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Gene expression markers in circulating tumor cells may predict bone metastasis and response to hormonal treatment in breast cancer

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Abstract. Circulating tumor cells (CTCs) have recently attracted attention due to their potential as prognostic and predictive markers for the clinical management of metastatic breast cancer patients. The isolation of CTCs from patients may enable the molecular characterization of these cells, which may help establish a minimally invasive assay for the prediction of metastasis and further optimization of treatment. Molecular markers of proven clinical value may therefore be useful in predicting disease aggressiveness and response to treatment. In our earlier study, we identified a gene signature in breast cancer that appears to be significantly associated with bone metastasis. Among the genes that constitute this signature, trefoil factor 1 (TFF1) was identified as the most differentially expressed gene associated with bone metastasis. In this study, we investigated 25 candidate gene markers in the CTCs of metastatic breast cancer patients with different metastatic sites. The panel of the 25 markers was investigated in 80 baseline samples (first blood draw of CTCs) and 30 follow-up samples. In addition, 40 healthy blood donors (HBDs) were analyzed as controls. The assay was performed using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) with RNA extracted from CTCs captured by the CellSearch system. Our study indicated that 12 of the genes were uniquely expressed in CTCs and 10 were highly expressed in the CTCs obtained from patients compared to those obtained from HBDs. Among these genes, the expression of keratin 19 was highly correlated with the CTC count. The TFF1 expression in CTCs was a strong predictor of bone metastasis and the patients with a high expression of estrogen receptor β in CTCs exhibited a better response to hormonal treatment. Molecular characterization of these genes in CTCs may provide a better understanding of the mechanism underlying tumor metastasis and identify gene markers in CTCs for predicting disease progression and response to treatment.

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Introduction

Metastases are the leading cause of mortality in patients diagnosed with cancer (1). Cancer metastasis occurs when tumor cells dissociate from the primary tumor and migrate to distant organs through the peripheral bloodstream or lymphatic drainage. Circulating cells with the characteristics of tumor cells of epithelial origin, or circulating tumor cells (CTCs), have been detected in the blood and bone marrow of patients with breast, prostate and colon cancer (2). These cells have been detected in patients with metastatic disease, as well as in those whose tumors are apparently localized. The identification and characterization of such cells and the determination of their clinical significance have attracted attention in the field of cancer research (3-5).

The CellSearch™ system is the first rare cell isolation technology that demonstrated its clinical validity in predicting progression-free and overall survival of metastatic breast cancer patients based on CTC enumeration (2). Further characterization of CTCs, such as assessment of CTC gene expression markers, may provide insight into the mechanisms of metastasis and the optimal treatment modalities for the patients. In a previous study, 55 mRNAs abundantly expressed in CTCs were identified, suggesting that this approach is feasible (5).

Breast cancer comprises 22.9% of all cancers in women worldwide. It is responsibe for ~13.7% of all the cases of cancer-related mortality in women and ~70% of breast cancer patients with bone metastases eventually succumb to the disease (6). Attempts have been made to identify molecular markers that may predict the site of metastasis in breast

cancer (7-9). For example, in an earlier study, we identified a 31-gene signature from primary tumor tissues that was shown to be significantly associated with bone metastasis of breast cancer; among these, trefoil factor 1 (TFF1) was identified as the most differentially expressed gene associated with bone metastasis (6).

In this study, we investigated breast cancer gene expression markers in CTCs as potential predictive markers for the site of metastasis and the response to treatment. We analyzed the genes selected from previous studies in the baseline CTC samples of 80 metastatic breast cancer patients (first blood draw prior to the initiation of chemotherapy) and the follow-up CTC samples of 30 patients. In addition, 40 healthy blood donors (HBDs) were used as controls. The assay was performed by using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) with RNA extracted from the CTCs captured by the CellSearch™ System.

Materials and methods

Patient samples and characteristics. This study was coordinated by Mayo Clinic, Mayo Validation Support Services, Rochester, MN, USA. The study was approved by the Institutional Review Board (IRB). All the patients were enrolled using IRB-approved protocols and provided written informed consent. Between 2009 and 2011, a total of 80 patients who were treated for metastatic breast cancer with specific chemotherapy, hormonal and radiation therapy were enrolled. Detailed patient clinicopathological information is presented in Table I. This study was approved by the Institutional Review Board (IRB) at the Mayo Clinic. All patients were enrolled using IRB-approved protocols and provided written informed consent.

Blood collection and sample preparation. Two 7.5-ml blood samples were drawn from each patient and collected into evacuated 10-ml blood collection tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ, USA) and 10-ml CellSave tubes (Veridex, Raritan, NJ, USA). The samples were maintained at room temperature and processed within 36 h of collection. The CellTracks®AutoPrep® system was used in conjunction with the CellSearch®CTC kit and the CellSearch®profile kit (Veridex) to enrich and enumerate CTCs. The enriched CTC samples were analyzed with CellTracks®Analyzer II and the number of CTCs in the sample was determined. For CTC profiling, the AutoPrep tube with the sample from the CellTracks AutoPrep system was removed and placed into the MagCellect Magnet for a 10-min incubation. With the tube still in the MagCellect Magnet, the supernatant liquid was aspirated with a Pasteur pipette without disrupting the ferrofluid bound cells. A 350-μl aliquot of RLT lysis buffer with β-mercaptoethanol (Qiagen, Valencia, CA, USA) was added to the ferrofluid bound cells and vortexed for 30 sec to lyse the cells. The cell lysate was briefly centrifuged at 800 x g to pellet ferrofluid and insoluble debris.

CTC RNA isolation. CTC-derived RNA was isolated using RNeasy Micro kit (Qiagen) according to the manufacturer's instructions, with the following modifications: To each cell lysate, 4 μl of Polyinosinic:polycytidylic acid [Poly(I:C)] was added and vortexed for 30 sec. An equal volume of 70% ethanol was added to the sample and mixed by pipetting. The sample was loaded onto a micro-column, provided in the kit, and centrifuged for 15 sec at 8,000 x g (the time and speed were maintained in the following steps). RW1 wash buffer (700 μl) was added to the column and centrifuged. RPE buffer (500 μl) was added to the column and centrifuged and 500 μl of 80% ethanol was added to the column and spun for 2 min to dry the column. The columns were added to a new collection tube and centrifuged for 5 min at maximum speed. RNA was eluted in 14 μl of RNase-free water by a spin for 1 min at 10,000 x g. Subsequently, the extracted RNA was quantified on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) according to the manufacturer's instructions and stored at -80˚C until later use.

cDNA synthesis, pre-amplification and qRT-PCR analysis. First-strand cDNA was synthesized using 10 ng of total RNA and High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). The cDNA was amplified with the ABI TaqMan PreAmp method (Applied Biosystems) and reagents according to the manufacturer's instructions. The selected candidate genes and the housekeeping control genes were evaluated using the qRT-PCR assay with the pre-amplified material. PCR amplification was performed on the ABI PRISM 7900HT Sequence Detection system (Applied Biosystems) using the 384-well block format with a 10-μl reaction volume. The concentration of the primers and the probes was 4 and 2.5 μmol/l, respectively. The reaction mixture was incubated at 95˚C for 10 min to activate AmpliTaq®, followed by 40 cycles at 95˚C for 15 sec for denaturing and at 60˚C for 1 min for annealing and extension. In addition, the primers and probes were optimized towards the same amplification efficiency according to the manufacturer's protocol. The sequences for the primers and probes for the 22 breast cancer-specific genes and 3 control genes are listed in Table II, in the 5'-3' direction. All the oligonucleotides, primers and probes, were manufactured by Biosearch Technologies (Novato, CA, USA). The probes were modified by fluorophore dye labeling at the 5' ends and BHQ labeling at the 3' ends and were synthesized according to the manufacturer's instructions.

Data analysis. qRT-PCR data were analyzed by a manual threshold of 0.2 and a baseline of 5-15 to obtain cycle threshold (Ct) values for both channels. The results were considered valid when the Ct value of actin was ≤25 and no template control had undetectable Ct. By using this threshold, only one of the 80 patient CTC samples (1.2%) was excluded from further analysis. The significance of the gene markers was evaluated by univariate and multivariate analysis using R software, A Language and Environment for Statistical Computing (R Foundation for Statistical Computing 2012, Vienna, Austria).

Results

Patient characteristics. The clinical and pathological characteristics of the patients are summarized in Table I. All the patients suffered from metastatic breast cancer and received their treatments at Mayo Clinic. The information

Table I. Patient characteristics.

Characteristics	n (%)
Age (years)	
Mean	59
Range	32-79
Family history	
Yes	24 (30%)
No	55 (70%)
ER	
Negative	9 (12%)
Positive	66 (88%)
PR	
Negative	17 (24%)
Positive	54 (76%)
HER2/neu	
Negative	40 (80%)
Positive	10 (20%)
T stage	
1	(5 (13%)
2:2A:2B	5 (13%):6 (16%):5 (13%)
3:3A:3C	5 (13%):3 (8%):3 (8%)
4	6 (16%)
Baseline metastases	
Bone	
Yes	60 (75%)
No	20 (25%)
Lung	
Yes	30 (38%)
No	50 (62%)
Brain	
Yes	13 (16%)
No	67 (84%)
Treatment	
Chemotherapy	
Yes	62 (78%)
No	17 (22%)
Radiation	
Yes	12 (15%)
No	67 (85%)
Hormonal	
Yes	15 (19%)
No	63 (81%)
Follow-up	
Disease progression	
No	44 (60%)
Yes, new metastases	3 (4%)
Yes, progression	26 (36%)
Baseline CTC count (n=80)	
25th percentile	0.0
50th percentile	2.0
75th percentile	33.5
95th percentile	341.4
Follow-up CTC count (n=30)	
25th percentile	0.0
50th percentile	1.0
75th percentile	5.5
95th percentile	59.0

ER, estrogen receptor; PR, progesterone receptor; CTC, circulating tumor cell; HER, human epidermal growth factor receptor.

included site of metastatic disease, type of treatment (chemotherapy, radiation and hormonal therapy) and response to treatment. A total of 78% of the patients were treated with chemotherapy, whereas 15 and 19% of the patients were treated with radiation and hormonal therapy, respectively, after being diagnosed with metastatic disease. A total of 60 patients (75%) had bone metastasis, 30 (38%) had lung metastasis and 13 (16%) had brain metastasis. Of the 80 patients, 23 presented with metastatic disease at multiple sites. In order to evaluate their response to treatment, disease progression was monitored approximately every 3 months by one or more of the following: computed tomography scan, positron emission tomography scan, magnetic resonance imaging, X-ray specific to the site of metastasis or concern, ultrasound or biopsy. The diagnosis of progression or lack thereof was confirmed by an oncologist based on the review of the test results.

CTC enumeration. Blood samples of 7.5 ml were obtained from the patients and collected into CellSave tubes. The number of CTCs in each blood sample was identified and counted with the CellSearch system. CTCs were identified in the 7.5-ml blood sample of 48 patients (61%). Of these, 32 patients (66.7%) had ≥5 CTCs. One patient, MAY_B_031, had >1,100 CTCs. The average number of CTCs at baseline in the 80 patients was 66 (Table I). No CTCs were detected in any of the HBDs.

Gene expression analysis of CTCs. A total of 25 gene markers were selected and analyzed in the 80 baseline samples, the 30 follow-up samples and the 40 HBD samples. The 25 markers included 22 breast-specific genes, 1 epithelial cell-specific gene, 1 leukocyte-specific gene and 1 housekeeping gene (Table II). The qRT-PCR assays for individual genes were optimized by testing various primers and probes in breast cancer cells, including the MCF7 and SKBR3 cell lines, as well as HBD samples (data not shown). The results demonstrated that 12 genes were specifically expressed in CTCs, without detectable expression in the HBD samples (Table III). Among the highly expressed genes in CTCs, 27 (33.8%) and 25 (31.3%) patients expressed TFF1 and mammaglobin, respectively. In addition, the keratin 19 (KRT19) gene was highly correlated with the CTC count, consistent with previously reported findings (10). The scatter plot of the correlation between KRT19 and the rank order

Table II. Quantitative reverse transcriptase polymerase chain reaction primers and probes.

Gene	RefSeq	5'-3' Sequence (forward, reverse and probe)
ERβ	NM_001437.1	ACCTGTAAACAGAGAGACACTGA AGCGCAGAAGTGAGCAT ACCGTTGCGCCAGCCCTGTTACT
MAGE-A3	NM_005362.3	GAAGGAGAAGATCTGCCAGT TGCTGACTCCTCTGCTCA ATTGCCCAGCTCCTGCCCACA
SERPINB5[a]	NM_002639.2	AGATCATAGAGCTTCCTTTTCA AGTTGTTTTTCAATCTTCTCCA TCTCAGCATGTTCATCCTACTACCCA
ERBB4	NM_005235.1	ACAGTCAGAGAGATAACAGGTTT ACAGGCCACTATAGAGTACTCTT ATGGCCACCAAACATGACTGACT
PR	NM_000926.2	TCTTGATAACTTGCATGATCTTGT AGACATCATTTCTGGAAATTCA AATACATTTATCCAGTCCCGGGCACT
TFF3	NM_003226.2	GTGGGCCTGTCTGCAA ACTCCTTGGGGGTGACAT AGGACAGGGTGGACTGCGGCTA
TFF1	NM_003225.2	GCCCAGACAGAGACGTGT TCGAAACAGCAGCCCTTA TGGCCCCCCGTGAAAGACAGA
MG	NM_002411	AGTTGCTGATGGTCCTCATGC CACTTGTGGATTGATTGTCTTGGA CCCTCTCCCAGCACTGCTACGCA
SPDEF	NM_012391	CGCCCACCTGGACATCTGGA CACTGGTCGAGGCACAGTAGTGA GTCAGCGGCCTGGATGAAAGAGCGG
EGFR	NM_005228	TCCTTCTTAAAGACCATCCA GATCTGCAGGTTTTCCAA TGGTTATGTCCTCATTGCCCTCA
S100A16	NM_080388.1	CCCTGCTGGAGAGGAGGC GACATCTCCCTGCTTCGCC TGAGGCAGCAGGCCCCGC
PKP3	NM_007183.2	ACCTGTCTCGGAACGCTAGGA GGCAGCTTCTCGATCAGGTG GGACGAGATGTCCACGAAGGTGGTGA
SCGB1D2	NM_006551.3	TGCTACCAGGCCAATGCC GGCAAGACTTAACTTGAACAGAGGTT GCCCAGCTCTTGTTTCTGAGCTGTTAGACTT
FOXA1	NM_004496.2	CCAGCGACTGGAACAGCTACTAC CTGAGTTCATGTTGCTGACCG ACACGCAGGAGGCCTACTCCTCCGT
AGR2	NM_006408.3	CAGATACAGCTCTGTTGCTTGACA GACAGACAGAAGGGCTTGGAGA AGAAAGCTCTCAAGTTGCTGAAGACTGA
PIP	NM_002652.2	AGGACAACACTCGGAAGAT TGCATTCTTTCAATTCTGTTT ACATTCCCAAGTCAGTACGTCCAA
CEA	NM_004363.2	CAATAATTCCATAGTCAAGAGCA CAACCAGCACTCCAATCAT TGCATCTGGAACTTCTCCTGGTCT

Table II. Continued.

Gene	RefSeq	5'-3' Sequence (forward, reverse and probe)
TNRC9	NM_001080430	TACGGCTACAGCAAGTTTGGA TGGTGTGTGGAATGTCTGCT ATATGGCTGAGGCGAACAATGCGT
LAD1	NM_005558.3	ACTCGCAGTGCCAGCAT ACCCCGAGACTTGACAGATT TGAAGTTGGGAGAGAAGCTGGAGAGA
FGFR3	NM_022965.3	CGTACTGTGCCACTTCAGTGT AGTAAGGGGCCCCTGTGT ATGACGAAGACGGGGAGGACGA
IGFBP5	NM_000599.3	AGCAAGTCAAGATCGAGAGA ATCTTGGGGGAGTAGGTCT AGGAGCCCACCACCTCTGAGAT
KRT19	NM_002276	AGCAGGTCCGAGGTTACT TCCAAGGCAGCTTTCAT TCTTGAGATTGAGCTGCAGTCACA
ACTB	NM_001101	ACAGGATGCAGAAGGAGAT TCCACACGGAGTACTTGC ATCAAGATCATTGCTCCTCCTGAG
TACSTD1	NM_002354	GTAAAAGTTTGCGGACTGC AATACTCGTGATAAATTTTGGATC TCAGAAGGAGATCACAACGCGTTA
BST1	NM_004334	AGCAGCGGAACAAGAA AGTTAATAAAAAGGTCATAGTCTGA AGCCATCTGGGAAGCCTTTAAAGT

[a]Also referred to as mammary serine protease inhibitor (maspin). ER, estrogen receptor; MAGE-A3, melanoma-associated antigen 3; ERBB4, erythroblastic leukemia viral oncogene homolog-4; PR, progesterone receptor; TFF, trefoil factor; MG, mammaglobin; SPDEF, SAM pointed domain-containing Ets transcription factor; EGFR, epidermal growth factor receptor; PKP, plakophilin; SCGB1D2, secretoglobin family 1D member 2; FOXA1, forkhead box protein A1; AGR2, anterior gradient 2 homolog; PIP, prolactin-induced protein; CEA, carcinoembryonic antigen; TNRC9, trinucleotide repeat-containing 9; LAD, ladinin; FGFR, fibroblast growth factor receptor; IGFBP, insulin-like growth factor binding protein; KRT, keratin; ACTB, β-actin; TACSTD, tumor-associated calcium signal transducer; BST1, bone marrow stromal cell antigen 1.

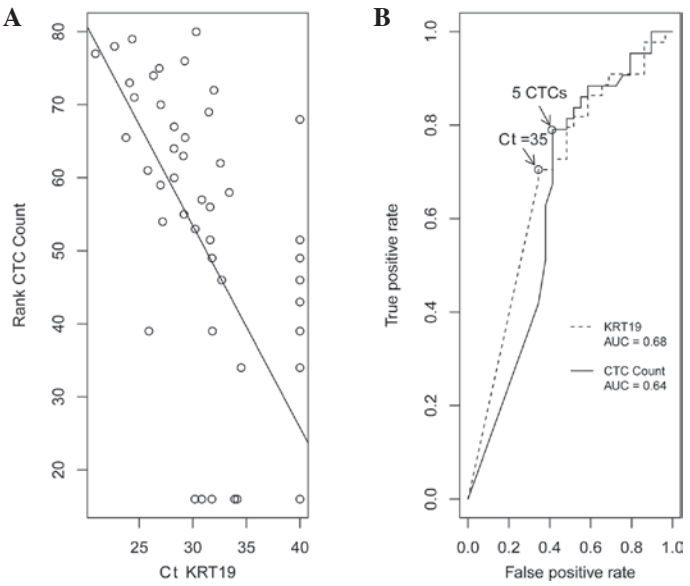

Figure 1. Keratin 19 (KRT19) gene expression is highly correlated with circulating tumor cell (CTC) count. (A) Scatter plot of the correlation between the Ct of KRT19 and the rank order of the CTC count (correlation coefficient, 0.7). (B) Receiver operating characteristic curve of using baseline measures of either the Ct of KRT19 or the CTC count to discriminate between patients with and those without disease progression at follow-up. Ct, cycle threshold; AUC, area under the curve.

Table III. Gene markers specifically expressed in circulating tumor cells (CTCs).

Gene	No. of CTC samples detected	Correlation coefficient between Ct and CTC count	P-value
TFF1	27	-0.53	5.13E-07
ERBB4	12	-0.55	1.59E-07
CEA	10	-0.48	9.31E-06
IGFBP5	17	-0.47	1.21E-05
MAGE-A3	7	-0.43	9.35E-05
MG	25	-0.40	2.36E-04
TNRC9	5	-0.38	6.33E-04
PIP	16	-0.35	1.76E-03
PR	5	-0.29	9.44E-03
SERPINB5	3	-0.28	1.31E-02
SCGB1D2	1	-0.16	1.62E-01
EGFR	4	-0.09	4.55E-01

TFF, trefoil factor; ERBB4, erythroblastic leukemia viral oncogene homolog-4; CEA, carcinoembryonic antigen; IGFBP, insulin-like growth factor binding protein; MAGE-A3, melanoma-associated antigen 3; MG, mammaglobin; TNRC9, trinucleotide repeat-containing 9; PIP, prolactin-induced protein; PR, progesterone receptor; MASP, maspin; SCGB1D2, secretoglobin family 1D member 2; EGFR, epidermal growth factor receptor; Ct, cycle threshold.

Table IV. Contingency table of baseline hormonal treatment and disease progression.

Hormonal treatment	No progression[a]	Progression
A, Patients with no detectable ERβ expression in CTCs[b].		
Yes	3	2
No	27	19
B, Patients with detectable ERβ expression in CTCs (Ct<34)[c].		
Yes	9	1
No	4	7

[a]One sample was excluded due to the lack of available information on hormonal treatment. CTCs, circulating tumor cells. [b]Fisher's exact test P=1. [c]Fisher's exact test P=0.02. ER, estrogen receptor; Ct, cycle threshold.

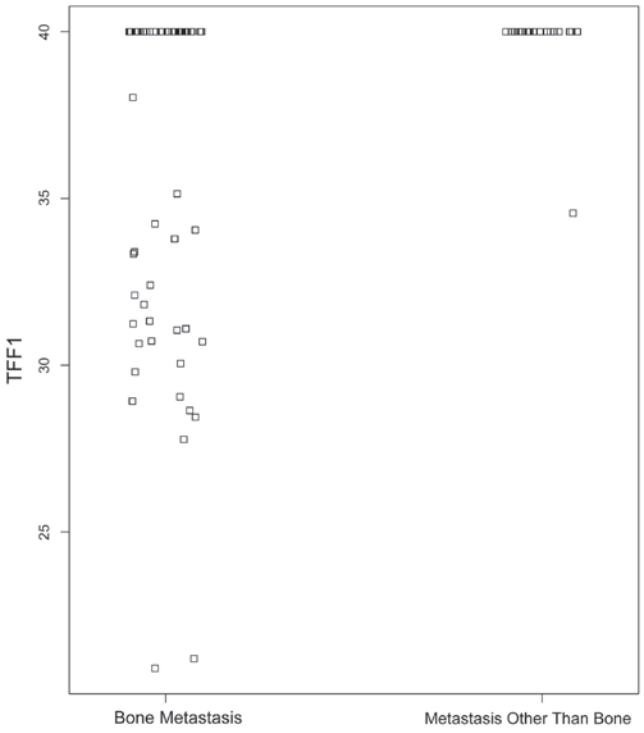

Figure 2. The Ct value of trefoil factor 1 (TFF1) expression stratified by bone metastasis at the base line measures. TFF1 association with bone metastasis is independent of correlated to circulating tumor cell count. Ct, cycle threshold.

of the CTC count is presented in Fig. 1A; the correlation coefficient was 0.7. A receiver operating characteristic curve was generated using the baseline measurements of either the KRT19 expression [area under the curve (AUC=0.68)] or the CTC count (AUC=0.64), to distinguish between patients with and those without disease progression (Fig. 1B). The expression of β-actin and bone marrow stromal cell antigen 1 (BST1) was prominent in the samples purified through the CellSearch® system (data not shown). The correlation of expression between these two genes was 0.953. Since β-actin is expressed in all cell types, whereas BST1 is specific to leukocytes, the detection of BST1 expression demonstrated that the cells purified through the CellSearch System exhibited a significant amount of leukocyte carryover, as previously demonstrated (11). The highly correlated expression profile between β-actin and BST1 also suggested that the leukocyte carryover contributed to the expression of β-actin.

TTF1 expression is associated with bone metastasis. In our earlier study, TFF1 was identified as the most differentially expressed gene associated with bone metastasis (6). Consistently, the results of this study demonstrated that TFF1

expression in CTCs is strongly associated with bone metastasis and this association is independent of the CTC count (Fig. 2). A total of 26 (43.3%) of the 60 patients with bone metastasis exhiibited a high expression level of TFF1. By contrast, only 1 patient (5%) with metastasis at a site other than bone exhibited detectable levels of TFF1 expression. In addition, there was no significant correlation between any other single gene expression and lung or brain metastasis among the 22 genes tested (P>0.5, data not shown).

Association of estrogen receptor β (ERβ) expression with treatment response. ERβ expression and its potential role as a predictor of hormonal treatment response in breast cancer has been well established based on studies of primary breast cancer tissues (12-14). To assess the involvement of ERβ expression in CTCs, 73 patients were analyzed. One sample exhibiting no disease progression was excluded during data analysis due to the lack of available information on hormonal treatment (Table IVA). The ERβ expression was found to be correlated with disease progression following hormonal treatment. A total of 51 of the remaining 72 patients (70%) had undetectable ERβ gene expression levels in CTCs after 40 cycles of PCR (Table IVA). In this group of patients, there was no significant difference in disease progression between the patients with and those without hormonal treatment. By contrast, 21 of the 72 patients (29%) exhibited ERβ expression (Ct<35) in CTCs (Table IVB). In this group, the patients with high ERβ expression in CTCs exhibited significantly improved outcomes with hormonal treatment (P=0.02). In this group, 9 of 10 patients (90%) did not exhibit disease progression following hormonal treatment. However, 4 of 11 patients (36%) that received hormonal treatment did not have progression and 7 of 11 patients (64%) that did not receive the treatment had disease progression. Furthermore, there was no correlation between the expression of ERβ and the CTC count.

Discussion

The detection of CTCs in the blood of cancer patients has been shown to be a prognostic factor. In addition, post-therapy changes in the CTC count are associated with disease progression in patients with metastatic breast cancer (2,14,15). qRT-PCR is a sensitive and specific method for the assessment of gene expression and is commonly used in research and clinical applications. In this study, CTC enrichment and specific gene expression evaluation by qRT-PCR were used in combination (2,16,17). We demonstrated that CTCs were detected in 61% of the study population; among these, 66.7% had ≥5 CTCs and 33.3% had 1-4 CTCs in the 7.5-ml blood samples. Moreover, we evaluated specific gene expressions in CTCs and our results suggested that CTC-derived gene expression markers may be used as specific indicators for the research and treatment of breast cancer.

The genes selected for this study were specific to breast epithelial cells and their upregulation had been previously associated with breast cancer tumorigenesis (3,18). The detection of several markers in the isolated CTCs confirmed that the CTCs originated from the breast and revealed that the majority of the CTCs maintained the properties of breast cancer cells. Previous studies by Ignatiadis *et al* (19) and Xenidis *et al* (20) reported that the presence of KRT19 mRNA-positive CTCs prior to the initiation of adjuvant therapy was associated with a shortened disease-free survival and that the presence of KRT19 mRNA-positive CTCs was associated with early clinical relapse and disease-related mortality in 167 node-negative breast cancer patients. In our study, ~50% of the patients exhibited KRT19 overexpression. Therefore, similar to the CTC count, the measurement of the KRT19 expression level in CTCs may be used to predict disease progression. However, the significance of this association has not been fully elucidated.

It has been documented that breast cancer spreads to distant organs, particularly regional lymph nodes, bone, liver, lung and brain (1). TFF1 has been shown to be associated with breast cancer bone metastases. In this study, we demonstrated that 43.3% of the patients with bone metastasis had a high expression level of TFF1 in the CTCs, with only 5% of the patients with metastases in sites other than bone exhibiting detectable TFF1 expression in the CTCs. Furthermore, the strong correlation between the TFF1 expression in CTCs and bone metastasis appears to be independent of the CTC count.

Various observations have been made on the relationship between ERβ expression and response to endocrine therapy. Lee *et al* (13) reported that the increased expression of ERβ is associated with increased likelihood of response to endocrine therapy. Hartman *et al* (14) reported that overexpression of ERβ mRNA was observed in the tumors of breast cancer patients who relapsed while receiving adjuvant hormone treatment. A positive correlation was also reported between the expression of ERβ and epidermal growth factor receprtor, frequently associated with endocrine insensitivity (21). The contradictory results may be attributed to the differences in the patient characteristics between the different study cohorts. In our study, 31% of the patients exhibited ERβ overexpression, which was significantly associated with endocrine sensitivity. In addition, there was no significant correlation between the expression of ERβ and that of other markers, including ERα. Further investigations are required to determine whether ERβ is of clinical value in the prediction of the response to hormone therapy and disease progression.

We also observed that BST1, a leukocyte-specific marker, was expressed in the majority of the HBD and the metastatic breast cancer patient samples. This result was consistent with the detection of leukocytes in the CTC-enriched populations, which may affect the analytical sensitivity of CTC-specific genes and represent a challenge regarding the detection of genes that are not specifically expressed in CTCs, although they may be of important diagnostic or therapeutic value. Further improvements on the CTC technology or development of additional negative selection methods for the reduction of leukocytes are required for CTC molecular characterization.

In summary, clinical oncology is challenged by the lack of predictive models for therapy selection and response to treatment that are simple, non-invasive and cost-effective. CTC technologies may represent a promising tool that enables enumeration and molecular characterization of metastatic cancer cells and estimate the prognosis and therapeutic response of cancer patients. Ongoing investigations continue to accumulate knowledge on the molecular and cellular processes implicated in the clinical behavior of cancer. Although in need of further validation, the findings

of the present study may benefit patients through the earlier detection of organ-specific metastasis and the design of personalized treatment strategies, leading to improved patient management and outcomes.

References

1. Weigelt B, Peterse JL and van 't Veer LJ: Breast cancer metastasis: markers and models. Nat Rev Cancer 5: 591-602, 2005.
2. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW and Hayes DF: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 351: 781-791, 2004.
3. Smirnov DA, Zweitzig DR, Foulk BW, Miller MC, Doyle GV, Pienta KJ, Meropol NJ, Weiner LM, Cohen SJ, Moreno JG, Connelly MC, Terstappen LW and O'Hara SM: Global gene expression profiling of circulating tumor cells. Cancer Res 65: 4993-4997, 2005.
4. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, Doyle GV, Matera J, Allard WJ, Miller MC, Fritsche HA, Hortobagyi GN and Terstappen LW: Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol 23: 1420-1430, 2005.
5. Sieuwerts AM, Mostert B, Bolt-de Vries J, *et al*: mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. Clin Cancer Res 17: 3600-3618, 2011.
6. Smid M, Wang Y, Klijn JG, Sieuwerts AM, Zhang Y, Atkins D, Martens JW and Foekens JA: Genes associated with breast cancer metastatic to bone. J Clin Oncol 24: 2261-2267, 2006.
7. Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordón-Cardo C, Guise TA and Massagué J: A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 3: 537-549, 2003.
8. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL and Massagué J: Genes that mediate breast cancer metastasis to lung. Nature 436: 518-524, 2005.
9. Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, Minn AJ, van de Vijver MJ, Gerald WL, Foekens JA and Massagué J: Genes that mediate breast cancer metastasis to the brain. Nature 459: 1005-1009, 2009.
10. Saloustros E, Perraki M, Apostolaki S, Kallergi G, Xyrafas A, Kalbakis K, Agelaki S, Kalykaki A, Georgoulias V and Mavroudis D: Cytokeratin-19 mRNA-positive circulating tumor cells during follow-up of patients with operable breast cancer: prognostic relevance for late relapse. Breast Cancer Research 13: R60, 2011.
11. Reinholz MM, Nibbe A, Jonart LM, Kitzmann K, Suman VJ, Ingle JN, Houghton R, Zehentner B, Roche PC and Lingle WL: Evaluation of a panel of tumor markers for molecular detection of circulating cancer cells in women with suspected breast cancer. Clin Cancer Res 11: 3722-3732, 2005.
12. Speirs V: Oestrogen receptor beta in breast cancer: good, bad or still too early to tell? J Pathol 197: 143-147, 2002.
13. Lee GS, Choi KC, Kim HJ and Jeung EB: Effect of genistein as a selective estrogen receptor beta agonist on the expression of calbindin-D9k in the uterus of immature rats. Toxicological Sci 82: 451-457, 2004.
14. Hartman J, Ström A and Gustafsson JA: Estrogen receptor beta in breast cancer - diagnostic and therapeutic implications. Steroids 74: 635-641, 2009.
15. Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, Matera J, Allard WJ, Doyle GV and Terstappen LW: Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res 12: 4218-4224, 2006.
16. O'Hara SM, Moreno JG, Zweitzig DR, Gross S, Gomella LG and Terstappen LW: Multigene reverse transcription-PCR profiling of circulating tumor cells in hormone-refractory prostate cancer. Clin Chem 50: 826-835, 2004.
17. Ring AE, Zabaglo L, Ormerod MG, Smith IE and Dowsett M: Detection of circulating epithelial cells in the blood of patients with breast cancer: comparison of three techniques. Br J Cancer 92: 906-912, 2005.
18. Ghadersohi A and Sood AK: Prostate epithelium-derived Ets transcription factor mRNA is overexpressed in human breast tumors and is a candidate breast tumor marker and a breast tumor antigen. Clin Cancer Res 7: 2731-2738, 2001.
19. Ignatiadis M, Kallergi G, Ntoulia M, Perraki M, Apostolaki S, Kafousi M, Chlouverakis G, Stathopoulos E, Lianidou E, Georgoulias V and Mavroudis D: Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, mammaglobin A, and HER2 in early breast cancer. Clin Cancer Res 14: 2593-2600, 2008.
20. Xenidis N, Ignatiadis M, Apostolaki S, Perraki M, Kalbakis K, Agelaki S, Stathopoulos EN, Chlouverakis G, Lianidou E, Kakolyris S, Georgoulias V and Mavroudis D: Cytokeratin-19 mRNA-positive circulating tumor cells after adjuvant chemotherapy in patients with early breast cancer. J Clin Oncol 27: 2177-2184, 2009.
21. Knowlden JM, Gee JM, Robertson JF, Ellis IO and Nicholson RI: A possible divergent role for the oestrogen receptor alpha and beta subtypes in clinical breast cancer. Int J Cancer 89: 209-212, 2000.