Liquid Biopsy: Is There an Advantage to Analyzing Circulating Exosomal DNA Compared to cfDNA or Are They the Same?

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Introductory Statement

Cancer is one of the leading causes of death worldwide. This life-threatening disease requires novel strategies for the early detection and therapy response prediction. Circulating DNA was first described 70 years ago. However, only the recent evolution in the PCR-based sequencing techniques allowing mutant DNA from small-volume "liquid biopsies" such as blood, urine, or saliva. In this article, we aim to summarize the fast-growing evidence for cfDNA and exosomal DNA as minimally invasive diagnostic markers in solid tumors and to highlight their opposing diagnostic advantages and disadvantages.

Introduction

Although the cancer death rate has declined by about 1.5% annually in both men and women for the last decade, it remains the second leading cause of death worldwide (1). In the United States, the annual incidence of all types of cancer accounts for around 1,735,350 new cases, and 609,640 deaths are estimated to be cancer-related (1). However, there is still a lack of accurate, efficient and inexpensive screening tests that could help to diagnose many cancer types at an early stage and to decrease its mortality significantly. For this reason, there is an urgent clinical need for additional complementary molecular biomarkers that can be utilized for early diagnosis or therapeutic response prediction. A "liquid biopsy" offers the great advantage that it is significantly less stressful for the patient and may also provide much more comprehensive information about tumor biology. Besides, a "liquid biopsy" can be taken from the blood at several times and thus help to establish a molecular profile on the dynamic tumor behavior. Therefore, blood-based liquid biopsies have come into the spotlight of translational research as a minimally invasive screening test for the diagnosis and clinical monitoring of cancer. Two of the most important biomarker classes in the field of "liquid biopsy" are cell-free DNA (cfDNA) and exosomes. Exosomes are a class of extracellular vesicles of endosomal origin with a size range of 40–150 nm that are released by all cells. They have a lipid bilayer membrane and are enriched in exosome-related proteins such as CD9, CD63, CD81 or TSG 101 whereas they are devoid of markers such as calnexin (CANX; refs. 2–4). Exosomes derive from the intracellular endosomal compartment in a ceramide-triggered process (5). In contrast, "microvesicles," "ectosomes," or "shed vesicles/particles" have been thought to originate by direct budding from the plasma membrane (6). In addition to RNA and proteins, exosomes also transport DNA that reflects the mutational status of their parental cells. In this article, we will discuss the complementary utility of these two fractions of circulating DNA and their clinical relevance.

The Origin of cfDNA and Exosomal DNA

There are some fundamental differences between the origin and traits of cfDNA and exosomal DNA. Cell-free DNA can be detected in healthy individuals as well as in patients with nonmalignant or malignant disease. Under physiologic conditions, nonmutated cfDNA might derive from nonmalignant cells undergoing apoptosis or being exposed to natural cell stress. Moreover, elevated copy numbers of nonmutated cfDNA have been observed in septic patients, patients suffering from severe trauma or patients with myocardial infarction (7).

In contrast, mutated cfDNA is predominantly described in patients with a known malignant disease. In this case, it is assumed that cell-free DNA results from apoptotic and necrotic tumor remnants that are insufficiently cleared by infiltrating phagocytes (8). Another source for mutant cell-free DNA is discussed as being secreted by circulating tumor cells directly into the bloodstream. A recent study has investigated the size distribution of mutated cell-free DNA in comparison with nonmutated cell-free DNA (9). Mouliere and colleagues have determined that mutant cfDNA is generally more fragmented than nonmutant cfDNA, with a maximum enrichment of tumor cfDNA in fragments between 90 and 150 bp, as well as enrichment in the size range of 250 to 320 bp (9). Moreover, the authors of this study could show that size-selected sequencing could increase the sensitivity for detecting genomic cancer alterations in cfDNA (9), which might be of particular significance when translating cfDNA analysis into the clinical routine.

While cfDNA was first described seventy years ago, the existence of exosomal DNA has long been doubted and has only been proven for less than a decade. However, nowadays it...
Diagnostic Value of cfDNA and Exosomal DNA

The analysis of cfDNA has become a very standardized procedure and has a high clinical significance for the minimally invasive diagnosis of tumors or as a prenatal blood test. Nowadays, the use of PCR-based assays that can simultaneously assess multiple regions of driver genes enables screening tests to detect low-prevalence mutations in cancer patients in a cost-effective and high-throughput procedure (13). A recent study presented a multi-analyte blood test that primarily targets cell-free DNA from 16 selected driver genes and a panel of 8 selected tumor proteins (13). This test was applied to examine the blood of 1,005 patients with a tumor disease including cancers of the ovary, liver, stomach, pancreas, esophagus, colorectum, lung, or breast. Cohen and colleagues reported a median sensitivity of 70% among the eight cancer types evaluated.

Further analysis of subgroups revealed that the results were strongly dependent on both tumor type and tumor stage. The highest sensitivity was observed for ovarian cancer (98%), whereas for breast cancer, the multi-analyte test reached a sensitivity of 33%. Likewise, the sensitivity of stage I cancers amounted to 43% and increased to 78% in stage III cancers (13). Regarding these impressive results, one may ask whether the evaluation of circulating exosomal DNA can provide an additional clinical value.

Currently, there are only a few studies with conflicting results that have directly compared the diagnostic significance of cfDNA with exosomal DNA. A recent study has addressed this question in patients with melanoma or mastocytosis (14). For this approach, the authors established a protocol for the separation of different extracellular vesicle populations including oncosomes, microvesicles, and exosomes as well as for the separation of cfDNA. In human colorectal adenocarcinoma cells harboring the BRAFV600E mutation, they could prove that BRAF and BRAFV600E mutations are present in all extracellular vesicle fractions including exosomes as well as in the cfDNA fraction. However, Klump and colleagues could detect much higher copy numbers of wild-type and mutant DNA in the cfDNA fraction in comparison with the extracellular fraction. Next, 2 mL of serum was used from 6 patients with stage IV melanoma diagnosed positively for the BRAFV600E allele, and 3 patients with systemic mastocytosis diagnosed positively for the cKITD816V allele. The results of this analysis show that mutant BRAF and cKIT were detected in cfDNA and exosomal DNA. However, despite a higher yield of total dsDNA from the exosome fraction, there was an approximately 10-fold higher amount of copy numbers of the wild-type and mutant BRAF and cKIT in the cfDNA fraction (14). These results would suggest that the analysis of cfDNA is sufficient to determine the mutational status of a tumor from a liquid biopsy. However, the authors of this study also note that their observation most probably cannot be extrapolated to patients with early-stage disease (14).

Furthermore, there is still a lack of evidence for other cancer types. The analysis of cell-free DNA often raises the challenge that the prevalence of mutated cell-free DNA in tumor patients can be very low. Especially in patients with an early tumor stage, in which the tumor is potentially resectable and thus curable, the detection rate of cell-free DNA is often low. Moreover, the detection sensitivity of cfDNA depends on the signal-to-noise ratio owing to fragments of DNA from cancer cells (mutation) mixed with DNA from normal cells (no mutation; ref. 15). Many different factors can influence this signal-to-noise ratio, such as the administration of chemotherapy, which can induce a direct tumor breakdown before blood collection, the cell lysis after blood collection, or the technical processing of the blood samples. Taking these challenges into consideration, the analysis of exosomal DNA can likely offer an additional clinical advantage. Exosomes are very robust due to their bilayered lipid membrane that protects the encapsulated cargo against degradation (5). Even a cycle of freezing and thawing of exosomes hardly affects the integrity of exosomes. Likewise, exosomes can be stored at 4°C for 48–96 hours or at −70°C for a long time without losing their biological activity (5, 16). These features may also help to increase the detection rate of mutated tumor DNA from the blood as shown by two recent studies.

When comparing exosome-derived DNA to cfDNA in liquid biopsies of patients with pancreatic ductal adenocarcinoma (PDAC), Allison and colleagues could observe a higher detection rate of KRAS mutations in exosomal DNA than in cfDNA by droplet digital PCR (ddPCR; ref. 17). Especially in patients with locally advanced or metastatic pancreatic cancer, the KRAS mutant call rate from exosomal DNA was much higher (80% and 85%, respectively) than the KRAS-mutant call rate from cfDNA (30.8% and 57.9%, respectively). In a complementary study, Bernard and colleagues have evaluated the clinical utility of cfDNA and exosomal DNA in serial blood samples from patients with localized and metastatic pancreatic cancer (18). They report that the KRAS mutation detection rate for localized and metastatic pancreatic cancer was nearly equivalent when profiling cfDNA and exosomal...
DNA. However, concordance among tissue from 22 surgically resected primary pancreatic tumors was 95.3% for exosomal DNA and only 68.2% for cfDNA. Likewise, concordance from 12 samples derived by fine-needle aspirates was 83.3% for exosomal DNA and only 68.8% for cfDNA. Moreover, only exosomal DNA level after neoadjuvant therapy was significantly associated with disease progression whereas cfDNA did not show correlations with outcomes in 34 patients with potentially resectable pancreatic cancer (18).

Eventually, the selective accumulation of tumor-derived exosomes by tumor-specific surface markers might improve the signal-to-noise ratio by depleting the fraction of exosomes and cfDNA from nonmalignant cells. The subsequent analysis of purified mutant DNA could lead to a more detailed insight into the mutation profile of the primary tumor. Melo and colleagues have described this approach in their study when they have demonstrated, that the proteoglycan “glypican-1 (GPC1)” is predominantly expressed on the surface of exosomes of pancreatic carcinomas both in vitro and in vivo (19).

One of the key findings of this study was that the authors divided exosomes into a GPC1-positive and GPC1-negative fraction using FACS sorting. Subsequently, mutant KRAS was analyzed at the mRNA level. In this analysis, the investigators were able to detect the mutated KRAS mRNA exclusively in the GPC1-positive fraction. In contrast, no mutated KRAS mRNA was detectable in the GPC1-negative fraction (19). This approach could be very promising in the future to increase the detection rate of mutated nucleic acids in the blood. In fact, nowadays, there are active efforts to develop "lab-on-a-chip" solutions to capture tumor-specific exosomes by cancer-associated surface markers such as glypican-1 from a small sample of blood (20). Soon, these medical devices might facilitate the applicability of exosomes in the clinic, for example, by enriching tumor exosomes from a high background of nonmalignant exosomes and directly subjecting them to further bioanalysis. Also, the exosome-selected enrichment of mutated DNA based on tumor-specific markers could help to minimize false-positive results in healthy individuals without a detectable tumor disease.

Conclusion

Both cfDNA and circulating exosomal DNA are powerful diagnostic tools to unravel the mutational landscape of cancer from a liquid biopsy by a minimally invasive method. So far, it remains elusive whether the DNA profiling of one of those two fractions is superior to the other one. On the one hand, the analysis of cfDNA offers the advantage that the method has already been established much longer and might already be used in routine clinical diagnostics in the near future. Furthermore, the isolation of cfDNA is not dependent on a labor-intensive working step, but can be almost entirely automated. In contrast, ultracentrifugation remains the gold standard for the extraction of exosomal DNA. This method is very elaborate and is not suitable as a high-throughput procedure. Therefore, it will be difficult to translate the profiling of exosomal DNA into routine clinical diagnostics, as long as there exist no efficient alternatives to exosome preparation by ultracentrifugation, such as biofluidic devices for high-throughput analysis.

On the other hand, the unselective processing of cfDNA from serum and plasma may result in the loss of relevant information of the tumor biology and the mutational tumor landscape. It can even be assumed that the unselective lysis of liquid biopsies for the routine isolation of cfDNA involves a simultaneous extraction of exosomal DNA, which may cause a bias in data interpretation. Therefore, differentiated DNA analysis from both fractions may potentially increase the diagnostic potential significantly. To clarify this question, however, further investigations are needed that directly compare the clinical significance of exosomal DNA and cfDNA in translational studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

C. Kahlert received DFG KA 3511/3-1. This work was supported in part by the Roland Ernst Stiftung für Gesundheitswesen (0515). Received January 4, 2019; revised February 6, 2019; accepted March 4, 2019; published first May 1, 2019.

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Cancer Res 2019;79:2462-2465. Published OnlineFirst May 1, 2019.

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