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Circulating Cell Free DNA as Blood Based Biomarker in Breast Cancer

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Abstract

The prevalence of circulating cell free DNA (cfDNA) in the peripheral blood of healthy persons and patients with several different diseases is well known since decades. Especially in cancer patients high amounts of cfDNA can be found because of the higher cellular turnover in cancer cells. CfDNA integrity reflects the ratio of longer to shorter DNA fragments and therefore represents the relation between non-apoptotic and apoptotic cell death. For calculation of cfDNA and cfDNA integrity different non-specific repetitive DNA sequences have been used.

In patients with breast cancer, several studies demonstrated the ability of cf DNA and cfDNA-integrity to discriminate between healthy persons and patients with breast cancer. However it remains still unclear if the use of non-tumour-specific cf DNA can be helpful in breast cancer diagnostic and monitoring of therapy. Next generation sequencing for somatic tumour specific mutations like PI3K or TP53 gene mutations in circulating cf DNA has successfully demonstrated to be accurate and feasible in patients with breast cancer. Tracking tumour- specific cf DNA may become a valuable tool for monitoring therapy and residual disease in breast cancer patients.

Keywords: Cell free DNA; DNA integrity; Breast cancer; Tracking tumour cells

Introduction

With over 1.3 million annually new diagnosed cases, breast cancer is the most common diagnosed cancer in women worldwide [1,2]. Although enormous improvements in diagnostics and treatment of early breast cancer during the last decades significantly improved the outcome of patients, breast cancer still remains the most frequent cause of cancer death in women. Approximately 500.000 women worldwide die from breast cancer every year [1,2]. Whereas radiological screening programs have been successfully applied in detecting breast cancer in earlier stages, no valuable blood biomarkers have been yet identified for that purpose [3,4].

The known tumour markers in clinical use are neither validated systematically for detection of early relapses nor for monitoring of neoadjuvant treatment in early breast cancer [4-6].

Whereas multiple studies demonstrated the efficacy of the established tumour markers carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA15-3) in monitoring disease dynamics in metastatic breast cancer, there is still no evidence for the efficacy of CEA and CA 15–3 in early stages [4].

The development of blood tests for monitoring of residual disease, early detection at first diagnosis, local or distant recurrences and monitoring of therapy especially in locally confirmed breast cancer remains a major unsolved clinical problem. Several potential serum and plasma glycoprotein biomarkers are under investigation [7-14]. However no blood based biomarker has been yet demonstrated to be specific, sensitive and reliable enough to be used for clinical use in early stage breast cancer [4,15].

On the other hand, there is a substantial progress in the development of tissue based diagnostic, prognostic and predictive tests based on the growing knowledge of molecular tumour biology. Molecular testing e.g. for Her2 gene amplifications in Her2/Neu-IHC indifferent cases is clinical standard. Furthermore especially multigene Arrays like the Oncotype-DX™, the Endopredict™ and the Mammaprint™ assay are going to become a widely accepted part of the diagnostic repertoire for therapeutic decision making in early stage breast cancer [16-18]. Therefore multiple efforts were made to transfer methods of molecular testing in patients with solid tumours from tissue to peripheral blood (liquid biopsy).

One potential source for the examination of DNA of solid tumours from peripheral blood is the detection of circulating tumour cells (CTC). Mainly FACS-based tests for CTC analysis are available. In patients with metastasized breast cancer it could be demonstrated that high pretherapeutic CTC counts as well as a persistent high number of CTC after chemotherapy are associated with a shorter time to progression and a worse overall survival [19]. However, hence the isolation and identification of circulating tumour cells is difficult and the reliability and reproducibility is problematic tests are still not recommended for routine use.

Although in 2013 FDA approved the CellSearch™System for the detection of CTC and estimation of prognosis in patients with metastatic breast cancer, the assay could fail to detect cells that have lost their epithelial surface markers [20].

Another more easily accessible, reliable and reproducible source of molecular information is free plasma or serum DNA (cf DNA).

Free DNA in the peripheral blood circulation

The existence of cell free DNA (cf DNA) circulation in the peripheral blood is well known since over 60 years and studies dealing with free DNA in the serum of cancer patients have been published already in the 70th [21-22]. Cell-free extracellular nucleic acids are usually forming complexes with proteins or membrane-bound structures.

The majority of cell free plasma DNA in healthy persons belongs to DNA repeat sequences including long and short interspersed nucleotide elements. ALU-sequences are about 300 base pairs long and belong to the class of short interspersed elements (SINEs) [23-26]. The physiological release of DNA into plasma or serum is known to be mainly of apoptotic origin. Other sources for cf DNA-release in various disease states are necrosis, other cell death modes with mixed morphological or biochemical properties of apoptosis and necrosis, lysis of blood cells, viruses and bacteria [26-28].

Fragments resulting from apoptosis are usually 150-180 base pairs ore a multiple thereof long whereas longer DNA fragments may result mainly from necrosis [29,30]. Physiologically dying cells are normally removed from circulation by phagocytosis. Therefore mean circulating DNA levels in healthy persons are low. However, depending on the method used, the variety of plasma cf DNA concentrations in healthy persons is high and ranks from less than 10 ng/mL to more than 150 ng/ml [23,31].

Higher levels of cf DNA have been found in patients with autoimmune diseases and cancer as compared with healthy individuals [27,32].

Measuring cf DNA and calculating cf DNA integrity in patients with cancer

Several methodical aspects of measuring cf DNA from peripheral blood remain still unclear. There is no standard procedure defined. One item is the optimal source of DNA isolation.

Usually plasma samples are preferred as compared to serum. It could be demonstrated that during serum separation, the lysis of cellular blood components may cause an artificial increase in cf DNA integrity. Especially delayed blood sample processing resulted in elevated cf DNA levels [24-26].

Several approaches have been used to measure cf DNA in plasma and serum, including measurement of non-coding DNA sequences like repetitive ALU sequences [33-37], or LINE1 (long interspersed nucleotide elements) [38] as a surrogate for whole cf DNA. These repetitive DNA sequences are known to be distributed everywhere in the genome, with approximately 1.4 million copies per genome for the ALUs [32,39].

In order to develop a more robust and reliable method to measure the ratio of longer to shorter DNA fragments (DNA-Integrity). Umetani et al. [38] described primers and a quantitative PCR method to measure ALU 115 and ALU 247. In this setting the smaller ALU 115 fragments were an integral part of the larger ALU 247 fragments [34,37]. As mentioned before, during apoptotic cell death, DNA is cleaved by specific endonucleases to nucleosomal or to subnucleosomal fragments smaller than 180 bp, while during necrotic cell death longer fragments are produced by a non- specific cleavage [29,30]. Following this hypothesis, ALU 247 is then supposedly a marker of necrotic cell death, while ALU 115 is associated with either form of cell death.

As elevated cellular proliferation and, in parallel, elevated rates of diverse forms of cell death are characteristic biological features of tumor growth [24], elevated levels of cf DNA and a higher portion of longer DNA fragments (DNA integrity) are supposedly useful blood markers for cancer detection [30]. Concerning the so-called DNA integrity that potentially mirrors the relation between the necrotic and overall cell death rate, different calculations have been used. Umetani et al.[35] calculated the ratio of the concentrations of longer DNA fragments (ALU 247) to shorter DNA fragments (ALU 115) [34,37] while Wang et al. [40] use a more sophisticated formula based on Cp value differences. For the calculation of the DNA integrity index according to Wang et al. [40](DNA), the difference between the Cp value of a standard pool of human genomic DNA (which was measured with every PCR plate) and the Cp value of each sample for ALU 115 and for ALU 247 to obtain ΔCp 115 and ΔCp 247 was used. These two ΔCp values were subtracted (ΔCp115−ΔCp247) to obtain ΔΔCp. Subsequently, DNA integrity was calculated using the formula: $e\ (-\Delta\Delta Cp \times \ln(2))$ [40].

Both investigators demonstrated significantly higher portions of long fragments in the plasma and the serum of cancer patients than in healthy controls.

Elevated levels of circulating cell-free DNA (cf DNA) have been detected in etiologically different diseases, like trauma, stroke, burns, sepsis, autoimmune diseases, and finally also cancer [14,41-44].

This broad prevalence of diseases with elevated non-tumor-specific cf DNA levels is a limitation for diagnostic specificity to a certain extent [45]. Nevertheless cf DNA has been identified to be a highly sensitive tool for cancer detection [34,38,46].

Multiple studies have indicated elevated absolute levels of cell-free DNA (cf DNA) in breast cancer [34], colorectal cancer [34,28], lung cancer [27,47], testicular cancer [48], prostate cancer [49], ovarian cancer [45] and other solid tumors [50,51]. Data from those studies suggest that altered or elevated cf DNA are present in more than 50% of cancer patients [51]. In patients with colorectal cancer serum cf DNA integrity was demonstrated to be significantly increased in patients with stage III/IV disease as compared to patients with stage I/II disease and healthy controls [37]. Persistent high cf DNA integrity in patients with nasopharyngeal carcinoma after radiotherapy was associated with reduced disease free survival [52].

Results from these studies suggest thatcf DNA and cf DNA integrity might be valuable as diagnostic and predictive biomarker in cancer treatment.

cf DNA in patients with breast cancer

Results from several studies revealing the diagnostic relevance of free circulating DNA in patients with breast cancer are available. However, reliability and comparability of these studies is problematic.

The number or patients included are relatively low (N = 30-283) and most studies are limited to patients with locally defined breast cancer. Furthermore the number of healthy persons as controls was comparably low and either no or only few patients with benign breast diseases were included. Furthermore the groups of patients with

benign diseases were very heterogeneously (fibroadenoma, mastopathia, and other benign breast diseases). Additionally, as mentioned before, different methods for measuring cf DNA levels were used. Some investigations based on serum [46] others on plasma samples [53,54] or both [35]. Different DNA extracting methods were used. In most studies material was stored at -20°C others cryopreserved samples at -80° C [35].

Several different methods were used in order to quantify the amount of circulation DNA.

Usually real-time quantitative PCR was used for the quantification of repetitive or other ubiquitous gene sequencessuch as GAPDH-gene [55], ß-globin-gene [46], hTERT [53], LINE1 [38] and ALU 115/ ALU 247 [34,35,56-57].

However results from all studies demonstrated significantly higher cf DNA levels in patients with breast cancer than in healthy persons [35,46,53-55,58,59]. The absolute DNA levels in patients are not comparable due to the use of different methods and therefore differ from 1.9 ng/mL (ALU247-method)[35] up to 63,0 ng/mL (ß-globin-method)[46].

Some studies included an additional control group of patients with non-malignant breast diseases. Kohler et al. [54] using the ß-globin-primer based method were able to discriminate significantly not only between breast cancer patients and healthy controls but also between healthy controls and benign diseases and between breast cancer and benign diseases ($p<0.001$). Hashad et al. [53] using the hTERT-based method were also able to discriminate between all groups ($p<0.001$) (healthy controls; benign diseases; breast cancer).

However in our study based on ALU 115/ALU 247 we did not see relevant differences in ALU-levels of patients with locally defined breast cancer and patients with benign diseases but interestingly significant higher ALU 115/ALU 247 levels in patients with locally confined breast cancer than in healthy controls [35,60]. Another study based on using the GAPDH-gene primer also did not observe a significant difference between malignant and benign breast lesions [55]. This discrepant observation is probably due to the use of non-tumor-specific DNA sequences as diagnostic tool. As described above chronic inflammation is an alternative source of free circulation DNA [33]. Potentially benign breast diseases might be associated with local inflammatory processes. A further study on 20 patients with newly diagnosed breast cancer and 17 healthy volunteers also revealed significantly higher cf DNA levels in breast cancer patients but additionally demonstrated that neither in premenopausal patients with breast cancer nor in young healthy women menstrual cycle phase had an influence on cf DNA levels [61].

Most studies were restricted to patients in early disease stages. Our data revealed significantly higher ALU 247 but not ALU 115 levels in patients with metastatic breast cancer as compared to patients with locally confined breast cancer ($p<0.0001$) [35].

There is also an association of cf DNA level, tumor size and lymph node involvement in locally confirmed breast cancer. Hashad et al. [53] (hTERT-method) found a positive correlation between higher cf DNA levels and tumor size, lymph node involvement and grading. Agostini et al. [58] using the ALU-method confirmedthis finding whereas two other studies only revealed an association of increasing cf DNA and tumor size [38,54,55]. In our own study population there was no clear correlation between cf DNA levels and TNM stage so far [35]. Gal et al. reported comparable results [46].

Catarino et al.[60] described a strong decrease of cf DNA after breast cancer surgery (105,2ng/mL versus 59 ng/mL, p=0-001) but there are no clinical follow up data available [59].

With regard to the predictive and prognostic value there are no prospective studies by now publishes demonstrating an association between cf DNA level and overall survival.

In our study with 65 patients with locally confined breast cancer under neoadjuvant chemotherapy treatment ALU 115 and ALU 247 was not able to identify to provide statistically significant information for predicting response. However there was a stronger ALU 115 decrease in patients achieving a pathological complete remission than in the other groups [57,60].

DNA integrity in patients with breast cancer

Umetani et al. [38] evaluated the DNA integrity in 51 healthy females and 83 women with primary breast cancers. On behalf of mean DNA-Integrity a significant discrimination between healthy persons and patients with Stage II, III and IV breast cancer was possible. Mean serum DNA integrity was positively correlated to size of invasive cancer and significantly higher in patients with lymphovascular invasion or lymph node metastasis [34].

Within our study population DNA Integrity was able to differentiate between healthy controls and benign diseases and between benign diseases and both LBC and MBC. Interestingly, the DNA integrity of healthy individuals and of patients with malignant diseases did not differ, which is in contrast to the findings of Umetani, who reported a clear discriminative difference [34]. In our hands similar results were obtained for the alternative formula for DNA Integrity according to Wang et al. [40], presenting significant differences between healthy controls with benign diseases as well as between MBC withbenign breast diseases and locally confined breast cancer. However, there was no or only a weak correlation with either ALU 115 or ALU 247 on the one hand and DNA integrity on the other hand. Interestingly, it was possible to differentiate between benign and malignant diseases by use of both types of DNA integrities, a feature which is an important criterion for diagnostic markers [35].

Recently a larger study with 283 patients with primary breast cancer (N=82) and metastatic breast cancer (N=201) as well as 100 healthy controls has been published [61,62]. For determination of cf DNA integrity a different method measuring ALU and LINE1 repetitive DNA was used. cf DNA integrity was able to discriminate between healthy controls, primary breast cancer and metastatic breast cancer. There was also a positive correlation between cf DNA integrity and progression free survival ($p<0.0025$) as well as overall survival ($p<0.0001$).

Concerning the predictive value of DNA-integrity, there a data available from our own study with breast cancer under neoadjuvant chemotherapy [57]. DNA integrity calculated using both above mentioned formulas did not show any correlation with therapeutic response neither pretherapeutic values nor during the chemotherapy [57,60].

The direct comparison of all cited studies remains difficult due to the use of different methods to isolate and quantify cf DNA. Therefore the value of use of cf DNA and cf DNA integrity as a biomarker for breast cancer is still unclear.

Thus, further clinical evaluations within prospective clinical studies under standardized conditions are crucial to determine the relevance of both cf DNA and DNA integrity as a diagnostic or predictive tool in breast cancer.

Detecting and tracking of somatic breast cancer specific mutations in circulating free DNA

Circulating cf DNA is carrying tumor-specific sequence alterations respective tumor specific mutations corresponding to mutations of the original tumor. Advances in targeted next generation sequencing technology made a detection of tumor specific DNA from peripheral blood recently possible [63].

First studies dealing with circulating tumor DNA in breast cancer mainly focusing on PI3K, TP53 and Her2 gene mutations. Board et al [64]. published a study with 46 patients with metastasized and 30 patients with localized operable breast cancer. PIK3K mutations were detected in 13/46 patients with metastatic breast cancer. In 41 cases the matched tumor and plasma concordance referring the same mutation in tissue and plasma was 95%. Whereas PIK3K mutations were present in 14 of 30 patients with localized breast cancer, no mutation was detected in the corresponding plasma [63].

Dawson et al. [65] examined 30 women with metastatic breast cancer with genomic alterations within the PIK3K or the TP53 gene suitable for monitoring. Circulating Tumor DNA was successfully detected in 20 of 30 women. The course of circulating tumor DNA levels during therapy additionally demonstrated a more consistent correlation to treatment response than CA15-3 measurement and CTC count [64].

A further study presented at the ASCO annual meeting 2014 focused on the monitoring of PIK3K and TP53 mutations in cf DNA in 20 patients with primary breast cancer receiving neoadjuvant chemotherapy. Next generation sequencing identified at least one mutation in 12 of 20 tumors. Five of 20 patients relapsed at a median of 8.1 (5-16.6) months post-surgery. Tumor specific mutation was detected in 9 of 12 cases in cf DNA samples at baseline. There was no correlation between baseline tumor specific cf DNA and risk of relapse. However all patients with detectable tumor specific cf DNA during the first six months post-surgery follow-up relapsed. None of the disease free patients had a detectable mutation in cf DNA post-surgery indicating clearance by the primary treatment [65].

Interestingly two other studies investigating Her2 gene amplification in cf DNA could not demonstrate a clear association between tumor-specific DNA and disease status [66-68].

Other efforts to increase the rate of detectable tumour specific mutations are under investigation. Klevebring et al. [69] e.g. recently demonstrated that even exome sequencing on cell-free DNA is possible and might be a potential tool for disease monitoring in metastatic breast cancer [68].

However PCR based tracking of tumor specific cf DNA is feasible, accurate and reproducible. cf DNA based monitoring of tumor-specific somatic mutations may become a clinically relevant tool for monitoring of residual disease and therapeutic response in patients with breast cancer.

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