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Malignant pleural effusions for cancer genotyping: A matter of *trans*-pleural traffic of cell-free tumor DNA

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ABSTRACT

Body cavity fluids accumulating in progressive malignancies are potential subjects of regular clinical testing for cancer-related features. Besides the cellular component, the supernatant of the fluid proved to gain diagnostic impact as the cell-free DNA (cfDNA) fraction ideally reflects general molecular features of the related tumorous process, e.g. in lung carcinoma. Thus, malignant pleural effusions can be used for lung cancer genetic profiling and this might remain the only source for testing in critical cases. The cfDNA concentration of the pleural effusion depends on many factors in both benign and malignant conditions. Further to direct pleural metastatic spread, the redirection of tissue lymphatic circulation, tumor angiogenesis, inflammatory processes and other variables may contribute to or enhance the enrichment of the effusion tumor DNA from the earliest stages of carcinogenesis. Our review addresses the traffic of cfDNA in the pleural space and the diagnostic utility of effusion cfDNA from the perspective of the complex pleural pathophysiology.

1. Introduction

Predictive molecular testing of solid cancer requires samples of high-quality nucleic acids representing the malignant process. A representative tumor specimen obtained by tissue biopsy or surgical sampling is the basis of standard histopathological diagnosis also defining the subtype, the grade, selected biological factors (such as cell proliferation) and often the extension (pathological stage) of the tumor. Moreover, molecular classification, including the determination of a growing number of genetic factors - required in support of therapeutic decisions - is performed using standard formaldehyde fixed and paraffin-embedded (FFPE) samples [1]. The complexity of oncological diagnostics is best reflected by lung carcinoma protocols where a large set of information in addition to histology is required, obtained by proteomics, and genetic technology platforms [2,3]. As a major challenge, the access to lung tissue samples is limited and tumor biopsy frequently ends with samples of inadequate quality or quantity. Moreover, repeated testing is needed for long-term disease control, thus, well tolerable non-invasive sampling alternatives are increasingly considered [4,5]. Cancer-related circulating free DNA (cfDNA) and other nucleic acids (mRNA, miRNA, etc.) isolated from the peripheral blood gained wide acceptance in our days as accessible sources for clinical testing, also called liquid biopsy (LB) [6–8]. However, the limitations of the blood-based cfDNA approach motivate the use of other available liquid resources, such as the urine, the cerebrospinal fluid and effusions from body cavities lined by serous membranes [9]. As such, pleural effusions have been evaluated as a unique opportunity to follow diverse malignant processes involving the lungs and the pleural cavity, in the first-line lung adenocarcinomas [10,11]. Clinical reports conclude that the pleural fluid supernatant, collected from the earliest stages of the malignant process carries tumor-derived cfDNA which may proceed to clinically or morphologically significant pleural tumor invasion [12,13].

The conditions which enable the generation of malignant pleural effusion (MPE) and how these may influence the accumulation, partition, and clearance of cfDNA are key issues, the understanding of which contributes to the proper management of the disease and a better

Abbreviations: cfDNA, cell-free deoxyribonucleic acid; mRNA, messenger RNA; miRNA, micro-RNA; FFPE, formaldehyde-fixed and paraffin embedded; LB, liquid biopsy; BPE, benign pleural effusion; MPE, malignant pleural effusion; VAF, variant allele frequency; PCR, polymerase chain reaction.

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interpretation of molecular genetic test results using this formerly underestimated sample source.

2. The pleural fluid: basic pathophysiology

The pleural fluid is a low (less than 1.5g/L) protein content fluid of 10–20 mL volume in healthy adults which enables the flexible sliding of the visceral and parietal plates of the pleura by keeping them together under negative pressure [14]. The surface mesothelial layer isolates the pleural place similar to the vascular endothelial layer but also acts as an active player in *trans*-serosal traffic, enabling fluid diffusion and intercellular leakage as well as performing increased vesicular transport (transcytosis) following activation in a dynamic fashion. The flow of water and solutes, including macromolecules (such as albumin, glycoproteins, phospholipids and nucleic acid fragments) follows virtual submesothelial channels located between the loose connective tissue fibres, while restrictions of diffusion are almost negligible. The fluid is physiologically filtrated from the capillaries of the parietal subpleural space and is cleared by lymphatic absorption, similarly directed toward the parietal pleura and the intercostal veins. Therefore, the fluid under physiological conditions dominantly derives from the plasma of the systemic circulation and has only a limited relation with the lung parenchyma. The reabsorption from the parietal pleural surface is directly enabled by the submesothelial lymphatic collecting vessel system which has free *trans*-mesothelial openings (stomata) to drain the transudate according to the actual forces from the thoracic cavity. In contrast, the visceral pleura is mainly connected with the bronchial blood circulation and the lymphatic drainage follows the direction through the peribronchial lymphatic vessels toward the hilar lymph nodes [14,15].

Diverse pathological conditions of the lung or the pleura itself easily disrupt the intrapleural balance and result in the accumulation of effusions in the pleural cavity [16]. The factors grossly involved in the generation of extrapleural fluid may be divided into four basic categories: change in the transpleural pressure balance, impairment of the lymphatic drainage, electrolyte-coupled liquid absorption and increase in mesothelial or capillary endothelial permeability [17]. However, the rate of absorption can be raised to 40 times before significant extra volumes are detected. The accumulation is the result of excess pulmonary interstitial fluid mostly arriving through the visceral pleura due to local or systemic pressure relations, such as the elevation of the hydrostatic pressure or the loss of the oncotic pressure. Pleural effusions, also called hydrothorax, may develop due to benign, non-cancerous processes (benign pleural effusion, BPE) or to cancer (malignant pleural effusion, MPE). The symptomatic form of BPE is most frequently associated with left ventricular heart failure (20%), liver cirrhosis (10%), or nephrotic syndrome (10%) [18]. Pulmonary thromboembolism may locally raise visceral intraparenchymal pressure following vessel obstruction [19]. Further, permeability is influenced by cytokines and inflammatory mediators associated with infective (25%) or autoimmune (5%) lung pathology. Finally, after the exclusion of the known etiologies, idiopathic forms of pleural effusion make approx. 5% of all BPE cases [20].

The normal pleural fluid contains cells of diverse nature and function which shows significant variability depending on the cause and duration of the effusion. In general, the physiological cell density is approx. $1.7 \times 10e3$/mL which is composed of macrophages (75%) and lymphocytes (20%) and further, mesothelial cells, neutrophils, and eosinophils [21]. The inflammatory component appeared to be significantly higher in smokers and patients with chronic lung disease [22].

The biochemical composition of the fluid is characteristic and rather constant. The normal range of total protein and albumin content, the pH, glucose and LDH levels are well established and serve as reference values in diverse pathological conditions.

3. Malignant pleural effusion

By definition, MPE arises as a result of malignant processes involving the lung parenchyma or the pleural plates (approx. 1/3 of all pleural effusions), thus, it may occur with or without the direct cancerous involvement of the pleura. MPE is most frequently a complication of progressive lung carcinoma, which affects 40% of the patients during the progression of the disease [16,23]. MPE as such is a sign of advanced clinical stage and is generally of unfavourable prognosis. Progressive forms frequently show resistance to chemo- and biological therapies [24]. Lung adenocarcinomas show MPE with a prevalence of 29–37% and the related survival time is strongly reduced compared to non-MPE patients (approx. 3 months) [25]. Besides lung cancer, metastatic lung processes originating from a broad spectrum of malignancies (all frequent cancer types, including breast, ovarian, gastric, and prostate cancer, as well as melanoma and lymphoma), may present as solitary metastases or pleural carcinosis, both enabling the generation of pleural fluid accumulation [26,27]. Mesothelioma, a rare primary malignancy arising from mesothelial cells lining the pleural cavity is another important manifestation of MPE [28,29]. In addition, effusions may present as paraneoplastic syndrome through complex and yet uncleared mechanisms.

Approximately 55–60% of cancer patients with lung manifestations develop MPE [30]. This so-called „wet pleural involvement" is equivalent to an overall poor prognosis and limited treatment options [24]. The generation and the extent of MPE is the result of multiple parallel mechanisms the actual effect of which varies dynamically and the individual contribution of which is difficult to measure separately.

The following mechanisms can be considered:

1. Tumor growth and local invasion may result in the obstruction of the lymphatic vessels, therefore the drainage of the tumor area is blocked. The delayed resorption of the interstitial fluid returns to the fluid circulation directing toward the visceral pleura according to the local pressure conditions [31]. Lung tumors with peripheral/subpleural localization (adenocarcinomas more frequently) are likely to communicate with the pleural cavity more easily.
2. Vasoactive substances are generated by the cancer cells or by the activated host environment and act in an autocrine fashion [14,30,32]. Major factors increase the capillary permeability simultaneously, such as tumor cell-derived proteases (e.g. matrix-metalloproteinases), vascular chemokines (CCL2, OPN), and pro-inflammatory molecules (IL-2, IL-8, TNFa, interferons) [33]. The specific role of acute inflammatory cells (primarily neutrophils, macrophages, and mastocytes) in this process was also suggested [34]. The net result of these mechanisms is the increased release and alternative drainage of parenchymal interstitial fluid continuously filtrated to the pleural cavity.
3. The incomplete endothelial lining and a defect basal membrane is a general feature of newly formed tumor vasculature induced by ANG-1/2, VEGF, CXCR4, and other growth factors [35]. The result of the stress stimuli (e.g. tumor hypoxia) is the rapid generation of irregular, fenestrated vessels and a network of leaky sinusoid-like capillaries, enabling the direct contact of tumor cells with the intraluminal space and the free distribution of soluble (cell-free) tumor-derived substances. Moreover, angiogenic stimulation of subpleural capillary formation also appears to interact with MPE formation [36].
4. Activating mutations of driver oncogenes are associated with pleural manifestation. Mutation of the MAPK-pathway (*EGFR*, *KRAS*, and *BRAF* oncogenes) are common in lung adenocarcinoma patients with MPE. Specific driver mutations may be associated with enhanced tumor cell migration and metastatic seeding. As such, activation of nuclear factor-kB (NF-kB) and Stat 3 was suggested to be important in the predisposition of pleural involvement [37]. According to Agalioti et al. [38], KRAS mutant cancer promotes the early

development of MPE through the specific induction of an inflammatory, angiogenic and vasoactive response within the pleural space.

In general, MPE is principally related to the special pleural biology influencing tumor-cell seeding and/or the filtration/resorption of the effusion fluid. Thus, the concept of „pleural homing" has emerged which covers all aspects of the host side (mesothelial cells, pleural macrophages, eosinophils, myeloid-derived suppressor cells, vascular endothelium, etc.) interacting in a highly complex fashion.

4. The pleural effusion as a liquid sample for cancer diagnosis

The pleural fluid is relatively easy to access for diagnostic sampling, moreover, thoracocentesis is a common curative procedure to treat symptomatic forms of effusions. The preparation of the fluid by centrifugation results in two fractions: sediment cells – used for cytology - and a cell-free supernatant traditionally reserved for biochemical assays. Thus, effusions can be tested both for cellular composition and for biological/biochemical components. High-protein or hemorrhagic contents refer to significant vascular/inflammatory involvement within the pleural space, while a decrease in pH and glucose and increase of LDH levels are associated with inflammation, enhanced metabolism and cellular turnover. Moreover, microbiological testing informs about the microbial etiology of fluid congestion.

Probably the most important clinical challenge is the clarification of the neoplastic origin of pleural effusions, especially in unilateral pleural involvement [24,31]. The cytomorphological analysis of the sediment is a potent tool to exactly identify the cellular composition and to demonstrate malignant cells in the background of the pleural process [39]. For this purpose smears, cytospins, liquid cytology preparation are in clinical use, similar to other sample types, such as the cerebrospinal fluid, saliva or urine.

Effusion derived tumor cells enriched for testing can be the subject for phenotypic studies, e.g. by fluorescence image analysis or flow cytometry. More recently, cell-block processing of the sediment was introduced to enable standardized tissue-like conditions similar to formaldehyde-fixed and paraffin-embedded (FFPE) sample procedures including immunohistochemistry [40]. Cellular samples appeared to be effective for molecular diagnostics following genomic DNA extraction, covering all clinical aspects, including progression-associated genetic variants, such as *EGFR*, *KRAS*, or *BRAF* gene mutations in non-small cell lung carcinoma. However, studies agreed that cell sediment-based testing was limited in sensitivity (from 40 to 87% positive predictive value for involvement) for diagnostic purposes [16,41].

Besides the sediment, the clinical value of the fluid supernatant was gradually uncovered. With the development of sequencing technology the accurate testing of minimal amounts of even highly degraded/fragmented nucleic acid fraction released in cell-free fluids became possible. The general interest turned to the body cavity fluids soon after peripheral blood DNA testing was introduced. Today there is firm evidence that the remaining MPE supernatant after the separation of the cellular component is rich in DNA fragments (cell-free DNA) which is applicable for PCR amplification and sequencing by most current technologies, including next-generation sequencing (NGS).

In gross agreement with other communications, a set of cfDNA fragments with a mean size of 160bp and 320–390 bp range could be regularly captured and identified during our studies (Fig. 1.) [41].

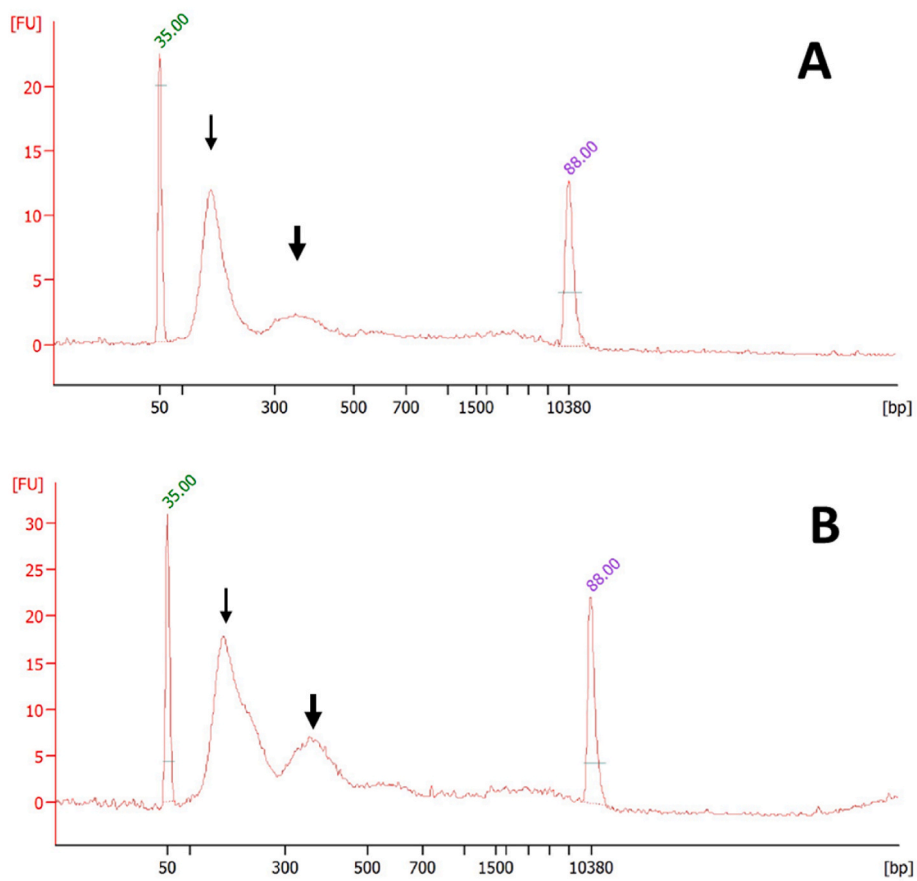

Fig. 1. Size distribution of the cfDNA fraction isolated from the peripheral blood plasma (A) and the pleural effusion (B) of a lung adenocarcinoma patient. The cfDNA fraction peaks at 160bp (thin arrow) and 320-390bp (thick arrow) fragment size as determined by the Bioanalyzer 2100 instrument (Agilent Technologies, Santa Clara, CA). Note the higher DNA yield in the MPE supernatant, determined by the height and area under the curves.

The size distribution generally supports the idea that free-DNA is released from dead or dying cells, mostly of ischemic/necrotic origin, although the contribution of apoptotic mechanisms was also proposed [6,42]. Whether and how active shedding/release of exosomes of living cancer foci contribute to the cfDNA-fraction is still debated [43,44].

Comparative analyses demonstrated that MPE cfDNA was as informative as tumor tissue-derived genomic DNA or peripheral blood cfDNA and superior to MPE sediment cellular DNA to represent clinically relevant gene mutations in lung adenocarcinoma [45–48]. In general, the cellular sediment is a mixed cell population where the low-frequency tumor can easily be suppressed. Due to the low cell counts, the number of smears is limited and thus, the whole diagnostic process is difficult to control. These limiting reasons explain why the mutation detection rate in the cell-free MPE supernatant could be consequently higher than that in the cell pellet. In line with the previous statement, significantly higher variant allele frequencies (VAF) could be achieved from the cfDNA fraction compared to the cellular DNA [41,49]. Of note, the cfDNA isolation proved to be highly effective resulting in significantly higher cfDNA concentrations compared to the genomic DNA isolated from the sediment (17.23 vs. 2.4 ng/μL; r: 0.81, $p < 0.05$) [41]. Interestingly, the amount of cfDNA was independent of the effusion cell density observed in the microscope during the cytological examination. Although the latter finding should be validated on a much larger sample cohort, it indicates that cfDNA accumulates in the effusion fluid by another alternative, highly complex mechanisms further to direct cancer cell damage within the pleural space.

5. Release and clearance of cell-free tumor DNA in the pleural cavity

In parallel with the formation of the pleural fluid, the disease-related accumulation of cfDNA is to be expected. The cell-free fraction represents both the normal tissue compartment of the pleura (mainly mesothelial cells, macrophages) and the cellular processes associated with the actual pleural and pulmonary pathology (lymphocytes, neutrophils, eosinophils, cancer cells). Accordingly, one can calculate with a basic level of cfDNA representing normal genotype in every effusion sample. How far tumor-related genetic alterations can be demonstrated in a particular MPE is determined by the total amount of cfDNA and by the actual admixture of cancer cell-derived sequences. The influence of further variables, such as individual biological features of the tumor, the changes of vascular permeability reflected by protein content or true extravasation, the pH shift, or the inflammation of the sub-pleural lung parenchyma is likely to happen but experimental or clinical studies on this issues are still missing.

In advanced cancer presenting with pleural involvement (carcinosis) and MPE, a direct release of viable tumor cells and accumulation of DNA fragments from spoiled tumor cells (apoptosis, necrosis) is simultaneously expected. However, minor initial foci of pleural carcinosis are unlikely to show significant cellular shedding and cytological positivity despite the fact of dissemination while DNA fragments may be already released in a measurable mass. This explains why tumor-derived cfDNA can be detected before the occurrence of massive tumor cell infiltration of the pleura. More advanced disease stages may also present with the occlusion of the subpleural lymphatic vessels (lymphangitis carcinomatosa), contributing to the redirection of the subpleural lymphatic flow and thus, extending the total of the fluid mass. In the case of manifest pleural carcinosis, the MPE is expected to be enriched in both cellular/genomic and cell-free DNA fractions.

However, MPE frequently occurs without obvious signs of pleural metastasis which calls for alternative explanations. As mentioned earlier, variations in the tissue environment, vascularization, local pressure relations, inflammatory and cytokine effects should all be considered. Reversal of the lymphatic drainage and increased hydrostatic pressure due to the blockade of the major lymphatic vessels and hilar/regional lymph nodes, or the pulmonary vein branches may redirect tumor-derived cfDNA-rich fluid through the visceral pleura toward the thoracic cavity. Besides the direct propagation of the tumor mass along the vessels, lung tissue atelectasis as a frequent consequence of airway obstruction adjacent to tumor areas should also have a significant effect on pleural fluid recharge.

Tumor-related inflammatory and vasogenic mechanisms appear to be major players in MPE generation, also promoting the continuous supply of small cfDNA fragments from the tumor parenchyma. Inflammatory vasoactive substances increase both endothelial permeability and vasodilatation while cancer and surrounding supportive stromal cells secrete angiogenic factors. On the other hand, progressive areas of the tumor suffer from hypoxia and necrosis, followed by angiogenesis. Freshly formed capillaries of the tumor bed are known to form irregular and leaky networks, therefore, tumor-derived waste metabolites and cellular residues (including cfDNA) are easily redirected according to the local forces, under specific circumstances toward the visceral pleura (Fig. 2.).

The total of the presented synergic processes drives the progressive accumulation of the tumor-derived cfDNA (ctDNA) in the effusion fluid. Local factors in the thoracic cavity seem to drive ctDNA flow in a specific manner that is independent of its release to the peripheral blood. From this perspective, the correlation between the effusion and the peripheral blood-derived cfDNA becomes an exciting issue. Reports evaluating patients' samples generally agreed that higher concentrations of cfDNA in MPE than in PB could be demonstrated. This will be simple to explain in the case of direct carcinomatous involvement of the pleura

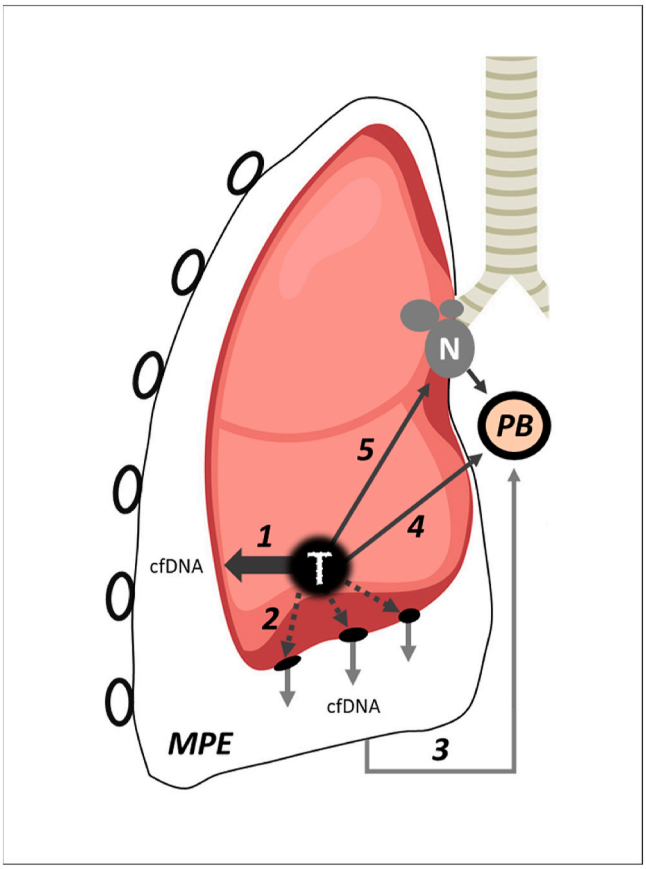

Fig. 2. Schematic presentation of the major routes leading to release and accumulation of tumor-derived cfDNA in the pleural fluid and peripheral blood. 1. tumor cfDNA rich interstitial fluid is redirected toward the pleural cavity, 2. clinical or subclinical pleural metastatic tumors release cfDNA, 3. pleural fluid enriched in cfDNA is re-entering the circulation, 4. cfDNA enters the circulation through venous peripheral blood, 5. cfDNA is carried by the lymphatic fluid toward the major lymphatic duct and the central vein.

(carcinosis). Considering the proximity and the reversed lymphatic flow, the local accumulation of tumor-derived transudate logically results in the relative enrichment of tumor-related substances compared with the peripheral blood. On the other hand, the dynamic clearance of cfDNA from the pleural fluid should be also considered: the pleural fluid turnover may be rather slow due to the actual pressure and filtration relations while the circulating blood volume is continuously renewing. As deduced from pleural physiology, large volumes of the thoracic fluid are absorbed by the parietal pleura through a channelling system connected with the intercostal lymphatic circulation. Therefore, a significant fraction of tumor cfDNA released to the pleural space may enter the circulation together with the reabsorbed effusion fluid, a way that was underestimated until date. How far the high cfDNA containing MPE contributes to the circulating cfDNA pool is still completely unclear, but cfDNA traffic between the different tissue and fluid compartments seems to be crucial. The theoretical ways of pleural cfDNA release and clearance are schematically summarized in Fig. 2.

6. MPE based clinical testing and molecular follow-up of lung adenocarcinoma

MPE derived cfDNA was the subject of profound investigation in the past and had become established for molecular testing in the clinic. The fluid supernatant requires the same handling and workload as described for blood-based analysis [50]. The yield of MPE tumor DNA proved to be appropriate and even more concentrated compared to the blood plasma, expressed by the VAF of specifically targeted gene sequences as already discussed. The clinical utility of the effusion derived nucleic acids is further increased by extended genetic targeting, as such, copy number alterations could be captured with improved efficacy [11]. Moreover, cytologically negative effusions were optimally used for mutation detection [12]. Results from the past decade suggest that the next-generation sequencing of MPE supernatants represent mutational profiles with the highest complexity and are applicable similar to plasma-derived cfDNA for both initial and follow-up clinical testing [45,47,48]. Sequencing results also accurately reflect the dynamics of different genetic subclones by the comparison of gene variant VAF in the particular samples.

Early reports efficiently demonstrated the *EGFR* mutation status by PCR using MPE derived cfDNA as a promising approach for lung carcinoma predictive testing [49,51,52]. Since then, the power of NGS-based mutation profiling simultaneously covering major clinically relevant gene variants (including *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, *MET*, *RET* genes) from effusion cfDNA was also demonstrated [10,41,53]. The general principle of the cfDNA approach also allows gene fusion studies similar to plasma-derived cfDNA, however, large-scale clinical data with pleural effusion samples are still missing [54]. Besides the many genetic issues attempts to estimate tumor mutational burden (TMB) predictive for immune-checkpoint inhibitor therapies were also addressed [55].

The extent of the pleural effusions is usually associated with the efficacy of the actual anti-tumor therapy. Therefore, as an early sign of systemic therapy failure, the rapid re-accumulation of the pleural fluid is frequently observed. Repeated pleurocentesis also enables regular MPE testing for cellular and soluble factors associated with progression. Effusion derived cfDNA safely represents gene variants associated with therapy failure and test results enable treatment repositioning by the use of reference databases. Currently, of great clinical importance, variant T790 M (exon 20) of the *EGFR* gene can be captured as a secondary mutation associated with *anti*-EGFR (cetuximab) therapy failure in lung adenocarcinoma, which indicates the necessity of an abrupt change to a third-generation *EGFR* tyrosine kinase inhibitor therapy.

7. Considerations on sample quality for cfDNA-based genotyping

From the beginning of the blood-based free-DNA area, critical voices call for standardization of the pre-analytical phase covering sampling, shipping and sample handling issues. It is generally accepted that short-term processing of the sample does not require specific conservation of the MPE fluid and similar to the PB EDTA-supplemented tubes are appropriate for sending. However, if submission takes longer than several hours transfer tubes with conservation liquid (e.g. PaxGene) for sequencing should be used, again, the procedure is the same as for sending peripheral blood samples.

Claims are formulated to work on quality issues, with a special focus on the standardization of all aspects of cfDNA sequencing originating from diverse liquid sample types. Compared to tissue-based testing, the control of dilution/contamination by DNA from non-tumor cells in a cell-free solution might be especially challenging [56]. In the meantime, efforts on standardization are also palpable [57], but clear recommendations for the clinical use including when and how to test, as well as how to integrate the result in the daily clinical work are still in progress [58].

8. Conclusions

Alternative sources of tumor-derived nucleic acids to identify and follow-up progression-related genetic/genomic changes are gaining importance as patient survival times extend and repeated invasive tumor sampling is not favoured. Pleural effusions are frequent and typical for progressive or advanced forms of lung carcinoma. As stated, further to the cellular fraction, the MPE is enriched in tumor-derived DNA fragments directly excreted to the pleural cavity by several parallel mechanisms.

cfDNA extracted from MPEs is particularly suitable for tumor genetic analysis. Detection rates of individual gene variants are comparable with those obtained from the tumor tissue or the peripheral blood. Liquid biopsy may reflect a more complex mutational profile compared to individual tumor samples especially in advanced diseases with multiple neoplastic foci. Accordingly, MPE drained and collected from patients with multiple lung processes may optimally reflect this biological heterogeneity within the same effusion. The cfDNA yield isolated from the MPE fluid supernatant proved to be unexpectedly high and serves with optimal test material even if the cellular composition does not suggest malignant involvement of the pleural cavity.

Author contributions

G.M.: conceptualization, supervision, visualization; writing - original draft preparation; A.M.: conceptualization, methodology, visualization; writing - original draft preparation; L.T.: conceptualization, methodology, investigation, writing - review and editing; S.L.C. and A.L.: methodology, investigation; N·B.: conceptualization, methodology, investigation, writing - review and editing, supervision. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the University of Debrecen.

Data availability statement

The data presented in this study are available on request from the corresponding author.

Declaration of competing interest

The authors declare no conflict of interest.

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