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RESEARCH ARTICLE

Diagnostic relevance of plasma DNA and DNA integrity for breast cancer

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Abstract Levels of ALU 115, ALU 247, DNA integrity ([1, 2]) and of the tumour markers CA 15–3 and CEA were analysed in the blood of 152 patients. Plasma levels of ALU 115 and ALU 247 were significantly higher in patients with locally confined (LBC; $N=65$), metastatic breast cancer (MBC; $N=47$), and benign diseases ($N=12$) than in healthy controls ($p<0.001$ for all comparisons). DNA integrity, CEA, and CA 15–3 were significantly higher in MBC than in benign controls and LBC but could not identify LBCs. The best discrimination of LBC from healthy controls was achieved by ALU 115 and ALU 247 (AUC 95.4 and 95.5 %) and of MBC from all control groups by CA 15–3 and CEA (AUC 83.2 and 79.1 %). Plasma DNA is valuable for the detection of LBC, while established tumour markers are most informative in MBC.

Keywords Plasma DNA · DNA integrity · Breast cancer · Neoadjuvant chemotherapy

Introduction

With 1.38 million new cases in 2008, breast cancer still represents the most frequently diagnosed cancer in women worldwide [3]. About 458,000 women die due to this disease each year [3]. An increased incidence of breast cancer (60 % of all cases) is known for developed countries like Western/Northern Europe, North America, and Australia, which is also due to early stage detection as a result of screening programs [3]. Whereas radiological screening programs (mammography) have demonstrated to be useful in detecting breast cancer in earlier stages, no valuable blood biomarkers have been identified for that purpose up to now. [4].

Several studies analysing the benefit of using the established tumour markers cancer antigen 15–3 (CA 15–3) and carcinoembryonic antigen (CEA) in breast cancer have been published. Whereas multiple investigations demonstrated the efficacy of CEA and CA 15–3 in monitoring the course of metastatic breast cancer, this has not yet been addressed in the neoadjuvant setting [5]. While a few studies support an effect of these markers on earlier relapse detection in breast cancer after curable surgery [6], there is still no evidence that CEA and CA 15–3 are valuable tools for early breast cancer detection and screening [7]. Therefore, there is yet a need for reliable biomarkers as an aid in breast cancer screening, early detection of local or distant relapse, and monitoring and predicting response to primary systemic chemotherapy.

Elevated levels of circulating cell-free DNA (cfDNA) have been detected in diseases of different origins, such as trauma, stroke, burns, sepsis, autoimmune diseases, and also cancer [8–12]. This broad prevalence of diseases with potentially elevated cfDNA levels limits to a certain extent the diagnostic specificity [13]. However, cfDNA has been identified to offer high sensitivity for cancer detection [1, 14, 15] and to indicate a high prognostic and predictive value in various solid tumour diseases [16]. Several approaches have been used to measure cfDNA in plasma and serum, including non-coding DNA (like repetitive ALU sequences [1, 2] or LINE1 (long interspersed nucleotide elements) [14]). These repetitive DNA sequences are known to be distributed everywhere in the genome, with approximately 1.4 million copies per genome for the ALUs [17, 18].

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Umetani et al. described primers and a quantitative PCR method to measure ALU 115 and ALU 247 in which the smaller ALU 115 fragments were an integral part of the larger ALU 247 fragments [1, 2]. 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 [19, 20]. 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 tumour growth [21], elevated levels of cfDNA and a higher portion of longer DNA fragments (DNA integrity) are supposedly useful blood markers for cancer detection [20].

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. simply calculate the ratio of the concentrations of longer DNA fragments (ALU 247) to shorter DNA fragments (ALU 115) [1, 2], while Wang et al. [22] use a more sophisticated formula based on Cp value differences. Both groups demonstrate significantly higher portions of long fragments in the plasma and the serum of cancer patients than in healthy controls. However, they do not compare their results with each other or with established protein tumour markers.

The present study was conducted to find out whether quantitative levels of ALU 115 and ALU 247 and the two DNA integrity formulas are powerful biomarkers for the diagnosis of breast cancer as well as for tumour characterization and staging purposes. Furthermore, we compared these biomarkers with the already established and routinely used cancer biomarkers CEA and CA 15–3 to identify their specific relevance in the clinical setting.

Patients and methods

Patients

Between 2007 and 2011, plasma samples of 112 breast cancer patients were collected at the time of diagnosis and before the therapy started. Forty-seven of the patients suffered from metastatic breast cancer (MBC); 65 had a locally confined breast cancer (LBC; UICC stages II and III). Additionally, we collected plasma samples of 40 controls, including 28 healthy female controls and 12 patients with benign breast diseases.

In all breast cancer patients, complete relevant histopathological staging (subtype, grading, oestrogen receptor status, progesterone receptor status, Her2/neu-status) was pretherapeutically assessed. Further clinical and radiological staging—including mammography, ultrasound, chest X-ray, abdomen ultrasound, and bone scintigraphy—were performed. In the neoadjuvant setting, the histology was done by fine needle biopsy or vacuum biopsy and underwent a clinical classification according to the TNM system. In all other cases, a complete pathological and clinical TNM status was available. In breast cancer patients, venipunctures were regularly performed before starting a neoadjuvant or palliative systemic chemotherapy in controls before any therapy was started.

The study was approved by the local ethics committee. Patients were intensively informed of the study; prospective and written informed consent was obtained from all patients before study entry.

Plasma preparation for qPCR

Plasma samples (4.4 ml) and serum samples (10 ml) were collected in K2-EDTA and gel separation tubes, respectively (Sarstedt, Nürnbrecht, Germany). All samples were centrifuged within 1 to 2 h after venipuncture. Plasma and sera were separated, aliquoted, and cryopreserved at −80 °C.

DNA isolation was done with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Initially, 400 μl of plasma sample and 400 μl of lysis buffer were added to a vial containing 20 μl of Qiagen protease. After the mixture of the reagents and 30 min of incubation at 56 °C, 400 μl of 100 % ethanol was pipetted into the vials and mixed. Subsequently, a vacuum pump was used to wash the two washing buffers (750 μl each) through spin columns. Afterwards, the spin columns were centrifuged, 50 μl of lysis buffer was added, and one more centrifugation followed to elute the DNA from the column filter. Five microliters of this eluate was used as a template for the qPCR.

Quantitative PCR of ALU repeats

For the qPCR of the ALU repeats, we used the same primers as described by Umetani et al. [1] ("Electronic supplementary material 1"). The reaction mixture for the qPCR contained 5 μl of template, 0.25 μl of uracile DNA glycosylase (UNG, Roche Diagnostics, Mannheim, Germany) to prevent carryover contamination, 2 μl of each primer (forward and reverse), 6.75 μl of PCR grade H_2O, and 4 μl of Mastermix SYBR Green (Roche Diagnostics), resulting in 20 μl of reaction volume.

Real-time PCR amplification was performed using the LightCycler® 480 Instrument II (Roche Diagnostics, Mannheim Germany). It started with 10 min of incubation time for the uracil–DNA–glycosylase at 40 °C, followed by 10 min of UNG inactivation time at 95 °C. The real-time PCR amplification was conducted with 45 cycles of denaturation (at 95 °C for 10 s), annealing (at 62 °C for 15 s), and extension (at 72 °C for 15 s). To determine the absolute quantitative amount of DNA in the samples, a standard curve was calculated. We used serial dilutions of 20 to 0.076 ng/ml of DNA (Roche Diagnostics) in ten dilution steps. The standard curve for ALU

115 had an efficiency of 1.95; for ALU 247 the efficiency was 1.84 ("Electronic supplementary material 2"). Additionally to the samples, a negative and a positive control, two patient plasma pools with high and low DNA levels as well as three dilution step samples of the standard curve were performed with every plate for quality control. All measurements were done in duplicates (description according to MIQE standards; see "Electronic supplementary material 3").

Calculation of the DNA integrity index

DNA integrity was calculated according to two different algorithms according to Wang et al. [22] and Umetani et al. [1, 2]. For the calculation of the DNA integrity index according to Umetani et al. (DNA Int 1), the ratio of the concentration of ALU 247 sequences to the concentration of ALU 115 sequences was calculated. This ratio can theoretically vary between 0 and 1 as the ALU 115 sequences are represented within the annealing sites of ALU 247 [1]. Assuming that DNA fragments originating from apoptosis are mainly sized below 180 bp, a high index would indicate a considerable contribution of non-apoptotic cell death, such as necrosis.

For the calculation of the DNA integrity index according to Wang et al. (DNA Int 2), 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: $e^{(-\Delta\Delta Cp \times \ln(2))}$.

Determination of established tumour markers

CA 15–3 and CEA were measured by enzymatic chemiluminescent immunoassay (ECLIA) on the ElecSys 2010 immunoassay analyser of Roche Diagnostics, Germany, in sera of breast cancer patients.

Statistics

The concentrations of all measured markers before the start of a therapy in the breast cancer groups as well as the measurements of the healthy and benign group were considered for statistical evaluation.

Medians, percentiles, and ranges are presented in tables for biomarker concentrations within the different groups. Dot plots show the individual marker distribution. Discriminative power between the groups was tested by overall analysis of variance on ranks of data followed by the Ryan–Einot–Gabriel–Welsch multiple-range test to assess the significance of differences between single groups. Additionally, results are illustrated in receiver operating characteristic (ROC) curves.

The correlation of biomarkers with disease characteristics, such as TNM stage and receptor status (oestrogen receptor, progesterone receptor, and Her2/neu receptor), was done by Wilcoxon test or Kruskal–Wallis test. The correlation of biomarkers with each other was done by Spearman rank–correlation test.

A p-value of <0.05 was considered to be statistically significant. All calculations were performed with SAS software (version 9.2, SAS Institute Inc., Cary, NC, USA).

Results

Clinical data of patients with primary breast cancers

Clinical and histopathological data of patients suffering from breast cancer and controls, including age, tumour subtype, grading, receptor-status, Her2/neu status, clinical and/or pathological TNM, and UICC stage, and radiological results are given in Table 1.

Table 1 Patient characteristics

Patients and controls, *N* (age, median)		
Locally confined breast cancer	65	47.0
Metastatic breast cancer	47	60.8
Benign breast diseases	12	45.5
Healthy controls	28	41.7
Characteristics of patients with locally confined breast cancer, *N* (%)		
T stage		
1	8	12.3
2	39	60.0
3	14	21.5
4	3	4.6
X	1	1.6
N stage		
0	17	26.1
1	40	61.6
2	1	1.6
3	3	4.6
X	4	6.1
Histology		
Invasive ductal carcinoma	57	87.7
Invasive lobular carcinoma	4	6.1
Adenocarcinoma	2	3.1
Unknown	2	3.1
Histopathological classification, *N* (%)		
Oestrogen receptor positive/negative	38/27	58.5/41.5
Progesterone receptor positive/negative	32/33	49.2/50.8
Her2/neu receptor positive/negative	21/44	32.3/67.7
Triple negatives/non-negatives	21/44	32.3/67.7

Biomarker values in different patient groups; diagnostic value

Plasma levels of ALU 115 were discriminated significantly between the single groups by Ryan–Einot–Gabriel–Welsch multiple-range test ($p < 0.0001$). Median values in healthy females (1.8 ng/mL) were significantly lower than in patients with benign diseases (27.4 ng/mL) and in patients with LBC (15.9 ng/mL) and with MBC (22.3 ng/mL).

Similar results were obtained for ALU 247. Overall significance for the discrimination of the single groups was $p < 0.0001$. There was a significant difference between median ALU 247 levels in healthy controls (1.9 ng/mL) and benign diseases (22.3 ng/mL), LBC (16.8 ng/mL), and MBC (29.8 ng/mL). In addition, ALU 247 levels significantly discriminated between benign diseases and MBC as well as between LBC and MBC (Table 2; Fig. 1a, b).

For both DNA integrities, overall significances for the discrimination of the single groups were $p = 0.0003$ and $p < 0.0001$, respectively. DNA integrity index 1 (DNA Int 1), representing the ratio of ALU 247 to ALU 115, was able to distinguish between healthy controls (median 1.2) and benign diseases (0.9) and between benign diseases and both LBC (1.1) and MBC (1.2). DNA integrity index 2 (DNA Int 2) showed significant differences between healthy controls (1.0) and benign diseases (0.7) as well as between benign diseases and LBC (0.8) and MBC (1.2), respectively (Table 2; Fig. 1c, d).

With respect to the established marker CEA, locally confined tumours could not be distinguished from the control groups of healthy women and from those with benign breast diseases. However, women with MBC (6.0 ng/mL) had significantly higher median CEA levels than healthy women (1.0 ng/mL), women with benign breast diseases (0.7 ng/mL), and patients with LBC (1.3 ng/mL). Comparable results were obtained for CA 15–3 that also revealed highly significant differences of median values in patients with MBC (61.3 U/mL) and all other groups, such as healthy women (17.6 U/mL), patients with benign diseases (17.3 U/mL), and patients with LBC (19.1 U/mL). Similar to CEA, CA 15–3 was not able to discriminate between locally confined tumours and either control group. Overall significances for the

Table 2 Biomarker values in different patient groups

Marker	Group	Number of cases	Median	Minimum	Maximum	Comparison with		
						Benign (p-value)	Localized breast cancer (p-value)	Metastatic breast cancer (p-value)
ALU 115 (ng/mL); overall $p < 0.0001$	Healthy	28	1.8	0.1	3.2	Sig.	Sig.	Sig.
	Benign	12	27.4	1.4	89.2		Not sig.	Not sig.
	Localized breast cancer	65	15.9	0.7	871.8			Not sig.
	Metastatic breast cancer	47	22.3	3.3	827.1			
ALU 247 (ng/mL); overall $p < 0.0001$	Healthy	28	1.9	0.3	4.4	Sig.	Sig.	Sig.
	Benigns	12	22.3	1.4	63.8		Not sig.	Sig.
	Localized breast cancer	65	16.8	0.8	577.6			Sig.
	Metastatic breast cancer	47	29.8	5.1	835.6			
DNA-Int 1; overall $p = 0.0003$	Healthy	28	1.2	0.5	9.3	Sig.	Not sig.	Not sig.
	Benign	12	0.9	0.5	1.1		Sig.	Sig.
	Localized breast cancer	65	1.1	0.6	1.7			Not sig.
	Metastatic breast cancer	47	1.2	0.6	1.9			
DNA-Int 2; overall $p < 0.0001$	Healthy	28	1.0	0.3	1.9	Sig.	Not sig.	Not sig.
	Benign	12	0.7	0.4	1.0		Not sig.	Sig.
	Localized breast cancer	65	0.8	0.4	1.5			Sig.
	Metastatic breast cancer	47	1.1	0.5	2.1			
CEA (ng/mL); overall $p < 0.0001$	Healthy	27	1.0	0.2	4.2	Not sig.	Not sig.	Sig.
	Benign	12	0.7	0.2	3.4		Not sig.	Sig.
	Localized breast cancer	62	1.3	0.2	14.1			Sig.
	Metastatic breast cancer	41	6.0	0.3	2,608			
CA 15–3 (U/mL); overall $p < 0.0001$	Healthy	27	17.6	5.6	26.9	Not sig.	Not sig.	Sig.
	Benign	12	17.3	8.2	41.1		Not sig.	Sig.
	Localized breast cancer	62	19.1	6.3	258.0			Sig.
	Metastatic breast cancer	41	61.3	10.0	319,000			

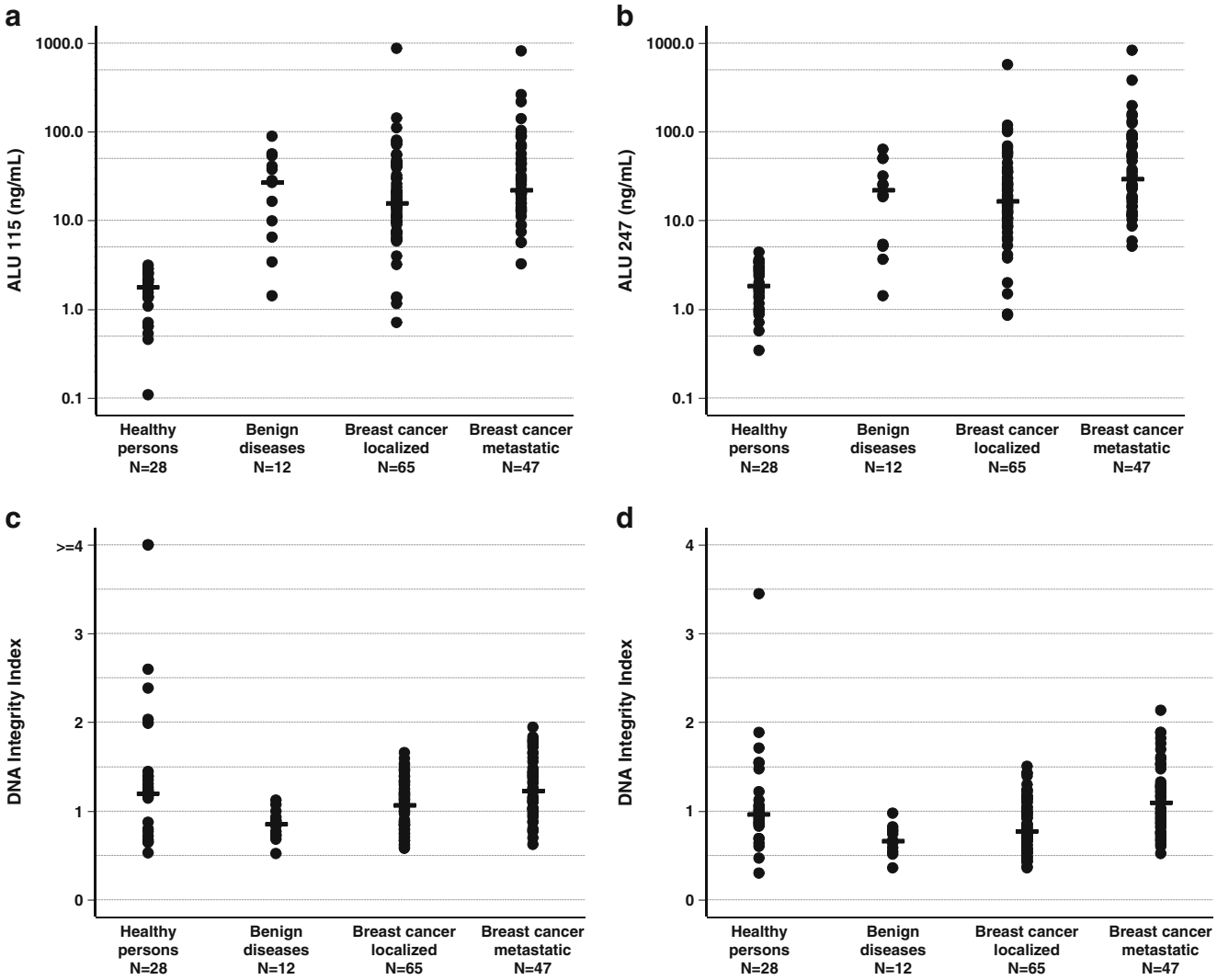

Fig. 1 Value distribution and medians of ALU 115 (**a**), ALU 247 (**b**), DNA integrity index 1 (**c**), and DNA integrity index 2 (**d**) in the plasma of healthy persons, patients with benign breast diseases, patients with locally confined and metastatic breast cancer

discrimination of the single groups were $p < 0.0001$ for CEA and CA 15–3, respectively (Table 2; "Electronic supplementary materials 4 and 5").

For a comparison of LBC with healthy persons, the diagnostic efficiency was highest for ALU 115 and ALU 247, reaching an area under the curve (AUC) of ROC curves of 95.4 and 95.5 %, respectively. AUCs of CA 15–3 and CEA were 56.9 and 59.3 % only, of DNA Int 1 39.8 %, and of DNA Int 2 35.4 %. Sensitivities for cancer detection at 95 % specificities were 93.8 % (ALU 115), 92.3 % (ALU 247), 0 % (DNA Int 1 and 2), 30.6 % (CA 15–3), and 8.1 % (CEA) (Fig. 2a).

For a comparison of MBC from either control group, the diagnostic efficiency was highest for CA 15–3 and CEA, reaching AUCs in ROC curves of 83.2 and 79.1 %, respectively. AUCs for ALU 115, ALU 247, and DNA Int 1 and 2 were slightly lower with 73.0, 76.4, 64.5, and 71.1 %, respectively. Sensitivities for MBC detection at 95 % specificities were 19.1 % (ALU 115), 29.8 % (ALU 247), 2.1 % (DNA Int 1), 17.0 % (DNA Int 2), 48.8 % (CA 15–3), and 56.1 % (CEA) (Fig. 2b).

Correlation with disease characteristics (TNM and UICC stage, receptor status)

Information on the clinical TNM and receptor status (oestrogen, progesterone, and Her2/neu receptor) was gained by pretherapeutic biopsy. When locally confined tumours (T stage 1 and 2) were compared with locally advanced tumours (T stage 3 and 4), none of the markers were able to differentiate between these groups. Concerning the nodal (N) stage, CEA was higher in lymph node positive (N1–3) as compared with lymph node negative (N0) patients ($p = 0.045$), while the other markers showed no differences. In addition, ALU 247 correlated with the progesterone receptor status, with higher levels for the receptor-positive group ($p = 0.041$). No differences of biomarker levels were observed between patients

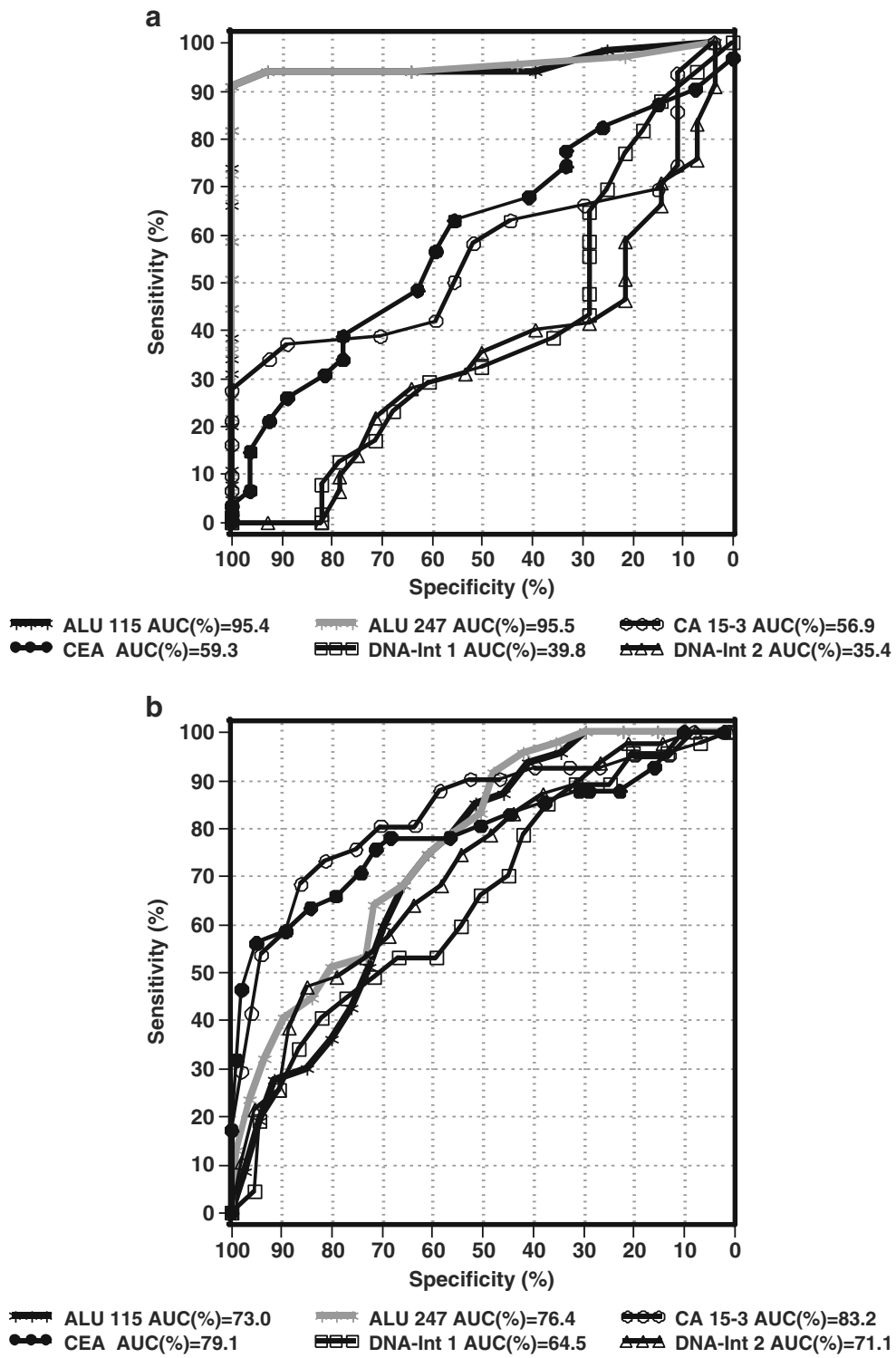

Fig. 2 ROC curves of plasma ALU 115, ALU 247, DNA integrity index 1 and 2 as well as of serum CA 15–3 and CEA for the discrimination between **a** locally confined breast cancer and healthy control groups as well as between **b** metastatic breast cancer and all control groups (healthy, benign, locally confined breast cancer)

with Her2/neu receptor-positive or -negative tumours, and neither between oestrogen receptor-positive and -negative tumour patients. When comparing the patients with a triple-negative receptor status (oestrogen receptor, progesterone receptor, and Her2/neu receptor negative) with all other patients, no significant difference of marker values was found.

Correlation of the markers with each other

Biomarker levels showed highly significant positive correlations with each other for the conventional tumour markers, ALUs, and DNA integrity indices. A highly significant correlation was found between both DNA integrity indices for LBC

and MBC as well as for both ALUs when compared with each other. The conventional tumour markers showed a very good correlation with ALU 115 and ALU 247, particularly in metastatic patients (see Table 3).

Discussion

In order to establish new serum biomarkers for breast cancer, we investigated the diagnostic value of cell-free DNA in breast cancer patients.

Multiple studies have indicated elevated absolute levels of cell-free DNA (cfDNA) in breast, colorectal, lung, testicular, prostate, and ovarian cancer among others [1, 2, 17, 23–27]. Results from these studies suggest that cfDNA levels might be valuable in order to determine the tumour cell turnover.

In addition, DNA integrity—as the relation of longer to smaller DNA fragments—has been elevated in the plasma [2] and the serum of cancer patients [2]. As other studies reported inconsistent data not supporting these results [13, 28, 29], we tried to verify the clinical validity of DNA integrity in plasma as a diagnostic tool for breast cancer. As diverse algorithms are used for the calculation of the DNA integrity index, we used the two most popular methods to compare their clinical validity and to determine whether the combination of the indices, the absolute cfDNA levels, and the conventional markers CEA and CA 15–3 increases the diagnostic sensitivity.

In line with most studies that have investigated absolute levels of cfDNA in plasma, we found significantly higher levels of plasma cfDNA in cancer patients as compared to healthy controls. Unfortunately, no significant difference was obtained from this comparison of benign and malignant diseases. Indeed this is not really surprising as benign breast diseases often occur together with inflammation, and inflammation is known to increase cfDNA levels in the blood as well [9]. On the other hand, cfDNA levels can be low in cancer patients due to low cell death rates and a low half-life time of cfDNA in the plasma as a result of high DNA clearance [30].

With DNA Int 1 [1, 2], it was possible 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 et al., who report a clear discriminative difference [1].

Similar results were obtained for DNA Int 2 [22], presenting significant differences between healthy controls with benign diseases as well as between MBC with

Table 3 Correlation between the biomarkers

	CA153	CEA	ALU 115	ALU 247	DNA Int 1	DNA Int 2
All patients						
CA 15–3		0.399	0.361	0.412	0.234	0.273
		<.001	<.001	<.001	0.005	0.001
		142	142	142	142	142
CEA	0.399		0.282	0.323	0.158	0.199
	<.001		0.001	<.001	0.061	0.017
	142		142	142	142	142
ALU 115	0.361	0.282		0.977	−0.168	−0.003
	<.001	0.001		<.001	0.039	0.965
	142	142		152	152	152
ALU 247	0.412	0.323	0.977		0.021	0.148
	<.001	<.001	<.001		0.798	0.069
	142	142	152		152	152
DNA Int 1	0.234	0.158	−0.168	0.021		0.752
	0.005	0.061	0.039	0.798		<.001
	142	142	152	152		152
DNA Int 2	0.273	0.199	−0.003	0.148	0.752	
	0.001	0.017	0.965	0.069	<.001	
	142	142	152	152	152	
Locally confined breast cancer						
CA 15–3		0.047	0.024	0.045	0.221	0.134
		0.715	0.855	0.729	0.084	0.298
		62	62	62	62	62
CEA	0.0473		−0.087	−0.052	0.119	0.161
	0.715		0.503	0.686	0.355	0.212
	62		62	62	62	62
ALU 115	0.024	−0.087		0.961	−0.377	−0.146
	0.855	0.503		<.001	0.002	0.246
	62	62		65	65	65
ALU 247	0.045	−0.052	0.961		−0.141	0.629
	0.729	0.686	<.001		0.263	0.619
	62	62	65		65	65
DNA Int 1	0.221	0.119	−0.377	−0.141		0.832
	0.084	0.355	0.002	0.263		<.001
	62	62	65	65		65
DNA Int 2	0.134	0.161	−0.146	0.629	0.832	
	0.298	0.212	0.246	0.619	<.001	
	62	62	65	65	65	
Metastatic breast cancer						
CA 15–3		0.713	0.536	0.554	0.104	0.123
		<.001	0.001	0.001	0.519	0.443
		41	41	41	41	41
CEA	0.713		0.430	0.464	0.045	0.018
	<.001		0.005	0.002	0.781	0.910
	41		41	41	41	41
ALU 115	0.536	0.430		0.962	−0.060	0.207
	0.001	0.005		<.001	0.688	0.163
	41	41		47	47	47

Table 3 (continued)

	CA153	CEA	ALU 115	ALU 247	DNA Int 1	DNA Int 2
ALU 247	0.554	0.464	0.962		0.167	0.355
	0.001	0.002	<.001		0.262	0.014
	41	41	47		47	47
DNA Int 1	0.104	0.045	−0.060	0.167		0.734
	0.519	0.781	0.688	0.262		<.001
	41	41	47	47		47
DNA Int 2	0.123	0.018	0.207	0.355	0.734	
	0.443	0.910	0.163	0.014	<.001	
	41	41	47	47	47	

benign breast diseases and LBC. Notably, both formulas showed a high correlation with each other in all patients ($R=0.75$), LBC ($R=0.83$), and MBC ($R=0.73$) despite the level of absolute values being different. However, there was no or only a weak correlation with either ALU 115 and 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 tool for diagnostic markers. This information was not obtained with cfDNA or the conventional tumour markers. However, due to elevated DNA integrity levels in some healthy controls, its use as diagnostic markers is limited in the individual patient case.

It has to be pointed out that the levels of DNA integrity were often above the value of 1.0. This is theoretically implausible as, according to Umetani et al. [1], the annealing sites of ALU 115 are represented within the annealing sites of ALU 247, implying that ALU 115 is always present when ALU 247 can be measured. Several reasons may explain this phenomenon: Lower absolute cfDNA levels could be caused by the shorter denaturation, annealing, and extension times in the qPCR. However, this argument cannot clarify why the longer ALU fragments were measured more often, as a shorter extension time during qPCR would preferably affect the amplification of longer DNA fragments. Alternatively, primer binding to DNA could have been impaired. To improve primer binding, we additionally included different add-ons (DMSO and BSA) to the PCR setting; however, results were unchanged.

To assure the quality of pPCR measurements, plasma pools with high and low DNA levels were included in every run, resulting in quite constant levels in the inter-run comparison. Interestingly, the level of ALU 247 was higher even in both pools compared to the level of ALU 115 in the same pools. This finding confirms that the ALU 247 levels were elevated compared to the ALU 115 levels not only in cancer patients. In a recently published paper about cfDNA in patients with testicular germ cell cancer using another primer pair (where the annealing sites of the short DNA fragment were also within the longer DNA fragment), calculated DNA integrity levels often were above 1, too [23]. Nevertheless, it should be mentioned that both levels of ALU sequences were highly elevated in the plasma of cancer patients compared to healthy controls.

The direct comparison of our results with other studies remains difficult due to the use of different types of blood samples (serum or plasma). Furthermore, DNA isolation in the different studies [31] is not always comparable as the amount of isolated DNA varies highly between the different extraction kits [27]. Additionally, many different primers are used to determine the DNA integrity, impairing further the direct comparison of different studies [27]. In fact, studies using single-copy sequences have also been successfully applied for the quantification of DNA integrity [29]. Thus, further clinical validation of these assays is crucial to determine the relevance of both cfDNA and DNA integrity as a diagnostic tool under routine conditions [17].

As it is generally requested that new promising biomarkers are compared with already established ones [17], we included the breast tumour markers CA 15–3 and CEA in our evaluations. As expected, they had significantly higher values in MBC than in all other control groups. However, these markers could not distinguish between LBC and healthy controls. There was a highly significant correlation of CA 15–3 and CEA with each other in MBC but not in LBC patients. The same applies to comparisons of these tumour markers with ALU 115 and ALU 247, while there was only a slight correlation with DNA integrity in the all patients group. As a consequence, tumour markers performed best for the detection of MBC. However, for the detection of LBC, ALU 115 and ALU 247 were considerably better indicators, showing the potential diagnostic impact of these new markers for the early detection of breast cancer patients.

Conclusion

Although DNA integrity could not improve the diagnostic performance of the established markers, ALU concentrations were highly promising for the detection of locally confined breast cancers and surpassed the conventional biomarkers CEA and CA 15–3 by far for this indication. For the detection of MBC, CA 15–3 and CEA showed the overall best diagnostic profile.

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Conflicts of interest None

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