

Positive Correlation of Cell-free DNA in Plasma/Serum in Patients with Malignant and Benign Breast Disease

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Abstract. *Background:* Circulating cell-free (ccf) DNA is measurable in healthy individuals and in higher concentration in patients with benign and malignant breast disease (BD). *Patients and Methods:* In paired plasma and serum samples ccf DNA was extracted and quantified by real-time quantitative PCR for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. *Results:* The concentration of ccf DNA in serum was higher in patients with benign and malignant BD ($p=0.023/p=0.001$) compared to healthy controls, whereas ccf DNA in plasma was higher in patients with malignant BD compared to patients with benign BD or healthy controls ($p=0.012/0.007$). The ccf DNA correlated significantly between plasma and serum samples in patients with benign ($p=0.01$; $R: 0.677$) as well as malignant BD ($p=0.01$; $R: 0.713$). *Conclusion:* The positive correlation between ccf DNA in plasma and serum in patients with benign as well as malignant BD, might have a diagnostic value for discriminating between malignant and benign BD.

The discovery of circulating cell-free (ccf) DNA in the blood created a new perspective in medicine. It is known that ccf DNA is present in low concentrations in healthy individuals (1-6), and in elevated concentrations in patients with graft rejection, stroke, trauma, and burns as well as in pregnant women with pregnancy associated disorders (6-12). Also in cancer patients elevated levels of ccf DNA have been observed (2, 4, 5, 13-22). Many studies have found evidence that tumour DNA is released into the circulation by leakage during necrosis and apoptosis (5, 13, 21-25). Ccf DNA can be recovered from plasma and serum. The concentration of ccf DNA in serum, however is several folds higher than that

found in plasma. The ccf DNA in serum does not exclusively reflect tumour-DNA but may also contain a large fraction of DNA which has been released from blood cells during the clotting process (2-4, 13, 22, 26). Elevated concentrations of ccf DNA in benign and malignant breast lesions compared to healthy controls have been published in previous studies (27, 28). Here we put emphasis on the correlation between ccf DNA in serum and plasma.

There is scant literature on a possible correlation of ccf DNA in plasma and serum. It seems that ccf DNA in plasma and serum correlate in benign and malignant prostatic disease (3) and in paired samples of different unselected tumour patients (29).

The goal of this study was to find a possible correlation between the concentration of ccf DNA in the plasma and serum in patients with benign and malignant breast disease. Therefore ccf DNA in paired serum and plasma samples was quantified in patients with breast cancer and benign breast lesions as well as in healthy controls.

Patients and Methods

From January until November 2005, 115 women were recruited at the Department of Obstetrics and Gynecology, University of Basel, Switzerland. The study was approved by the local institutional ethical review board. All the women signed informed consent and two peripheral blood samples were subsequently taken from each woman to quantify the ccf DNA in the serum and the plasma. The blood samples were collected before core biopsy (14-gauge needle, Magnum® Core high speed, Bard Medica, Karlsruhe, Germany) was performed for histological identification of the breast lesions. The control group was composed of 50 healthy female blood donors who agreed to have blood samples taken but did not have any breast lesion and in whom no breast biopsy was performed. A total of 107 paired samples, 49 paired samples of healthy controls, 29 paired samples of patients with benign breast diseases and 29 paired samples of patients with breast cancer were obtained from the 115 women. In 8 cases one of the two samples was missing.

No women reported a history of breast biopsy due to benign or malignant breast lesions or a history of previous cancer treatment. All the patients diagnosed with breast cancer

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Table I. Circulating cell-free DNA in serum and plasma of healthy controls, patients with benign breast disease and patients with breast cancer.

	Control group (1) n=49 mean (min-max)	Benign breast lesion (2) n=29 mean (min-max)	Breast cancer (3) n=29 mean (min-max)	p (1:2/1:3/2:3)
Age (mean)	54 (33-79)	42 (20-91)	59.5 (34-96)	0.001 / 0.12 / 0.000
Serum GE*/ml	14764 (2198-87450)	30826 (3292-130104)	41600 (3644-192482)	0.023 / 0.001 / 0.253
Plasma GE*/ml	1508 (298-12030)	1368 (404-13682)	2546 (603-20636)	0.657 / 0.012 / 0.007

*Genome Equivalents/ml.

underwent breast surgery with sentinel node lymphadenectomy or axillary lymphadenectomy according to the stage of the disease. The biopsy specimens and the surgical breast cancer samples were all examined at the Institute of Pathology, University Hospital Basel.

From each woman two blood samples (approximately 10 ml) were obtained by venipuncture into untreated (serum sample) and EDTA-coated glass tubes (plasma sample) (Sarstedt, Sevelen, Switzerland) and were sent to the Laboratory for Prenatal Medicine and Gynaecological Oncology, University of Basel. The blood samples were processed immediately by centrifugation at 1600 xg for 10 minutes. The plasma and serum layers were transferred to new Eppendorf tubes and centrifuged once more at maximum speed (16000 xg) for 10 minutes. The ccf DNA was extracted from 400 µl plasma or serum sample by using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Rotkreuz, Switzerland) following the manufacturer's protocol. The DNA preparations were eluted in 100 µl elution buffer. The eluted pure DNA was stored at -20°C until further use.

Five µl of DNA elution were used as template for the TaqMan® (Applied Biosystems, ABI, Rotkreuz, Switzerland) real time PCR analysis. The amount of ccf DNA was quantified using the following primer pairs and VIC® (Applied Biosystems, ABI, Rotkreuz, Switzerland) labelled TaqMan MGB-probe for the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (GenBank Accession No. J04038) (forward): 5' CCCCACACACATGCACTT ACG 3' (reverse): 5' CCTAGTC CCAGGGCTTGATT 3' and probe 5' (MGB) (minor groove binding) GTG AAC GTG GAT GAA GTT GG (VIC) 3'.

The TaqMan (real-time) PCR conditions were standardized as described previously in previous publications (30, 31) using 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The ccf DNA equivalents were calculated according to reproducible standard dilution curves using a known concentration of human genomic DNA. The concentrations of ccf DNA were expressed as genome equivalents (GE).

Statistical analysis. The data were analyzed with SPSS® software (SPSS Inc., Chicago, USA). The Mann-Whitney U-test was used to compare the levels of ccf DNA in the groups categorized as normal healthy individuals, patients with benign breast lesions and breast cancer patients. The Spearman rank test was used to analyze the relationship between levels of ccf DNA and breast cancer characteristics.

Results

The mean age of the women and the mean concentration of ccf DNA in serum and plasma is shown in Table I. The women with benign breast lesions were significantly younger than the women with breast cancer or the healthy individuals.

In the serum the mean concentration of ccf DNA was significantly higher in the patients with benign breast lesions or breast carcinomas ($p=0.023$ and $p=0.001$, respectively) as compared to the healthy controls, but between the patients with benign breast lesions and the breast cancer patients the mean concentration of ccf DNA in the serum was not statistically different.

In the plasma the mean concentration of ccf DNA was significantly higher in the patients with breast cancer as compared to the patients with benign lesions or the healthy controls ($p=0.012$ and 0.007 , respectively). The mean concentration of ccf DNA was not statistically different between the patients with benign breast lesions and the healthy controls. Furthermore, additional statistical analysis with adjustments for patient age did not change the results.

In the group of benign breast disease there were 12 cases (41.4%) of fibroadenoma and 17 cases (58.6%) of other benign conditions. In Table II the tumour characteristics from all 29 breast cancer patients are listed.

A positive correlation between ccf DNA in the plasma and the serum in the whole study population ($p=0.01$; R:0.540), in the patients with benign breast disease ($p=0.01$; R:0.677) and in the breast cancer patients ($p=0.01$; R:0.713) was shown (Table III). There was no correlation between the ccf DNA in the plasma and serum in the healthy controls ($p=ns$; R:-0.044), (Table III, Figure 1).

Discussion

In previous studies we showed that in plasma significantly higher concentrations of ccf DNA could be measured in patients with breast cancer compared to patients with benign breast lesions and healthy controls (27), whereas the

Table II. Breast cancer characteristics.

	n=29	%
Histological type		
ductal	23	79.3
ductulolobular	3	10.3
lobular	2	6.9
tubular	1	3.5
Primary tumour		
T1	16	55.2
T2	9	31.0
T4	4	13.8
Grade		
G1	5	17.2
G2	15	51.7
G3	9	31.1
Positive lymph nodes	11	37.9
ER / PR		
ER pos / PR pos	21	72.4
ER pos / PR neg	4	13.8
ER neg / PR neg	4	13.8
ER neg / PR pos	0	0
HER-2/neu amplification	7	24.1
Distant disease	1	3.5

ER: estrogen receptor; PR: progesterone receptor; HER-2/neu: human epidermal growth factor receptor 2.

Table III. Correlation of paired samples of ccf DNA from plasma and serum.

	n	Correlation	p
The whole study population	107	0.540	0.01
Patients with benign breast disease	29	0.677	0.01
Patients with breast cancer	29	0.713	0.01
Healthy control group	49	-0.440	ns

serum healthy individuals had significantly lower levels of ccf DNA than patients with malignant or benign breast disease (28).

In the present study a positive correlation between the ccf DNA in the plasma and the serum in the study population as a whole ($p=0.01$; R:0.540) was revealed as well as in the patients with benign breast disease ($p=0.01$; R:0.677) and breast cancer ($p=0.01$; R: 0.713) but not in the healthy controls ($p=ns$; R:-0.044). While a number of quantitative studies on ccf DNA in plasma and serum have been reported (6, 15, 32, 33), only a minimal number of studies (3, 29) have investigated a possible correlation between quantitative levels of ccf DNA in plasma and serum. Boddy *et al*. (3) investigated the ccf DNA in both plasma and serum in patients with malignant and benign prostatic disease. Umetani *et al*. (29) reported a positive correlation of the ccf

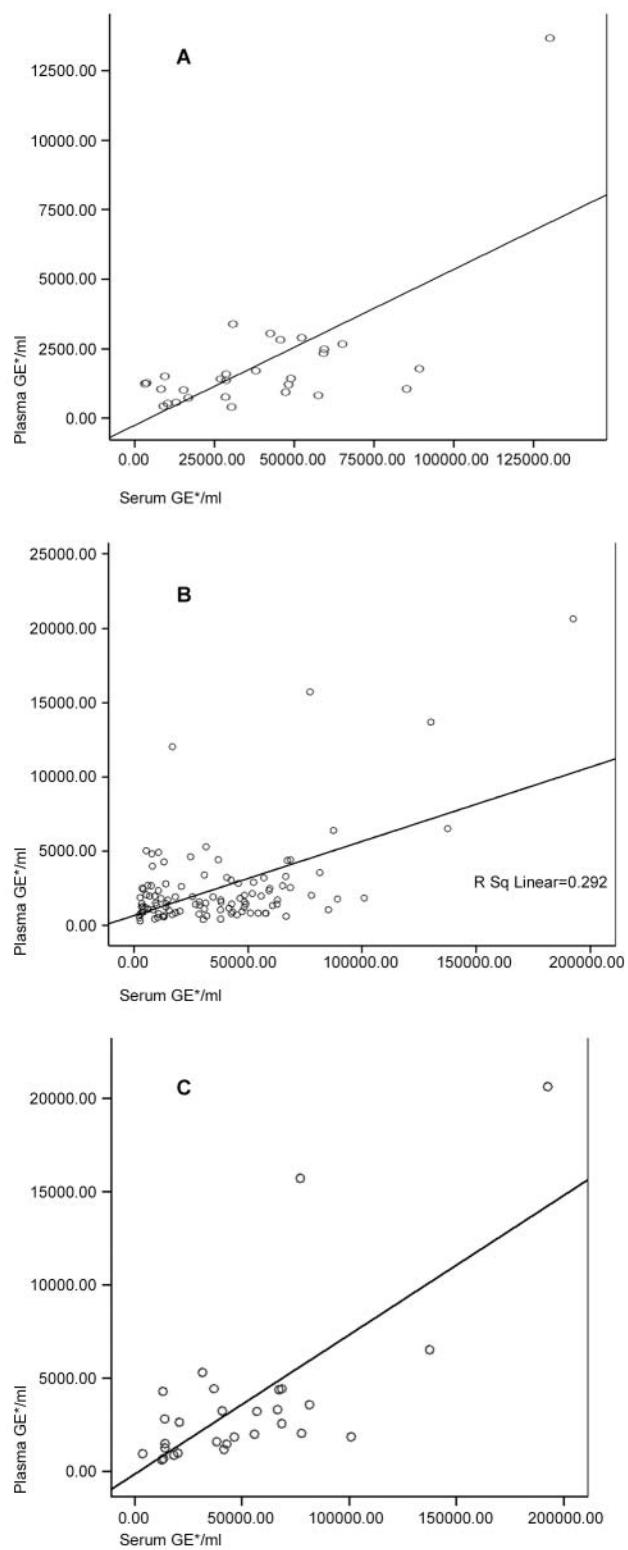


Figure 1. Positive correlation between ccf DNA in plasma and serum in the whole study population (A), in patients with benign breast disease (B) and in patients with breast cancer (C). *Genome Equivalents/ml.

DNA in paired serum and plasma specimens in unselected tumour patients (colorectal cancer, breast cancer, thyroid cancer and thyroid adenoma). Their data confirm ours since they found a positive correlation. A lack of correlation between the ccf DNA in the plasma and serum in healthy controls has also been observed by others (3, 34, 35).

A positive correlation in the ccf DNA in paired plasma and serum samples in tumour patients, suggests that studies on ccf DNA can be done either in plasma or in serum, bearing in mind that the concentration of ccf DNA in the serum is higher due to DNA derived from white blood cells released during the clotting process.

According to the presented and previously published data (27, 28) the concentration of ccf DNA was higher in the patients with malignant disease compared to patients with benign disease. Less necrosis, apoptosis and cell turn-over in benign lesions might explain these observations. Furthermore elevated ccf DNA in the plasma was related to breast cancer whereas elevated ccf DNA in the serum was related to benign breast lesions and breast cancer. Combining the plasma and serum concentrations of ccf DNA may have the potential to distinguish between malignant and benign conditions.

Elevated concentrations of ccf DNA can be measured in cancer patients (16, 18, 21, 24, 36-38). The exact mechanism of how DNA is shed into the circulation needs to be investigated. Different hypotheses for the release of DNA have been proposed such as direct leakage during cellular necrosis or apoptosis, lysis of circulating tumour cells or even active release. The most widely accepted hypothesis is that the tumour sheds DNA into the circulation as a result of tumour necrosis and apoptosis (1, 2, 20-23, 39).

The results of our study showed that ccf DNA levels were higher in the serum than in the plasma, confirming the data of others (3, 15, 29, 32, 33) and in line with other groups the ccf DNA could be measured in individuals without breast disease and in a higher concentration in patients with benign and malignant breast disease (2-4, 13, 18, 19, 21, 24, 28, 37-39).

In conclusion, there is a positive correlation between ccf DNA in plasma and serum. The combination of ccf DNA levels measured in plasma and serum from patients with breast diseases has good potential to increase the retrievable information from the underlying diseases.

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