J Korean Med Sci 2000; 15: 194-8
ISSN 1011-8934

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The Detection of Circulating Breast Cancer Cells in Peripheral Blood by Reverse Transcriptase-Polymerase Chain Reaction

Some circulating cancer cells in the blood play a central role in the metastatic process and may have a major influence on patient progress. Their numbers can be very small and techniques for their detection need to be both sensitive and specific. Polymerase chain reaction (PCR) has been successfully used to detect small numbers of tumor cells in cancer. We used a reverse transcriptase-polymerase chain reaction (RT-PCR) to detect circulating breast cancer cells in venous blood samples before operations and assessed cytokeratin-19 (CK-19) and cytokeratin-20 (CK-20) as target mRNA markers in the blood of healthy donors (n=6) and breast cancer patients (n=30) with American Joint Committee on Cancer stages 0 to IIIa. CK-19 mRNA was expressed in all blood samples of healthy donors and patients. But CK-20 was the only mRNA marker not detected in the blood from healthy donors. Seven of 30 (23%) venous blood isolates of breast cancer patients yielded a CK-20 mRNA with positive results. There was no correlating CK-20 mRNA expression with stage and axillary lymph node status. In conclusion, CK-19 showed no diagnostic value as a mRNA marker in the detection of circulating cancer cells by RT-PCR assay because this was expressed in the blood of healthy donors. CK-20 mRNA was an useful marker to detect circulating cancer cells in breast cancers.

Key Words: *Breast Neoplasms; Keratin; Reverse Transcriptase Polymerase Chain Reaction*

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Received: 3 November 1999
Accepted: 7 January 2000

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INTRODUCTION

In patients with operable breast cancer, the number of axillary nodes with metastases is an important prognostic factor that allows selection of those who might benefit from adjuvant medical treatment (1). But, other than lymph node metastases, prognostic factors that suggest high probability of recurrence and need for adjuvant therapy have not been well established (2). Breast cancer has been accepted by many investigators as a systemic disease (3, 4). Thus, the detection of circulating tumor cells in the peripheral blood of breast cancer patients may be a major factor that influences their prognosis. Serial sectioning and immunohistochemistry staining of axillary lymph nodes in breast cancer patients have increased the detection of micrometastases ranging from 9% to 33% (5). However, immunohistochemistry despite having the sensitivity to detect 1 tumor cell in 10^5 to 10^6 normal cells is very labor-intensive and has a limited specificity due to a specific antigen expression. Recently, reverse transcriptase-polymerase chain reaction (RT-PCR) has been developed as an ultrasensitive method (sensitivity: 1 cancer cell in 10^7 normal cells) to detect micrometastases in peripheral blood of patients with breast cancer (6-9) and various epithelial cell cancers (10). In breast cancer, Datta et al. (11) have developed a method based on RT-PCR amplification of cytokeratin mRNA for detection.

In this study, we detected the circulating cancer cells with CK-19 and CK-20 as mRNA markers in the peripheral blood of patients with varying stages of breast cancers using RT-PCR. Then we assessed the usefulness of CK-19 or CK-20 as a target mRNA marker.

MATERIALS AND METHODS

Blood preparation

Peripheral blood was obtained from 6 healthy female donors and 30 breast cancer patients without evidence of distant metastasis and who were operated at Korea

University Hospital in 1998. Ten milliliters of peripheral blood was sampled at the ante-cubital vein prior to surgery.

Total RNA isolation

0.5 mL of blood samples were homogenized in 0.5 mL of TRI REAGENT (MRC Inc., Cincinnati, OH, U.S.A.) and total RNA was extracted according to the manufacturer's instructions. Protein was removed by adding 0.2 mL of chloroform. Then, they were centrifuged at 14,000 *g* for 20 min at 4℃ after vigorous vortexing. The supernatant was transferred to a fresh tube and 0.3 mL of isopropranol was added for RNA precipitation. The extracted RNA was washed with 70% ethanol, dried and solubilized in diethylpyrocarbonate-treated water and stored at -20℃ until use.

RT-PCR

cDNA was synthesized from 50 ng (10 μL) of extracted RNA in reaction mixture containing 0.02 mM deoxynucleotide triphosphate (dNTP), 25 ng/μL oligodithiothreitol (dT), 2 unit/μL RNAsin and 5 μL of 5× reverse transcriptase reaction buffer. The mixture was incubated at 42℃ for 45 min, heated to 95℃ for 5 min, and chilled at 4℃ for 5 min.

The PCR conditions for CK-19 was set up as follow: one cycle of denaturing at 95℃ for 5 min, followed by 35 cycles of 95℃ for 30 sec, 62℃ for 30 sec, and 72℃ for 30 sec before one cycle of a final extension at 72℃ for 5 min.

The PCR conditions for CK-20 was set up as follow: one cycle of denaturing at 95℃ for 5 min, followed by 35 cycles of 95℃ for 1 min, 55℃ for 1 min, and 72℃ for 1 min before one cycle of a final extension at 72℃ for 5 min for each first and second round PCR. Five to 8 μL aliquots of the PCR products were electrophoresed on 2% agarose gels and detected by direct UV visualization after bromide staining. The first PCR product for CK-20 exhibited a 349-bp fragment and the second PCR product for CK-20 exhibited a 309-bp fragment.

Table 1. The primer sequence of beta-microglobulin and synthetic oligonucleotide primers for CK-19 and CK-20 RT-PCR

mRNA	Primers 5'-3'	Product size (bp)
beta-micrglobulin		
	F: TGA CGG GGT CAC CCA CAC TGT GCC	
	R: CTA GAA CCA TTG GGG TGG ACG ATG	281
CK-19	F: GCG GGA CAA GAT TCT TGG TG	
	R: CTT CAG GCC TTC GAT CTG CAT	214
CK-20 (First PCR)		
	1F: CAG ACA CAC GGT GAA CTA TGG	
	1R: GAT CAG CTT CCA CTG TTA GAC G	349
CK-20 (Second PCR)		
	2F: CTG TTT GTT GGC AAT GAG AAA ATG G	
	2R: GTA TTC CTC TCT CAG TCT CAT ACT	303

Primers for beta-microglobulin, CK-19 and CK-20

The primer sequence of beta-microglobulin and synthetic oligonucleotide primers for CK-19 and CK-20 RT-PCR are listed in Table 1.

Statistical analysis

The Student's t test was used to compare discrete variables. Significance was defined as $p<0.05$. All statistical analysis were made using a commercial statistical software package (Statistica 6.0 for Windows: Statsoft Inc., Tulsa, OK, U.S.A.).

RESULTS

To determine the specificity of the RT-PCR assay, peripheral blood from 6 healthy donors was evaluated for target mRNA marker expression. In our study, CK-19 mRNA was totally expressed in blood cells from healthy donors and 30 breast cancer patients (Fig. 1).

CK-20 mRNA was expressed in 23.3% (7/30) of breast cancer patients but it was not expressed in healthy donors. CK-20 was the only reliable mRNA marker for the detection of circulating tumor cells in the blood because it was not expressed in blood cells from healthy donors (Fig. 2).

CK-20 mRNA expressions according to stages of breast cancer were 20.0% in carcinoma in situ (1/5), 20.0% in stage I (1/5), 25.0% in stage IIA (2/8), 20.0% in stage IIB (1/5) and 28.6% in stage IIIA (2/7). There was no correlating expression of CK-20 mRNA with stage of breast cancer ($p>0.05$) (Fig. 3).

Expressions of CK-20 mRNA according to axillary lymph node metastases were 23.5% in patients without axillary lymph node metastases and 23.0% in patients with axillary lymph node metastases. Also there was no correlating expression of CK-20 mRNA with axillary lymph node metastases ($p>0.05$) (Fig. 4).

DISCUSSION

As 12-37% of small breast cancers below 1 centimeter, which are mammographically detected, have been already

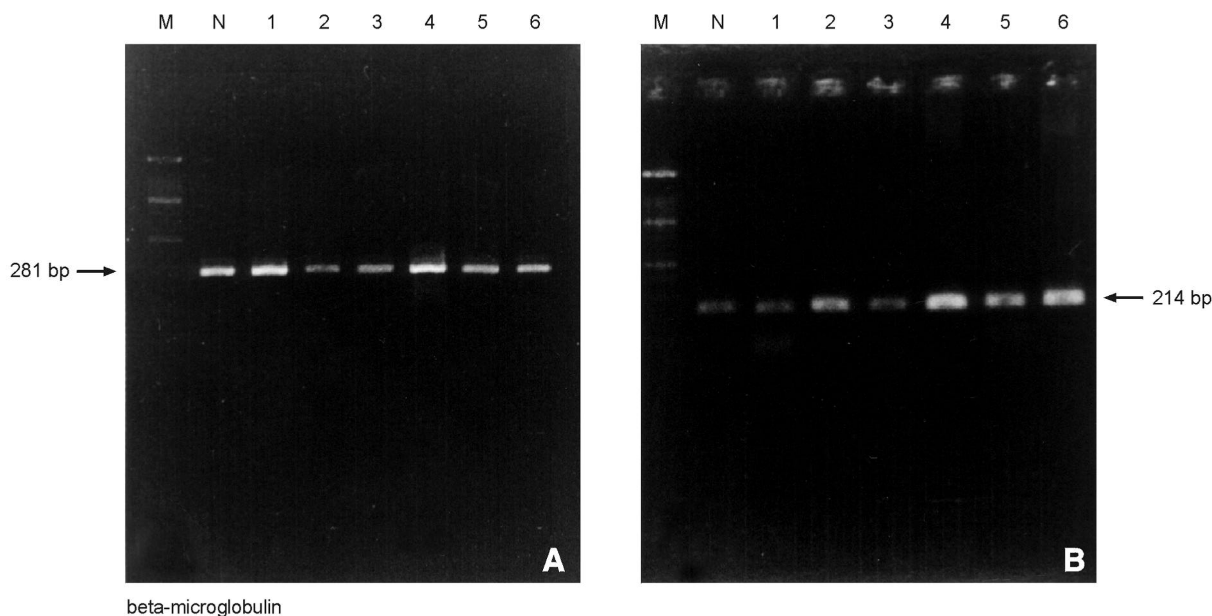

Fig. 1. RT-PCR products for CK-19 from peripheral blood of breast cancer patients and normal healthy donor. The bands indicate an adequate cDNA synthesis of both normal healthy donor and breast cancer patients (**A**). CK-19 (214 bp) was identified in both breast cancer patients (1-6) and normal healthy donor (N) after separation of products on an agarose gel and staining with ethidium bromide (**B**). 100-bp DNA ladder are shown in lane M. M, M.W. markers; N, normal healthy donor; bp, base pairs.

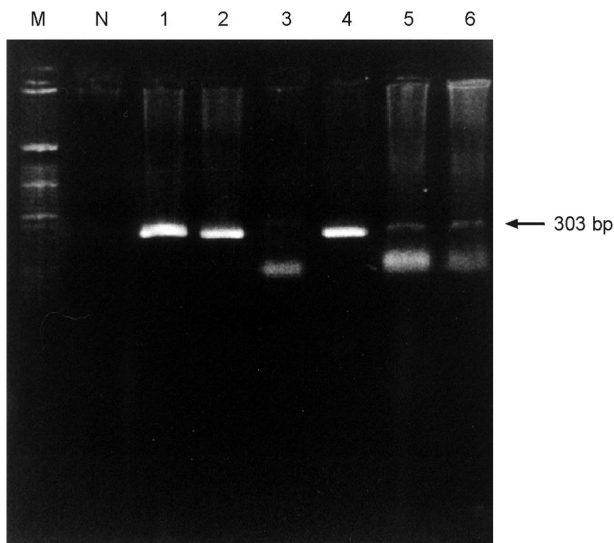

Fig. 2. RT-PCR products for CK-20 from peripheral bloods of breast cancer patients and normal healthy donors. The positive amplification with RT-PCR (a single band of 303 bp) was identified after separation of products on an agarose gel and staining with ethidium bromide (patients 1, 2, 4, 5, 6). Lane N and Lane 3 were not identified at any band of 303 bp. 100-bp DNA ladder are shown in lane M. M, molecular weight markers; N, normal healthy donor; bp, base pairs.

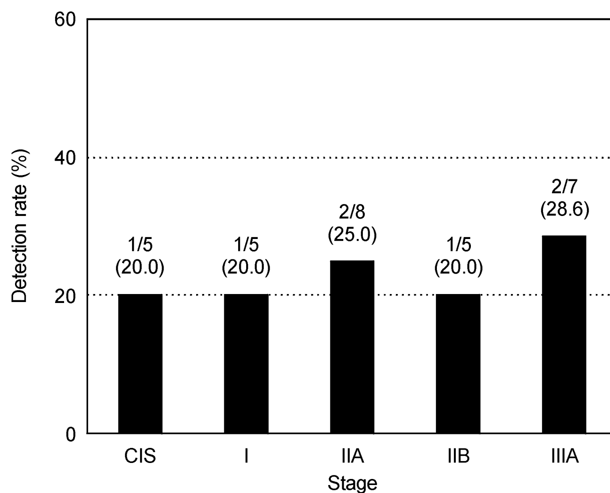

Fig. 3. The detection rates of CK-20 according to stages. CIS, carcinoma in situ.

metastasized at diagnosis, early detection is important in cancer treatment (12, 13). The detection of micrometastasis using RT-PCR may be valuable in assessing the prognosis and planning of treatment. Although the immunohistochemistry of peripheral blood is sensitive, the RT-PCR of peripheral blood is more superior than it in terms of sensitivity and work load (7, 9, 11). The sensitivity of RT-PCR is so high that even a trace of chromosomal DNA in the RNA sample may result in gene amplification products.

Recently, the assay of carcinoembryonic antigen (CEA), CK-19, CK-20, gastrointestinal tumor-associated antigen (GA733.2) and mucin-1 (MUC-1) in peripheral blood using RT-PCR method has been developing rapidly for early detection of micrometastasis as mRNA markers in

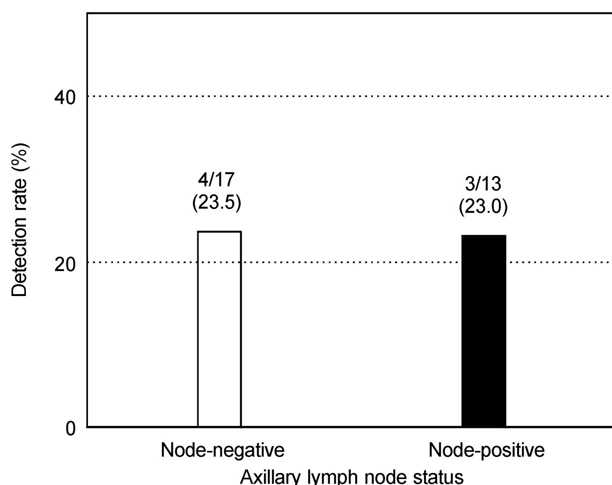

Fig. 4. The detection rate of CK-20 according to lymph node status.

cancer patients (8, 14, 15). The cytokeratins are predominantly expressed in epithelial cells and malignant epithelial cells generally retain intermediate filaments of these cytokeratin. Consequently, the cytokeratins have been used to characterise neoplastic cells of epithelial origin (16).

It has been shown that CK-19 is expressed not only in breast cancer but also in other cancers such as pancreatic and gastric cancers. Datta et al. (11) reported that CK-19 was not expressed in normal blood cells using RT-PCR and described their use for early detection of hematogenous breast cancer dissemination. However, some recent publications have suggested that CK-19 expression is seen in a significant number of peripheral blood samples from healthy donors (17, 18). Aihara et al. (19) reported that the contamination of epidermal cells at needle veno-puncture or during collection of sample might explain the discrepancy between the two reports. By using a venous catheter and collecting blood sample after discarding the first 1 milliliter, they could avoid false positivity due to epithelial cells contamination. In our report, in spite of using this method, CK-19 was expressed 100% in both normal healthy donors and breast cancer patients. Therefore, CK-19 shows no diagnostic value as a mRNA marker for the detection of circulating cancer cells because it was also expressed in the blood of healthy donors.

CK-20 has recently been reported as an mRNA marker in the detection of micrometastasis by RT-PCR analysis, mainly in patients with gastrointestinal malignancies (16, 20, 21). Burchill et al. (17) suggested that CK-20 was not expressed in normal hematopoietic cells unlike other cytokeratin genes (CK-8 and CK-19), but expressed in almost all cases of colorectal adenocarcinoma. Bostick et al. (8) reported that CK-20 was the only mRNA marker to be not expressed in the lymph nodes or peripheral blood of patients without breast cancer by RT-PCR analysis and the detection rate of CK-20 in breast cancer patients was 75%. In our study, CK-20 was expressed in 7 of 30 (23.3%) breast cancer patients, and the detection rate of CK-20 was low compared with their results. But CK-20 was not expressed in normal healthy donors. Therefore we thought that CK-20 may be specific for the detection of circulating cancer cells as mRNA marker in peripheral blood even if it's sensitivity is low.

Although Datta et al. (11) demonstrated that CK-19 was expressed only in stage IV breast cancer patients, there were no trials concerning correlating CK-20 expression with stage of breast cancer. Our results show that the detection rates of CK-20 according to stage of American Joint Committee of Cancer (AJCC) were not significantly different and suggest that the detection rate of CK-20 by RT-PCR in peripheral blood clearly does not depend on tumor stages from carcinoma in situ (CIS) to IIIA. But further research is needed to get more defined results because our experimental samples were not large enough.

So far, the correlation between the expression rates and axillary lymph node metastases has not been investigated in breast cancer patients. Although there was a slight difference in detection rate between patients with and without axillary lymph node metastasis, it was statistically not significant in our study. But, the definite answer to the prognostic significance of CK-20 which was assessed by RT-PCR assay will require extensive follow-up to evaluate the recurrence rate and survival of patients.

In conclusion, we suggest that CK-19 showed no diagnostic value as mRNA marker for the detection of circulating breast cancer cells because this was expressed in the blood of healthy donors. CK-20 was an useful marker for the detection of circulating breast cancer cells in the peripheral blood, although it did not correlate with stage of breast cancer and status of axillary lymph node. For a final evaluation of application of CK-20 assay in the detection of single tumor cells in breast carcinoma, the need for larger samples from patients with this disease is mandatory.

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