Real-time quantitative RT-PCR detection of circulating tumor cells from breast cancer patients

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Abstract. Circulating tumor cells (CTCs) were recognized as novel tumor biomarker for prognostic and predictive purposes in various cancers. Various detection technologies and devices have been developed to enumerate and characterize CTCs. Most of those approaches are based on the positive enrichment strategy and immunocytological techniques. However, the sensitivity of these approaches proved to be limited in metastatic tumors and the detection of early tumor cell dissemination was problematic. In the present study, we developed a novel CTC detection method by real-time RT-PCR technique in combination of negative enrichment strategy. The developed enrichment approach could recover more than 75% of spiked breast cancer cells from peripheral blood. The detection limit of duplex real-time RT-PCR assay using KRT19 and ERBB2 as targeted genes was consistently one breast tumor cell. Moreover, CTC detection by duplex real-time RT-PCR assay had higher detection sensitivity than that by immunostaining, especially in early breast cancer. In summary, the results of the present study indicated the potential clinical utilities of CTCs identification on breast cancer by duplex real-time RT-PCR in combination with negative enrichment.

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Introduction

Breast cancer is the most common cancer in women worldwide and the leading cause of death from cancer among women globally. According to the GLOBOCAN 2012 data (1), World Health Organization International Agency for Research on Cancer, there were nearly 1.7 million new cases diagnosed in 2012 which is ~12% of all new cancer cases and 25% of all cancers in women. Fortunately, the increasing application of effective therapies such as adjuvant medical therapy made it possible for the mortality rate to decline over the last decades. However, it also poses a challenge for clinicians to avoid over-treatment, insufficient treatment or incorrect treatment due to lack of useful prognostic, diagnostic and monitoring biomarkers (2).

Routine clinical therapies for breast cancer are based on the classical clinical and pathological features from immunohistochemistry evaluations, radiologic evaluations and serum tumor markers. Unfortunately, these features are incapable of providing enough information on the ongoing metastasis as early as possible for predicting the clinical outcome with high accuracy and reproducibility. As an emerging tumor biomarker, circulating tumor cell (CTC) analysis is a promising new diagnostic field for micrometastasis, although it depends on the emergence of increasingly advanced and sensitive technologies to isolate and characterize human CTCs (3). Multiple independent studies have demonstrated that CTCs can be recognized as novel tumor biomarkers for prognostic and predictive purposes in metastatic breast cancer (4-8).

Various detection technologies and devices have been developed to enumerate and characterize CTCs (9-13), but no gold standard could define the absolute accuracy, sensitivity and specificity in detecting CTCs. For the isolation of CTCs, a variety of currently used methods were based on various properties of CTCs compared with leukocytes and broadly divided into two isolation/enrichment strategies (positive or negative strategy). The positive strategy was based on epithelial cell adhesion molecule (EpCAM) or cytokeratin expression profile or cell size, while the negative strategy was developed by targeting and removing the normal blood cells. For the detection of CTCs, immunocytological approaches

Table I. Oligonucleotide primer and probe sequences used in the present study.

Gene symbol	Accession number	Primer-probe	Sequence (5'-3')	Tm (˚C)	Amplicon size (bp)	Fluorophore-quencher
ERBB2	NM_004448	Primer_F	CCTGGCCGTGCTAGACAATGG	58.5	138	
		Primer_R	GGGTTCCGCTGGATCAAGACC	58.2		
		Probe	CGCTGAACAATACCA	69		VIC-MGB-NFQ
KRT19	NM_002276	Primer_F	CAGATCGACAATGCCCGTCTGG	59	149	
		Primer_R	TGCATCTCCAGGTCGGTCCTG	59		
		Probe	AGATGACTTCCGAAC	69		FAM-MGB-NFQ
EPCAM	Nm_002354	Primer_F	GCTGGCCGTAAACTGCTTTGTG	58.5	115	
		Primer_R	TGCCTTCATCACCAAACATTTGGC	58.6		
		Probe	AATCGTCAATGCCAG	69		VIC-MGB-NFQ
MUC1	NM_002456	Primer_F	GGTGCTGGTCTGTGTTCTGG	58.9	136	
		Primer_R	GTACTCGCTCATAGGATGGTAGG	58.3		
		Probe	CCATTGTCTATCTCATTGC	69		NED-MGB-NFQ

and molecular techniques such as RT-PCR are the most widely used. To date, the CellSearch® system is the only approved assay by FDA for prognosis in metastatic breast, prostate and colon cancer which captures the CTCs by immunomagnetic beads coated with antibodies targeting EpCAM and identify CTCs by fluorescent probes for intracellular protein cytokeratin 8, 18 and 19. However, the CellSearch® system was shown to have a lower sensitivity in early breast cancer (14).

In the present study, we developed a new CTC detection approach based on negative enrichment strategy and real-time quantitative RT-PCR technique. The approach applied in this study for breast cancer CTC detection showed both high sensitivity and specificity, especially in early breast cancer.

Materials and methods

Ethical statement. Human blood samples were obtained from the Affiliated Hospital of Academy of Military Medical Sciences (Beijing, China). All patients and healthy donors enrolled in the present study signed the Consent Forms approved by the Ethics Review Committee of the Academy of Military Medical Sciences.

Materials. Fetal bovine serum (FBS), RPMI-1640 media, DMEM media, TRIzol reagent, glycogen, Ambion TUBRO DNA-free kit, SuperScript™ III First-Strand Synthesis system for RT-PCR, TaqMan® Universal Master Mix II kit, rabbit anti-cytokeratin (Pan) polyclonal antibody, Alexa Fluor 594 labeled goat anti-rabbit IgG antibody, Dynabeads CD45, Hoechst 33342 and Mitotracker® Red CMXRos were obtained from Life Technologies (Grand Island, NY, USA). T-25 cell culture flasks, 50- and 15-ml tubes were purchased from Corning Inc. (Corning, NY, USA). Ficoll-Paque Plus was purchased from GE Healthcare (Uppsala, Sweden). BD Vacutainer® evacuated blood collection tube with acid citrate dextrose anticoagulant were from Becton-Dickinson (Franklin Lakes, NJ, USA).

Cell lines and culture conditions. Human breast cancer cell line SK-BR-3, MCF-7, MDA-MB-453, ZR 75-1, human promyelocytic leukemia cell line HL-60, human Brukitt's lymphoma cell line Raji and human T-cell leukemia cell line Jurkat were obtained from the Cell Resource Center (IBMS, CAMS/PUMC, Beijing, China). The SK-BR-3, ZR 75-1, HL-60, Raji and Jurkat cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS, the MCF-7 and MDA-MB-453 cell lines were maintained in DMEM medium supplemented with 10% FBS. All cell cultures were maintained at 37˚C in a humidified atmosphere with 5% CO_2.

Tumor cell enrichment. We developed the tumor cell enrichment method based on negative enrichment and density gradient centrifuge strategy that updated our previously published method (15). Briefly, 1 ml of peripheral blood collected in BD Vacutainer tube was diluted with PBS to 15 ml and subsequently incubated with 100 μl Dynabeads CD45 for 30 min at room temperature with gentle tilting and rotation. Then, the whole blood sample was carefully layered on 7.5 ml Ficoll-Paque Plus in a 50-ml centrifuge tube, followed by spinning at 350 x g for 5 min at 4˚C. Supernatants were transferred into a centrifuge tube followed by spinning at 650 x g for 5 min. Cell pellet was stained in glass slide and subsequently subjected to fluorescent microscope observation, or extracted mRNA and performed real-time RT-PCR detection.

Primers and probes design. Primers and probes (Table I) were designed with AlleleID 6.0 (Premier Biosoft) and synthesized by Life Technologies Corp. (Beijing, China). Nucleotide sequences used for design of probe-primers were retrieved from NCBI database and the designed probe-primers were aligned by BLAST to confirm gene specificity.

RNA isolation and complementary DNA synthesis. Total RNA from enriched cells was extracted using TRIzol reagent according to the manufacturer's manual. To enhance the precipitation of RNA from small quantity of cell samples, glycogen (final concentration 250 μg/ml) was added to the cell lysate before phase separation. The contaminating DNA from RNA preparation was removed using Ambion® TURBO DNA-free kit according to the manufacturer's protocol.

Complementary DNA (cDNA) synthesis was performed using the SuperScript™ III First-Strand Synthesis system for RT-PCR. Briefly, the following conditions were performed in a total volume of 20 μl: 1 μl oligo(dT) primer and 1 μl 10 mM dNTP mix were mixed with 8 μl mRNA, incubated for 5 min at 65˚C for 5 min, and then placed on ice for at least 1 min. Then, 10 μl of cDNA synthesis mix was added [2 μl 10X RT buffer, 4 μl 25 mM $MgCl_2$, 2 μl 0.1 M DTT, 1 μl RNaseOUT™(40 U/μl), SuperScript™ III RT (200 U/μl)] to each RNA/primer mixture and incubated for 50 min at 50˚C and the reactions at 85˚C for 5 min was terminated. The sample was chilled on ice and 1 μl of RNase H was added to the tube and incubated for 20 min at 37˚C. The product of cDNA synthesis reaction was stored at -20˚C or used for real-time PCR immediately.

Multiplex real-time PCR. The real-time PCR was performed using the TaqMan® Universal Master Mix II kit with the StepOnePlus™ real-time PCR instrument. The PCR experiments were performed according to the protocol and cycling conditions outlined in the manual. The final concentrations of each primer and probe in the real-time PCR reaction were 0.4 and 0.2 μM, respectively if not otherwise indicated.

Immunostaining and identification of enriched CTCs. Immunostaining of CTCs were performed as previously described with some modifications (15). Briefly, enriched cells were fixed by 2% paraformaldehyde on glass slides and then permeabilized with 0.1% Triton X-100, followed by incubation with rabbit anti-cytokeratin (Pan) polyclonal antibody for 1 h. Slides were washed three times with PBS, followed by incubated with Alexa Fluor 594 labeled goat anti-rabbit IgG antibody for 1 h. After being washed three times with PBS, slides were applied with mounting media containing DAPI (Vector Laboratories) and subsequently subjected to image analysis using laser confocal scanning microscope FV1000 (Olympus). For identification of CTC, each positive CTC had to meet the following criteria: cell size >4 μm, cells were intact with round to oval morphology with visible DAPI stained nucleus, and positive for cytokeratin staining.

Spiking study. Several validation experiments by spiking study were performed to establish the accuracy of the method to detect cancer cells in blood. To validate the recovery rate of the enrichment method, liver human breast cancer cells were labeled with Hoechst 33342 and Mitotracker® Red CMXRos for 1 h. Different numbers of cells were counted with a fluorescent microscope (BX53; Olympus, Tokyo, Japan) and spiked into 1 ml blood from healthy donor. The samples were treated by the enrichment isolation procedure and recovered cancer cells were enumerated using a fluorescent microscope (BX53; Olympus) by an observer in a blinded manner. To validate the accuracy of real-time RT-PCR, liver breast cancer cells were spiked into different blood cells or cell lines (Raji, HL-60, Jurkat and isolated leukocytes) respectively, and the mixed samples were detected the expression of tumor marker genes by real-time RT-PCR assay.

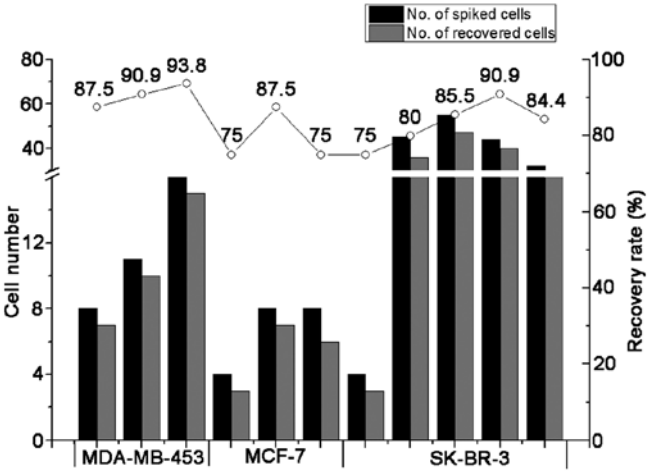

Figure 1. Validation of recovery efficiency of the new enrichment strategy by spiking study. Live human breast cancer cells MDA-MB-453, MCF-7, SK-BR-3 were labeled with Mitotraker® Red CMXRos and Hoechst 33342, respectively. The labeled cells were counted under a microscope and spiked into 1 ml bloods from healthy donor, respectively. After enrichment, the recovered labeled cells were enumerated under a microscope.

Patients and specimens. Fifteen healthy donors and sixteen breast cancer patients were enrolled in the present study. Peripheral blood (2 ml) was drawn from the median cubital vein into a BD Vacutainer tube (with acid citrate dextrose anticoagulant), and to avoid potential epithelial cell contamination, the first 2 ml of blood were discarded before each collection of blood samples. The blood sample was equally divided into two aliquots and processed immediately. Then, the enriched CTCs from two aliquots were identified by immunostaining or real-time RT-PCR analysis, respectively.

Results

Validation of recovery efficiency of the new enrichment strategy by spiking study. A series of blinded spiking studies were performed to validate the developed enrichment method of detecting breast cancer cells in blood. Live human breast cancer cell lines MDA-MB-453, MCF-7 and SK-BR-3 were labeled with Mitotracker® Red CMXRos and Hoechst 33342 as described in Materials and methods. After counting under a microscope, the labeled cells were spiked into 1 ml fresh peripheral blood collected from healthy donors to simulate the blood of tumor patients. The blood samples were treated with the enrichment procedure and the recovered tumor cells were enumerated using a fluorescent microscope (BX53; Olympus) by an observer in a blinded manner. As shown in Fig. 1, >75% cells (range from 75 to 93.8%, mean 84.1%) could be recovered from spiked MDA-MB-453, MCF-7 and SK-BR-3 cells. These results indicated that our enrichment method was able to efficiently enrich and recover spiked

Table II. Ct values of ERBB2, KRT19 and EpCAM transcripts in serial dilutions of RNA extracted from the cell line SK-BR-3, MCF-7 and ZR 75-1.

		SK-BR-3		MCF-7		ZR 75-1	
Gene	Cell no.	Ct (mean ± SD)	% positive replicates	Ct (mean ± SD)	% positive replicates	Ct (mean ± SD)	% positive replicates
ERBB2	10,000	18.9±1.28	100	25.58±0.14	100	20.96±1.02	100
	1,000	22.99±1.83	100	29.28±0.14	100	24.54±1.28	100
	100	26.91±1.96	100	32.30±0.08	100	28.01±1.54	100
	10	29.96±1.92	100	33.77±0.09	100	30.97±1.74	100
	1	32.98±1.57	100	34.29±0.01	100	32.96±1.94	100
	0.1	34.59±1.40	100	34.24±0.10	100	33.37±2.04	100
KRT19	10,000	17.98±0.88	100	22.28±1.80	100	17.59±3.62	100
	1,000	22.64±1.08	100	27.33±2.33	100	22.18±4.91	100
	100	26.75±1.34	100	30.99±2.01	100	26.12±5.07	100
	10	30.08±1.23	100	33.65±2.00	100	29.72±4.82	100
	1	33.64±1.09	100	34.27±2.25	100	32.52±3.18	100
	0.1	37.74±2.34	86	35.96±0.00	50	34.26±2.29	67
EpCAM	10,000	20.94±1.78	100	19.84±0.40	100	16.63±1.10	100
	1,000	25.18±1.49	100	23.53±0.29	100	20.35±1.52	100
	100	28.30±0.38	93	27.11±0.04	100	23.90±1.84	100
	10	30.71±0.85	86	30.87±0.29	100	27.51±2.09	100
	1	32.05±1.38	86	34.05±0.37	100	31.53±1.94	100
	0.1	33.29±2.06	71	34.30±0.17	100	32.88±2.67	83

breast cancer cells from peripheral blood, with a high degree of accuracy.

Development of multiplex real-time RT-PCR detection of breast CTCs. For the real-time RT-PCR detection of breast CTCs, MUC1, EpCAM, ERBB2 and KRT19 were selected as candidate marker genes. To evaluate if the selected genes were appropriate marker genes for RT-PCR assay, we first detected the expression of these genes in different breast tumor cells and human blood cells. The results showed that all these selected genes were expressed highly in breast tumor cell lines SK-BR-3, MCF-7 and ZR 75-1 (data not shown). However, the expression of MUC1 gene could also be detected in Raji, Jurkat and isolated leukocytes when the cell number was >50,000 (Fig. 2). These results indicated that MUC1 may not be a suitable marker gene for detection of CTCs in peripheral blood.

Then, we tried to determine the sensitivity of the real-time RT-PCR detection of breast CTCs. For this, serial dilutions of total RNA from breast cancer cell line SK-BR-3, MCF-7 and ZR 75-1 were performed to detect ERBB2, KRT19 and EpCAM gene, respectively. The detection limit using ERBB2 or KRT19 (Table II) as targeted gene was consistently one tumor cell RNA of SK-BR-3, MCF-7 or ZR 75-1, which indicated that ERBB2 and KRT19 could potentially detect a single breast cancer cell. But, the sensitivity of real-time RT-PCR detection of CTCs using EpCAM gene as targeted gene was not consistent in all tested cell lines (Table II). Based on these results, we chose the ERBB2 and KRT19 as the marker genes to develop the real-time RT-PCR detection of breast CTCs.

Table III. Comparation of amplification efficiency of KRT19 and ERBB2 in a duplex assay and corresponding singleplex assays.

Gene	Assay	Slope	R^2
KRT19	Duplex	-3.54	0.973
	Singleplex	-3.88	0.977
ERBB2	Duplex	-3.51	0.98
	Singleplex	-3.61	0.925

To test if the contaminated leukocytes after negative enrichment affect the sensitivity and specificity of real-time RT-PCR detection of CTCs, various numbers of SK-BR-3 cells were spiked into a serial diluted leukocytes and detected by real-time RT-PCR assay. As shown in Fig. 3, the Ct number of breast CTCs without contaminated leukocytes was exactly equal to that of equal breast CTCs with the different numbers of contaminated leukocytes, which indicated the contaminating leukocytes did not affect the amplification of ERBB2 and KRT19 genes in breast CTCs.

Based on the above results, we decided to develop the duplex real-time RT-PCR detection of breast CTCs using KRT19 and ERBB2 primer-probe sets. Next, we compared the sensitivity of the duplex and singleplex assays by detecting total RNA of 10-fold serial dilutions of SK-BR-3 (from 10^5 to 10^{-1}). For the duplex reactions, the primers and probes were

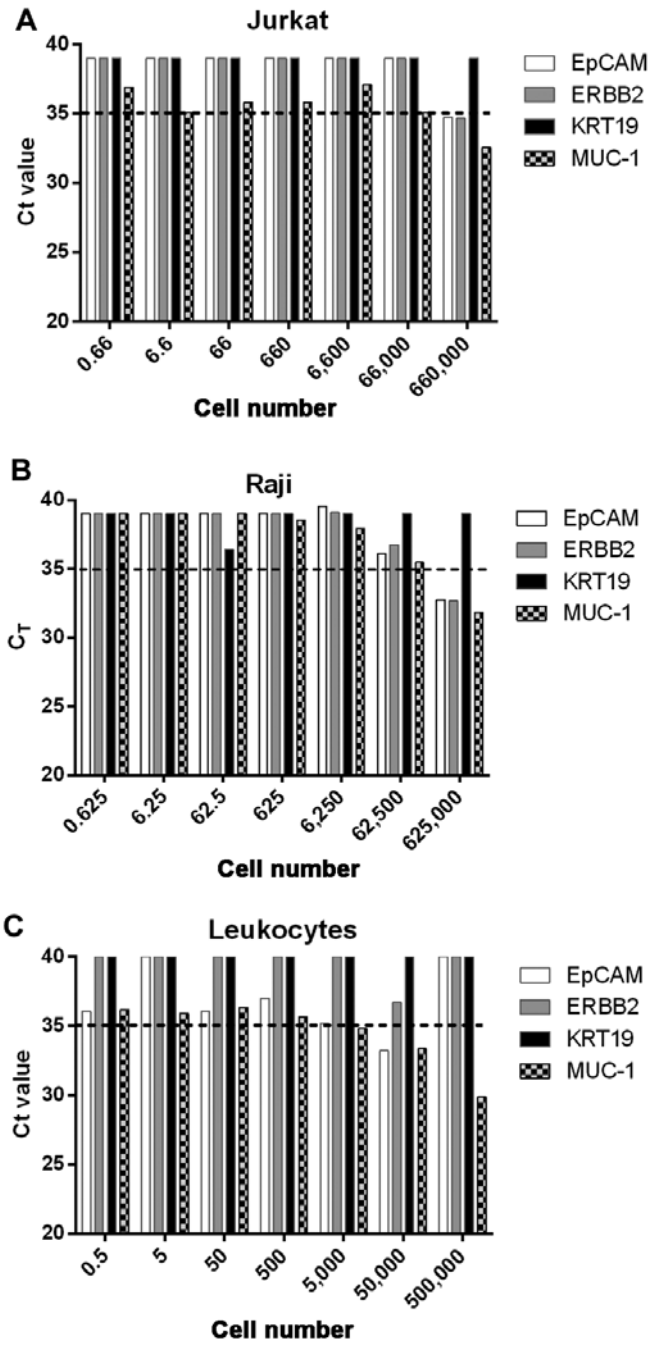

Figure 2. Gene expression of EpCAM, ERBB2, KRT19 and MUC1 in cell line (A) Jurkat, (B) Raji and (C) leukocytes. To evaluate the potential contribution of gene transcript levels derived from human blood cells, transcript levels of EpCAM, ERBB2, KRT19 and MUC1 were detected with real-time RT-PCR assay.

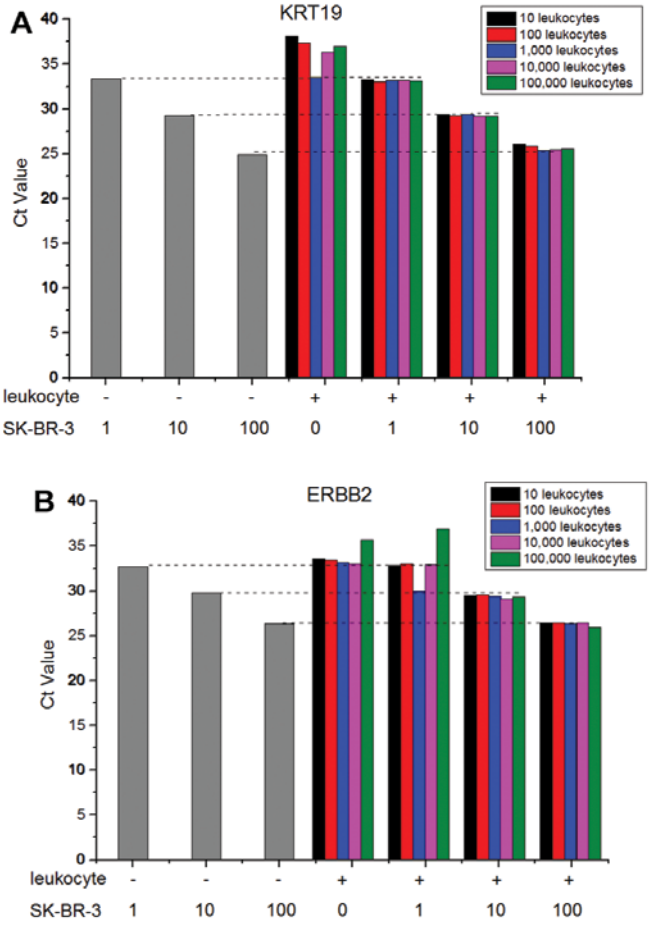

Figure 3. Influence of contaminated leukocytes on the amplification of (A) KRT19 and (B) ERBB2 in breast CTCs. Different number of SK-BR-3 cells (1, 10 and 100) were spiked into different number of leukocytes (10, to 100,000), respectively. After extraction of total RNA, the KRT19 or ERBB2 transcript in different samples was amplified, respectively.

each used at 0.2 μM, and for the singleplex reactions, the probe concentration was set at 0.2 μM, while the primer concentration was set at 0.4 μM. The amplification plots achieved with duplex assay overlapped with those from the singleplex assays (data not shown), and equivalent Ct value, slope and R^2 were achieved for each gene regardless of whether a duplex or a singleplex assay was performed (Table III). These results indicated that the developed duplex assay had equivalent sensitivity with singleplex assays.

Enrichment and detection of CTCs from breast cancer patients. Having proved that the breast tumor cells could be recovered efficiently by negative enrichment and detected quantitatively by duplex real-time RT-PCR assay, we applied the approaches to the detection of CTCs in the whole blood samples. In the present study, 15 healthy donors (Table IV) and 16 breast cancer patients (Table V) including 2 stage I, 6 stage II, 3 stage III and 5 stage IV were enrolled. Tumor-node-metastasis (TNM) staging of breast cancer patients was performed according to seventh edition of cancer staging manual by the American Joint Committee on Cancer (AJCC). Blood samples from breast cancer patients were collected before drug therapy and subsequently subjected to enrichment and CTC counting. To evaluate the results of real-time RT-PCR assay, enriched CTCs from another aliquot of those blood samples were also identified by immunostaining with anti-cytokeratin (pan) antibody and counted under fluorescence microscopy (Table V). Fig. 4 shows some representative enriched breast circulating tumor cells which were intact with round to oval morphology with visible DAPI stained nucleus and positive for anti-cytokeratin (pan) staining.

For the quantification of enriched CTCs by real-time RT-PCR assay, total RNAs extracted from a serial of concen-

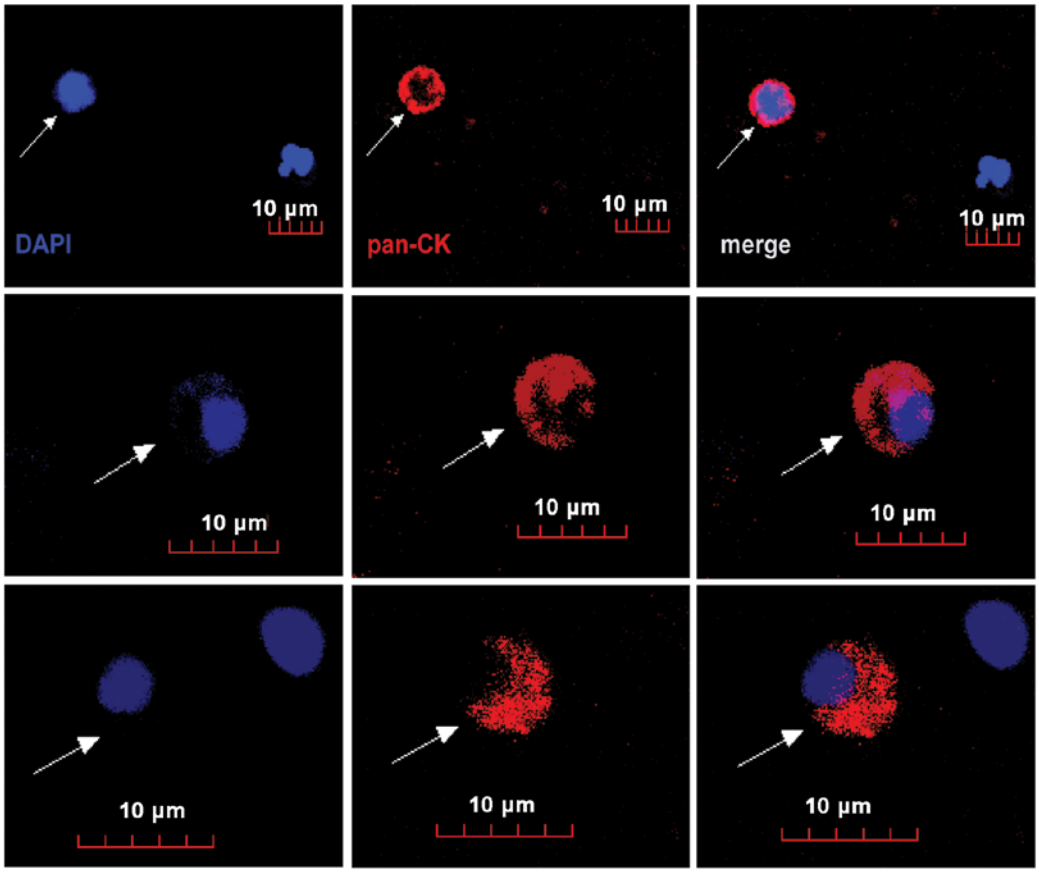

Figure 4. Images of breast CTCs from peripheral blood of patient BCA27. Enriched CTCs were subjected to immunofluorescent staining with anti-cytokeratin (pan) antibody (red) and nuclear counterstain DAPI (blue). The stained CTCs were visualized with confocal microscope FV1000 (Olympus).

Table IV. Quantification of CTCs of blood samples from healthy people.

Healthy sample no.	CTCs identified by ICC	CTCs idenfied by KRT19	CTCs identified by ERBB2
HD01	0	0	0
HD02	0	0	0
HD03	0	0	1
HD04	0	0	0
HD05	0	0	0
HD06	0	0	0
HD07	0	2	0
HD08	0	1	0
HD09	0	1	0
HD10	0	0	1
HD11	0	0	0
HD12	0	0	1
HD13	0	2	1
HD14	0	0	0
HD15	0	0	0

trations of SK-BR-3 cells served as a template for an external calibration curve to calculate the quantity of breast CTCs. Among the 15 healthy donors, 8 people were detected with 1 or 2 CTCs by KRT19 and/or ERBB2 genes. For this reason, we set 5 as a cut-off value of positive CTCs for all real-time RT-PCR assay. For the immunocytochemistry staining, no healthy donors could be detected with CTC. Thus, a positive patient was defined as one whose CTC count in 1 ml blood was >2 for immunocytochemistry staining.

Distributions of positive CTC cells among healthy donors and patients are demonstrated in Fig. 5 and summarized in Table VI. According to the cut-off value, the positive CTC detection rate was zero in healthy donors by real-time RT-PCR assay or immunocytochemistry staining. For immunocytochemistry staining, the positive detection rates for all histologic cancers according to stage I-II, III and IV were 28.5, 75 and 80%, respectively. For real-time RT-PCR assay by KRT19 gene, the positive detection rates for stage I-II, III and IV were 71, 100 and 80%, respectively. For real-time RT-PCR assay by ERBB2 gene, the positive detection rates for stage I-II, III and IV were 57, 100 and 100%. Combining the results of real-time RT-PCR assay by KRT19 and ERBB2 gene together, the positive detection rate for stage I-II, III and IV reached 85.7, 100 and 100%, respectively. These results indicated that breast CTCs detection by duplex real-time RT-PCR assay had higher detection sensitivity than that by immunostaining.

Discussion

In the present study, we developed a novel circulating breast tumor cell detection approach using duplex real-time RT-PCR

Table V. Quantification of CTCs of blood samples from patients with breast cancer.

Sample no.	Type of breast cancer	TNM stage	Age (years)	Surgery status	Recurrence	IHC index			CTCs identified by ICC	CTCs identified by KRT19	CTCs identified by ERBB2
						ER	PR	HER-2			
BCA21	Invasive breast carcinoma	T2N1M0, IIB	70	Post		+++	+++	++	1	17.5	36.8
BCA22	Invasive ductal carcinoma	T2N2M0, IIIA	39	Post		+++	+	+	7	99.6	65.8
BCA23	Invasive breast carcinoma	T4N2M0, IIIB	62	No	Yes	-	-	+++	9	55	18.6
BCA24	Invasive ductal carcinoma	T1N2M1, IV	46	Post		++	-	+++	1	50.4	22.4
BCA25	Ductal carcinoma *in situ*	T2N0M0, IIA	31	No		-	+	++	0	29.8	18.4
BCA26	Invasive ductal carcinoma	T2N2M0, IIIA	60	Post		-	-	+++	3	42.6	19.2
BCA27	Invasive ductal carcinoma	T1N2M1, IV	56	No		-	+	-	11	21.2	11
BCA28	Invasive lobular carcinoma	T1N1M0, IIA	57	No		+	+	++	3	2.4	4.8
BCA29	Invasive ductal carcinoma	T4N2M1, IV	59	Post	Yes	-	-	+++	23	2.6	15.2
BCA30	Invasive ductal carcinoma	T1N0M0, I	32	Post		+	+	+++	1	11.2	28.6
BCA31	Invasive ductal carcinoma	T2N0M0, IIA	48	No		-	-	-	0	10.2	0.2
BCA32	Invasive breast carcinoma	T1N1M0, IIA	60	No		+++	+	++	1	10.9	8.2
BCA33	Invasive ductal carcinoma	T1N0M0, I	36	Post		+	-	+	0	7	0
BCA34	Invasive ductal carcinoma	T4N2M1, IV	59	Post	Yes	+++	+++	++	19	20.6	11.6
BCA35	Invasive ductal carcinoma	T3N1M1, IV	40	No		+	+	+++	21	70.6	13.86
BCA36	Invasive lobular carcinoma	T2N1M0, IIA	44	No		N/A	N/A	N/A	3	3.1	20.78

IHC, immunohistochemistry; ICC, immunocytochemistry; ER, estrogen receptor; PR, progesterone receptor; N/A, Not applicable.

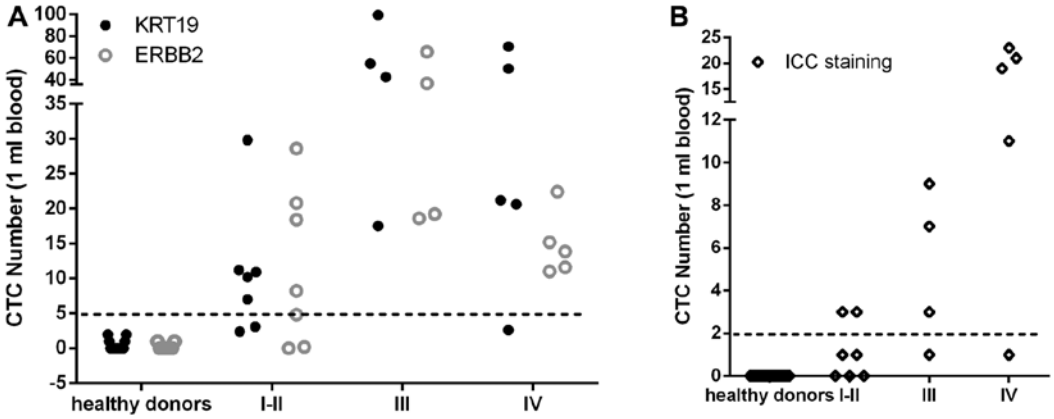

Figure 5. Distribution of CTCs identified by real-time RT-PCR assay and immunocytochemistry staining. Fifteen healthy donors and 16 breast cancer patients before subjected to drug therapy were enrolled and CTCs were identified in peripheral blood by (A) duplex real-time RT-PCR assay and (B) immunocytochemistry staining, respectively. For real-time RT-PCR assay, a cut-off value of >5 cancer cells per 1 ml blood was defined as positive. For ICC staining, the positive cut-off value was set >2 cancer cells per 1 ml blood.

Table VI. Analysis of CTC detection by real-time PCR and immunostaining.

		CTCs >5		CTCs >2
Pathological type	Number	Identified by KRT19 Positive (%)	Identified by ERBB2 Positive (%)	Identified by ICC staining Positive (%)
Healthy donors	15	0 (0)	0 (0)	0 (0)
I-II	7	5 (71)	4 (57)	2 (28.5)
III	4	4 (100)	4 (100)	3 (75)
IV	5	4 (80)	5 (100)	4 (80)

technique in combination with negative enrichment strategy. Our results showed that the developed negative enrichment approach could efficiently enrich and recover breast tumor cells from peripheral blood with a high degree of accuracy, and the duplex real-time RT-PCR assay using KRT19 and ERBB2 as targeted genes could consistently detect one breast tumor cell even in the environment containing relative large quantities of contaminating leukocytes.

Since CTCs are rare cells in peripheral blood, isolation of CTCs represents a major technological challenge and enrichment prior to the actual detection procedure could improve the sensitivity of CTCs detection. The most widely used CTC isolation technologies rely on positive enrichment by antibody-based capture of CTCs which express epithelial cell surface markers such as the epithelial cell adhesion molecule (EpCAM) that are absent from normal leukocytes. But, not all CTCs express the EpCAM antigen. Moreover, EpCAM expression tends to change dynamically during the epithelial-mesenchymal transition (EMT) process in the metastatic cascade (16-18). To avoid the bias of selecting cells by virtue of EpCAM expression, negative enrichment by removing leukocytes has been advocated which has the potential to purify CTCs irrespective of presumed cell surface markers and the advantages such as keeping the CTCs in an intact/untargeted form over positive enrichment in isolating rare cells (18-21).

To determine the performance of the system in the negative enrichment process, the overall level of enrichment, recovery rate and sensitivity were the common quantitative measures. Different negative enrichment approaches based on immunomagnetic separation demonstrated different overall level of enrichment, ranging from 2.7 to 5.66 log for fresh peripheral blood, and different recovery rate by spiking experiment, range from 36 to 85% (22). Although a completely negative selection methodology might achieve a higher enrichment performance, it also might reduce the recovery rate (22). In the present study, we tried to perform the negative enrichment process with as few steps as possible to balance between enrichment level and recovery rate. We used a density gradient centrifugation step instead of the red blood cell lysis step to reduce the possible harm and loss to CTCs (23,24). Here, we achieved an average 3 log enrichment (data not shown), and such concentration is sufficient to perform further RT-PCR analysis of CTCs with less background contamination. Furthermore, we achieved a high recovery rate (range from 75 to 93.8%, mean 84.1%) to increase the overall sensitivity of the CTCs detection.

Another major challenge for CTC identification was the prevailing difficulty of finding an mRNA marker which could distinguish normal expression in blood from that due to the presence of CTCs. Several mRNA markers have been used for RT-PCR-based detection of CTCs and evaluated for their sensitivity, specificity and clinical potential in breast cancer, such as CK19 (25), mammaglobin (26), HER2 (27), MUC1 (28). The fact that few markers provide adequate sensitivity individually makes combination of markers a good choice for CTCs detec-

tion (29,30). In the present study, we screened the sensitivity and specificity of CK19, HER2, EpCAM and MUC1 for breast CTCs detection, and found that the real-time RT-PCR assay using KRT19 and ERBB2 as targeted genes could consistently detect a tumor cell in various breast cancer cell lines. In addition, duplexing assay using KRT19 and ERBB2 improved the sensitivity of CTCs detection in breast cancer, especially in early breast cancer compared to that of immunocytochemistry staining.

In summary, our CTC detection approach that combines negative enrichment with real-time quantitative RT-PCR assay using KRT19 and ERBB2 as targeted genes demonstrated high sensitivity, specificity and potential clinical utility in breast cancer.

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