Imaging, Diagnosis, Prognosis

Clinical Cancer Research

Loss of Heterozygosity at Tumor Suppressor Genes Detectable on Fractionated Circulating Cell-Free Tumor DNA as Indicator of Breast Cancer Progression

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Abstract

Purpose: LOH on circulating DNA may provide tumor-specific information on breast cancer. As identification of LOH on cell-free DNA is impeded by the prevalence of wild type DNA in blood of cancer patients, we fractionated plasma DNA, and determined the diagnostic and prognostic value of both fractions.

Experimental design: Our cohort of 388 patients with primary breast cancer before chemotherapy was selected from a multicenter study (SUCCESS). Postoperative plasma was fractionated in low- and high-molecular weight DNA by two different column systems. In both fractions, LOH was determined by a PCR-based microsatellite analysis using a panel of 8 polymorphic markers. Circulating tumor DNA in plasma from 30 patients after chemotherapy was additionally analyzed. The significance levels were adjusted for multiple comparisons.

Results: More patients (38%) had LOH at all markers in the fraction containing short DNA fragments than in the fraction containing the long DNA molecules (28%, $P = 0.0001$). In both fractions 32.85% of LOH were concordant. LOH at the markers D3S1605, D10S1765, D12S1725, D13S218, and D17S855 significantly correlated with tumor stage, tumor size, and lymph node metastasis, positive progesterone, and HER2 receptor status. Most importantly, LOH at D12S1725 mapping to *cyclin D2* correlated with shorter overall survival ($P = 0.004$).

Conclusions: The improved detection of LOH on cell-free DNA provides important information on DNA losses of tumor suppressor genes *TIG1*, *PTEN*, *cyclin D2*, *RB1*, and *BRCA1* in breast cancer. In particular, loss of the *cyclin D2* gene might become an important prognostic marker easily detectable in the peripheral blood. *Clin Cancer Res; 18(20); 5719–30. ©2012 AACR.*

Introduction

Worldwide, breast cancer is the most common carcinoma detected in women, accounting for 23% of all new cancer cases in women. On the basis of the heterogeneous features and different subtypes of this disease, the therapy is adapted to disease stage and usually consists of a combination of surgery as well as cytostatic drugs, endocrine therapy, and radiotherapy (1). In addition, new approaches on the field of targeted therapies have been introduced by the administration of monoclonal antibodies (e.g., against HER2). Estrogen receptor (ER)- or progesterone receptor (PR)-positive tumors detected by immunohistochemical techniques can be treated with endocrine therapy. Immunohistochemistry (IHC) and FISH can reveal the overexpression or amplification of HER2, which can therapeutically be targeted by antibodies or small molecule tyrosine kinase inhibitors, such as trastuzumab and lapatinib (2). Early detection of breast cancer depends on mammography screening and is assisted by numerous diagnostic biomarkers, which are not sufficiently cancer sensitive (1). To improve early diagnosis and reduce the mortality of the patients, new diagnostic and prognostic biomarkers are urgently needed. The serial, minimally invasive assessment of cell-free tumor DNA, which circulates in high levels in blood of patients with cancer (3–5), could become such a biomarker. The determination of DNA losses of tumor suppressor genes could be an independent diagnostic and

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Translational Relevance

Accurate identification of LOH on cell-free DNA from blood plasma is often restricted by technical limitations, such as poor quality and quantity of tumor-specific DNA, and contamination by normal DNA. To advance the identification of LOH on circulating DNA, we optimized the DNA extraction method and fractionated plasma DNA in high- and low-molecular weight DNA. By this DNA fractionation, the exposure of LOH in the plasma samples could be improved. Validating our improved method in the context of the multicenter SUCCESS trial showed that the detection of DNA losses of the tumor suppressor genes *TIG1*, *PTEN*, *cyclin D2*, *RB1*, and *BRCA1* on circulating cell-free DNA was associated with a more aggressive biology of breast cancer. In particular, the observed *cyclin D2* loss was a strong indicator of an unfavorable prognosis. Thus, the improved detection of circulating tumor DNA might provide clinically relevant information on the variable biology of breast cancer.

prognostic biomarker useful in identifying subgroups of cancer patients and their prognosis.

The physiologic and pathologic processes that lead to increasing DNA levels in blood of patients with cancer are still not well understood and are subject of discussion. The high concentrations of circulating DNA are thought to be related to the apoptosis and necrosis of cancer cells in the tumor microenvironment (6). An active release by living cancer cells has also been discussed (7, 8). The uncontrolled cell proliferation leading to a high cell turnover in the tumor might cause this increased cell death. Tumor cells that circulate in the blood can also contribute to the release of DNA (9). However, circulating tumor DNA is diluted by varying concentrations of normal DNA and the overall content of cell-free DNA in serum or plasma is therefore not a cancer-specific marker. The main source of this DNA in blood is most probably leucocytes, which constitute two-third of the cells in blood and have a short turnover of 3 days, thus contributing the vast majority of degraded DNA (4).

Genetic alterations, such as LOH, on cell-free tumor-derived DNA can be examined by PCR-based microsatellite analyses. Although this method has usually been used by different groups, a great variability in frequency of LOH on cell-free DNA has been reported and is a current subject of discussion (4). Besides the described concordances between LOH on plasma/serum DNA and LOH in the matched primary tumors, also discrepancies were discovered (4). Generally, the number of LOH detected in plasma or serum was lower than in the paired tumor tissues (10). The contradictory microsatellite data derived from blood and tumor tissue might be caused by the low prevalence of tumor-derived DNA in blood of patients with cancer (4, 10, 11). Apart from killing of neoplastic cells, necrosis may also cause death of stromal cells surrounding the primary tumor. Moreover, inflammation processes in other tissues also release their DNA into the bloodstream. However, at least part of cell-free DNA originates from tumor cells because it contains numerous cancer-specific aberrations (9). Hence, the pool of circulating DNA in peripheral blood may be derived from different sources, such as tumor, stromal, inflammatory, and mononuclear cells (6). The observation that tumor-associated blood DNA is diluted by wild type DNA has been discussed for different tumor entities (11–13). This wild type DNA may camouflage genetic alterations and consequently, impede the detection of circulating tumor DNA. Therefore, a reliable detection of genetically altered DNA fragments is still challenging.

To advance assay sensitivity, we optimized the detection of cell-free tumor DNA in blood plasma by extending the commonly used DNA extraction method in the current protocols (14). Here, we show that circulating DNA detected in blood plasma of breast cancer patients by this new approach might become a valuable prognostic marker associated with more aggressive breast cancer and unfavorable prognosis.

Materials and Methods

Patients with primary breast cancer and healthy women

During October 2005 to February 2010, blood plasma samples were collected from 388 patients participating in a multicenter study (SUCCESS), which includes 251 German centers. The study was approved by relevant ethical boards involved in Germany. The tumor stage at primary diagnosis was classified according to the revised American Joint Committee on Cancer tumor-node-metastasis classification (15). Histopathologic grading of the primary tumors was conducted according to the Bloom–Richardson system (16). For the diagnosis of lymph node metastasis, single-embedded lymph nodes were screened up to 3 levels. In all patients treated with breast conservation, external beam radiation therapy was administered. Chest wall irradiation following mastectomy was conducted in patients with more than 3 involved lymph nodes or T3 and T4 tumors. All patients and healthy controls gave their informed consent.

DNA extraction and fractionation

Preparation of blood samples and peripheral blood mononuclear cells were conducted centrally. Wild type DNA was extracted from leukocytes and cell-free DNA from cell-free blood plasma of 388 patients before chemotherapy. From 30 patients plasma samples were also available after chemotherapy. For the fractionation in high- and low-molecular weight DNA, in a first step DNA was extracted from blood plasma using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. However, the flow-through of the QIAamp mini column was collected and further processed. In a second step, DNA was extracted from this flow-through using Wizard Plus SV columns (Promega) and 2 volumes of 6 mol/L guanidine thiocyanate. The DNA extracted from the QIAamp DNA Mini Kit contained high-molecular weight DNA (long DNA

fragments, >1000 bp) and was denoted as the first fraction. The DNA derived from the flow-through contained low-molecular weight DNA (short DNA fragments, <1000 bp) and was denoted as the second fraction. Quantification and quality of the extracted DNA were determined spectrophotometrically using the NanoDrop Spectrometer ND-1000 (NanoDrop).

PCR-based fluorescence microsatellite analysis

Note that 10 ng of wild type DNA derived from leukocytes and of the low- and high-molecular weight DNA from plasma were amplified in a 10 μL-reaction volume containing PCR Gold buffer, 2.5 mmol/L $MgCl_2$ (Applied Biosystems), 20 nmol/L dNTPs (Roche), 0.4 pmol/L of primer sets (Sigma), and 0.2 units of AmpliTaq Gold DNA-Polymerase (Applied Biosystems). Leukocyte DNA of each patient served as reference DNA. The following 8 microsatellite markers were used: D3S1605, D10S1765, D11S4200, D12S1660, D12S1725, D13S218, D16S421, and D17S855 (Table 2). Each sense primer was fluorescence-labeled (HEX, FAM, or TAMRA) at the 5′end. The reaction was started with activation of the DNA polymerase for 5 minutes at 95°C, followed by 40 cycles of PCR amplification. For optimizing the PCR, 0.1 mmol/L TMAC was added to the reaction (14, 17, 18). To confirm the microsatellite alterations, each PCR was repeated at least twice.

To determine the lowest portion of tumor-specific DNA, which can be flawlessly detected, additional, serial dilution experiments were carried out. For this study we mixed and amplified known quantities and increasing proportions of normal leukocyte DNA and plasma DNA with confirmed LOH.

Evaluation of PCR products

The fluorescence-labeled PCR products were separated by capillary gel electrophoresis and detected on an automated ABI 3130 DNA analyzer (Applied Biosystems). Fragment length and fluorescence intensity were evaluated by the GeneScan software. The 500-ROX size marker (Applied Biosystems) served as an internal standard. The incidence of LOH was determined by dividing the ratio of intensities of the 2 alleles from a plasma sample by the ratio of intensities of the 2 alleles from the corresponding leukocyte sample, which served as reference DNA. LOH was interpreted if the final quotient was less than 0.6 or more than 1.67. Homozygous and nonanalyzable peaks were designated as noninformative cases.

Statistical analyses

The statistical analyses were conducted using the SPSS software package, version 18.0 (SPSS Inc.). The χ^2 or 2-tailed Fischer exact test was used to identify potential associations between LOH frequency and the clinical and histopathologic risk factors of the patients with breast cancer. The contingency coefficients were calculated by the comparison of the LOH incidences at each marker in both fractions. For nonparametric comparisons, univariate analyses of the Mann–Whitney *U* test of 2 independent variables were used. To compare the LOH incidences at all markers in the low- and high-molecular weight DNA fractions, Wilcoxon test of 2 dependent variables was conducted. Binary logistical regression was carried out for analyzing the association of LOH frequency at combined markers with the clinicopathologic data. Univariate and multivariate analyses were conducted for the prognostic factor of overall survival using the Cox regression model. Kaplan–Meier plots were drawn on to estimate overall survival, and the Log rank test was applied for statistical analyses. Missing data were handled by pairwise deletion. For multiple testing the significances were verified by the Holm–Bonferroni method. A P value ≤ 0.05 was considered as statistically significant. All P values are 2-sided.

Results

Patient design

Patients have been invited to take part in a prospectively randomized adjuvant multicenter phase III treatment study (SUCCESS). Eligible patients were defined as women with histologically confirmed invasive breast cancer (stages pT1-4, pN0-3, M0). The SUCCESS study compared the disease-free survival after randomization in patients treated with 3 cycles of epirubicin (100 mg/m^2)-fluorouracil (500)-cyclophosphamide (500, FEC) chemotherapy followed by 3 cycles of docetaxel (100 mg/mg^2, D) versus 3 cycles of FEC followed by 3 cycles of gemcitabine (1,000 mg/m^2 d1,8)-docetaxel (75 mg/m^2 DG). After chemotherapy, patients were randomized to receive 2 (q 3 months × 24 months) versus 5 years of zoledronate (q 3 months × 24 months followed by q 6 months × 36 months). Women with hormone receptor positive disease received endocrine treatment. As determined by IHC, a cutoff level of more than 10% was used for positive hormone receptor status. For the expression of HER2, the DAKO score from 0 to 3+ was determined according to the recommendations of American Society of Clinical Oncology, and by IHC and FISH analyses.

For this retrospective study, we chose 388 patients from the SUCCESS study based on their HER2 receptor status, to assemble a collective that showed a well-balanced distribution of established risk factors including hormone receptor status, lymph node status, tumor stages, and grading. Of this cohort, 55% of the patients were receptor-negative whereas 71% of the patients had lymph node metastases. Although 74% and 26% of patients had tumor stages 1–2 and 3–4, respectively, approximately half of patients had histopathologic grade 3 (Table 1, Fig. 1). This highly selected cohort does not represent the population of the SUCCESS study (19).

Blood plasma samples were taken from these patients about 1 or 2 months after surgery before initiation of chemotherapy and from 30 patients after chemotherapy. The median follow-up time was 2.5 years (range 2 months to 4.5 years). All patients analyzed had histologic proven epithelial cancer. Metastatic spread in M0 patients was excluded by chest radiology, liver ultrasound scan and bone

Table 1. Relationship of the incidence of LOH at 8 microsatellite markers in the combined plasma DNA fractions with the clinicopathologic parameters

Parameters	Patients *n* (%)	D3S1605 *n* (%) Inf.	D10S1765 *n* (%) Inf.	D11S4200 *n* (%) Inf.	D12S1660 *n* (%) Inf.	D12S1725 *n* (%) Inf.	D13S218 *n* (%) Inf.	D16S421 *n* (%) Inf.	D17S855 *n* (%) Inf.
Total	388 (100)								
Age	56 years (range 26–75)								
Histology									
Ductal	256 (67)	10 (5)	22 (10)	19 (11)	35 (18)	33 (16)	13 (8)	26 (19)	22 (11)
		186	227	180	196	203	161	137	198
Lobular	61 (16)	2 (5)	11 (21)	6 (13)	10 (22)	11 (23)	7 (19)	7 (23)	9 (18)
		39	52	48	45	48	37	30	49
Others	67 (17)	3 (6)	7 (12)	8 (14)	6 (12)	3 (6)	5 (15)	4 (11)	2 (4)
		50	59	56	51	52	34	37	52
Tumor stage									
pT1-2	283 (74)	**7 (3)**	**19 (8)**	21 (10)	35 (16)	**21 (9)**	**13 (8)**	26 (17)	21 (10)
		211	**249**	212	215	**228**	**169**	156	218
pT3-4	100 (26)	**8 (13)**	**20 (23)**	11 (16)	16 (21)	**25 (34)**	**12 (19)**	11 (23)	12 (15)
		63	**88**	71	76	**74**	**63**	48	81
Lymph node metastasis									
Negative	110 (29)	**0 (0)**	7 (7)	11 (13)	16 (21)	**8 (9)**	7 (10)	12 (19)	12 (14)
		80	98	88	75	**90**	69	63	86
Positive	273 (71)	**15 (8)**	33 (14)	22 (11)	35 (16)	**39 (18)**	18 (11)	25 (18)	21 (10)
		194	239	195	217	**212**	163	141	212
Grading									
1,2	199 (52)	6 (4)	21 (12)	18 (13)	26 (18)	28 (18)	14 (11)	22 (21)	21 (14)
		139	172	144	147	153	127	106	152
3	185 (48)	9 (7)	19 (12)	15 (11)	25 (17)	19 (13)	10 (10)	15 (15)	12 (8)
		134	165	139	144	149	103	98	145
Estrogen receptor									
Negative	116 (30)	3 (4)	10 (10)	10 (11)	15 (17)	10 (11)	8 (11)	11 (17)	7 (7)
		83	103	89	86	88	70	66	96
Positive	268 (70)	12 (6)	30 (13)	23 (12)	36 (18)	37 (17)	17 (11)	26 (19)	26 (13)
		192	235	195	206	215	162	138	203
Progesterone receptor									
Negative	149 (39)	**1 (1)**	16 (12)	11 (10)	19 (17)	15 (13)	9 (10)	14 (16)	12 (10)
		105	135	115	113	116	90	89	123
Positive	235 (61)	**14 (8)**	24 (12)	22 (13)	32 (18)	32 (17)	16 (11)	23 (20)	21 (12)
		170	203	169	179	187	142	115	176
HER2 receptor									
Negative	204 (55)	5 (5)	20 (14)	15 (12)	24 (19)	**28 (21)**	13 (11)	17 (19)	**22 (17)**
		111	147	122	124	**135**	115	92	**130**
Positive	167 (45)	10 (7)	19 (11)	18 (12)	26 (17)	**16 (10)**	12 (11)	18 (17)	**9 (6)**
		155	179	154	158	**157**	110	105	**157**

NOTE: The significant differences in bold are shown as *P* values in Table 2.

Abbreviation: Inf, informative cases.

scan. Table 1 summarizes the clinical and histopathologic parameters of the breast cancer patient cohort.

Quantification of cell-free plasma DNA

To improve the detection rate of LOH on cell-free DNA in blood plasma, we extended the commonly applied DNA extraction method, which uses the QIAamp DNA Mini kit. The flow-through of the QIAamp mini column was collected and DNA was extracted from this flow-through using Wizard Plus SV columns. The DNA extracted from the QIAamp DNA Mini kit contained rather high-molecular weight DNA (long DNA fragments) and was denoted as the first fraction. The DNA derived from the flow-through contained low-molecular weight DNA (short DNA fragments) and was denoted as

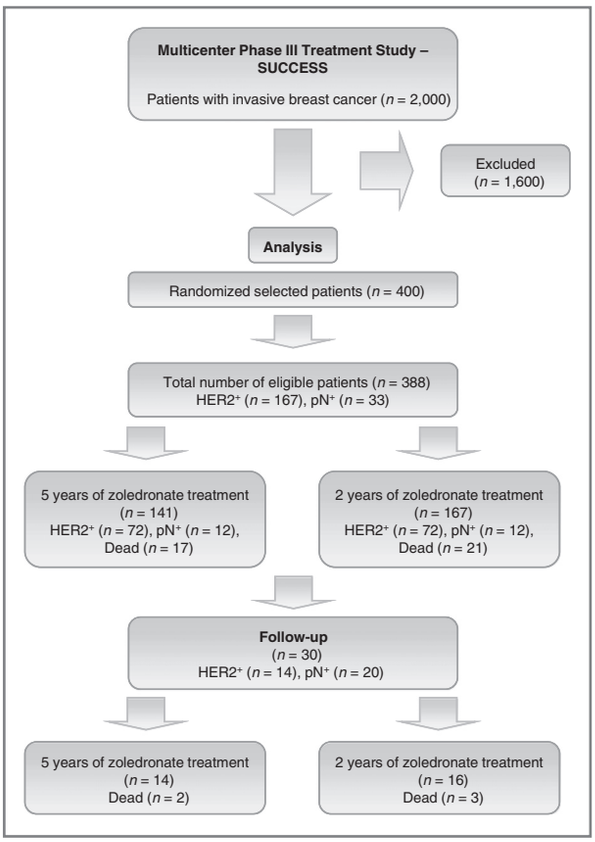

Figure 1. CONSORT diagram showing the number of patients analyzed from the multicenter study (SUCCESS).

the second fraction (14). We verified the fractionation by additionally spiking a plasma sample with a DNA marker and gel electrophoresis. In the first fraction, predominantly large signals were visible on the gel indicating that small DNA molecules got partially lost by the Qiagen extraction method and in the second fraction short DNA fragments could be seen (data not shown).

Spectrophotometrical quantification of the DNA concentrations in both plasma fractions of our patient cohort revealed a wide range of DNA yields. In the high-molecular weight DNA fraction the range of DNA concentrations was between 40 and 6030 ng/mL of plasma with a median value of 139 ng/mL. In the low-molecular weight DNA fraction the range of DNA concentrations was between 45 and 1510 ng/mL of plasma with a median value of 81 ng/mL. No significant correlation was detected between the DNA concentrations in both plasma fractions and aging of the patients.

The LOH frequency is higher on the low-molecular weight plasma DNA than on the high-molecular weight plasma DNA

In both fractions, LOH on cell-free DNA was determined by a PCR-based fluorescence microsatellite analysis using a panel of 8 polymorphic markers (D3S1605, D10S1765, D11S4200, D12S1660, D12S1725, D13S218, D16S421, and D17S855) mapping to tumor-associated genes on different chromosomal loci (Table 2).

To determine the lowest portion of tumor-specific DNA, which can be flawlessly detected, serial dilution experiments were at first conducted for each marker. In serial admixtures plasma DNA with confirmed LOH (10, 9, 8, 6, 4, 2, and 0 ng) was mixed with increasing proportions of wild type DNA from leukocytes up to a final amount of 10 ng, and amplified by PCR. The LOH ratios were determined by calculating the ratio of intensities of the 2 alleles from the plasma sample (or the mixture) of high- or low-molecular weight DNA and corrected by that from the corresponding leukocyte sample. LOH was interpreted if the final quotient was less than 0.6 or more than 1.67. Figure 2 shows exemplarily the serial dilution steps of the markers D10S1765 and D13S218 in the low- and high-molecular weight DNA. On the basis of the cutoff value of 0.6, the transition point of LOH to the retention of heterozygosity was generally attained at a mixture of 90% plasma DNA and 10% wild type DNA. For the marker D13S218, the transition point was at an admixture of 20% leukocyte DNA to the high-molecular weight plasma fraction. These findings are similar to our findings on serial dilution experiments using plasma samples from prostate cancer patients. In that study we detected, that an admixture of 5% normal DNA to plasma DNA was already sufficient, to lead to a retention of heterozygosity (14).

Our analyses show that significantly more patients (38% of the patients) had LOH at one or more microsatellite markers in the fraction containing short DNA fragments than in the fraction containing the long molecules (28%, $P = 0.0001$). The overall LOH frequency in all analyses

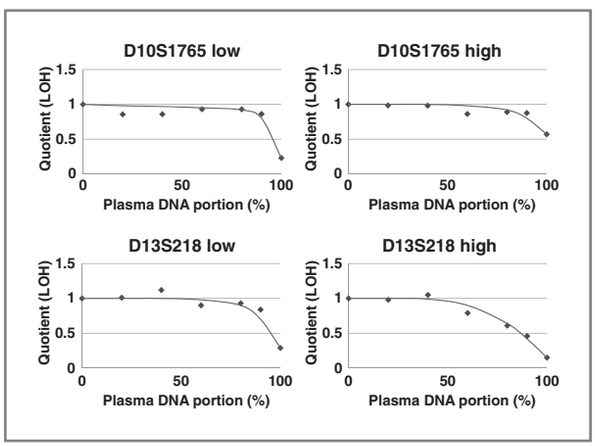

Figure 2. The diagrams show admixtures of leukocyte DNA (wild type) to low- and high-molecular weight plasma DNA fractions (low, high) amplified by primers binding at the markers D10S1765 and D13S218. An admixture of 10% leukocyte DNA to plasma DNA led to transition of the LOH to heterozygosity for D10S1765 in the low- and high-molecular weight DNA fraction. For D13S218 an admixture of 10% and 20% leukocyte DNA to low- and high-molecular weight plasma DNA led to transition of the LOH to heterozygosity, respectively. LOH was interpreted if the final quotient was less than 0.6 or more than 1.67.

Table 2. Summary of the associations between marker-specific LOH and clinicopathologic parameters as evaluated by the Man–Whitney *U* and Log-rank test

Microsatellite markers			*P* values					
LOH at	**Chromosomal loci**	**Gene products**	**pT**	**pN**	**Tumor size**	**PR$^+$**	**HER2$^+$**	**OS**
D3S1605 H	3q25.31-32	TIG1	0.008*	0.066	/	0.129	/	/
D3S1605 L			0.082	0.056	/	0.010	/	/
D3S1605[a]			0.004*	0.011	/	0.010	/	/
D10S1765 H	10q23.3	PTEN	0.0001*	/	0.009	/	/	/
D10S1765 L			0.004*	/	0.025	/	/	/
D10S1765[a]			0.0001*	/	0.006*	/	/	/
D11S4200 H	11p13	CD44	/	/	/	/	/	/
D11S4200 L			/	/	/	/	/	/
D11S4200[a]			/	/	/	/	/	/
D12S1660 H	12q21.2	GLIPR1	/	/	/	/	/	/
D12S1660 L			/	/	/	/	/	/
D12S1660[a]			/	/	/	/	/	/
D12S1725 H	12p13.32	Cyclin D2	0.003*	0.375	0.077	/	0.219	0.176
D12S1725 L			0.0001*	0.014	0.010	/	0.028	0.007
D12S1725[a]			0.0001*	0.037	0.0001*	/	0.012	0.004*
D13S218 H	13q12-14	RB1	0.002*	/	/	/	/	/
D13S218 L			0.039	/	/	/	/	/
D13S218[a]			0.013	/	/	/	/	/
D16S421 H	16q22-23	E-Cadherin	/	/	/	/	/	/
D16S421 L			/	/	/	/	/	/
D16S421[a]			/	/	/	/	/	/
D17S855 H	17q21	BRCA1	/	/	/	/	0.170	/
D17S855 L			/	/	/	/	0.003*	/
D17S855[a]			/	/	/	/	0.002*	/
D3S1605*D12S1660 H			/	/	/	/	/	/
D3S1605*D12S1660 L			0.158	/	/	/	/	/
D3S1605*D12S1660[b]			0.019	/	/	/	/	/
D10S1765*D12S1725 H			0.999	/	/	/	/	/
D10S1765*D12S1725 L			0.023	/	/	/	/	/
D10S1765*D12S1725[b]			0.002	/	/	/	/	/
D10S1765*D16S421 H			0.046	/	/	/	/	/
D10S1765*D16S421 L			0.074	/	/	/	/	/
D10S1765*D16S421[b]			0.050	/	/	/	/	/
D12S1660*D12S1725 H			0.999	/	0.999	/	/	/
D12S1660*D12S1725 L			0.148	/	0.553	/	/	/
D12S1660*D12S1725[b]			0.003	/	0.038	/	/	/
D12S1725*D16S421 H			0.047	/	/	/	/	/
D12S1725*D16S421 L			0.043	/	/	/	/	/
D12S1725*D16S421[b]			0.006	/	/	/	/	/

NOTE: /, no significant *P* value; *, significant *P* value in accordance with the Holm–Bonferroni method.

Abbreviations: H, high-molecular weight DNA; L, low-molecular weight DNA; PR, progesterone receptor; HER2, human epidermal growth factor receptor; OS, overall survival; TIG1, Tazarotene-induced gene 1; PTEN, phosphatase and tensin homologue; GLIPR1, glioma pathogenesis-related protein 1; RB1, retinoblastoma 1.

[a]Combined plasma DNA fractions.

[b]Combined markers.

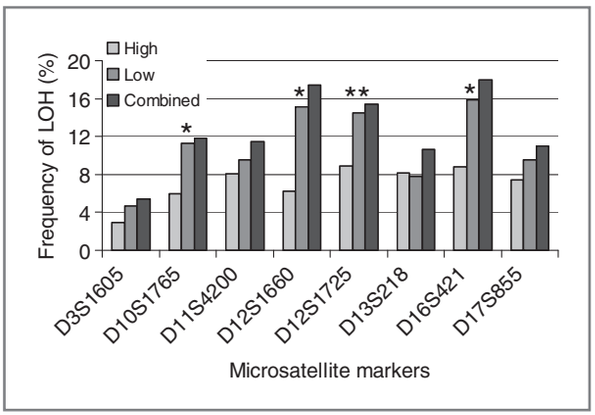

Figure 3. Comparison of the frequencies of LOH detected at 8 different microsatellite markers in high- and low-molecular weight and combined plasma DNA fractions. The frequency of LOH was calculated by division of the number of LOH by the number of the informative cases. Statistical significance (*) and borderline significance (**) of the higher LOH frequency in the low-molecular weight plasma DNA fraction were determined by the Wilcoxon test: D10S1725, $P = 0.003$; D12S1660, $P = 0.0001$; D12S1725, $P = 0.068$; D16S421, $P = 0.009$.

increased from 7% in the fraction containing long DNA fragments up to 11% in the fraction containing short DNA fragments. Figure 3 depicts the distributions of LOH in the high- versus the low-molecular weight plasma DNA fraction, as well as the combined high- and low-molecular weight DNA fractions. With the exception of marker D13S218, the LOH incidences at all other markers were higher in the low- than in the high-molecular weight DNA fraction. As determined by the Wilcoxon test, the frequency was higher at D10S1765 ($P = 0.003$), D12S1660 ($P = 0.0001$), D12S1725 ($P = 0.068$), and D16S421 ($P = 0.009$, Fig. 3). These findings show that the DNA extraction of the flow-through derived from QIAamp mini columns improved the detection rate of LOH compared with the nonextended method using the QIAamp DNA Mini kit, which is commonly used by laboratories. In addition, we compared the LOH incidence at each marker in both fractions and calculated the contingency coefficients for D3S1605 (0.535), D10S1765 (0.354), D11S4200 (0.364), D12S1660 (0.520), D12S1725 (0.420), D13S218 (0.354), D16S421 (0.427), and D17S855 (0.358, Supplementary Table S1). The overall concordance of the LOH patterns detected in both fractions was 32.85% (data not shown), indicating that in most cases LOH detected in the low- or high-molecular weight DNA could not be retrieved in the corresponding other fraction. As a control we also conducted microsatellite analysis of fractionated plasma DNA from 30 healthy women and detected no LOH at these 8 markers indicating that these markers were tumor specific (data not shown).

LOH frequency in plasma of patients before and after chemotherapy

From 30 patients plasma samples were also available after chemotherapy. To obtain information on changes in the levels of tumor DNA during chemotherapy, we compared the LOH frequency of these 30 patients before and after chemotherapy. Twenty of the 30 patients had lymph node metastases. Tumor recurrence was observed in 6 patients after a mean time period of 27 months. Eighteen women with hormone receptor-positive disease received endocrine treatment. In addition, 4 mg of zoledronate were administered to 16 and 14 patients for 2 and 5 years, respectively (Fig. 1).

In the high-molecular weight DNA 15 and 17 LOH were found at all 8 markers before and after chemotherapy, respectively. Of these LOH, only 3 LOH (19%) were concordant before and after therapy. In the low-molecular weight DNA 25 and 26 LOH were detected before and after chemotherapy, respectively. Of these LOH, 7 LOH (27%) were concordant. LOH persisting before and after chemotherapy were detected at the markers D11S4200 (*CD44*), D12S1660 (*GLIPR1*), D13S218 (*RB1*), and D16S421 (*E-cadherin*). Fourteen patients, who displayed retention of heterozygosity before therapy, had also no LOH after therapy. In the other patients, the number of LOH either increased or decreased after chemotherapy (Fig. 4). Taken together, these findings show the marked heterogeneity of LOH patterns before and after chemotherapy.

The cohort of 30 patients and the follow-up time were too small to analyze the potential associations between baseline values of marker-specific LOH or their chemotherapy-induced changes and the different treatment arms or patient response to chemotherapy.

Association of LOH on circulating cell-free DNA with established risk factors

The Mann–Whitney *U* test was used for statistical evaluations of LOH at single markers. For multiple testing, the significances were verified by the Holm–Bonferroni method. The binary logistical regression was carried out for statistical assessments of LOH at combined markers. Moreover, the events (LOH and retention of heterozygosity) at each marker in the low- and high-molecular weight plasma DNA were combined. Accordingly, statistical correlations of the number of LOH at each marker in each plasma DNA fraction (low- and high-molecular weight DNA) and the combined fractions of the samples from 388 patients with their clinical and histopathologic data were conducted (Table 1).

Table 2 summarizes the significant associations between these parameters and risk factors with the corresponding *P* values. Increased LOH frequencies at 5 (D3S1605, D10S1765, D12S1725, D13S218, and D17S855) of the 8 markers significantly correlated with clinicopathologic risk factors, such as advanced tumor stages, tumor sizes, and lymph node metastasis, as well as positive PR and HER2 receptor status. After adjustment by the Holm–Bonferroni method, the correlations with advanced tumor stages, tumor sizes, and HER2 receptor status still remained significant (as indicated by * in Table 2). In addition, our statistical analyses of the LOH frequencies at

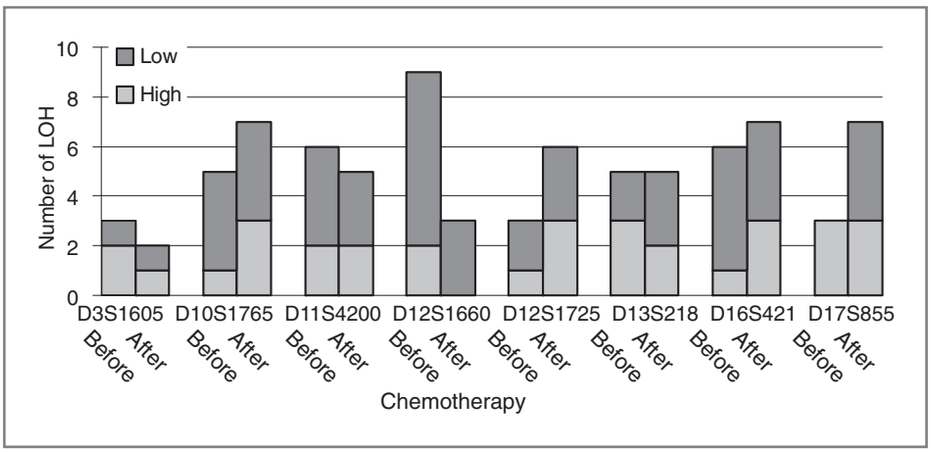

Figure 4. Comparison of the number of LOH detected at 8 different microsatellite markers before and after chemotherapy. The frequency of LOH was calculated by division of the number of LOH by the number of the informative cases.

combined marker sets also showed significant associations with advanced tumor stage and size, but they were less significant than those evaluations of the incidences of the single markers. In most cases the associations of LOH at single and combined markers in the combined plasma DNA fractions were more significant than those in the high- or low-molecular weight DNA fractions alone (Table 2). However, no adjustment of the level of significance for multiple comparisons was conducted. Furthermore, significant correlations with parameters, such as ER/PR$^+$ or ER/PR$^-$, triple negative tumors and histology, were not detected.

Prognostic value of LOH on circulating cell-free DNA

To assess the prognostic value of tumor-specific, cell-free DNA in blood plasma, Kaplan–Meier and Log-rank models, as well as univariate and multivariate Cox regression models were carried out. The mean follow-up time of the cancer patients was 2.5 years (range 2 months to 4.5 years). The REMARK criteria for prognostic studies (20) have been taken into consideration.

As shown in Fig. 5, the LOH incidence at the marker D12S1725, which maps to *cyclin D2*, correlated with overall survival when the LOH frequency was considered in high-molecular weight DNA ($P = 0.176$, Log-rank test; $P = 0.216$,

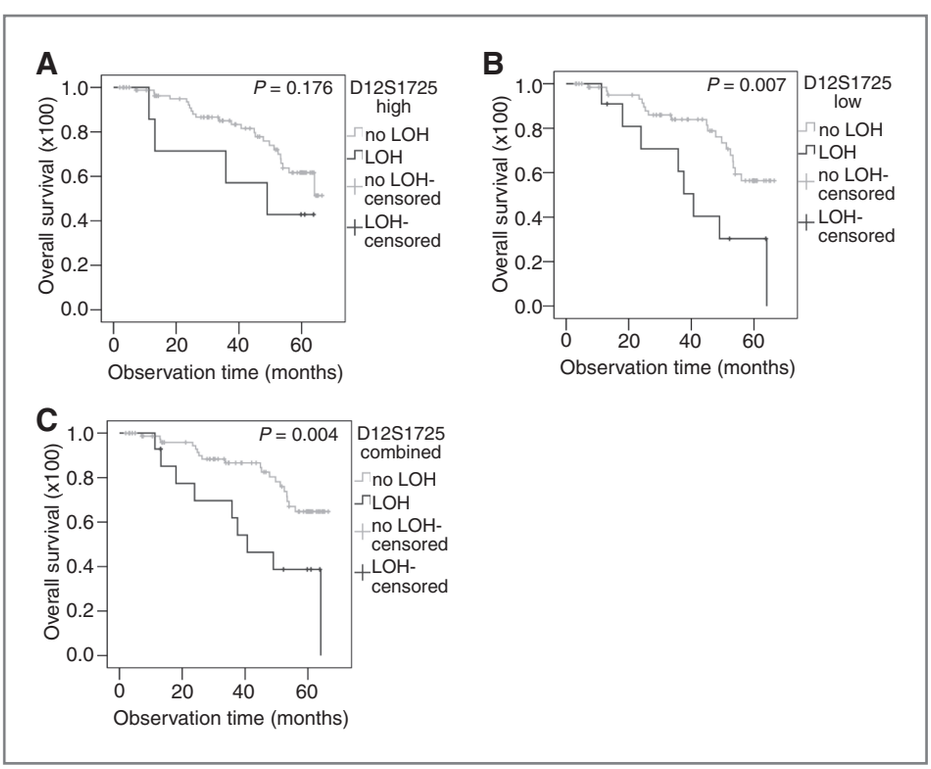

Figure 5. LOH at the marker D12S1725 significantly correlates with overall survival. Kaplan–Meier analyses of primary breast cancer patients with the occurrence of LOH at the microsatellite marker D12S1725 in high- (A) and low-molecular weight plasma DNA (B) and combined fractions (C) were conducted. Top curves show patients with retention of heterozygosity at the marker, and bottom curves patients with LOH at the marker (see also Table 1).

univariate Cox regression; A), low-molecular weight DNA ($P = 0.007$, 0.009, B), or combined plasma DNA fractions ($P = 0.004$, 0.006, C). After adjustment by the Holm–Bonferroni method the correlations with the combined plasma DNA fractions still remained significant (as indicated by * in Table 2). In patients who harbored LOH and retention of heterozygosity at the marker D12S1725 in their plasma, the mean overall survival periods were 43 and 55 months (95% confidence interval (CI): 27–59 and 52–59) for high-molecular weight DNA (Fig. 5A), 41 and 55 months (95% CI: 29–53 and 51–59) for low-molecular weight DNA (Fig. 5B), and 42 and 57 months (95% CI: 31–54 and 53–61) for combined fractions (Fig. 5C), respectively. The prognostic value of the LOH incidence at D12S1725 was somewhat affected by the duration of the zoledronate treatment (high-molecular weight DNA, $P = 0.163$; low-molecular weight DNA, $P = 0.01$ and combined plasma DNA fractions $P = 0.009$). To date, the effectiveness of therapy in the SUCCESS study has still not been evaluated, therefore, its association with the LOH-positive markers could not be determined.

Besides, this LOH frequency was also associated with advanced tumor stage, tumor size, lymph node metastasis, and HER2 receptor status (Table 2). Therefore, we adjusted the significant correlation of this LOH incidence with the overall survival detected in the univariate analyses by conducting multivariate Cox regression analyses. After adjustment for prognostic clinical factors (Table 1), the LOH incidence at the marker D12S1725 only remained significant for overall survival in association with the positive HER2 status. However, the *P* values were much less significant for the low-molecular weight DNA ($P = 0.021$ vs. 0.009, multivariate vs. univariate analyses) and combined plasma DNA fractions ($P = 0.012$ vs. 0.006) than in the univariate analyses. Although we detected that ER-negative patients had a significantly shorter overall survival than ER-positive patients ($P = 0.0001$, Supplementary Fig. S1), there was no significant correlation of the overall survival with the LOH incidence at the marker D12S1725, as well as LOH at the other markers, in association with the ER status.

Discussion

In the current study we investigated the LOH incidence at 8 microsatellite markers mapping to known tumor suppressor genes in the plasma of breast cancer patients. For our retrospective study, we chose a patient cohort that was randomly selected from the SUCCESS study (19). Therefore, we cannot exclude that this patient selection may have introduced a bias, which is a common limitation for all translational cohort studies frequently conducted as part of larger clinical trials. Our findings showed numerous significant correlations between clinicopathologic risk factors of the patients and the LOH-positive markers D3S1605, D10S1765, D12S1725, D13S218, and D17S855. The high LOH frequencies detected at these markers were associated with more aggressive carcinomas, and in particular, LOH at D12S1725 mapping to the *cyclin D2* gene was associated with reduced survival of the breast cancer patients.

Furthermore, our extended DNA extraction of the flow-through derived from QIAamp mini columns showed detection of additional LOH, which could not be found in the nonextended method using the QIAamp DNA Mini kit commonly used by laboratories. The LOH detection rate was higher in the low- than in the high-molecular weight plasma DNA fraction and supports our previous studies showing that the screening of genetic alterations can be improved by the use of short DNA fragments (14). Moreover, the combination of both plasma DNA fractions showed that the LOH data could be further improved and thus, might have implications for practical plasma-based diagnostic and prognostic tests of cell-free DNA. The detection of LOH in one plasma DNA fraction and not in the other fraction can be explained by the presence of wild type DNA. The higher LOH frequency in the low-molecular weight DNA fraction alludes to the higher content of tumor-associated DNA in this fraction. It would be of interest to examine whether the marker-specific LOH patterns of the low- and high-molecular weight DNA fractions reflect those of the primary tumor. However, no tumor tissues have been collected in the SUCCESS study. To date, with the exception of our recent report (14), there is no further study measuring the LOH frequency on long and short DNA fragments. In this respect, Wang and colleagues showed that circulating mutated k-ras molecules were enriched in nucleosomal DNA fragments in serum of colorectal cancer patients using a column-based, modified Guanidine/Promega Resin method (21), which we adopted in the second step of our fractionation for the extraction of short DNA fragments. They suggested that a method, which can preferentially isolate small DNA molecules should be used, to enhance assay sensitivity for detection of somatic mutations or epigenetic modifications in circulating DNA (21). A further study measured the variation in length of soluble plasma DNA fragments by electron microscopy and indicated that a significant amount of this DNA is probably derived from apoptosis in neoplastic and/or normal cells (22). Moreover, abnormal proliferation of malignant and benign cells was proposed to be accompanied by an increase in apoptotic cell death and that small, fragmented DNA may accumulate in the blood circulation (23). These studies and our findings showing an enhancement of LOH rate in the low-molecular weight plasma DNA point up that tumor-specific plasma DNA seems to rather consist of short fragments.

Our statistical evaluations showed that the LOH incidence at the marker D12S1725 was associated with advanced tumor stage, increased tumor size, presence of lymph node metastases, and positive HER2 status. Moreover, our Cox regression analyses revealed that the overall survival of all patients with an increased LOH incidence at this marker was significantly more reduced than the overall survival of HER2-positive patients with this increased marker-specific incidence. Although ER-negative patients had a significantly shorter overall

survival than ER-positive patients, there was no significant correlation of the overall survival with the LOH incidence at the marker D12S1725, in association with the ER status, suggesting that LOH at this marker, mapping to *cyclin D2*, may be of prognostic value.

The D-type cyclins D1, D2, and D3 and their associated cyclin-dependent kinases are critical components in cell proliferation. Cyclin D2 is implicated in cell differentiation and its loss may cause deregulation of the G1/S checkpoint of the cell cycle. It has been reported that inactivation of cyclin D2 by promoter hypermethylation correlates with clinicopathologic features of tumor aggressiveness in breast cancer (24). As far as we know, there are no studies investigating the LOH incidence at this marker on cell-free DNA or tumor tissue of cancer patients. Our data show, for the first time, the relationship of LOH at D12S1725 with tumor load, tumor progression, for example, lymph node metastases, and poor prognosis, suggesting that the deregulation of the cell cycle plays a crucial role in this process. Moreover, we found a significant correlation of increased LOH frequency at this marker with positive HER2 status. It has been reported that HER2-overexpressing breast carcinomas are histologically undifferentiated, high-grade tumors with a high-proliferation rate and a clinically poor outcome (2). Our findings are consistent with the observation that increased genetic instability, which is reflected by LOH, has been found to be associated with the aggressive features of HER2-positive breast tumors (25). On the basis of the low number of triple negative patients with cancer in our cohort, statistical evaluations of marker-specific LOH in these carcinomas were not considered to be appropriate.

In HER2-positive tumors we also revealed frequent LOH events at the marker D17S855 mapping to *BRCA1* gene. The gene product of this tumor suppressor gene has been involved in a number of cellular processes including DNA repair and recombination, cell-cycle checkpoint control, chromatin remodeling, ubiquitination, and apoptosis (26). Deletions of BRCA1 have recently been implicated in local recurrence and bad prognosis (10, 27–29). Because repression of BRCA1 may cause increased genomic instability, its loss may contribute to the aggressive features of HER2-positive breast tumors.

Furthermore, we found that an increased LOH frequency at the marker D3S1605 mapping to *tazarotene-induced gene 1* (*TIG1*) correlated with advanced tumor stage, positive lymph node, and PR^+ status. As retinoid-inducible type II tumor suppressor gene, *TIG1* is induced by the synthetic retinoid tazarotene. TIG1 has been reported to inhibit growth and invasion of cancer cells. Silencing of *TIG1* promoter by hypermethylation is common in human cancers and was associated with worse clinical outcome (30, 31). Our findings give evidence that not only inactivation of TIG1 by promoter hypermethylation (32), but also loss of TIG1 by LOH may play a role in breast cancer and be involved in tumor progression and lymph node metastasis.

In addition, we detected an association between increased LOH frequency at the marker D10S1765 located on the chromosomal area of *PTEN* (*phosphatase and tensin homologue*) and increased tumor stage and size, indicating that the lipid and protein phosphatase activity of PTEN plays an essential role in the pathogenesis of this cancer. *PTEN* is a well-known tumor suppressor that inhibits cell proliferation and migration by antagonizing the phosphatidylinositol 3-kinase (PI3K) signaling pathway (33). In many primary and metastatic human tumors, *PTEN* is inactivated by mutations, deletions, or promoter hypermethylation (34, 35). Repression of PTEN results in the activation of the PI3K-Akt pathway, which gives rise to increased cell survival. The loss of PTEN expression may cause malignant transformation and be associated with invasiveness and metastasis of breast cancer (34). To date, LOH at the marker D10S1765 was investigated in other tumor entities, and its significance in regulating cell proliferation has been reported (36–38). Here, we show, for the first time that advanced breast tumors harbor DNA loss at the *PTEN* locus.

Finally, we found that the high LOH incidence at the marker D12S218 flanking the chromosomal region encoding for *retinoblastoma 1* (*RB1*) was associated with higher tumor stages, indicating that RB1 may be important in advanced breast carcinomas. It has been reported that *RB1* gene is inactivated in about 20% of breast carcinomas (39). Genetic aberrations of this region have been described to frequently occur in breast cancer and to associate with tumor progression, poor prognosis (10, 40), and especially with the inability of cells to differentiate (39). RB1 acts as a master regulator by interacting and controlling numerous proteins. It mediates cellular replication and regulates the cell-cycle initiating entry into the S-phase and inhibiting cell cycle progression (41).

Apart from the microsatellite analyses at the time of primary surgery, we also looked at the changes in the LOH pattern in serial plasma DNA samples taken before and after chemotherapy. Only 19% of LOH in the high- and 27% in the low-molecular weight plasma DNA were concordant before and after therapy, indicating considerable therapy-induced changes in LOH patterns. To date, several clinical studies have described microsatellite alterations in follow-up sera of patients with different cancer entities (42, 43). A similar observation has also been described in patients with small cell lung cancer (SCLC) patients. Following chemotherapy, changes in the LOH pattern on cell-free DNA were detected and associated with disease recurrence (44). The persistence of microsatellite alterations in plasma DNA of postmastectomy patients with breast cancer has been correlated with a poor prognosis (28). On the basis of the limited number of analyzed plasma DNA samples, we cannot give evidence on the clinical significance of altered LOH occurrence on cell-free DNA observed after chemotherapy in our present study. Changes in the LOH pattern after chemotherapy could indicate that the cell-free DNA might be primarily derived from circulating tumor cells or occult micrometastatic deposits being resistant to

systemic treatment. The half-life time of circulating DNA is rather short (9) and DNA released from the primary tumor should not be present in the peripheral blood after 6 months of adjuvant therapy. Thus, this difference in cellular sources releasing cell-free DNA at different time points may also explain the limited overlap of the marker-specific LOH patterns observed in our and other studies before and after chemotherapy. Comparative analysis of the genetics of circulating tumor cells and cell-free DNA in cancer patients are now feasible and they will shed more light into this important subject.

In conclusion, our study revealed that fractionation of plasma DNA in high- and low-molecular weight DNA improves the detection of cell-free tumor DNA in blood of breast cancer patients. The high LOH frequencies detected at the markers mapping to the known tumor suppressor genes *TIG1*, *PTEN*, *cyclin D2*, *RB1*, and *BRCA1* were associated with more aggressive HER2-positive carcinomas, higher rate of lymph node metastases, and reduced survival, indicating that the improved detection of circulating tumor DNA might provide clinically relevant information on the variable biology of breast cancer. Currently, we try to improve our fractionation method to precisely determine the sizes of DNA fragments in both plasma DNA fractions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H. Schwarzenbach, W.J. Janni, B. Rack, K. Pantel
Development of methodology: H. Schwarzenbach
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W.J. Janni, B. Rack, K. Pantel
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Schwarzenbach, J. Kropidlowski, W.J. Janni, K. Pantel
Writing, review, and/or revision of the manuscript: H. Schwarzenbach, W.J. Janni, B. Rack, K. Pantel
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Rack
Study supervision: H. Schwarzenbach, K. Pantel
Laboratroy analysis of LOH: C. Eichelser
Patient and Biomaterial Recruitment: W.J. Janni

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