Review

Cell-free DNA in the Circulation as a Potential Cancer Biomarker

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Abstract. *In the course of the search for new biomarkers, circulating cell-free DNA (ccf-DNA) has become a popular target of interest. An elevated level of ccf-DNA has been detected in the circulation of cancer patients in comparison with healthy controls. Since ccf-DNA in cancer patients often bears similar genetic and epigenetic features to the related tumor DNA, there is evidence that some of the ccf-DNA originates from tumoral tissue. This, and the fact that ccf-DNA can easily be isolated from the circulation and other body fluids of patients, makes it a promising candidate as a non-invasive biomarker of cancer. Yet ccf-DNA-based cancer tests have not come to fruitful clinical applications. This review evaluates the potential of ccf-DNA alterations as a biomarker for cancer management by addressing the question of how large the gap between ccf-DNA and the ideal cancer biomarker is.*

Worldwide, cancer is the third most common cause of death, directly following cardiovascular diseases and infectious parasitic diseases. According to data published by the World Health Organisation (WHO) in the world cancer report, cancer deaths will increase dramatically in the forthcoming years. Whereas there were 7.4 million cancer deaths in 2004, it has been estimated that in 2030, there will be 11.8 million dying as a consequence of cancer (1).

During the last few years, much research has been carried out to find new cancer biomarkers with the aim of reducing cancer mortality. However, most of the cancer biomarkers currently available are not sensitive or specific enough to be applied in routine clinical approaches. Additionally, for many cancer types, such as lung or breast cancer, invasive procedures are still necessary to obtain material for pathological analyses. To simplify cancer management, much effort has been made in the search for biomarkers which allow non-invasive assessment, screening, disease classification and monitoring.

Circulating cell-free DNA (ccf-DNA) represents such a non-invasive biomarker, as it can easily be isolated from human plasma, serum and other body fluids (2). Mandel and Métais demonstrated the existence of ccf-DNA in human plasma as early as 1947 (3). The fact that there is an elevated level of ccf-DNA in the circulation of cancer patients in comparison with healthy controls was primarily discovered 30 years later by Leon *et al*. (4) and was confirmed in numerous studies (5-7). After the findings by Leon *et al*., it took more than a decade until it was shown that ccf-DNA often exhibits the same alterations as DNA derived from related tumoral tissue (8). A huge variety of alterations, such as mutations in oncogenes and tumor suppressor genes (9), microsatellite variances (10), and epigenetic alterations, such as promoter hypermethylation (11), have since been reported and plenty of studies have been conducted investigating the potential of ccf-DNA as a non-invasive diagnostic tool for cancer management (12, 13).

Although ccf-DNA was discovered more than half a century ago, ccf-DNA-based cancer tests have not yet been developed for clinical application. The main progress has been observed in the field of prenatal medicine, where ccf-DNA has been successfully used for fetal Rhesus D genotyping (14) and for the detection of paternally inherited genetic disorders (15) from maternal plasma and serum. However, the applicability of ccf-DNA as a biomarker for cancer management still needs extensive evaluation. This review discusses the potential for commonly analyzed ccf-DNA alterations to be used as biomarkers and compares their characteristics with that of the ideal cancer biomarker.

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Alterations of ccf-DNA and their Potential for Use as Cancer Biomarkers

Quantitative alterations in ccf-DNA. The fact that there is an elevated ccf-DNA concentration in the serum of cancer patients when compared with healthy controls was first observed in the late 1970s using radioimmunoassay (4). Although elevated levels of ccf-DNA in serum and plasma were detected in various studies and in many cancer types, interpretation of such results requires special consideration. Firstly, a certain level of ccf-DNA can also be observed in healthy individuals. Therefore, it is crucial to gain closer insight into the mechanisms that lead to ccf-DNA release and to establish a baseline that allows a reproducible discrimination between healthy and diseased individuals. In addition, it has been shown that there are various parameters influencing ccf-DNA levels, such as sample preparation (16) and speed and effectiveness of clearance of ccf-DNA from the circulation (17). Finally, an elevated level of ccf-DNA was found not only in the circulation of cancer patients, but also in patients with other physiological conditions, such as myocardial infarction (18), physical trauma (19) and inflammatory disorders (20), which makes it difficult to evaluate the extent to which ccf-DNA in the circulation of a patient is cancer specific. Nevertheless, research on how qualitative ccf-DNA changes could be used in a clinical approach is ongoing, as there are promising results for the use of ccf-DNA content in combination with other well-known tumor markers (21).

Qualitative alterations in ccf-DNA. Cancer is a disease mainly caused by the accumulation of genetic and epigenetic alterations and both have been observed in DNA isolated from tumoral tissue and in the corresponding ccf-DNA (22). In recent years, the research in this field has mainly been driven by these findings, since they promise to provide a basis for the development of new approaches in which cancer-specific ccf-DNA alterations may be used as a non-invasive biomarker.

Mutations in oncogenes and tumor suppressor genes. The regulation of cell proliferation, cell differentiation and apoptosis is subjected to special control mechanisms that keep these processes in balance and thereby allow a regular course of the cell cycle. Such mechanisms are controlled by those genes that are involved in the regulation of the cell cycle, specifically DNA repair genes, tumor suppressor genes and diverse growth-regulating genes. Genetic alterations in these genes can cause disturbance of the cell cycle, which often results in cell transformation and thereby promotes carcinogenesis (23).

Many groups have tried to discover mutations in such genes in cancerous tissues and confirm them in corresponding plasma or serum ccf-DNA. Analyses were mainly carried out for well-known proto-oncogenes such as *KRAS*, and tumor suppressor genes, such as *TP53* and *APC* (9). Garcia *et al.* detected mutations in exons 5-8 of the *TP53* gene in 73.2% of patients suffering from primary breast carcinoma. Among these, 42.9% exhibited molecular changes in plasma DNA (24). Another study which evaluated the same *TP53* exons detected *TP53* mutations in 36% of breast cancer patients and 65.1% of these patients also had mutations in their plasma DNA (25). *TP53* mutations in cancerous tissue and corresponding plasma were also found in other types of cancer including colorectal and ovarian cancer (26, 27).

Several studies also confirmed the presence of *KRAS* mutations in tissue and in ccf-DNA of patients. In pancreatic carcinoma patients, the *KRAS* gene was verifiably found to be mutated in 80% to 90% of the cases. Castells *et al.* showed that these mutations were also present in 27% of plasma DNA samples of patients with pancreatic ductal adenocarcinoma (28). Kopreski *et al.* determined *KRAS* mutations in colorectal cancer patients: 83% of the patients in whom *KRAS* mutations were found in the cancerous tissue also had mutations in their plasma ccf-DNA. However, in this study, *KRAS* mutations were also found in the plasma of some patients that had high risk factors but did not test positively for colorectal cancer (29). To increase the sensitivity, some groups therefore studied *KRAS* mutations and tumor marker levels simultaneously. As the detection of mutations in ccf-DNA is completely independent of the results obtained by using a common tumor marker, such a combined detection has the advantage of having a much higher sensitivity. Combining the detection of *KRAS* mutations in ccf-DNA and the analyses of the tumor marker CA19-9, *KRAS* mutations were found in 70.7% of patients with pancreatic carcinoma, while no *KRAS* mutation was detected in healthy controls. Additionally, the level of CA19-9 was elevated in 73.2% of pancreatic carcinoma patients. In total, 90.2% of the patients tested positively (30).

Microsatellite alterations. Beside mutations in oncogenes and tumor suppressor genes, microsatellite alterations, such as microsatellite instability (MSI), and loss of heterozygosity (LOH) have been observed for ccf-DNA as well (10, 31). Microsatellites are highly polymorphic repetitive sequences, consisting of multi-nucleotide repeats. As a consequence of mutations in DNA repair genes, some microsatellites show abnormal length referred to as MSI. LOH occurs when, due to mutations, the normal function of one allele is lost. In cancer, such LOH is often found in tumor suppressor genes, which most likely contributes to neoplastic transformation. As MSI and LOH are both suggested to play a fundamental role in carcinogenesis, numerous studies have tried to find such alterations in ccf-DNA. Testing a panel of 12 microsatellite markers, Beau-Faller and colleagues found

alterations in 88% of the plasma samples of lung cancer patients, whereas all control samples were negative for such changes (32). Using two markers, one to detect MSI (D21S1245) and another one to find LOH (*FHIT* locus) microsatellite alterations were observed in 56% of non-small cell lung cancer tumors and in 40% of the related plasma samples (33). Goessl *et al*. conducted a study to identify microsatellite alterations in renal malignancies. In 80% of all renal malignancies, a deletion of DNA sequences on chromosome 3p which led to LOH occurred. By the use of several highly polymorphic microsatellite markers spanning the chromosomal region between 3p26 and 3p14, they demonstrated that there is LOH in one locus in 63% and in more than one locus in 35% of plasma samples of cancer patients (34).

Epigenetic alterations. The epigenetic code bears information additional to that of the genetic code. Epigenetic modifications are known to play a role in many cellular processes, including chromatin remodeling, imprinting, gene silencing, X chromosome inactivation and carcinogenesis (35). The best examined epigenetic modification doubtless is that of DNA methylation. In cancer, aberrant DNA methylation is often found in the promoter region or at regulatory sites of genes which are involved in cell cycle regulation, growth or apoptosis (36). While promoter *hyper*methylation of tumor suppressor genes results in gene silencing, promoter *hypo*methylation of proto-oncogenes can lead to gene activation (37). Concordant methylation patterns in tissue of primary tumors and corresponding plasma or serum have been found for a huge number of genes and various types of cancer including breast (38), ovarian (39), cervical (40), and lung cancer (41). However, the methylation pattern seems to be subject to factors such as age and gender. Previous studies on monozygotic and dizygotic twins revealed divergent methylation patterns with increasing age (42) and between genders (43). Additionally, there is no defined methylation signature for healthy individuals, which makes it difficult to really determine cancer-specific methylation patterns.

Ccf-DNA – The Ideal Cancer Biomarker?

In the course of technological and medical progress, the demand for biomarkers has increased enormously during the last decade. According to the FDA, the ideal cancer biomarker should meet multiple requirements in order to make it attractive for routine clinical use (44):

(a) The first premise is the direct association of the biomarker with the disease in general or at least with a specific disease state. Regarding the direct association of ccf-DNA with cancer, it should be mentioned that although

significantly elevated levels of ccf-DNA have been found in many studies and in patients with several cancer types, one has to consider that an undefined part of DNA present in the circulation is of non-tumoral origin and is thus not directly associated with the disease.

Considering qualitative alterations in ccf-DNA, it is obvious that at present, there are no cancer-specific alterations that show high enough sensitivity or specificity to be used as a marker in clinical applications. Therefore, the first hurdle that has to be overcome is to find cancer-specific genetic and epigenetic alterations which are not present in non-cancer-derived ccf-DNA. The question as to which alterations should be targeted for an approach is difficult to answer. Epigenetic alterations are highly frequent in cancer but their disadvantage is that, for example, methylation patterns are quite heterogenous between different cancer and tissue types and individuals (45). Genomic mutations seem to be excellent candidates, as they can be easily analyzed by high-throughput multiplex approaches. However, finding such cancer-specific mutations seems to be the critical point. Firstly, the mutational background of cancer is enormously complex, which makes it quite difficult to find cancer-specific mutations. Secondly, each cancer type probably possesses cancer-type specific mutations (46) which would limit the marker to a certain cancer type.

(b) The ideal cancer biomarker preferably should cover the whole continuum or at least a part of the cancer management process ranging from the assessment of predisposition to monitoring of disease recurrence (47). Although there are no clinical applications to date, it is undeniable that ccf-DNA has a huge potential as a cancer biomarker. Cancer specific ccf-DNA alterations theoretically could be implemented for the whole continuum of cancer management. As a risk assessment marker, a level could be set for the assessment of a probable cancer risk, enabling clinicians to take appropriate provisions before the onset of the disease. For cancer screening in asymptomatic patients, ccf-DNA alterations could be used to detect cancer at the earliest stage possible, thereby improving outcome. Determining the frequency of aberrant methylation of four candidate genes, adenomatous polyposis coli (*APC*), glutathione *S*-transferase P (*GSTP1*), ras association domain family 1 isoform A (*RASSF1A*), and retinoic acid receptor, beta 2 (*RARB2*) in the plasma of women with breast cancer, Hoque *et al*. was able to successfully detect 33% of early-stage tumors (48). Ccf-DNA may also be usable for categorization of disease stages, as was shown by Fujiwara *et al*., who demonstrated a significant correlation of a combination of plasma LOH microsatellite markers with progression of different clinical stages of disease in melanoma patients (49). As a prognostic marker, ccf-DNA alterations could be helpful in stratifying patients for treatment. Müller *et al*. evaluated several prognostic DNA methylation markers

in the serum of cervical and breast cancer patients of which two, *APC* and *RASSF1A*, proved to be independent prognostic parameters in breast cancer patients (40). Finally, as a marker of recurrence, ccf-DNA alterations could facilitate assessment for disease recurrence in individuals who previously suffered from cancer. It was shown that plasma tumor DNA levels are significantly higher in patients with colorectal cancer, and that there is a progressive decrease in the follow-up period in tumor-free patients, and increase in patients with recurrence or metastasis (50).

(c) In all stages the ideal biomarker should provide 100% sensitivity and 100% specificity. For several reasons, quantitative and qualitative ccf-DNA alterations seem to provide too low a sensitivity and specificity to reliably discriminate between cancerous and healthy individuals, as has been shown by several studies (28, 51, 52). Firstly, ccf-DNA is also present in the circulation of healthy individuals and the physiological factors which influence the levels of ccf-DNA are relatively unknown, which makes it difficult to establish a clear baseline and to interpret the results in a correct way. Secondly, even though mutations can be detected in cancerous tissue and the corresponding serum or plasma, some studies found mutations in healthy individuals as well, increasing the probability of false-positive detection (29). Nevertheless, a possibility to overcome low sensitivity and specificity may be the use of combined measurement of ccf-DNA alterations with common tumor markers. Various reports reported an improved sensitivity and specificity for a combined use of quantitative, as well as qualitative ccf-DNA alterations with well-known markers such as prostate-specific antigen (53), carcino embryonic antigen (21) and CA19-9 (30).

(d) On behalf of the user, as well as of the patient, the biomarker should be as non-invasive as possible. Unfortunately at present, many diagnostic tests for cancer require biopsy-proof for confirmation. Even though there is no routine ccf-DNA-based cancer test, the fact that ccf-DNA can easily be obtained from the patient by extraction from a simple blood (serum/plasma) or urine sample would make it an ideal non-invasive biomarker. Its non-invasive nature not only brings the advantage of easy access to the specimen for the clinician, but at the same time also a reduction of the physical and psychological stress to the patient.

(e) Since economical aspects gain importance when selecting clinical tools, the cost benefit of the biomarker should be reasonable. The major advantage of the use of ccf-DNA is its ease of access, which in comparison to biopsies, is concomitant with an enormous reduction of cost. However, for quantitative analysis, costs would be dependent on the method of choice and a reasonable cost benefit should be considered.

Conclusion

Considering all aspects, ccf-DNA seems only partially to meet the attributes that characterize the ideal cancer biomarker. Even though quantitative as well as qualitative ccf-DNA alterations are to a certain extent associated with cancer, one has to realize that at present none of these alterations can be considered absolutely cancer-specific and that the low sensitivity and specificity of known alterations do not allow use in a clinical setting. However, the attractiveness of using ccf-DNA as a biomarker lies in its non-invasive nature and a combined use with common already established tumor markers could be the first step to a clinical approach to its use.

Acknowledgements

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