COMBINED DETECTION OF BREAST CANCER MICROMETASTASES IN THE LYMPH NODES AND BONE MARROW USING REVERSE-TRANSCRIPTASE CHAIN REACTION AND SOUTHERN HYBRIDIZATION

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ABSTRACT

Objective: The presence of lymph nodes and bone marrow micrometastases of patients with breast carcinoma by immunohistochemistry (IHC) methods has been strongly correlated to early recurrence and shorter overall survival. The aim of this study was to detect micrometastases in matched sample pairs of lymph nodes and the bone marrow of primary breast cancer patients using a more sensitive method, and compare with other clinical parameters. Methods: Cytokeratin 19 (CK-19) gene mRNA expression was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern blot hybridization. Human breast cancer cell line T47D was mixed with bone marrow cells at different proportions. The positive detection rate was compared among RT-PCR, Southern blotting and IHC methods. Results: Cytokeratin 19 gene was expressed in all 6 positive control samples, while the expression wasn't seen in 18 negative control samples. CK-19 IHC positive cells were detected at a dilution of one T47D cell in 5 $\times 10^5$ bone marrow cells, while the sensitivity detected by PCR and Southern blot hybridization was at $1:5\times10^4$ and $1:10^6$, respectively. In the samples from the 35 patients, we found CK-19 positive cells in 2 cases (5.7%) by IHC. CK-19 gene expression signal was detected in 14/35 (40%) by RT-PCR, and 17/35 (48.6%) by southern blotting. Four cases were micrometastases positive both in lymph node and bone marrow (11.4%). There was no correlation between CK-1 9 detection and other clinical parameters. Conclusion: combined detection of micrometastases in lymph node and bone marrow by RT-PCR and Southern blotting, using CK-19 as a biological marker, is a highly sensitive method for breast cancer.

Key word: Micrometastases, Cytokeratin 19, Breast cancer, Reverse transcriptase-chain reaction, Southern blot hybridization

Thirty-five to 40% of patients with breast carcinoma, including up to 24% of patients with no evidence of metastases at the time of diagnosis, will relapse after primary therapy.[1, 2] The most reliable prognostic indices-axillary lymph node status and size of primary tumor cannot predict which particular individuals will progress. Bone marrow is a frequent and readily accessible site of metastases. In up to 80% of patients the relapse develops bone marrow metastases at some point in the process of their illness.[3] Current methods to detect bone involvement, such as X-ray, bone scanning, are too insensitive to detect minimal metastases because they depend on the destruction of the bone matrix.

In recent years, the PCR method has been used in various areas. Measurement of a tissue-specific gene transcript following PCR amplification, while retaining specificity, has been reported to increase the sensitivity, with detection of a single neuroblastoma cell in 10^7 peripheral blood mononuclear cells.[4] More recently, several reports have demonstrated that PCR is the most

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sensitive method to detect micrometastases in lymph nodes, bone marrow or peripheral blood.[5-11]

In this study, for the first time, we combined detection of micrometastases in both lymph nodes and bone marrow of patients with operable breast carcinoma using RT-PCR and Southern blot assay. We have also analysed samples by immunohistochemical (IHC) staining technique and compared with two other methods.

MATERIALS AND METHODS

Chemicals

MMLV SUPERSCRIPT™II reverse transcriptase kit and Trizol reagent was obtained from GIBCO BRL (Paisley, UK). Taq polymerase was from DYNAZYMES OY (Finland). 3'-end labeling biotin kit with streptavidin-AP was obtained from NEN™ LIFE SCIENCE (Boston, US). All other reagents were from Sigma (UK) unless indicated.

Cell Lines

T47D human breast cancer cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum glutamine, penicillin and streptomycin. When required, cells were harvested by trypsinization.

Patients and Specimens Collection

Lymph nodes and bone marrow specimens were collected from 35 patients at Beijing Cancer Hospital in Beijing from March to November 1998. Clinical details for 35 patients are given in Table 1. Each patient has shown no evidence of distant metastases disease by pre-operative investigation. The investigation included serum calcium, alkaline phosphatase and liver function tests, complete blood count and chest X-ray, liver ultrasound, isotope bone scan and skeletal survey. All patients gave their informed consent to the study.

Axillary lymph nodes were separated from the surgical specimen immediately after resection. Each lymph node was stripped carefully of adipose tissue and blood. They were cut into halves with a surgical knife. The surgical knife was cleaned each time by rinsing it in saline before it was used for the bisection of a lymph node. One half of each lymph node was fixed in 10% formalin for routine pathologic examination and IHC staining (1 slide), the other half was snap frozen in liquid nitrogen and kept at -70°C until RNA extraction. Two to 4 representative lymph nodes were taken from each patient.

The bone marrow was collected from patients under general anaesthesia just before their breast operation. Under sterile conditions a bone marrow aspirate was taken from sternum. To minimize the risk of contamination, a small (≤ 0.5 cm) incision was made in the skin with a scalpel before introducing the needle. Approximately 15 ml of bone marrow and venous blood were aspirated. The sample was separated and the mononuclear cells were obtained as described previously.[9] The cells were kept at -70°C.

For negative controls, 10 lymph nodes from patients with benign disease and 8 bone marrow samples from patients with hemato-malignant disease were taken. We also used 6 metastases positive lymph nodes as positive control.

Table 1. Clinical data on patients with operable breast cancer

	No. of patients
Total	35
Age (year)	
Range	40–75
Mean	58.8
Menopausal status	
Pre-	8
Post	27
Operation	
Modified mastectomy	34
1/4 mastectomy	1

Oligonucleotide Primers and Hybridization Probes

The primers and probes for CK-19 and β-actin were designed from previously published sequences[12, 13] and selected to maximize mismatching from pseudogene sequences. Both primers and probes were synthesized by Cybersy Bio. Com. (US). The CK-19 up-stream primer was 5'-AGG TGG ATT CCG CTC CGG GCA-3'; the down-stream primer was 5'-ATC TTC CTG TCC CTC GAG CA-3'; the probe was 5'-CGA GCA GAA CCG GAA GGA TGC TGA AGC CTG GTT CA-3'. The β-actin up-stream primer was 5'-ATC ATG TTT GAG ACC TTC AA-3'; the down-stream primer was 5'-CAT CTC TTG CTC GAA GTC CA-3'; the probe was 5'-GAC CTG GCT GGC CCG GAC CTG ACT GAC TAC-3'.

Sensitivity Assay

Suitable volumes of media containing 1 to 500 T47D human breast cancer cells were mixed with 5×10^6 normal bone marrow cells after cell separation to give a ratio of T47D cells to bone marrow cells of $1:5\times10^6$, $1:10^6$, $1:5\times10^5$, $1:10^5$, $1:5\times10^4$, $1:10^4$. These preparations were then used either to prepare smears for IHC staining or for

RNA extraction.

RNA Extraction

Total RNA was extracted from frozen samples using Trizol reagent following the kit protocol. The integrity of the RNA was checked electrophoretically and quantified spectrophotometrically. The samples were diluted to approximately 0.3 μg/μl in water and stored at -70°C.

Reverse Transcription and PCR Amplification

Total RNA (1 μg) was reverse transcribed in a 20 μl reaction mixture containing 4 μl 5 × first strand buffer, 2 μl 0.1mol/L DTT, 1 μl 10 mmol/L dNTP Mixture, 1 μl Oligo $(dT)_{12\text{-}18}$, 1 μl (200 units) of SUPERSCRIPT™II for 50 minutes at 42°C. The reaction was terminated by heating at 70°C for 15 minutes. From this cDNA solution, 1 μl was removed for subsequent PCR amplification by adding to each sample 24 μl of a solution containing 10 mmol/L Tris-HCL (pH 8.8), 50 mmol/L KCL, 1.5 mmol/L $MgCL_2$ 0.2 mmol/L dNTP mixture, down-stream and up-stream primer (50 pmol each), 1 μl (2.0 units) Taq polymerase. The PCR amplification was conducted on a gene-amp PCR system thermal cycler (Parkin-Elmer) with initial denaturation at 94°C for 3 minutes, followed by 35 cycles using this cycling profile: denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minutes and 30 seconds. A final extenuation step at 72°C for 10 minutes completed the reaction.

Southern Blot Hybridization

After electrophoresis of the PCR products, the gel was placed in 0.25 N HCL for 30 minutes and then in 0.4 N NaOH for 20 minutes. Downward transfer of the gel to the Nylon transmembrane (GeneScreen Plus, NEN™ Life Science Products, Inc. US) was performed overnight. The hybridization procedure was done according to the protocol of 3'-end labeling biotin kit. The membrane was exposed to REFLECTION autoradiography film (NEF 481, NEN Life Science Products, US) with intensify screens at room temperature for 5 to 20 minutes.

Immunohistochemistry

The IHC staining was performed to an S-P standard protocol. Sections of formalin-fixed, paraffin-embedded lymph nodes were dewaxed in xylene and alcohol. They were placed in a 30% hydrogen peroxide-10% methanol solution to block endogenous peroxidase, and washed with PBS. Each slide was then covered with 10% normal goat serum in PBS which was removed before incubation with the primary mouse anti-cytokeratin 19 antibody (ZYMED Lab. INC) overnight at 4°C. After washing with PBS, a biotinylated second antibody (goat anti-mouse, monoclonal) was applied and incubated for 30 minutes at 37°C. The HRP labeled third antibody was added and then washed with PBS, before freshly prepared 0.3% H_2O_2-diaminobenzidine solution was added for 10 minutes. The slides were then washed for 1 minutes with distilled water and the sections were counterstained with Mayer's hematoxylin for 15 seconds, then dehydrated, cleared and mounted. The IHC staining procedure of bone marrow smears was the same as that of the lymph node slides except without dewaxation and restaining with Mayer's hematoxylin.

RESULTS

Specificity and Sensitivity of IHC, RT-PCR, and Southern Blotting for Detection of CK-19 Expression

Initial experiments were performed to determine whether CK-19 gene expression could be detected in samples from patients with no evidence of breast or other epithelial malignancy. We examined 10 lymph nodes and 8 bone marrow samples and we were unable to detect any CK-19 positive cells by IHC staining with an anticytokeratin 19 antibody. RT-PCR, using primers to amplify CK-19 mRNA, in the RNA extracted from aliquots of the same samples was also performed. No signal corresponding to CK-19 mRNA was detected. Southern blotting, using specific probe to CK-19, couldn't see any signal either. At the same time, the signal to a housekeeping gene-β-actin, was very clear by both RT-PCR and Southern blotting, confirming the presence of amplifiable cDNA and ensuring that the absence of CK-19 product was not due to the lack of input RNA.

We assessed the sensitivity of both IHC, PCR and Southern blotting techniques by prepared smears or RNA from 5×10^6 normal bone marrow cells to which were added 5 to 500 T47D breast cancer cells. We were able to detect CK-19 immunopositive cells at a dilution of one T47D cell in 5×10^4 bone marrow cells on 3/4 separate occasions, but not at higher dilutions. The cytoplasm staining was shown specifically on epithelial cells, and there was no cross-reactivity with other cell types in the bone marrow.

After PCR amplification of RNA from the various cell preparations, a 460 bp product indicating amplification of CK-19 mRNA was visualized by ethidium staining at a dilution of one T47D cell in 5×10^5 bone marrow cells. The Southern blot hybridization trace corresponding to CK-19 could be seen at a dilution of one T47D cell in 1×10^6 bone marrow cells (Figure 1).

Detection of Breast Cancer Cells in Lymph Node and

Bone Marrow

In the samples from the 35 patients with operable primary breast cancer and no lymph node metastases by routine pathologic examination, we found CK-19 positive cells in 2 cases (5.7%), including 1 in lymph node with 1.2 mm metastasis lesion and 1 in the bone marrow by IHC alone. CK-19 gene expression signals were seen in 8/35 (22.9%) lymph nodes and 8/35 (22.9%) bone marrow samples by RT-PCR, another positive signal was obtained in 3/35 (8.6%) lymph nodes and 2/35 (5.7%) bone marrow samples by Southern blotting. Two IHC positive patients were also positive by RT-PCR and Southern blotting. If positivity was considered in either lymph node or bone marrow, we detected CK-19 expression in 2/35 (5.7%) by IHC, 14/35 (40%) by RT-PCR, and 17/35 (48.6%) by Southern blotting. Four cases were micrometastases positive both in lymph node and bone marrow (11.4%) (Figure 2, Table 2).

The Relationship between CK-19 Positive and Other Clinical Parameters

There was no correlation between CK-19 detection and the size, histologic type, vascular invasion, and steroid receptor content of the primary tumor, and menopausal status. Although there is an increased tendency of CK-19 detection rate as the tumor size becomes bigger, the statistical value was not seen (P>0.05).

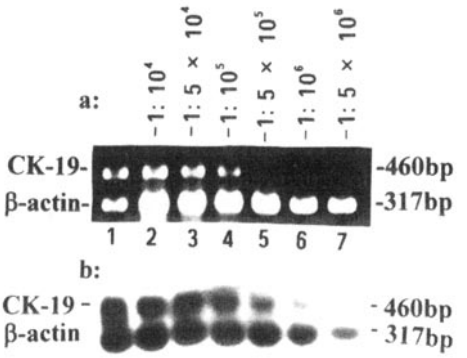

Fig. 1. Serial dilution study using T47D cells for comparison between detection sensitivity of RT-PCR and Southern blot methods. Detection sensitivity was determined by performing serial dilutions of T47D cells and preparing mixtures with 1 to 500 T47D cells in 5×10^6 normal bone marrow cells. Total RNA was extracted from these mixtures, and cytokeratin 19 mRNA and β-actin mRNA expression were studied on these samples by RT-PCR and Southern blot. a: PCR products were electrophoresed in a 1.2% agarose gel and ethidium-stained. b: autoradiograph of a Southern blot of the same PCR products.

Table 2. The results of combined detection of micrometastases in lymph node and bone marrow of 35 cases of primary breast cancer which axillary lymph node was negative for metastases by routine pathologic examination

Samples	Detecting methods		
	IHC (%)	RT-PCR (%)	RT-PCR+SB (%)
LN^+	1 (2.9)	8 (22.9)	11 (31.4)
BM^+	1 (2.9)	8 (22.9)	10 (28.6)
LN^+ and BM^+	0 (0)	2 (5.7)	4 (11.4)
LN^+/BM^+	2 (5.7)	14 (40.0)	17 (48.6)

LN: lymph node BM: bone marrow IHC: immunohistochemistry RT-PCR: reverse transcriptase polymerase chain reaction SB: Southern blotting

DISCUSSION

In this study, we have demonstrated that RT-PCR and Southern blotting using CK-19 primers and probe is a sensitive and specific technique for the detection of tumor cells in the lymph node and bone marrow of patients with breast cancer. This assay detected CK-19 expression from T47D cells serially diluted down to 1 T47D cell in 10^6 normal bone marrow cells. This level of sensitivity was 20 times greater than that achieved with IHC. Some articles reported that the sensitivity level of PCR method was from 1 in 10^5 cells to 1 in 10^7 cells.[6, 14–19] The discrepancy is because those different biological genes expressed various amounts in different kinds of cells, as well as depending on the efficiency of the primers and the design of the assay.

For the first time, we examined 35 matched lymph nodes and bone marrow sample pairs obtained from patients with breast cancer by RT-PCR and Southern blotting during primary surgery reflecting the cancer stage at diagnosis. Combined micrometastases detection of lymph nodes and bone marrow can further improve our

understanding about the patient's metastases status because of these two kinds of tissues are the two major metastases paths of breast cancer cells.

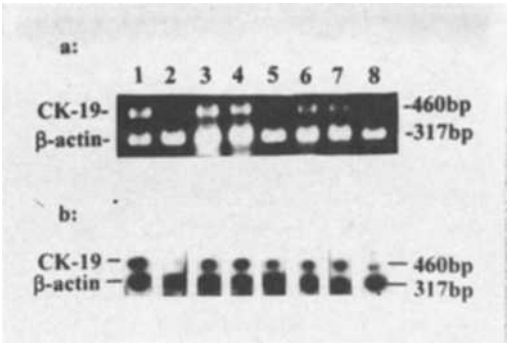

Fig. 2. Detection of breast cancer micrometastases in lymph node and bone marrow by cytokeratin 19 RT-PCR and Southern blotting methods. a: PCR were electrophoresed in a 1.2% agarose gel and ethidium-stained. b: autoradiograph of a Southern blot of the same PCR products. Lane 1 was positive control; Lane2 was negative control; Lane 3–5 were samples of lymph node; Lane 6–8 were samples of bone marrow.

There was report that micrometastases detection was related to the tumor size.[7] In this study, we have seen the tendency of relationship between micrometastases positive and tumor diameter but without a statistically significant value (P>0.05). Maybe this was because enrolled patients were not enough.

The specificity of RT-PCR relies on the detection of a unique or overexpressed gene in the tumor cell. Certain malignancies are characterized by unique targets; in malignant melanoma there is a specific tyrosinase gene expression, and prostate cancer cells overexpress prostate-specific antigen. No unique markers have been identified in breast cancer cells, although maspin is detected in all breast carcinoma biopsies and normal breast tissue. Maspin detection outside the breast has been seen in only 20-30% of samples from patients with metastatic disease.[20] We have, therefore, used an epithelial-specific marker, CK-19, as this has been reported to be presented in most benign and malignant epithelial tissue.[21] This was confirmed by our study in that no CK-19 signal was detected in the lymph nodes and bone marrow of patients with non-epithelial malignancies or benign diseases, and this was also proved by other researchers.[5–11]

Establishing the clinical relevance of staging based on RT-PCR positive lymph nodes and bone marrow is the next essential step that will needs to be addressed to be able to validate this screening method. Several previous studies have found that the pathologic behavior of patients with occult histopathologically positive nodes and bone marrow suggests that those RT-PCR positive patients may also have a worse prognosis.[22–26] But this still needs further long term follow-up of patients with RT-PCR positive and histologically negative specimens to be able to determine the clinical relevance of RT-PCR staging. If validated as a predictor of disease recurrence, the RT-PCR method may provide valuable prognostic information that would allow the clinician to make more valid adjuvant therapy decisions.

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