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Circulating HER-2 mRNA in the peripheral blood as a potential diagnostic and prognostic biomarker in females with breast cancer

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Abstract. Breast cancer is a prevalent malignant cancer worldwide, and a lack of defined biomarkers for early prognostication contributes to its high associated mortality rate, especially in human epidermal growth factor receptor 2 (HER-2)-positive breast cancer. In the present study, HER-2 mRNA levels in patients were detected prior to surgery and during neoadjuvant chemotherapy to explore its potential diagnostic and prognostic value. Blood samples were collected from 70 patients with breast cancer, including 50 HER-2-negative and 20 HER-2-positive patients, prior to and following surgery (postoperative, n=13; neoadjuvant chemotherapy, n=5); the control group included 35 samples from healthy individuals. The relative mRNA level of HER-2 in blood was determined by one-step reverse transcription-quantitative polymerase chain reaction. HER-2 expression curves of measurements taken during neoadjuvant chemotherapy were compared with the tumor size. A significant difference in the blood HER-2 mRNA level was observed between healthy women and patients with breast cancer (P<0.0001). A cutoff value of 1.512 was established for the circulating HER-2 level in healthy subjects based on the upper 95% confidence interval value of samples from the control group. The level of HER-2 mRNA in blood was associated with the HER-2 status, Ki-67 expression, and lymphovascular invasion in primary tumor tissue samples; however, there was no association with the lymph node status, tumor stage, tumor grade, tumor size, patient age, estrogen or progesterone receptor status of the primary tumor. HER-2 mRNA levels were associated with the response rate, as determined by primary tumor size, in patients who received neoadjuvant chemotherapy. In conclusion, baseline and early changes in peripheral blood HER-2 mRNA indicated that HER-2 mRNA may be a potential diagnostic biomarker for breast cancer and a prognostic marker for predicting the efficacy of neoadjuvant therapy.

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Introduction

Breast cancer is a significant health problem for women in China. According to the China Health Statistics Yearbook published in 2011, approximately 169,452 new patients with breast cancer were diagnosed and 44,908 breast cancer-associated mortalities occurred in China in 2008; furthermore, these numbers accounted for 12.2% of all newly diagnosed breast cancer cases and 9.6% of all mortalities from breast cancer worldwide (1,2). Therefore, it is important to identify predictive and prognostic factors for breast cancer, and to assess their potential impact for treatment selection. The discovery of human epidermal growth factor receptor 2 (HER-2)/neu gene amplification and its association with poor prognosis and an aggressive tumor phenotype has improved our understanding of the prognosis and therapeutic management of patients with breast cancer (3,4).

The HER-2/neu gene is an oncogene located on chromosome 17 that encodes HER-2/neu, a transmembrane glycoprotein with tyrosine kinase activity from the epidermal growth factor receptor family (5). In ~20% of patients with invasive breast cancer, the HER-2/neu gene is amplified or overexpressed (6,7). It can activate cellular signaling pathways such as PI3K-Akt and Ras-MAPK, leading to cell proliferation, growth, and survival (8). The amplification of HER-2 is associated with a poor prognosis, metastasis, chemoresistance, and an aggressive tumor phenotype (9). Trastuzumab, a recombinant humanized monoclonal antibody against HER-2, improves the survival of patients with HER-2-overexpressing tumors (10,11). However, in clinical practice, the definition of primary tumor HER-2 overexpression in breast cancer patients is controversial. In certain patients with primary trastuzumab resistance, HER-2 expression in the peripheral blood after primary tumor resection is difficult to detect. In rescue treatments for metastatic breast cancer, the anti-HER-2 therapies are ineffective in a proportion of the patients. In addition, biopsy results for certain types of metastasis are often unavailable,

and the HER-2 status cannot be determined. Furthermore, the use of anti-HER-2 treatments based on primary tumor HER-2 status is not adequate because of the treatment delay (12,13). Therefore, the identification of an indicator for the real-time monitoring of HER-2 status is urgently required.

In clinical practice, the main methods currently used to determine tissue HER-2 status are immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) (14). Only tumors with scores of 2+ or 3+ with a FISH ratio ≥2.0 are defined as HER-2-positive (15). In addition to determining HER-2 status in tissue specimens, there has been a high level of interest in liquid biopsy to determine the level of circulating HER-2, due to its accessibility and the possibility for the serial monitoring of the tumor response to therapy (16). The detection of HER-2 mRNA-positive circulating tumor cells (CTCs) in peripheral blood is considered a useful tool in the early diagnosis of breast cancer, and an independent prognostic factor for disease-free survival (DFS) (17,18). A number of previous studies have indicated that HER-2 mRNA in the peripheral blood of patients with breast cancer can be detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and that the detected levels were consistent with HER-2 status determined by IHC (19-21). To the best of our knowledge, a cutoff value for HER-2 mRNA as a marker of breast cancer has not been determined to date. Changes in the HER-2 mRNA level in peripheral blood may provide information for the selection of adequate therapeutic regimens, especially in patients exhibiting a poor response to chemotherapy.

In the present study, one-step RT-qPCR was used to detect circulating HER-2 mRNA, in order to determine its efficacy in indicating HER-2 expression status in breast cancer. The main aim of the present study was to determine the HER-2 mRNA status in the peripheral blood of patients with breast cancer prior to surgery and healthy individuals, to assess its potential diagnostic value in patients with breast cancer. For this purpose, we established an exact cutoff for HER-2 mRNA as a marker of breast cancer. In addition, the present study investigated whether the HER-2 mRNA level in the peripheral blood could predict the efficacy of neoadjuvant chemotherapy without trastuzumab.

Materials and methods

Patients. Peripheral blood was obtained from 70 patients with breast cancer without distant metastases, and 35 healthy control subjects (median age is 52 years, age range: 27 to 82 years, female, with no history of breast cancer at the Second Affiliated Hospital of Soochow University (Suzhou, China) between August 2016 and August 2017.All patients who participated in this study signed a document of informed consent. Study approval was obtained from the independent ethics committee at the Second Affiliated Hospital of Soochow University (Suzhou, China). The privacy of the patients involved was protected.

Patient characteristics. This study included 70 women with breast cancer, with a median age of 52 years (range: 27-82 years). The pathology type was invasive ductal carcinoma for all patients. The distribution of tumor sizes (T) was as follows: 42.9% (n=30) T1 (≤2 cm), 54.3% (n=38) T2 (>2 cm and <5 cm), and 2.8% (n=2) T3 (≥5 cm). Lymph node status was negative in 41.4% (n=29), positive in 50% (n=35), and unknown in 8.6% (n=6) of the patients. According to the World Health Organization grading system (22,23), 74.3% (n=52) of tumors were well differentiated (grade I) and/or moderately differentiated (grade II), 10% (n=7) were poorly differentiated (grade III), and 15.7% (n=11) were of unknown differentiation. None of the patients had distant metastases; 24.3% (n=17) had stage I cancer, 41.4% (n=29) had stage II, 21.4% (n=15) had stage III, and 12.9% (n=9) had an unknown cancer status. Clinical and pathological characteristics of the patients are listed in Table I. Among the 95 blood samples isolated from the patients with breast cancer, 70 (73.7%) were from preoperative patients, 13 (13.7%) were from postoperative patients without adjuvant therapy, and 12 (12.6%) were from patients receiving neoadjuvant therapy.

Neoadjuvant therapy. Three cycles of docetaxel (75 mg/m^2 on day 1 every 3 weeks), followed by three cycles of FEC (5'-fluorouracil 500 mg/m^2, epirubicin 75 mg/m^2, and cyclophosphamide 600 mg/m^2 on day 1 every 3 weeks).

MRI assessment. A total of 5 patients with confirmed breast cancer underwent MRI examinations at GE Signa Excite HD 3.0T scanner. All breast MRI scans were confirmed by two consultant radiologists, and where there was discordance the images were reviewed by a third consultant radiologist. The schedule of imaging was: Prior to antitumor treatment (time point zero; TP0), after three treatment cycles (TP3), and after six treatment cycles (TP6). However, due to the small sample of biopsy samples, the tissue sub-type could not be determined.

Sample collection. Peripheral blood (5 ml) was collected in vacuum blood collection tubes with EDTA (0.05 M). The whole blood samples were stored at 4˚C.

RNA isolation. Total RNA was collected using an RNA extraction kit for whole blood (Ezol kit, Suzhou GenePharma Co., Ltd.) according to the manufacturer's protocol. RNA purity was determined through the measurement of absorbance using a 96-well plate (Corning Incorporated, Corning, NY, USA) (A) at 260, 280, and 230 nm with NanoDrop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) (24). RNA concentration was determined from the A260. Qualifying samples were used to detect HER-2 expression by one-step RT-qPCR.

One-step RT-qPCR. HER-2 levels were measured using one-step HER-2 TaqMan RT-qPCR kits (Suzhou GenePharma Co., Ltd.), the fluorophore (SYBR Green) was purchased from Thermo Fisher Scientific, Inc. Reactions contained 2.5 μl 10x PCR buffer, 2.5 μl 5x RT buffer, 0.375 μl of each primer, 0.5 μl of each probe, 0.5 μl enzyme mix, and 8 μl blood RNA extract in 20 μl. Total RNA from MDA-MB-231 cells (from the American Type Culture Collection, Manassas, VA, USA) were used as the negative control. RT-qPCR cycling was performed on an ABI-Step One Plus system (Thermo Fisher Scientific, Inc.) as follows: 45˚C for 5 min, 95˚C for 30 sec, and 40 cycles of 5 sec at 95˚C and 50 sec at 62˚C. Fluorogenic signals were detected at the end of the annealing-extension steps. A threshold was automatically set and the threshold

Table I. Patient clinical and pathological characteristics.

	All patients		HER-2 mRNA positive		HER-2 mRNA negative		HER-2 value
Parameter	n	%	n	%	n	%	P-value
Patients enrolled	70	100%	22	31.4	48	68.6	
Age years (median) range)	52 (27-82)		51 (30-66)		53 (27-82)		0.131
Menopausal status							0.114
Premenopausal	36	51.4	12	17.1	24	34.3	
Postmenopausal	34	48.6	10	14.3	24	34.3	
Stage							0.367
I	17	24.3	9	12.9	8	11.4	
II	29	41.4	5	7.1	24	34.3	
III	15	21.4	5	7.1	10	14.3	
Unknown	9	12.9	3	4.3	6	8.6	
Tumor grade							0.666
I/II	52	74.3	16	22.9	36	51.4	
III	7	10.0	2	2.9	5	7.1	
Unknown	11	15.7	4	5.7	7	10.0	
Tumor size (cm)							0.663
1 (2≥T1)	30	42.9	10	14.3	20	28.6	
2 (2<T2<5)	38	54.3	11	15.7	27	38.6	
3 (5≤T3)	2	2.8	1	1.4	1	1.4	
Lymph node status							0.860
0	29	41.4	10	14.3	19	27.1	
1-3	20	28.6	5	7.1	15	21.4	
4-9	11	15.7	3	4.3	8	11.4	
≥10	4	5.7	2	2.9	2	2.9	
Unknown	6	8.6	2	2.9	4	5.7	
Lymphovascular Invasion							0.035[a]
No	37	52.9	9	12.9	28	40.0	
Yes	19	27.1	8	11.5	11	15.7	
Unknown	14	20.0	5	7.1	9	12.8	
Perineural Invasion							0.506
No	43	61.4	9	12.8	34	48.7	
Yes	7	10.0	4	5.7	3	4.3	
Unknown	20	28.6	9	12.8	11	15.7	
ER							0.526
Negative	21	30.0	5	7.1	16	22.9	
Positive	49	70.0	17	24.3	32	45.7	
PR							0.748
Negative	32	45.7	6	8.6	26	37.1	
Positive	38	54.3	16	22.9	22	31.4	
Ki-67							0.007[a]
≤14	16	22.9	5	7.1	11	15.7	
>14	54	77.1	17	24.3	37	52.9	
Sub-type							0.148
Luminal A	10	14.3	5	7.1	5	7.1	
Luminal B	39	55.7	12	17.1	27	38.6	
ERBB 2+	10	14.3	0	0.0	10	14.3	
Basal-like	11	15.7	5	7.1	6	8.6	
HER-2							0.039[a]
Negative	50	71.4	21	30.0	29	41.4	
Positive	20	28.6	1	1.4	19	21.7	

[a]P<0.05. HER-2, human epidermal growth factor receptor 2. HER-2, human epidermal growth factor receptor 2; ER, estrogen receptor; Progesterone receptor, PR; ERBB2, Erb-B2 Receptor Tyrosine Kinase 2.

cycle value (Cq) was determined (25,26). Two replicate assays within and between runs were performed. The sequences of the primers used for HER-2 were as follows: Forward, 5'-CCA GCTGGCTCTCACACTG-3'; and reverse, 5'-AGCCCTTAC ACATCGGAGAAC-3'; probe, 5'-FAM/AGGCCCGAGAGC GGTTGGTGT/BHQ1-3'. Sequences of the primers used for β-actin were as follows: Forward, 5'-GACCCAGATCATGTT TGAGACCTT-3'; and reverse, 5'-CCATCACGATGCCAG TGGTA-3'; probe, 5'-FAMCCATGTACGTTGCTATCCAGG CTGTGCBHQ1-3'.

Sensitivity evaluation of HER-2 mRNA RT-qPCR. To test the sensitivity of HER-2 detection by RT-qPCR on blood samples, the SKBR-3 cell line (ATCC), which expresses high levels of HER-2, was 10-fold serially diluted with PBS (pH 7.4) from $1x10^5$ cells/ml to 1 cell/ml using fluorescence-activated cell sorting (FACS; BD FACSARIA II; BD Biosciences, Franklin Lakes, NJ, USA) and spiked into 5 ml normal blood (27). Following RNA extraction, HER-2 mRNA RT-qPCR was performed in duplicate.

Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). Immunohistochemistry was performed according to previously described methods using a normal light microscope (magnification, x400) (28). Paraffin-embedded tissues from surgery were deparaffinized and pretreated in a microwave. The slides were incubated using monoclonal mouse anti-HER2 antibodies (1:200, Proteintech 60311-1-lg, China) for 1 h at 37˚C. After rinsing with PBS (pH 7.4), sections were treated with a horseradish peroxidase conjugated-goat-anti-mouse secondary antibody (1:2,000, Jackson ImmunoResearch, Inc., West Grove, PA, USA; cat no. 115-035-003) at room temperature for 1 h. Then, the slides were incubated with 3-diaminobenzidine solution for 20 min at room temperature. Patients with HER2+ breast cancer was diagnosed by FISH in the Second Affiliated Hospital of Soochow University as described previously (22).

Cutoff value determination. To determine the cutoff value for HER-2, a total of 35 normal blood and 70 blood samples from breast cancer patients prior to surgery were collected and sorted by FACS (27). MB-MDA-231 and SKBR-3 cells ($1x10^0$, $1x10^1$, $1x10^2$, $1x10^3$, and 10^4) were spiked into 5 ml normal blood. Samples were analyzed using the TaqMan HER-2 RT-qPCR kits (Suzhou GenePharma Co., Ltd). For each sample, Ct values for HER-2 were normalized to the Ct values of β-actin as an endogenous control to yield ΔCt data. For all of the 70 breast cancer blood samples and cell line-spiked samples, ΔCt values were normalized to the median ΔCt values of the 35 normal blood samples to obtain ΔΔCt data. HER-2 relative expression for each sample was calculated using the $2^{-\Delta\Delta Ct}$ formula (26,28).

Statistical analysis. Statistical analyses were performed using Statistical Package for Social Sciences software version 22 for Windows (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.01 (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test by using GraphPad Prism 6.01 (GraphPad Software, Inc., La Jolla, CA, USA) to compare the HER-2 mRNA levels in cell lines, peripheral blood and tissues. The Pearson χ^2 test was used to assess the associations between blood HER-2 status and clinical features. The Wilcoxon signed rank test was performed to examine the mean changes in paired samples. Receiver operating characteristic (ROC) curve analysis was performed to determine the relationship between the HER-2 mRNA level in blood samples, and the HER-2 FISH and IHC status of primary tumor tissues. Sensitivity, specificity, and Youden index values were calculated to assess the diagnostic performance of RT-qPCR for determining HER-2 status. HER-2 expression curves representative of measurements taken during neoadjuvant chemotherapy were compared with changes in tumor size. Cohen's Kappa coefficient and 95% confidence intervals (CI) were calculated to assess the agreement in HER-2 status between primary tumors and peripheral blood. All data are presented as the mean ± standard error. P<0.05 was considered to indicate a statistically significant difference.

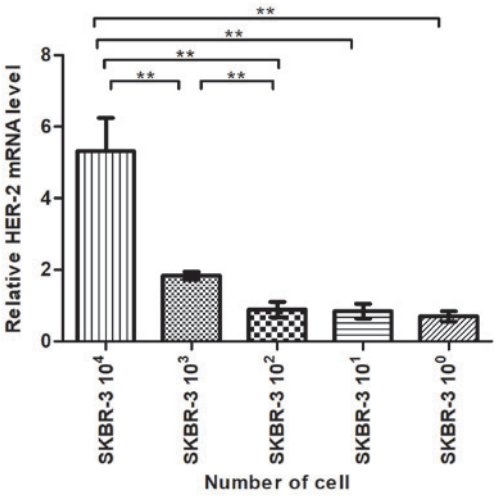

Figure 1. Evaluation of the HER-2 mRNA detection sensitivity of RT-qPCR. Relative expression values from serially diluted SKBR-3 cells are shown. The mean relative values were determined by RT-qPCR (one ANOVA, Tukey's Test, **P<0.01). RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Results

Sensitivity evaluation of RT-qPCR detection of HER-2 mRNA. The relative values obtained using serially diluted SKBR-3 cells are shown in Fig. 1. The mean relative RT-qPCR values obtained using $1x10^4$, $1x10^3$, $1x10^2$, $1x10^1$, and $1x10^0$ cells/ml were 5.32, 1.84, 0.89, 0.84, and 0.70, respectively; the results indicated that the relative Ct values of RT-qPCR decreased with a decreasing cell number. Therefore, it was determined that RT-qPCR demonstrated good sensitivity for the detection of HER-2 mRNA. Ct values from blank control reactions were negative for all experiments.

Peripheral blood HER-2 mRNA in preoperative patients and controls. Peripheral blood HER-2 mRNA status in samples from 70 preoperative patients and 35 healthy controls were determined by RT-qPCR. Tumor samples were HER-2-positive

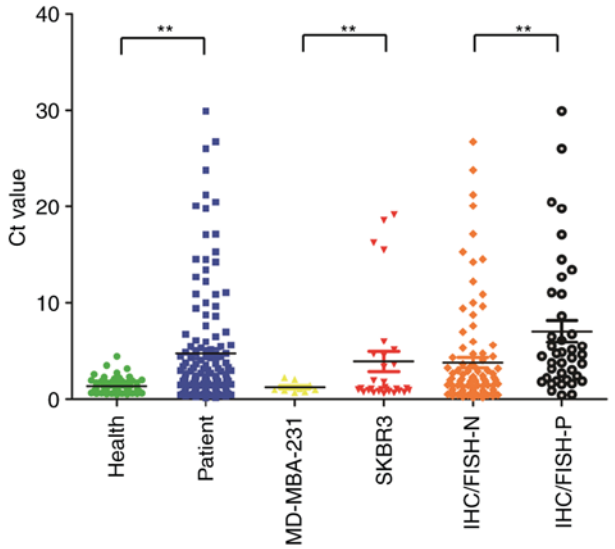

Figure 2. HER-2 mRNA expression in the different groups. MD-MBA-231: HER-2-negative breast cancer cell line. SKBR3: HER-2-positive breast cancer cell line. HER-2, human epidermal growth factor receptor 2; IHC, Immunohistochemistry; FISH, fluorescent *in situ* hybridization; IHC/FISH-N: HER-2-negative tissue as determined by IHC/FISH. IHC/FISH-P: HER-2-positive tissue as determined by IHC/FISH (one ANOVA, Tukey's Test, **P<0.01).

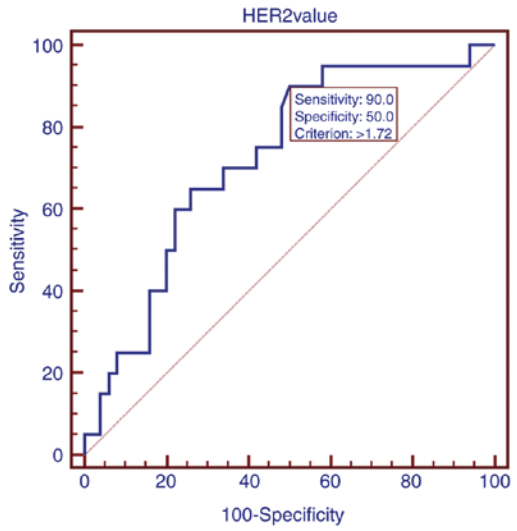

Figure 3. Comparison with HER-2 status determined by IHC/FISH in tumor tissue samples as a standard. AUC value: 0.723 (P<0.001). The optimized cutoff for peripheral blood with HER-2 mRNA positivity from preoperative patients was 1.72, with 90% sensitivity, 50% specificity, and a Youden index value of 0.40 for the distinguishing of HER-2 negative and positive tumors by one-step RT-qPCR compared with IHC/FISH. HER-2, human epidermal growth factor receptor 2; AUC, area under the curve; IHC, Immunohistochemistry; FISH, fluorescent *in situ* hybridization; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

in 20 (28.6%) patients and negative in 50 (71.4%) patients when analyzed by IHC and/or FISH (14,22,28-30).

The upper 95% CI value for normal blood was 1.512, which was set as the cutoff for the system analysis based on the $2^{-\Delta\Delta Ct}$ method (19,26,31). The system could distinguish tumor blood from normal blood, and tumor cells with high HER-2 expression from cells with low expression. The median values

Table II. Change of HER-2 mRNA level in patients pre- and postoperative based on cutoff of 1.512.

HER-2 mRNA (Preoperative)	HER-2 mRNA (Postoperative)			
	Negative		Positive	
	n	%	n	%
Negative (n=6)	2	33.3	4	66.7
Positive (n=7)	2	28.6	5	71.4

for the relative HER-2 mRNA level were 4.52 (0.39-29.92) for HER-2-positive, 1.89 (0.14-26.71) for HER-2-negative, and 1.12 (0.48-4.41) for healthy control samples (P<0.0001, one-way ANOVA; Fig. 2). Significant differences were observed between the peripheral blood samples with positive HER-2 mRNA expression from healthy controls and that from samples patients with from HER-2-positive (n=22, P<0.001, one-way ANOVA) or HER-2-negative (n=48, P<0.001, one-way ANOVA) tumor samples. Significant differences were observed between the patients with HER-2 mRNA-positive peripheral blood and those with HER-2-positive tissue (P<0.001), and between the peripheral blood from subjects with HER-2 negative tissue or samples before operation from preoperative patients with breast cancer (P<0.05). No significant differences were observed between HER-2 mRNA-positive and HER-2 mRNA-negative peripheral blood from patients prior to surgery (P>0.05).

Comparison with HER-2 status determined by IHC/FISH in tumor tissue samples as a standard. HER-2 mRNA level in blood was correlated with HER-2 status in primary tumor tissue samples (P<0.05, Pearson χ^2 test). Blood HER-2 mRNA level in patients with breast cancer and HER-2 status determined by IHC/FISH in tumor tissues was used to produce an ROC curve (Fig. 3), for which the area under the curve (AUC) was 0.723 (P<0.001). Based on the ROC curves, the optimized cutoff for peripheral blood HER-2 mRNA positivity in preoperative patients was 1.72, with 90% sensitivity, 50% specificity, and a Youden index value of 0.40 for one-step RT-qPCR distinguishing HER-2-negative and -positive tumors compared with IHC/FISH.

Combined pre- and postoperative analysis of HER-2 mRNA. The cutoff value for HER-2 mRNA positivity in circulating blood was 1.512 (P<0.0001). Five patients exhibited a positive peripheral blood HER-2 mRNA status prior to and following surgery (pre+/post+), while 2 were pre+/post-, 4 were pre-/post+, and 2 were pre-/post- (Table II). Thus, only 2 patients demonstrated a postoperative decrease in peripheral blood HER-2 mRNA, whereas 11 exhibited an HER-2 mRNA level that did not decrease.

Circulating HER-2 mRNA is a prognostic biomarker in patients receiving neoadjuvant chemotherapy. To analyze the effect of neoadjuvant chemotherapy on HER-2 mRNA expression in peripheral blood, the blood from five breast cancer patients

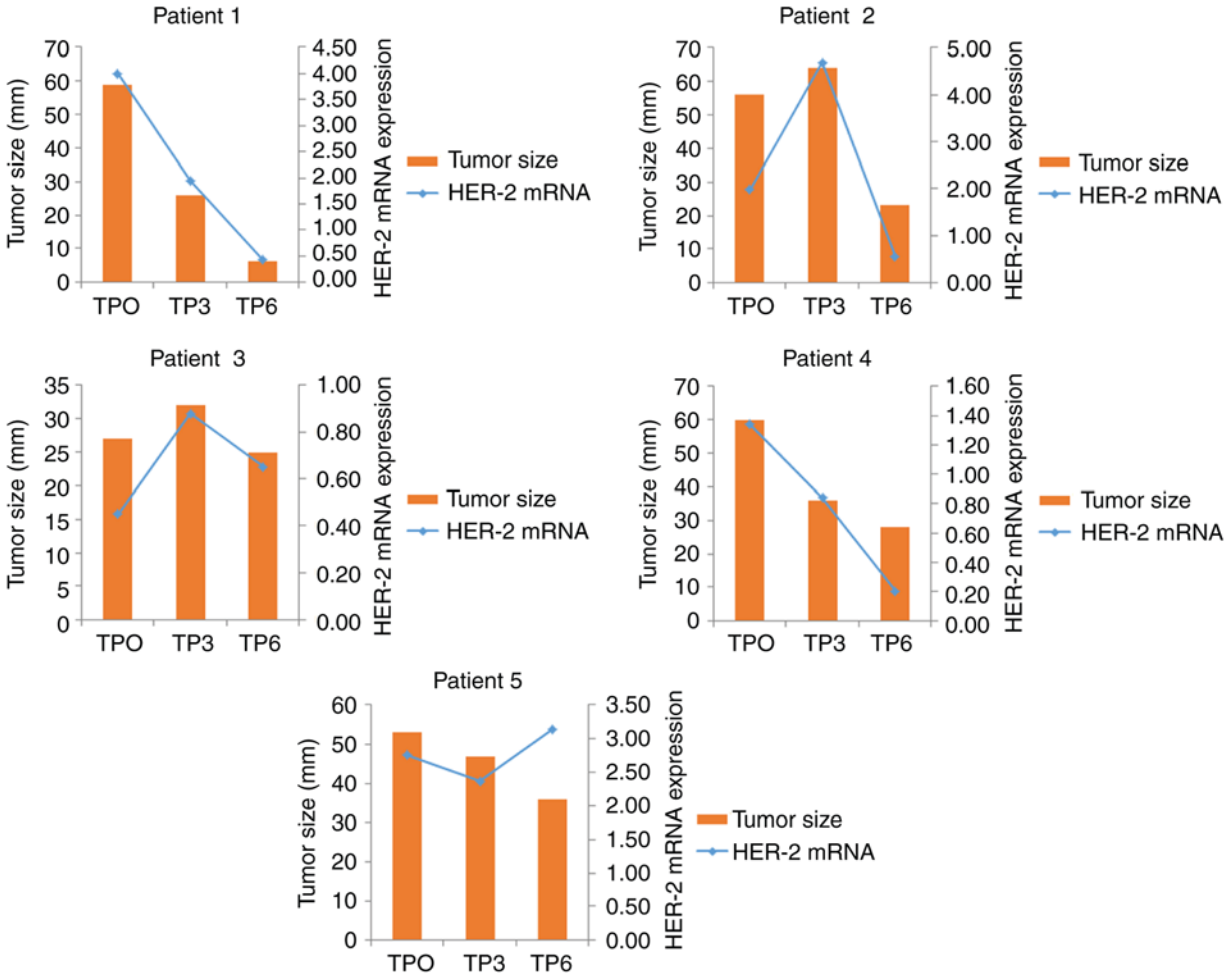

Figure 4. Circulating HER-2 mRNA expression is a prognostic biomarker for patients receiving neoadjuvant treatment. Patients received three cycles of 5-FU/epirubicin/cyclophosphamide with three sequential cycles of docetaxel and the diameter of the tumor was measured. HER-2, human epidermal growth factor receptor 2 TP, time point. n=5.

was monitored during neoadjuvant therapy (n=5). All patients received three cycles of 5-FU/epirubicin/cyclophosphamide with three sequential cycles of docetaxel (22,32). According to the treatment cycles, blood samples were divided into groups as follows: prior to antitumor treatment (time point zero; TP0), three treatment cycles (TP3), and six treatment cycles (TP6). Patients receiving neoadjuvant therapy were regularly monitored by MRI to evaluate treatment efficacy (32). In all patients who received neoadjuvant treatment, peripheral blood HER-2 mRNA and the original tumor size decreased (Fig. 4). This result suggests that the change in circulating HER-2 mRNA following neoadjuvant therapy was consistent with the results of imaging evaluation.

Discussion

Breast cancer is a highly heterogeneous malignant tumor, and HER-2 status changes during tumor progression, though these changes may not be detected in primary tumor histological examinations (33). Postoperative chemotherapy, endocrine therapy, and targeted therapy have been demonstrated to affect HER-2 expression (34). In clinical practice, the principal methods currently used to determine HER-2 tissue status are IHC and FISH, which can be used to assess HER-2 status at the time of diagnosis (35). In patients with metastatic breast cancer, biopsy samples for the metastases are not always available; furthermore, as breast cancer is highly heterogeneous and core needle biopsy (CNB) assesses only part of the tumor tissue, it may provide incomplete information for the diagnosis of metastatic breast cancer (36). Liquid biopsies can detect the HER-2 released into the peripheral blood from tumor tissues, even in patients with multiple tumor foci. Liquid biopsies also allow the detection of cancer-associated alleles in the blood and provide a genetic landscape for primary and metastatic tumors (16,37). A liquid biopsy detects all circulating HER-2 mRNA released by breast tumors, and can be used for quantification by RT-qPCR (31,38). Therefore, liquid biopsy results may be useful complementary information to the information obtained by histology. Furthermore, liquid biopsies can dynamically monitor changes in HER-2 levels in breast cancer following chemotherapy or targeted therapy, which provides prognostic information for clinical decisions (39).

HER-2 status in the peripheral blood can be converted from positive to negative following anti-HER-2 treatment in patients presenting with HER-2 positive primary tumor tissue, and these changes determine whether anti-HER-2 treatment should be continued (40). Koumarianou *et al* (41) demonstrated

that certain patients with breast cancer with HER-2 negative tissue benefited from treatment with trastuzumab. Therefore, liquid biopsies may be more convenient and accurate than CNB biopsy for tumor monitoring (42).

In the present study, a suitable cutoff for circulating HER-2 mRNA was established based on the circulating HER-2 mRNA levels in healthy controls (22 positive and 48 negative). The cutoff for circulating blood HER-2 was 1.512 (19). In previous studies, Savino *et al* (19) and Korantizis *et al* (20) have described that the circulating levels of HER-2 were associated with the HER-2 mRNA level of tissues. The results of the RT-qPCR analysis were correlated with those of tissue HER-2 status determined by IHC and/or FISH, though there was a deviation of ~4%. Compared with previous study, Savino *et al* (19) performed qPCR to detect peripheral blood HER-2 expression from 30 HER-2 positve breast cancer patients (IHC) (19,43). After establishing a cut-off value, 18 out of the 30 HER-2 positive patients were scored, indicating ~40% deviation (14). These two results indicated circulating HER-2 is a potential diagnostic marker although there were some differences observed, which may be derived from using a different patient cohort. It is established that although IHC pathology is conveys a high degree of accuracy, the results are not definitive and are open to interpretation (44,45). Therefore, the present study highlighted that HER-2 mRNA in peripheral blood may be an effective complementary assay in clinical practice. The prospective detection of HER-2 mRNA using one-step RT-qPCR on peripheral blood samples from patients with breast cancer prior to surgery or during treatment detected a significant difference in peripheral blood HER-2 mRNA level between normal samples and samples from breast cancer patients ($P<0.0001$). Xu *et al* (46) previously identified that ~43.3% of patients with breast cancer were positive for plasma HER-2 mRNA, whereas only 10% were positive in the control group ($P<0.001$). Oloomi *et al* (47) obtained similar results, with HER-2 positivity detected in 36.7% of patients with breast cancer, and reported significant differences between patients and healthy controls ($P<0.05$). However, Owrangi *et al* (21), indicated that there were no differences in the expression of HER2 in patients with cancer compared with healthy individuals, which may be a result of a different patient sample. Collectively these studies indicate that peripheral blood HER-2 mRNA is higher in breast cancer samples than in samples from patients without cancer, indicating that HER-2 mRNA in blood may be a dependable biomarker for identifying patients with breast cancer irrespective of the HER-2 status of the primary tumor. However, the exact level of circulating HER-2 mRNA in peripheral blood that identifies breast cancer, especially HER-2-positive cancer, was not determined in these studies.

The present study also identified an association between the level of HER-2 mRNA in blood and HER-2 tissue status ($P<0.05$) with high sensitivity and low specificity; the agreement between blood HER-2 mRNA detected by one-step RT-qPCR and tissue HER-2 status determined by IHC/FISH was 30.4% ($P<0.01$, Kappa coefficient). These results suggest that the detection of circulating HER-2 mRNA may be a useful predictive method for breast cancer diagnosis, complementary to tissue analysis. However, a previous study identified no correlation between blood HER-2 mRNA level and tissue HER-2 status (48). Additionally, no associations were observed between the blood HER-2 mRNA level, and the lymph node status, tumor grade, tumor stage, tumor size, patient age, menopausal status, or ER or PR status of the primary tumor in a previous study (49). Furthermore, the association between the level of HER-2 mRNA in blood and Ki-67 expression or the lymphovascular invasion status of primary tumors ($P<0.01$ and $P<0.05$, respectively) indicated a poor prognosis. The discrepancy in HER-2 status between peripheral blood and tissue could be attributed to differences in the two techniques, and the heterogeneity of breast cancer. No association was observed between peripheral blood HER-2 mRNA and other prognostic factors.

Information on peripheral HER-2 mRNA levels for predicting the efficacy of antitumor treatment in breast cancer is limited. In the present study, changes in HER-2 mRNA during neoadjuvant therapy were evaluated. Peripheral blood HER-2 mRNA in breast cancer patients was monitored during neoadjuvant therapy to correlate circulating HER-2 mRNA levels with therapeutic efficacy. The changes in circulating HER-2 mRNA during treatment were consistent with the results of MRI evaluation. As depicted in Fig. 4, all patients receiving neoadjuvant chemotherapy exhibited a decrease in circulating HER-2 mRNA after antitumor treatment, excluding patient 5. However, these results were opposite with the prognostic value of peripheral blood HER-2 mRNA detection during antitumor treatment using Docetaxel which may resulted from different patient cohort (20). The discrepancy between peripheral blood HER-2 mRNA level and tumor size after antitumor treatment in these patients may be attributed to differences in therapeutic responses and the heterogeneity of breast cancer. The detection of circulating HER-2 mRNA in patients receiving neoadjuvant chemotherapy suggested that patients benefited from neoadjuvant therapy for the treatment of breast cancer, and that circulating HER-2 mRNA could be used to predict breast cancer progression. Thus, the results demonstrated that HER-2 mRNA in peripheral blood could be used as a prognostic biomarker during neoadjuvant or adjuvant treatment.

To conclude, the present study has provided evidence from a patient cohort for the diagnostic value of circulating HER-2 for breast cancer; meanwhile, the cut-off value of 1.512 (mean of $2^{-\Delta\Delta Ct}$) was established which may be useful in clinical applications. Finally, five patients were analyzed and identified that circulating HER-2 was associated with the outcome of neoadjuvant chemotherapy, which may serve as a novel prognostic biomarker.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YW and QM performed the experiments, analyzed the data and wrote the manuscript. ZY, LS, JW, JR, BL, DX, RH, PZ provided technical assistance, analyzed the data and modified the manuscript. GJ designed and supervised the study. All authors are in agreement with the content of the manuscript.

Ethics approval and consent to participate

Study approval was obtained from the independent ethics committee at the Second Affiliated Hospital of Soochow University (Suzhou, China). The privacy of the patients involved was protected. Patients provided written informed consent.

Patient consent for publication

Study participants provided consent for the publication of the data and any associated images.

Competing interests

This study uses equipment from Shanghai GenePharma Co., Ltd. (Shanghai, China).

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