Detection of CK-19 mRNA in Peripheral Blood of Breast Cancer Patients by Reverse Transcription Polymerase Chain Reaction*

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Abstract **Objective** To evaluate the prognostic significance of the molecular detection of cytokeratin 19 (CK-19) mRNA-positive cells in the peripheral blood of women with operable breast cancer, and establish an early diagnostic method for breast cancer micrometastasis in peripheral blood. **Methods** Peripheral blood samples of patients with breast cancer (test group, n=65) and with benign breast tumor (control group, n=37)were taken. Then, separated nucleated cells and extracted total RNA,CK-19 mRNA was detected by nested reverse transcription polymerase chain reaction(nested RT-PCR) technique. **Results** Samples were diagnosed as CK-19mRNA positive when 460 bp band is appeared in nested RT-PCR end-product. The positive rates of CK-19mRNA is 36.92 % (24/65) in test group. None of CK-19mRNA was expressed in contral group. The difference between the two groups was statistically significant (P<0.001). **Conclusion** Analyses using RT-PCR for CK19 mRNA may prove to have clinical significance in the assessment of circulating tumour cells in peripheral blood of breast cancer patients. Application of this technique in a clinical population may improve diagnosis and monitoring of metastatic breast cancer and its validation is currently ongoing.

Key Words Breast cancer; Peripheral blood; Micrometastasis; RT-PCR; CK-19mRNA

Breast carcinoma is one of the most prevalent potentially lethal diseases in women, its incidence rates is 31%[1]. Approximately two thirds of these patients have histologically negative axillary lymph nodes metastasis. Still 30% of these node-negative patients will develop metastatic disease and die from it[2]. Especially in this patient population, detection of small numbers of breast cancer cells in bone marrow, peripheral blood or lymph nodes could provide a useful diagnostic and monitoring tool.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) as target mRNA markers in the blood of the patients with breast benign tumors and with breast cancer patients.

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MATERIAL AND METHODS

Clinical materials

During the period between May 2000 and April 2002, 65 female patients were diagnosed as having breast cancer and were treated at the Departments of tumor surgeon at First Affiliated Hospital of Lanzhou Medical College, China. Peripheral blood (4 ml in EDTA) was obtained from 65 patients with stage I-III breast cancer without evidence of distance metastesis(test group, n=65) and patients with benign breast tumor (control group, n=37)were taken.Every cases had not undergone chemotherapy radiotherapy before blood was drawn. According to UICC, patients in stage I-III were 25, 33, 7 cases respectively, and cases with the axillary lymph nodes metastases and no-metastases were 34, 31 case respectively.

RNA Extraction and cDNA

Total cellular RNA was extracted from the mononuclear peripheral blood cells by using RNA-clean according to the manufacturer´s instructions and dissolved in diethylpyrocarbonate-treated water.

RNA concentration was measured spectrophotometrically at 260 nM and the RNA samples were stored at −70° C until assaying. First-strand cDNA was synthesized by using Moloney-murine leukemia virus (M-MLV) reverse transcriptase. RNA (1 μg) was added to 200 units of enzyme, 2 μl 10× reaction buffer (500 mM Tris HCl, pH 8.3; 750 mM KCl; 100 mM DTT; 30 mM MgCl2), 1 μM deoxyribonucleoside triphosphates, 20 units RNAsin, 5μM random hexamers, and 1μM antisense primer to a final volume of 20 μl. The cDNA synthesis was performed at 37° C for 60 minutes. After heat inactivation at 95° C for 10 minutes, 2 μl cDNA were subjected to PCR analysis.

PCR and Gel Electrophoresis

Specific cDNA sequences were amplified in a reaction mix composed of 2 μl cDNA, 5 μl 10× PCR-buffer (100 mM Tris HCl, pH 8.3; 500 mM KCl; 15 mM MgCl2, 0.1% gelatin), 50 μM dNTPs, 400 nM each of specific sense and antisense primer, and 1.5 units AmpliTaq DNA polymerase in a total volume of 50 μl. All primers(for Ck-19:A: 5′-AGCAGAACCGGAAGGATG-3′, andB:5′-AGGCTGCGGTAGGTGGCAAT-3′; for β-actin: 5'-GTCAACGGATTTGGTCTGTATT-3'and 5'-AGT-CTTCTGGGTGGCAGTGAT-3') were synthesized. The cycling conditions for β-actin and Ck-19 were 40 cycles (300seconds at 94℃, 45 seconds at annealing temperature, and 45 seconds at 72℃). The RT-PCR products were analyzed in 2% agarose gels stained with ethidium bromide. A 1000 bp DNA ladder was used as a size marker. The presence of intact RNA was confirmed by a single-round RT-PCR using the housekeeping gene β-actin. A DNA-derived amplification product of CK-19 PCR is approximately 460 bp in length and could be clearly distinguished from the RNA-derived product. All reagent were provided by Shanhai Biological Engineering Technology and Service Co; Ltd, China.

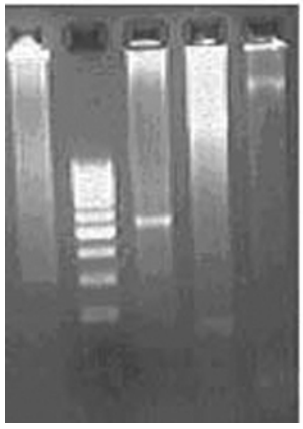
Statistical Analysis

Differences in characteristics and of groups were analyzed by the two-tailed chi-square or Fisher′s exact test, when appropriate. A $p<0.05$ was considered as significant. Data processing was performed by means of SPSS for Windows (SPSS Inc, Chicago, Illinois).

RESULTS

Marker Expression in Blood Samples from Patients with breast benign tumor and Patients with breast cancer

Table indicates that the presence of CK-19 mRNA-positive cells in peripheral blood from patients with breast cancer and benign breast tumor. CK-19mRNA was expressed in 36.92%(24/64) of breast cancer patients but it was not expressed in benign tumor patients. The difference was statistically significant ($P<0.001$). CK-19 mRNA expressions accordings to stages of breast cancer were



1. Negative contrast 2. DNA Marker 3. CK-19mRNA -Postive 4. β-actin 5. CK-19mRNA-negative

Fig. Characteristics of the Breast Cancer Patients with CK-19 mRNA Expression in Peripheral Blood.

Table Expression of ck-19 mRNA in Blood Samples from Patients with benign tumor and Patients with breast cancer

Groups	cases	Positive	positive rates(%)	P
Breast cancer	65	24	36.92	$P<0.001$
Breast benign tumor	37	0	0	
Breast cancer stage				
StageI	25	5	20.00	$P<0.05$
StageII	33	14	42.42	
StageIII	7	5	71.43	
Axillary lymph node				$P<0.05$
Positive	34	17	50.00	
Negative	31	7	22.58	

20.00% in stage I, 42.72% in stage II and 71.43% in stage III. There was correlating expression of CK-19 mRNA with stage of breast cancer($P<0.05$). Expressions rate of CK-19 mRNA was 50.00% with axillary lymph node metastases and 22.58% in patients without axillary lymph node metastases. The difference was significant ($P<0.05$). Expression of CK-19 mRNA in peripheral blood of the pas with breast cancer see Fig..

DISCUSSION

Breast carcinoma is one of the most prevalent tumors in women, the development of metastases is due to the migration of tumor cells from the original tumor to distant organs. This phenomenon probably occurs early during the evolution of the disease and even before the surgical excision of the primary tumor. Major prognostic factors are nodal status, tumor size, histological grade and hormonal receptor status. Theoretically, distant metastasis or recurrence is the result of tumor growth from micrometastasis already existing at the time of surgery. Therefore, efforts have been made to uncover micrometastasis in distant organs to characterize the cancer biologically and to determine subclinical staging. Recently, reverse transcriptase polymerase chain reaction (RT-PCR) has been developed as an ultrasensitive method. The detection of occult tumor cells may be improved by the use of RT-PCR amplification technique, which can identify cell-specific mRNA; this method can detect up to one tumor cell in 10 [7] normal peripheral blood mononuclear cells [3]. Several authors elaborated the techniques to detect cancer cells in circulating blood of many cancer patients by identifying target gene transcripts [4-6]. The aim of our study was to determine the sensitivity and specificity of CK-19 markers in detecting breast cancer cells by RT-PCR and study its clinical significance.

Now, there are no specific and definite markers in solid tumors. Several markers have been used to detect occult tumor cells in the bone marrow of patients with breast cancer. These markers represent proteins encoded by genes that are thought to be tissue specific and are expressed on epithelial cell but not on hematopoietic cells, such as bone marrow cells. The assay of CK-19 in peripheral blood using RT-PCR method has been developing rapidly as mRNA marker in cancer patients. It has been shown that CK-19, which expresses not only in brest cancer but also in other cancers such as gastric and colonic cancer, but din´t express in normal blood and lymph nodes, is one of the most frequently used markers[7]. Therefore, CK-19mRNA detected in peripheral blood of breast cancer may demonstrate the micrometastasis.

With immunocytochemistry alone Schonefeld et al [8] detected CK-19-positive cells in 4 of 75 (5%) peripheral blood samples and 14 of 65 (22%) bone marrow samples of patients with breast cancer; conversely, using RT-PCR they observed CK-19 expression in 19 (25%) of 75 peripheral blood samples and 23 (35%) of 65 bone marrow samples. Slade et al[9], using a semiquantitative RT-PCR assay, also reported that CK-19-positive cells was detected in 54% of peripheral blood from patients with breast cancer. Stathopoulou et, al[10] detected that CK-19-positive cells in the peripheral blood from stages I and II breast cancer by nested RT-PCR before adjuvant therapy was associated with reduced disease-free interval and overall survival. In multivariate analysis, detection of CK-19-positive cells in peripheral-blood was an independent prognostic factor for disease relapse and death. It our study also showed that the expression of CK-19-mRNA was associated with axillary lymph node metastases. Kahn et al[11] detected CK-19mRNA by the reverse transcriptase-polymerase chain reaction (RT-PCR) in blood from 109 patients with invasive breast cancer, CK-19 mRNA was detected in 7/23 patients with node-negative disease, in 21/58 with node-positive disease, and in 20/28 with distant metastases, there was a significant association between the expression of CK-19 and the distant metastatic with node-positive disease.In a way, these results indicate that micrometastasis is highly associated with axillary lymph node metastases and distant metastases.

During the follow-up period, 5 patients (20.83%) with micrometastasis developed distant metastases, only one patient without micrometastasis developed distant metastases. There was no statistically significant difference between two groups ($p>0.05$). This might have been due to fewer cases and shorter follow-up. Manhani et al.[12] studied serially collected blood samples of 53 patients with breast cancer before, during, and after adjuvant, neoadjuvant, and palliative chemotherapy to evaluate its effects on the expression of CK-19 measured by RT-PCR, the positive percentage of CK-19 decreased consistently from 43% (23/53) before

chemotherapy to 14.3% (7/49) and 18.9% (7/37) after 3 and 6 cycles, respectively, furthermore, there was a significant correlation between a negative CK-19 at three months and the response to chemotherapy, there is a significant trend for the occurrence of more negative RT-PCR results. In conclusion, the RT-PCR of CK-19 for micrometastasis in peripheral blood of breast cancer showed high sensitivity. To be useful as an independent prognostic factor of RT-PCR in breast cancer patients, further studies with more cases and longer-term follow-up are required.

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