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Detection of occult HER2 mRNA-positive tumor cells in the peripheral blood of patients with operable breast cancer: evaluation of their prognostic relevance

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Abstract To evaluate whether HER2 mRNA could be used as a marker of circulating tumor cells (CTCs) in women with operable breast cancer. A nested RT-PCR assay was developed and used for the detection of HER2 mRNA-positive CTCs. Blood from 216 women with early breast cancer obtained before adjuvant treatment was tested for HER2 mRNA-positive cells to assess their prognostic value. Nested RT-PCR for HER2 mRNA showed high sensitivity whereas no HER2 mRNA-positive cells could be identified in the blood of healthy donors. HER2 mRNA-positive CTCs were detected in 53 (24.5%) of 216 patients and HER2 mRNA detection was associated with reduced disease-free survival (DFS; $P < 0.0001$) and overall survival (OS; $P = 0.004$). In multivariate analysis, detection of HER2

mRNA-positive CTCs emerged as independent prognostic factor for DFS ($P = 0.0001$) and OS ($P = 0.003$). HER2 mRNA could be a valuable prognostic marker for the detection of CTCs in early breast cancer patients.

Keywords HER2 mRNA · Breast cancer · Circulating tumor cells · Prognosis

Introduction

Breast cancer is considered a systemic disease because early tumor cell dissemination may occur even in patients with small tumors; using immunocytochemistry or molecular techniques, several investigators have shown that epithelial cells can be identified in the bone marrow (disseminated tumor cells; DTCs) aspirates or the peripheral blood (circulating tumor cells; CTCs) of otherwise metastases-free patients with breast cancer [1]. The detection of occult tumor cells either in the bone marrow or the peripheral blood has been demonstrated to represent an independent prognostic factor for disease relapse and reduced survival [2–6]. Indeed, a meta-analysis involving 4,703 patients with stage I-III breast cancer confirmed the independent prognostic value of DTCs [7].

Several markers, corresponding to molecules encoded by genes that are thought to be tissue specific and expressed on epithelial but not on hematopoietic cells, have been used to detect occult tumor cells in the bone marrow or the peripheral blood of patients with breast cancer. Among these markers are cytokeratin-19 (CK19), mammaglobin, maspin, and carcinoembryonic antigen (CEA) [2–5, 8–11].

Immunophenotyping of DTCs and CTCs revealed that they often express the HER2/neu (p185-erbB2) oncoprotein

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[12–14]. The HER2/neu oncoprotein is a transmembrane glycoprotein receptor sharing sequence homology with the epidermal growth factor receptor [15, 16]. Amplification of HER2/neu, occurring in approximately 15–30% of human primary breast cancers, is associated with a higher probability of disease relapse [17, 18]. Braun et al. [14] using a double labeling assay showed that 60% of the studied patients had DTCs which co-expressed the cytokeratin-18 (CK-18) and HER2/neu molecules; the detection of these CK-18+/HER2+ cells was an independent prognostic factor for overall survival. A more recent study in 35 patients with stage I–III breast cancer showed the immunocytochemical detection of cytokeratin+/HER2+CTCs in 48.6% of the patients; the detection of these double-positive CTCs was also correlated with a significantly decreased relapse-free and overall survival [19].

There are only a few data in the literature concerning the value of HER2/neu oncoprotein as a marker of occult tumor cells in patients with breast cancer. A qualitative nested reverse transcriptase-polymerase chain reaction (RT-PCR) assay for the detection of HER2 mRNA has already been described [20, 21]. In the present study we evaluated a nested RT-PCR assay to detect HER2 mRNA-positive occult tumor cells in the peripheral blood (CTCs) of patients with operable (stage I and II) breast cancer; our findings demonstrate that the detection of HER2 mRNA-positive CTCs represents an independent prognostic factor associated with decreased disease-free interval and overall survival.

Methods

Cell lines

The human mammary carcinoma cell lines MCF-7, T47D, MDA-415 and SKBR3, as well as the COLO-205 colon adenocarcinoma cell line and ARH-77 myeloma cell line (all obtained from the American Type Culture Collection, Rockville, MD) were used; the MDA-415 cells do not express HER2/neu, the SKBR3 cells highly express the HER2/neu molecule while the MCF-7 and T47D cells present an intermediate expression of HER2/neu. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco Life Sciences, BRL, Grand Island, NY, USA) supplemented with 10% bovine fetal serum (Gibco), L-glutamine 2 mmol (Sigma Chemical Company, St. Louis, MI, USA) and pyruvate 1 mmol/l (Sigma). Cell cultures were maintained in 5% CO₂ air, and cells grown in monolayer were harvested by washing the dishes once with phosphate-buffered saline (PBS), pH 7.3, and then incubating the cells with PBS containing EDTA 0.53 mmol/l and 0.05% trypsin (Gibco) for 10–15 min at 37°C. The cells were washed with PBS and

then passed through 25-gauge 5/8 needles to dissociate them. Cells were counted and viability was assessed by trypan blue dye exclusion.

Patients and clinical samples

Peripheral blood (20 ml in EDTA) was obtained from: (i) 61 patients with stage III-IV breast cancer; (ii) 20 patients with operable (Astler-Coller stages B₂ and C) colorectal cancer; (iii) 31 healthy female blood donors, (iv) 12 women operated for a benign breast fibroadenoma and (v) 216 patients with operable (stages I and II) breast cancer approximately 3–5 weeks after the surgical procedure and before the initiation of any systemic (adjuvant chemotherapy or hormonotherapy) or loco-regional (adjuvant radiation) treatment. The stage of disease was determined according to the tumor-node-metastasis classification of the Union Internationale contre le Cancer and independent of the results of the RT-PCR assay. All patients had the primary breast tumor completely resected and dissection of level I and II axillary lymph nodes. In all patients immunohistochemical analysis of tumor tissue for the expression of estrogen (ER) and progesterone (PR) receptors as well as for HER2 molecule was performed according to standard techniques. Immunohistochemical detection of HER2 was performed using the CB11 monoclonal antibody (NCL-CB11, Novocastra Lab., London, UK) and scoring was based on the criteria recommended by DACO A/S (0–3 + scale). Only membrane staining intensity was taken into account. Patients with breast-conserving surgery received radiation therapy and patients with ER/PR-positive tumors received tamoxifen 20 mg/day for 5 years. All patients received adjuvant chemotherapy with 5-fluorouracil/epirubicin/cyclophosphamide (FEC; *n* = 111), sequential docetaxel and epirubicin/cyclophosphamide (T/EC; *n* = 61), cyclophosphamide/methotrexate/5-fluorouracil (CMF; *n* = 33) and other regimens (*n* = 11). No patient received adjuvant Herceptin since all patients were enrolled before the results of the adjuvant Herceptin trials were reported. Before the initiation of adjuvant treatment, the baseline diagnostic evaluation to exclude the presence of distant metastases included plain chest radiography, mammography, ultrasonography of the abdomen, computed tomography of the thorax and abdomen if indicated and whole body bone scan. No patient showed evidence of distant metastases at the time of enrollment onto the study. All patients were regularly followed every three months for the first 2 years, every 6 months for the three subsequent years and yearly thereafter. All patients gave their written informed consent to participate in the study, which was approved by the Ethics and Scientific Committees of our Institution.

All blood samples were obtained at the middle of vein puncture after the first 5 ml of blood were discarded. This

precaution was undertaken in order to avoid contamination of blood with epithelial cells during sample collection. Peripheral blood samples were diluted with PBS (vol/vol) and cells were dissociated by passing them through 25-gauge 5/8 needles. Peripheral blood mononuclear cells (PBMCs) were obtained by gradient density centrifugation using Ficoll-Hypaque 1,077 (Sigma) at 1,200 g for 30 min at 4°C. The interface cells were carefully collected, washed twice with 50 ml of sterile PBS and pelleted. Cell pellets were kept at –80°C until RNA extraction. Total RNA isolation was performed by using Trizol LS reagent (Gibco) according to the manufacturer's instructions. All RNA preparations and handling steps took place in a laminar flow hood, under RNase-free conditions. The isolated RNA was dissolved in diethylpyrocarbonate-treated water and stored at –80°C until used. RNA concentration was determined by absorbance readings at 260 nm with the Hitachi UV-VIS (U-2000) spectrophotometer (Tokyo, Japan). RNA integrity was tested by PCR amplification of the β -actin house-keeping gene. RNA extracted from breast cancer cell lines (MCF-7 and SKBR3) and COLO 205 cell line was used as positive and negative controls, respectively.

Nested reverse transcription-polymerase chain reaction (RT-PCR)

A previously described nested RT-PCR assay for the detection of HER2 mRNA [21] with minor modifications was used [22]. Reverse transcription of RNA was carried out with the Thermoscript RT-PCR system (Invitrogen, Paisley, UK). cDNA was synthesized according to the manufacturer's instructions. Two different PCR reactions, with the respective negative controls, were performed with each sample in order to amplify fragments of HER2 and β -actin. The sequences of primers used for HER2 (synthesized by Gencet, Paris, France) were as follows: 5'TCCTCC TCGCCCTCTTGC3' (sense Her-2-A); 5'GCGGGTCTCC ATTGTCTA3' (antisense Her-2-B); 5'AGCCGCGAGCA CCCAAGT3' (sense Her-2-C); 5'TTGGTGGCAGGTA GGTGAGTT3' (antisense Her-2-D). To amplify cDNA, 2 μ l were subjected to first PCR in 20 μ l buffer [10 mM of HCL, buffer (pH 8.3), 50 mM KCL and 2.5 mM MgCl₂] containing 1 mM deoxynucleotide triphosphate, 3 mM of primers (Her-2-A and Her-2-B) and 2.5 units platinum Taq DNA polymerase (Invitrogen). For the second round of amplification (nested RT-PCR) a 2 μ l aliquot of first PCR product was added to the same PCR buffer with 1 mM deoxynucleotide triphosphate, 3 mM of primers (Her-2-C and Her-2-D) and 2.5 units platinum Taq DNA polymerase. The nested RT-PCR was performed using a modified touchdown program as previously described [22]. All PCR products were electrophoresed in agarose 2% gel, stained with ethidium bromide and photographed under UV

conditions. In order to determine the sensitivity of the assay, MCF-7, T47D and SKBR3 cells were mixed with normal PBMCs in a cell ratio ranging from 1:10 to 1:10⁶, and total RNA was extracted from these cell dilutions and tested for HER2 mRNA by nested RT-PCR.

Double staining for cytokeratin and HER2 expression and confocal laser scanning microscopy

Alexa Fluor anti-rabbit IgG and FITC anti-mouse IgG were obtained from Molecular Probes, Inc. (Eugene, Oregon, USA). Polyclonal anti-pan-cytokeratin antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and anti-HER2 monoclonal antibody from Oncogene (Calbiochem, Germany). For morphological studies by confocal laser microscopy, cytopspins of mononuclear cells from patients with breast cancer were fixed with cold acetone:methanol 9:1 (vol/vol) for 20 min and stained for cytokeratins with polyclonal anti-rabbit antibody and for HER2 with monoclonal anti-mouse antibody for 45 min each. Subsequently, cells were incubated with secondary antibodies, Alexa Fluor anti-rabbit IgG and FITC-anti-mouse IgG for 45 min each. Slides were analyzed using a confocal laser scanning microscopy module (Leica, Lasertechnik, Heidelberg, Germany).

Fluorescence in situ hybridization for HER2

Cytopspins with PBMCs (5×10^5 cells/slide) were prepared and stored at –70°C until used. Then, frozen slides were left at room temperature for 15 min, immersed in 70% acetic acid for 30 s, washed and dehydrated in ETOH, immersed in cold acetone for 2 min and air dried. Thereafter, they were placed in 2 \times SSC solution (pH 5.3) for 2 min at 73°C. Proteolysis with pepsin was performed for 5 min at 37°C and, the slides were placed in 2 \times SSC solution for 1 min at room temperature with post fixation in formalin buffer solution for 5 min and in 2 \times SSC for 1 min and dehydrated in ETOH. For hybridization the PathVysion HER2 DNA probe (Vysis, Downers, IL, USA) was used at 85°C for 2 min and overnight at 37°C. The next morning, slides were placed in the post-hybridization wash buffer for 2 min at 72°C, 10 μ l of DAPI were added and the covered slides were stored at –20°C. The microscopic examination was performed using an Axioplane 2 Nikon microscope with Vysis filters and the images were processed with the Metasystem's ISIS FISH imaging system (Alltussheim, Germany). For HER2/neu probe we used single-band filters for orange, green and DAPI. Cells with more than four orange signals or a ratio of HER2/neu orange signals to CEP 17 green signals >2 in cases of aneuploidy were considered as FISH HER2-positive.

Statistical analysis

The main tools of analysis were logistic regression and the Cox proportional hazards model [23] for outcomes related to point events and time variables, respectively. To select those factors with an independent significant influence on outcomes, both analyses were carried out in a stepwise (unconditional backward) fashion [23]. Before the application of these methods, univariate analyses were performed for a preliminary exploration of marked associations. Univariate analyses included contingency tables, *t* or Mann-Whitney *U* tests, log-rank tests, and simple Cox regression analyses [23].

Results

Sensitivity and specificity of nested PCR for HER2 mRNA+ cells in the peripheral blood

In preliminary experiments, RNA extracted from the MCF-7, T47D, SKBR3, MDA-415, ARH-77 and COLO-205 cells was amplified by nested PCR using the set of HER2 primers as described in Materials and Methods. The MCF-7, T47D and SKBR3 cell lines were consistently positive (amplified product of 147 bp; Fig. 1, lanes 1–3), whereas the MDA-415, ARH-77 and COLO-205 were negative (Fig. 1, lanes 4–6); in addition, no HER2 amplified transcripts could be detected by nested RT-PCR using mRNA from SKBR3 amplified in the absence of Taq polymerase (Fig. 1, lane 7) as well as from genomic DNA (Fig. 1, lane 8), indicating that any contaminating DNA would not amplify using the above mentioned pair of primers.

The sensitivity of the detection of HER2 mRNA-positive cells was evaluated by mixing MCF-7 or SKBR3 cells with PBMCs from healthy blood donors in a cell ratio ranging from 0: 10^6 to 10^6 : 0 cells, which mimics the clinical setting for the detection of occult tumor cells in

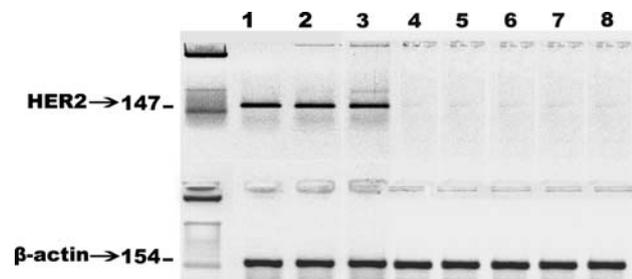


Fig. 1 Nested RT-PCR from total RNA of MCF-7 (lane 1), T47D (lane 2), SKBR3 (lane 3), MDA-415 (lane 4), ARH-77 (lane 5), COLO-205 (lane 6) cells lines, from SKBR3 cells amplified in the absence of Taq polymerase (lane 7) and from SKBR3 genomic DNA (lane 8). A standard β -actin RT-PCR assay was also used to demonstrate the presence of intact RNA

patients' peripheral blood. A representative experiment using the SKBR3 cell line showing a dose-dependent 147 bp signal corresponding to the HER2 mRNA, is presented in Fig. 2; conversely, the 154 bp signal, which corresponds to the β -actin gene, gave the same intensity in all dilutions (Fig. 2). In 3 of 3 identical experiments, this assay could detect 1 MCF-7 cell among 10^5 and 1 SKBR3 cell among 10^6 normal PBMCs.

No HER2 mRNA-positive cells could be detected in the peripheral blood of 31 healthy female blood donors, 12 patients operated for benign breast fibroadenomas as well as 20 patients with colorectal cancer; conversely, HER2 mRNA-positive cells could be detected in the blood of 18 out of 61 (29.5%) patients with advanced breast cancer. All samples obtained from healthy female blood donors, patients with colorectal cancer and patients with benign fibroadenomas or malignant breast tumors were positive for β -actin mRNA when tested with RT-PCR, indicating the presence of intact RNA and successful first-strand cDNA preparation.

Detection of peripheral blood HER2-positive cells by immunofluorescence

The presence of HER2(+) cells was further investigated by double staining experiments of cytospin smears, prepared from PBMCs of HER2 mRNA-positive patients, using anti-cytokeratins and anti-HER2 antibodies and confocal laser microscopy analysis. Figure 3 demonstrates representative CK- and HER2-positive cells (panel a and b, respectively) as well as double-stained CK⁺/HER2⁻ and CK⁺/HER2⁺ cells (panel C and D, respectively); as it was expected, the HER2 protein was localized in the cellular membrane while cytokeratin filaments were distributed all over the cytoplasm. HER2-positive cells could be detected by immunostaining in 15 (33%) of 45 patients; those included 14 of 28 cytokeratin-positive and one of 17 cytokeratin-negative patients. However, the expression of HER2 on

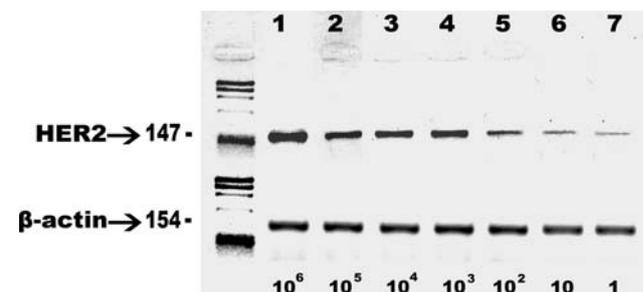


Fig. 2 Dose-response nested RT-PCR for HER2 mRNA. Total RNA obtained from serial dilutions of SKBR3 and normal PBMCs ($10^6/1-1/10^6$ cells) were analyzed by nested RT-PCR for HER 2 and β -actin mRNA transcripts

CK-positive cells was heterogeneous; in some patients all cytokeratin-positive cells were also HER2-positive whereas in others only a proportion of cytokeratin-positive cells co-expressed the HER2 molecule. In order to further characterize the HER2-expressing cells, peripheral blood smears from 5 patients, who had detectable HER2 mRNA-positive cells, were analyzed by FISH for HER2 gene amplification. A typical FISH HER2-positive case demonstrating gene amplification with a HER2 to CEP17 ratio >4 is shown in Fig. 4.

Detection of peripheral blood HER2 mRNA-positive CTCs in patients with operable breast cancer

Table 1 indicates the clinical characteristics of the 216 patients with stage I-II breast cancer according to the presence of HER2 mRNA-positive cells before the initiation of any systemic adjuvant treatment. The median age of the patients was 55 years and most of them (62.5%) were post-menopausal. One hundred and four (48.1%) patients had histological grade III tumors, measuring ≥ 2 cm (in 79.4% of them) while 141 (65.3%) presented lymph node

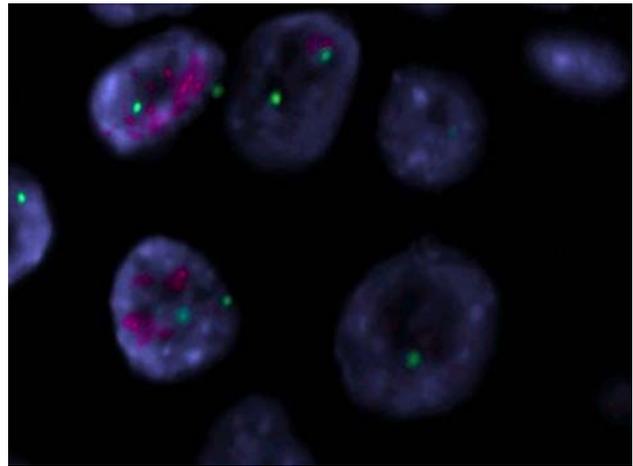
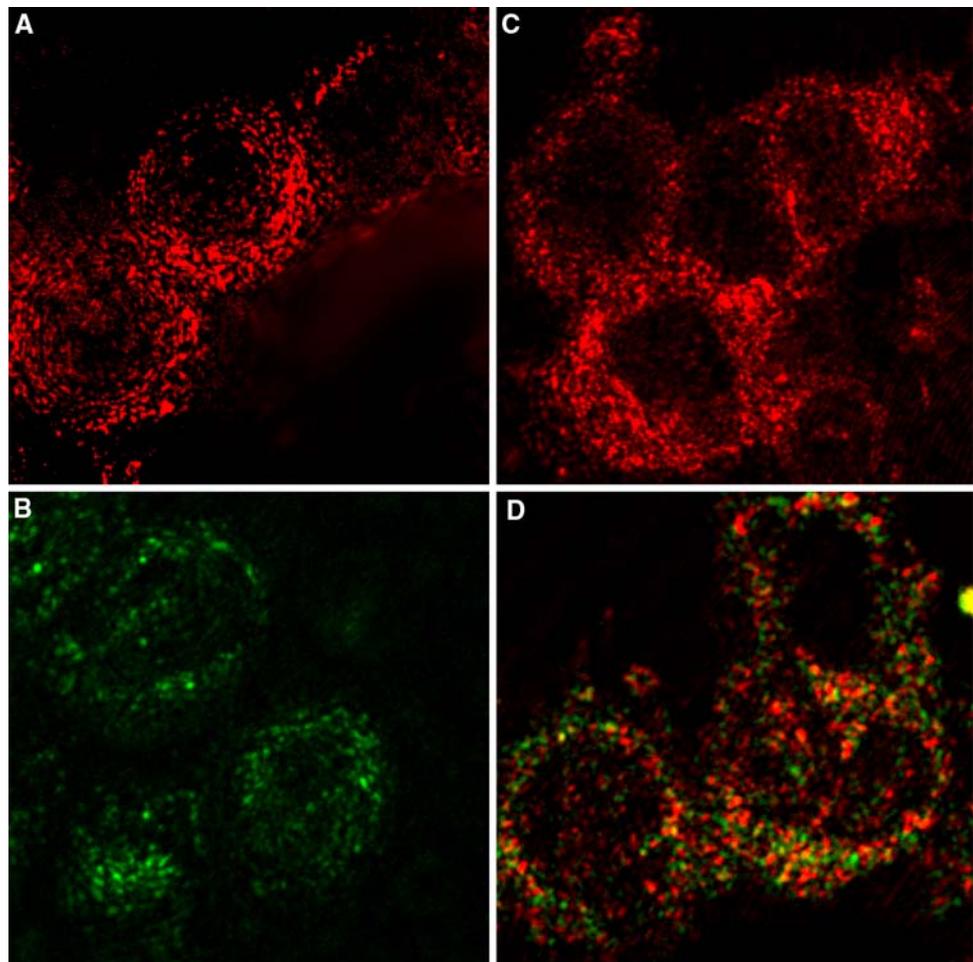


Fig. 4 Two breast cancer cells with HER2/neu gene copy amplification. Red dots represent the HER2/neu signal and green the CEP 17. The HER2 to CEP17 ratio is >4

involvement; in addition, ER-positive and HER2-positive (3+) tumors had 126 (60.3%) and 24 (11.1%) of the patients, respectively (Table 1). The median follow-up time for the entire group of patients was 78 months (range, 5–108).

Fig. 3 Confocal laser microscopy analysis of PBMCS cytospin smears prepared from breast cancer patients using anti-cytokeratins with Alexa Fluor (red colour) and anti-HER2 antibodies with FITC (green colour) **a** CK+ cells stained with anti-cytokeratin antibody ($\times 600$), **b** HER2+ cells stained with anti-HER2 antibody ($\times 600$), **c** Double stained CK+/HER2– cells ($\times 600$), **d** Double stained CK+/HER2+ cells ($\times 600$)



Circulating HER2 mRNA-positive cells could be detected in 53 (24.5%) out of 216 patients. In univariate analyses, there was no statistically significant association between the detection of HER2 mRNA-positive cells and the patients' menstrual status, stage of the disease, size and differentiation grade of the tumor, number of involved lymph nodes, estrogen or progesterone positivity and HER2 status of the primary tumor (Table 1).

Clinical relevance of the detection of peripheral blood HER2 mRNA-positive CTCs in patients with early breast cancer

Relapse

During the follow-up period, 51 (23.6%) patients developed distant metastases. The incidence of clinical relapses

was significantly higher in patients with detectable HER2-positive CTCs compared with those without before the initiation of any systemic treatment (49.1 vs. 15.3%; $P = 0.001$) (Table 2). The median disease-free interval (DFI) for patients with detectable HER2 mRNA-positive CTCs was 81 months (range, 7–104) whereas it has not been reached (range, 5–100 months) for patients without HER2 mRNA-positive CTCs (log-rank test; $P < 0.0001$) (Fig. 5a). Univariate analysis including standard clinicopathologic parameters demonstrated that the presence of HER2 mRNA-positive CTCs [Hazard ratio (HR): 3.717, 95% CI 2.15–6.44; $P < 0.0001$], the high histology grade (HR: 2.254, 95% CI 1.25–4.08; $P = 0.007$), ≥ 4 involved axillary lymph nodes (HR: 2.292, 95% CI 1.32–3.99; $P = 0.003$) were significantly associated with decreased DFI (Table 3). Multivariate analysis demonstrated that the presence of HER2 mRNA-positive CTCs, (HR: 4.045, 95%

Table 1 Patient characteristics according to the detection of HER mRNA-positive CTCs before the initiation of adjuvant therapy

Clinicopathologic parameter	All patients (<i>n</i> = 216) <i>n</i> (%)	Pre-chemotherapy		<i>P</i> -value
		HER2 mRNA ⁺ (<i>n</i> = 53) <i>n</i> (%)	HER2 mRNA ⁻ (<i>n</i> = 163) <i>n</i> (%)	
Age (median, range)	55 (28–79)	55 (28–74)	55 (30–79)	0.866
Menopausal status				
Premenopausal	81 (37.5)	21 (39.6)	60 (36.8)	0.745
Postmenopausal	135 (62.5)	32 (60.4)	103 (63.2)	
Disease stage				
I	68 (31.5)	14 (26.4)	54 (33.1)	0.399
II	148 (68.5)	39 (73.6)	109 (66.8)	
Tumor size				
<2.0 cm	44 (20.6)	7 (13.2)	37 (22.7)	0.170
≥ 2.0 cm	172 (79.4)	46 (86.8)	126 (77.3)	
Histology grade				
I/II	112 (51.8)	26 (49.1)	86 (52.8)	0.747
III	104 (48.1)	27 (50.9)	77 (47.2)	
Axillary lymph nodes				
0–3	153 (70.8)	38 (71.7)	115 (70.6)	0.999
≥ 4	63 (29.2)	15 (28.3)	48 (29.4)	
Estrogen receptor				
Negative	83 (38.4)	23 (43.4)	60 (36.8)	0.514
Positive	126 (60.3)	29 (54.7)	97 (59.5)	
Unknown	7 (3.2)	1 (1.9)	6 (3.7)	
Progesteron receptor				
Negative	132 (61.1)	30 (56.6)	102 (62.6)	0.504
Positive	76 (35.2)	21 (39.6)	55 (33.7)	
Unknown	8 (3.7)	2 (3.8)	6 (3.7)	
HER2/neu expression on primary tumor				
Negative (0–2 ⁺)	188 (87.0)	47 (88.7)	141 (86.5)	0.803
Positive (3 ⁺)	24 (11.1)	5 (9.4)	19 (11.7)	
Unknown	4 (1.9)	1 (1.9)	3 (1.8)	

CI 2.28–7.16; $P = 0.0001$), 4 or more involved axillary lymph nodes (HR: 5.743, 95% CI 2.17–15.19; $P = 0.0004$) and high histopathology grade (HR: 4.246, 95% CI 1.78–10.14; $P = 0.001$) emerged as independent factors associated with reduced DFS (Table 4).

Survival

During the follow-up period, 30 (13.8%) patients died because of disease progression. The incidence of death was 26.4% (14 out of 53 patients) and 9.8% (16 out of 163 patients) in patients with and without detectable HER2 mRNA-positive CTCs, respectively ($P = 0.005$) (Table 2). The median overall survival times for patients with and without detectable HER2 mRNA-positive CTCs have not been reached (range, 10–106) and (range, 5–108), respectively (log-rank test, $P = 0.004$; Fig. 5b). As shown in Table 3, the univariate analysis revealed that the detection of HER2 mRNA-positive CTCs (HR: 2.740; 95% CI 1.34–5.61; $P = 0.006$) and the involvement of four or more axillary lymph nodes (HR: 2.926; 95% CI, 1.43–6.00; $P = 0.003$) were significantly associated with decreased overall survival. Multivariate analysis revealed that detection of peripheral blood HER2 mRNA-positive CTCs (HR: 2.761, 95% CI: 1.35–5.66; $P = 0.003$) and involvement of ≥ 4 axillary lymph nodes (HR: 2.947, 95% CI: 1.44–6.04; $P = 0.006$) were independent prognostic factors associated with a decreased overall survival (Table 4).

Discussion

The early tumor cell dissemination away from the primary tumor as well as the failure of adjuvant treatment to eradicate them may account for the high risk of relapse in patients with operable solid tumors [1]. Several studies have shown that the detection of bone marrow (DTCs) [2–4] or peripheral blood [CTCs; 5–6] tumor cells is associated with an increased risk of relapse and reduced survival in patients with stage I/III breast cancer. In most of the studies, the detection of CTCs was performed using CK19 as a marker of epithelial cells. The development of a multimarker approach for the detection of CTCs may increase the specificity and the sensitivity of this procedure allowing for a more accurate identification of those patients with early breast cancer who are at increased risk for relapse and death.

Table 2 Incidence of clinical relapses and disease-related deaths according to the HER2 mRNA-positive cells before the initiation of adjuvant chemotherapy

HER2 mRNA status	Relapse <i>n</i> (%)	Non-relapse <i>n</i> (%)	<i>P</i> -value	Dead <i>n</i> (%)	Alive <i>n</i> (%)	<i>P</i> -value
Negative (<i>n</i> = 163)	25 (15.3)	138 (84.7)	0.001	16 (9.8)	147 (90.2)	0.005
Positive (<i>n</i> = 53)	26 (49.1)	27 (50.9)		14 (26.4)	39 (73.6)	

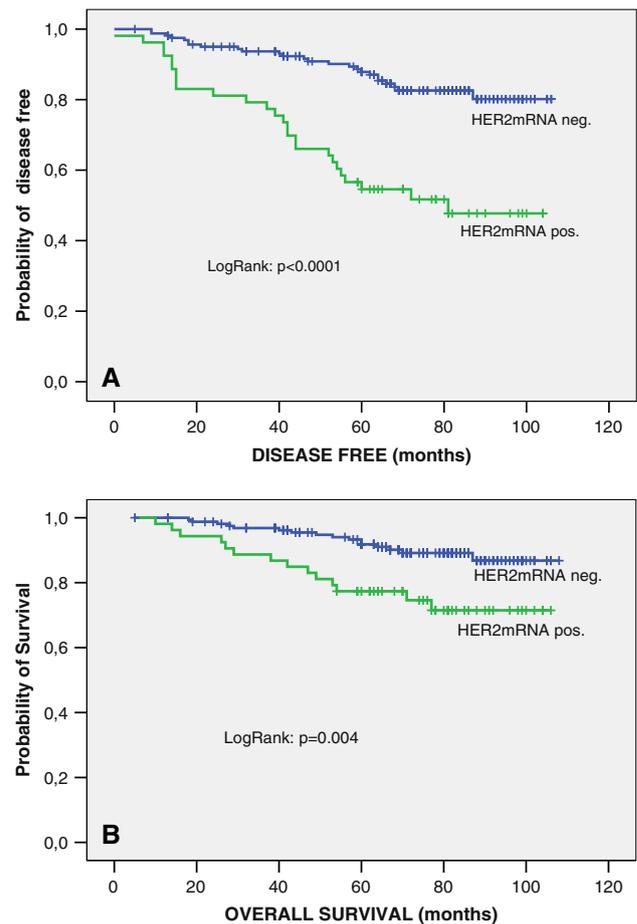


Fig. 5 Disease-free survival (a) and overall survival (b) of patients with early stage breast cancer according to the detection of HER2 mRNA-positive CTCs before adjuvant chemotherapy

The HER2 oncoprotein could be a useful marker for the detection of CTCs in patients with breast cancer.

Indeed, previous studies have demonstrated that DTCs [13, 14] and CTCs [12, 19] frequently express the HER2 oncoprotein. Using a double immunocytochemical staining technique, Solomayer et al. detected HER2 expression on DTCs in 20 out of 137 (14%) breast cancer patients [13] while Braun et al. found co-expression of HER2 in 31 out of 52 (60%) women with CK18-positive DTCs [14]. Moreover, Brandt et al. found cytokeratin/c-erbB-2 positive clustered CTCs in 19 out of 46 (41%) breast cancer patients [12], and Wulfing et al. reported immunocytochemical detection of cytokeratin+/HER2+CTCs in 48.6% of patients [19].

Table 3 Univariate analysis for DFS and overall survival of patients with early breast cancer

Parameter	Hazard ratio	95% CI	P-value
<i>DFS</i>			
Menopausal status (post vs. pre)	1.399	0.774–2.527	0.266
Disease stage (II vs. I)	1.666	0.872–3.183	0.122
Tumor size (>2 vs. ≤2 cm)	2.191	0.934–5.136	0.071
Histology grade (III vs. I/II)	2.254	1.247–4.076	0.007
Axillary lymph nodes (≥4 vs. <4)	2.292	1.318–3.986	0.003
Estrogen receptors (negative vs. positive)	1.713	0.989–2.967	0.055
Progesteron receptors (negative vs. positive)	1.090	0.607–1.958	0.773
HER2 mRNA (positive vs. negative)	3.717	2.145–6.442	<0.0001
<i>Overall survival</i>			
Menopausal status (post vs. pre)	1.094	0.520–2.299	0.813
Disease stage (II vs. I)	2.534	0.970–6.623	0.058
Tumor size (>2 vs. ≤2 cm)	7.954	1.083–58.395	0.041
Histology grade (III vs. I/II)	1.559	0.737–3.297	0.246
Axillary lymph nodes (≥4 vs. <4)	2.926	1.427–6.001	0.003
Estrogen receptors (negative vs. positive)	1.965	0.959–4.028	0.065
Progesteron receptors (negative vs. positive)	1.488	0.659–3.362	0.339
HER2 mRNA (positive vs. negative)	2.740	1.337–5.614	0.006

Table 4 Multivariate analysis for DFS and overall survival of patients with early breast cancer

Parameter	Hazard ratio	95% CI	P-value
<i>DFS</i>			
HER2 mRNA pre-chemotherapy (positive)	4.045	2.285–7.161	0.0001
Axillary lymph nodes (≥4 positive)	5.743	2.171–15.195	0.0004
Histology grade (III)	4.246	1.777–10.143	0.001
<i>Overall survival</i>			
HER2 mRNA pre-chemotherapy (positive)	2.761	1.347–5.658	0.003
Axillary lymph nodes (≥4 positive)	2.947	1.437–6.045	0.006

In the present study we evaluated a nested RT-PCR assay to detect peripheral blood cells expressing HER2 mRNA. Specificity experiments demonstrated that the 147 bp HER2 mRNA transcripts could be identified in breast cancer cells expressing the HER2 molecule (MCF-7, T47D and SKBR3 cells) but not in breast cancer cells which do not express HER2 (MDA-415) or in tumor cells from a colorectal (Colo 205) and myeloma (ARH-77) cell line which, also, do not express HER2. The specificity of the used RT-PCR assay was further demonstrated by the observation that no HER2 mRNA-positive cells could be identified in female healthy blood donors, patients with benign fibroadenomas as well as in patients with colorectal cancer. In addition, the detection of circulating HER2 mRNA-positive cells was independent of the prior surgical excision of the primary tumor since they could be identified in 20% of patients who were enrolled in a neo-adjuvant chemotherapy program before the initiation of any systemic treatment (data not shown) suggesting that HER2 mRNA-positive CTCs were not shed into the circulation

during the manipulations for the surgical excision of the primary tumor but rather their detection reflects their potential to migrate through the blood vasculature. It is noteworthy that the sensitivity of the used nested RT-PCR assay was dependent on the cell line ($1/10^5$ and $1/10^6$ normal PBMC for the MCF-7 and SKBR3 cells, respectively) suggesting that the degree of HER2 expression may be an important limiting factor for the detection of HER2 mRNA-positive cells. These findings are in the same range of sensitivity as the RT-PCR assays detecting CK-19 mRNA-positive cells [5, 24].

Double staining of CTCs with anti-pancytokeratin and anti-HER2 antibodies demonstrated that 50% of the patients with CK-positive cells, also displayed HER2-positive cells and, practically, in all but one patient, circulating HER2-positive cells co-expressed cytokeratins. However, in some patients distinct cell populations expressing cytokeratins (CKs) only or CKs and HER2 could be detected. This finding is in agreement with the already described heterogeneity of circulating occult tumor

cells [12, 19]. In addition, FISH analysis showed that CTCs present HER2/neu gene amplification, indicating the malignant nature of cytokeratin-positive cells as already has been reported [22, 25].

Previous studies have demonstrated that expression of HER2 molecule on DTCs and CTCs was independent of its expression on the primary tumor cells [12–14, 19]; the absence of any statistical correlation between the expression of HER2 molecule on the primary tumor cells and the detection of circulating HER2 mRNA-positive cells in our study further confirms the above observations. The reasons for the discrepancy of HER2 status between occult tumor cells and primary tumor cells are not obvious. It has been proposed that these cells may represent an early disseminated subclone of HER2-positive cells of the primary tumor, which is microscopically undetectable because of their very low frequency. Previous studies have demonstrated that cytokeratin/HER2 double-positive clustered peripheral blood cells have a high potential for locomotion suggesting that they might be precursor cells responsible for the development of DTCs [12]; furthermore, HER2-positive CTCs have a growth and survival advantage [12]. In addition, it has been shown that HER2 gene amplification can be acquired during breast cancer progression [26]. This hypothesis is further supported by the recently reported 14% discordance of HER2 overexpression between the primary tumor and the corresponding metastases in patients with breast cancer [27]. Despite all the above evidence, our data should be interpreted with caution since different methods have been used to detect HER2 expression in primary tumor cells and circulating HER2 mRNA-positive CTCs.

Based on these data we evaluated the detection of HER2 mRNA as a marker of CTCs in patients with operable breast cancer. Our findings demonstrated that 24.5% of patients with stage I-II breast cancer had detectable HER2 mRNA-positive CTCs before the initiation of any systemic adjuvant treatment. Moreover, the detection of HER2 mRNA-positive CTCs was not associated with other important clinical or pathological tumor parameters (Table 3). This positivity rate for HER2-expressing CTCs is in accordance with previous reports using immunocytochemical detection methods [12, 19]. Moreover, the pooled analysis of 4,703 early breast cancer patients reported detection of DTCs in 30.6% of patients at the time of primary surgery [7] and in another study co-expression of HER2 was found in 60% of patients presenting CK-positive DTCs [14]. Therefore, taken together and despite the different detection method used in our study, the HER2 positivity rate is in general agreement with the literature. Interestingly, the detection of HER2 mRNA-positive CTCs revealed important prognostic information since it was associated with reduced DFS and overall survival;

moreover, multivariate analysis revealed that the detection of HER2 mRNA-positive CTCs was an independent prognostic factor for reduced DFS and overall survival (Table 4). These findings are in agreement with the prognostic value of the detection of HER2 mRNA-positive CTCs after the completion of adjuvant chemotherapy in the same patient cohort which has already been published [28].

At the present time, the probability of cure for patients with operable breast cancer may be increased by reducing the incidence of distant metastasis. Since chemotherapy cannot in many cases eliminate the disseminated [29] or/and the circulating [30] tumor cells, it is important to target these occult tumor cells by using novel therapeutic approaches, such as monoclonal antibodies or other drugs acting on specific molecular targets. In a previous study, the administration of trastuzumab, a humanized anti-HER2 monoclonal antibody, resulted in a significant decrease of the HER2-positive CTCs [22]. Therefore, prospective trials targeting the residual HER2 mRNA-positive occult tumor cells in patients with early stage breast cancer could demonstrate the importance of a “secondary” adjuvant therapeutic approach.

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