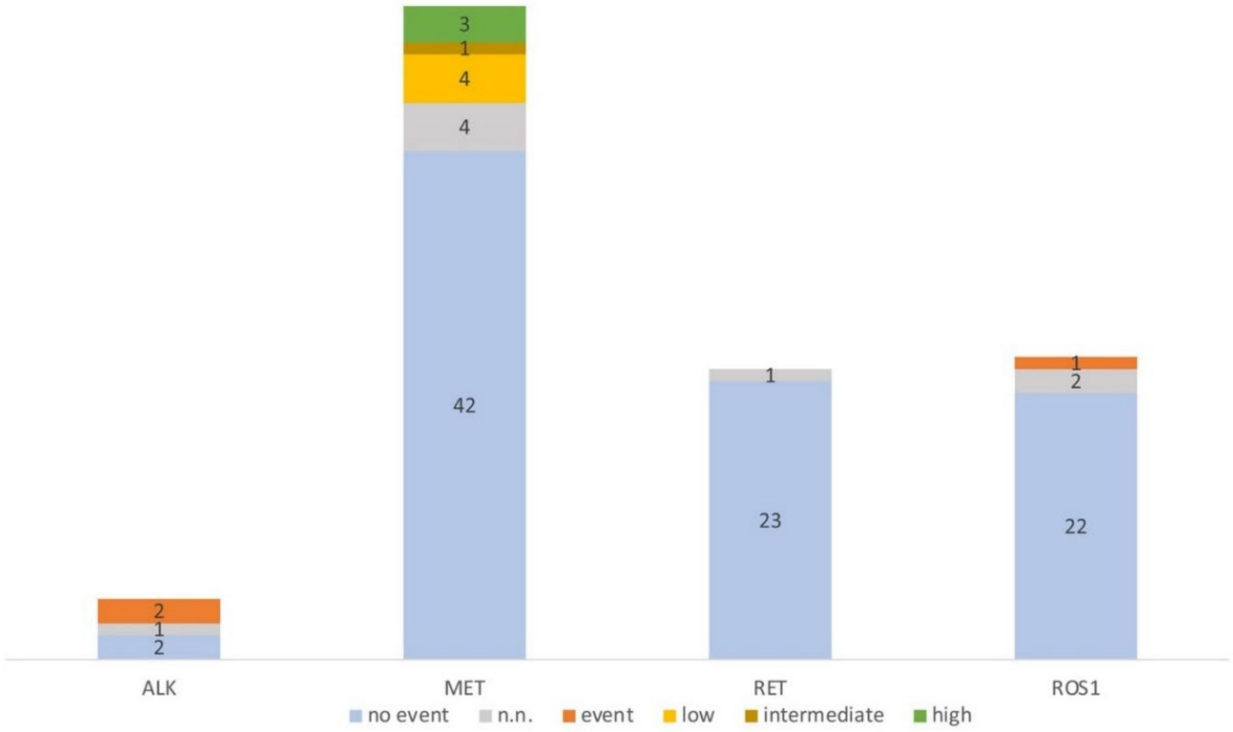
In the period between 2018 and 2019, 62 cytological lung cancer preparations (pleural effusion, pericardial effusion, ascites) from the pathology department of the University Hospital Cologne (in-house) were used for molecular diagnostics. Enough DNA for a fast-track analysis for hotspot mutation of *KRAS* Exon 2, *EGFR* exon 19 and 21 was available for 47 out of 62 samples (76%). Only four samples were not feasible for fast-track analysis, although they had a sufficient DNA concentration. A fast-track result was available for 92% (43 out of 47 samples). If DNA samples have a concentration of less than 5 ng/µL, the fast track is omitted, and only parallel sequencing is performed. In our study, 15 out of 62 samples did not reach this concentration limit (too low).

In 60 out of 62 samples of this series, parallel sequencing has been performed with a successful evaluation of 56 samples (93.3%, Figure 3A). Of the two remaining cases, in one of them, only fast track diagnostics had been performed before any further diagnostics had been cancelled because of clinical reasons. In the other case, only FISH analysis had been ordered by the clinician, as parallel sequencing was being performed simultaneously using a smear of a bronchial washing. As expected, most of the samples carried a *KRAS* mutation followed by *TP53* as a co-occurring mutation. In 4 out of 60 samples, no amplicons could be obtained in parallel sequencing, and coverage was insufficient.



(**A**)

Figure 3. *Cont.*



(**B**)

Figure 3. Molecular profiling of serous effusion samples with fast-track analysis, parallel sequencing and fluorescence in situ hybridization from 2018 to 2019. (**A**) Number of mutated and non-mutated samples for fast-track and parallel sequencing analysis. *KRAS* exon 2 and *EGFR* exon 19 and 21 within the fast track were analysed with high resolution melting and fragment length analysis. Too low: samples did not have >5 ng DNA/µL and were only analysed by parallel sequencing. N.n.: not detectable. In the parallel sequencing, 14/19 genes were analysed, of which the genes *BRAF*, *KRAS*, *EGFR*, *ERBB2*, *MET*, and *TP53* were mutated most frequently; only TP53: the sample had no mutation other than a TP53 mutation; TP53 co-mut: the samples had an additional mutation to the TP53 mutation; other: the samples had a mutation outside the before mentioned genes. (**B**) Diagram of the number of fluorescence in situ hybridization on cytological preparations for *ALK*, *RET*, and *ROS1* translocations and *MET* amplification. no event: no translocation or amplification, event: translocation or amplification, low/intermediate/high: low/intermediate/high amplification for *MET*. n.n.: not detectable.

In the same period, 108 FISH analyses were performed. The majority (n = 54) of FISH were *MET* amplification analysis (Figure 3B), followed by *ROS1* (25), *RET* (24), and *ALK* translocation (5) analysis. We detected two *ALK* translocations (40%), three *MET* high-level amplifications (5.6%), and one *ROS1* translocation (4%).

4. Discussion

Of a cohort of more than 5700 samples of pleural effusion, ascites, and pericardial effusion, which were processed in-house in 2018 and 2019, in 62 samples, molecular diagnostics for assessment of lung cancer were performed, 40 of these being primary diagnoses of pulmonary adenocarcinoma in advanced stage.

In 93.3% of these samples, parallel sequencing was successful. Fast-track testing for hotspot mutations of *KRAS* and *EGFR* gene was successful in a lesser proportion of 69.4% (43/62) of cases. Insufficient quantity of extracted DNA was the main reason for this draw-back. In cytology specimens, there is much more preanalytical variability than in histological specimen [26]. Different types of fixatives, sample recovery techniques from

different companies, and previously stained slides are used, which increases the diversity of the samples. To our knowledge, there are no standardized preanalytical protocols for cytological specimens. Since optimizing protocols can be very challenging in everyday work, standardized protocols may discourage laboratories from continuing to perform their preanalytical methods according to long-established patterns. In this study, we could show a very high success rate for samples that were processed in-house due to our optimized preanalytical protocols. Reviewing the cytological slides and choosing adequate areas for DNA extraction and for FISH analysis is time-consuming but essential for the high success rates. In preanalytics, only small modifications are required for integrating cytological preparations into the workflow. The extracted DNA from these samples is suitable for all subsequent PCR-based assays. The evaluation of the sequencing data proceeded according to the quality criteria given in the Supplementary Materials. The cytological preparations used here all had at least a tumor cell content of 10%. Nevertheless, special care must be taken when evaluating the analyses: The variant table of parallel sequencing is evaluated at a very low tumor cell content, even below an allele frequency of 5%. Visual inspection reduces the risk of false-negative results [27].

As a limitation of the study, the molecular results from the effusion samples were not compared with a histological specimen from the same patient to evaluate any possible discordance in mutation or fusion detection. Thus, the possible risk of false negative or false positive findings in the cytological specimen was not investigated. We could show earlier that parallel sequencing on biopsy and smear material, respectively, had a complete concordance with the parallel sequencing results [28].

If already stained slides were used for molecular diagnostics and sacrificed, it is recommended that they must be digitally scanned before usage to record the most representative and diagnostic slide [26]. Other entities, such as serous high-grade carcinoma, also benefit from the investigation of the BRCA status on cytologic specimens [29]. This may result in reduced risks and costs associated with additional surgical biopsies and faster turnaround times, which in turn influence therapy options and clinical management.

FISH testing for *ALK* and *ROS1* rearrangement has been decreasing in the last years due to the implementation of immunochemistry using cell block preparations for screening for *ALK* or *ROS1* rearrangement. FISH is still required for testing for *MET* amplification and for *RET* translocation as well as for *ALK* and *ROS1* translocation if no cell block is available or if immunochemistry screening is positive for one of the markers.

Other research groups have shown that *ALK* FISH on cell blocks compares favorably to immunohistochemistry and that cell blocks are not always absolutely necessary [30]. Smears and cytospins are equally suitable [31]. The result of immunohistochemistry is faster and cheaper to obtain in terms of probe and personnel costs compared to FISH. Therefore, cases are first stained by immunohistochemistry and FISH analysis is performed if positive. The same procedure applies to *ROS1* translocations.

In the future, RNA-based fusion detection on cytological specimens will play an increasingly important role [32]. It is a good complement to DNA-based and FISH diagnostics as described here and is now standard on FFPE material [11,33]. In our experience, fusion detection on cytological specimens is challenging but not impossible. For RNA-based fusion detection, the already stained preparations are less suitable than the native preparations.

Testing rates for lung cancer patients in Germany are high and good by international comparison. Nevertheless, only three out of four patients are tested for alterations in *EGFR*, *ALK*, *ROS1*, and *BRAF* [34]. Cytological preparations may potentially help to increase the testing rate, as they are less invasive than biopsy or surgery.

We could show that DNA-based genomic profiling of lung adenocarcinoma is feasible using routine preparations of effusion samples and has a high success rate. Careful marking of cytological preparations contributes to the success of molecular pathological analysis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jmp3020008/s1, Method Description 1: Fast Track Analysis, Method Description 2: Next Generation Sequencing, Method Description 3: fluorescence in situ

hybridization, Table S1: Primer Design Fast Track Analysis for *KRAS* Exon 2 and *EGFR* exons 19 and 21, Table S2: Parallel sequencing panel design, Table S3: Primer design for parallel sequencing of lung cancer; Table S4: FISH probes for *ALK*, *MET*, *RET* and *ROS1*.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Conflicts of Interest: Authors J.F. and M.E. declare no conflict of interest. R.B. has received honoraria for lectures and advisory boards from AbbVie, Amgen, AstraZeneca, Bayer, BMS, Boehringer-Ingelheim, Illumina, Janssen, Lilly, Merck-Serono, MSD, Novartis, Qiagen, Pfizer, Roche, and Targos MP Inc/DLS.

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