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

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ABSTRACT

Objective: The aim of this study was to detect micrometastases in bone marrow of primary breast cancer patients, 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 in different proportions. The positive detection rate was compared among RT-PCR, Southern blotting and immunohistochemistry (IHC) methods. Results: Cytokeratin 19 gene was expressed in all 6 positive control samples while the expression was not seen in 8 negative control samples. In all 54 patients 14 cases were CK-19 positive (25.9%) by RT-PCR, another positive signal was obtained in 5/54 (9.3%) of bone marrow samples by Southern blotting. The total positive cases are 19/54 (35.2%). CK-19 IHC$^+$ cells were detected at a dilution of one T47D cell in 5×10^4 bone marrow cells, while the sensitivity detected by PCR and Southern blot hybridization was at $1:5\times10^5$ and $1:1\times10^6$, respectively. This demonstrates that RT-PCR and Southern blotting was at least 20 times more sensitive than the IHC method. The micrometastases positive rate of the larger tumor size group (>5.0 cm) was significantly ($P<0.05$) greater than that of the smaller tumor size group (0–2.0 cm). Conclusion: detection of micrometastases in bone marrow by RT-PCR and Southern blotting, using CK-19 as a biological marker, is highly sensitive and it is a method to be used for anticipating the prognosis of breast cancer patients.

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

Thirty-five to 40% of all 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 of axillary lymph node status and size of primary tumor cannot predict in whom the disease will recur. Bone marrow is a frequent and readily accessible site of metastases. In up 80% of patients, the relapse develops starting from bone marrow metastases at some point in the process of their illness.[3] Current methods to detect bone involvement, such as X-ray and bone scanning are too insensitive to detect minimal metastases because they depend on the destruction of the bone matrix.

In recent years, PCR methods have been used in various areas. Measurement of a tissue-specific gene transcript following PCR amplification, while retaining specificity, has been reported to be highly sensitive, with the ability to detect a single neuroblastoma cell in 10^7 peripheral blood mononuclear cells.[4] More recently, several reports have demonstrated that PCR is the most sensitive method to detect micrometastases in the lymph node, bone marrow or peripheral blood.[5–8]

In this study, taking cytokeratin 19 (CK 19) as the biological marker, we have detected micrometastases in the bone marrow of patients with operable breast carcinoma using reverse-transcriptase chain reaction (RT-PCR) and Southern blotting assay. We have also compared the sensitivity of RT-PCR, Southern blotting and immunohistochemical staining methods.

MATERIALS AND METHODS

Chemicals

MMLV SUPERSCRIPT^{TM} II reverse transcriptase kit

Accepted for publication: July 15, 1999
This work was supported by a grant from Beijing Science & Technology Committee,
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and Trizol reagent were obtained from GIBCO BRL (Paisley, UK). Taq DNA 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 otherwise 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 Specimen Collection

The bone marrow specimens were collected from 54 patients at Beijing Cancer Hospital in Beijing from March to November 1998. Clinical details for 54 patients are given in Table 1. Each patient had shown no evidence of distant metastases 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. All patients gave their informed consent to the study.

The bone marrow was collected from patients under general anaesthesia just before their breast operation. Under sterile conditions bone marrow was aspirated from the sternum of the patients. 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 was aspirated. The sample was separated and the mononuclear cells were gathered as described previously.[9] The cells were kept at -70°C.

For negative controls, 8 bone marrow samples from patients with hemato-malignant disease were asprited. We have also obtained 6 metastases positive lymph nodes from patients with breast cancer as positive controls.

Table 1. Clinical data of patients with operable breast cancer

	Number of patients
Total	54
Manopausal status	
Pre-	18
Post	36
Operation	
Radical mastectomy	2
Modified mastectomy	51
1/4 mastectomy	1

Oligonucleotide Primers and Hybridization Probes

The primers and probes for CK-19 and β-actin were designed according to the previously published sequences[9,10] and selected to maximize mismatchings 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

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 $1:5\times10^6$, $1:10^6$, $1:5\times10^5$, $1:5\times10^4$, $1:10^4$. These preparations were then used either to prepare smears for immunohistochemistry 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 first strand buffer, 2 μl 0.1 M DTT, 1 μl 10 mM dNTP In mixture, 1 μl Oligo $(dT)_{12-18}$, 1 μl (200 units) of SUPERSCRIPT™II for 50 min at 42°C. The reaction was terminated by heating at 70°C for 15 min. From this cDNA solution, 1 μl was removed to be subsequently used for PCR amplification by adding each sample to 24 μl of a solution containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$ 0.2 mM 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 min, followed by 35 cycles using this cycling profile: denaturation at 94°C for 45 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min and 30 sec. A final extension step at 72°C for 10 min completed the reaction.

Southern Blot Hybridization

After electrophoresis of the PCR products, the gel was placed in 0.25 N HCl for 30 min and then in 0.4 N NaOH for 20 min. Downward transfer of the gel to the

Nylon transmembrane (Gene Screen 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 intensity screens at room temperature for 5 to 20 min.

Immunohistochemistry

The immunocytochemical staining was performed according to a S-P standard protocol. The primary mouse anti-cytokeratin 19 antibody was obtained from ZYMED Lab. Inc., US (No. 18–0190). The second and third antibodies were obtained from DAKO Crop. US)

RESULTS

Specificity and Sensitivity of Immunohistochemistry, 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. Eight bone marrow samples were tested. We were unable to detect any CK-19 positive cells by immunohistochemical staining with an anticytokeratin 19 antibody. RT-PCR, using primers to amplify CK-19 mRNA in RNA extracted from aliquots of the same samples was also performed. No signal corresponding to CK-19 mRNA was detected. Southern blot, using specific probe to CK-19, could not 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 the immunohstochemical, PCR and Southern blotting techniques by prepared smears or RNA from 5×10^6 normal bone marrow cells to which 5–500 T47D breast cancer cells were added. 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 cytoplasmic staining was shown specifically in 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 visualised by ethidium staining at a dilution of one T47D cell in 5×10^5 bone marrow cells. The Southern blotting 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 the Bone Marrow

Cytokeratin 19 gene was expressed in all 6 lymph nodes with metastasis proved pathologically. In the samples from the 54 patients with operable primary breast cancer, CK-19 gene expression signal was seen in 14/54 (25.9%) bone marrow samples by RT-PCR and another positive signal was obtained in 5/54 (9.3%) bone marrow samples by Southern blotting. The total positive cases was 19/54 (35.2%), in which 9 cases came from 19 lymph node metastases positive patients (47.4%) and 10 from 35 lymph node metastases negative patients (28.6%) (Figure 2, Table 2).

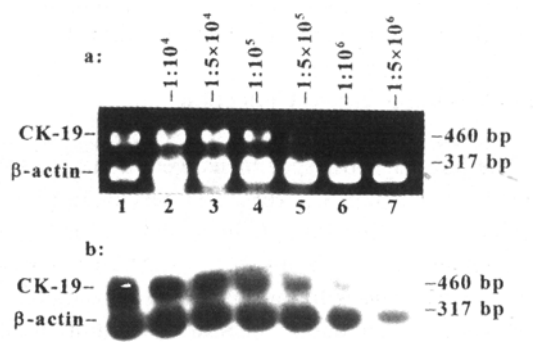

Fig. 1. Serial dilution study using T47D cells for comparison detection sensitivity between RT-PCR and Southern blotting methods. Detection sensitivity was determined by performing serial dilutions of T47D cells and preparing mixtures with 1 to 500 T47D cells in 5×10^6 normal bone marrow cells. Total 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 blotting. 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.

The Relationship between CK-19 Positive and Other Clinical Parameters

The micrometastases positive rate of the larger tumor size group (>5.0 cm, 9/11, 81.8%) was significantly ($P<0.05$) greater than that of the smaller group (0–2.0 cm, 3/24, 12.5%). There were no significant differences between the micrometastasis positive and negative group or among any of other clinical parameters such as histologic type, menopausal status, and steroid receptor content, although the positive rate was higher in the node-positive group (47.4%) than that in the node-negative group (28.6%) (Table 2).

DISCUSSION

In this study, we have demonstrated that RT-PCR and Southern blotting using CK-19 primers and probe are sensitive and specific techniques for detecting tumor cells in 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 immunohistochemistry. Some articles reported that the sensitivity level of PCR method was from 1 in 10^5 cells to 1 in 10^7 cells.[11–14] The discrepancy is due to different genes expressing various amounts in different kinds of cells, as well as depending on the efficiency of the primers and the design of the assay.

The CK-19-based RT-PCR and Southern blotting technique were applied to 54 bone marrow samples in different stages of breast cancer. Overall, CK-19 mRNA was detected in 35.2%. Nine of 11 bone marrow samples from patients with tumor size more than 5.0 cm in diameter were positive for CK-19 mRNA (81.8%), while 12.5% (3/24) of bone marrow samples from patients with tumor size less than 2.0 cm were positive. The increased frequency of tumor cells in material from patients with bigger tumor size could reflect the advanced stage of the disease.

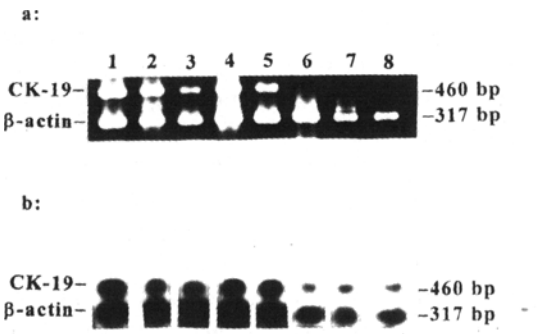

Fig. 2. Detection of breast cancer micrometastases in the bone marrow by cytokeratin 19 RT-PCR and Southern blotting methods. a: PCR products were electrophoresed in a 1.2% agarose gel and ethidium-stained. b: autoradiograph of a Southern blot of the same PCR products. Lane 1 was positive control; Lane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

Table 2. The relationship between bone marrow micrometastases and other clinical parameters

Parameter	No. of cases	No. of micrometastases	(%)
Lymph node micrometastases			
Positive	19	9	47.4
Negative	35	10	28.6
Menopasual status			
Pre-	18	9	50.0
Post	36	10	27.8
Tumor diameter (cm)			
0–2.0	24	3	12.5
2.1–5.0	19	7	36.8
>5.0	11	9	81.8*
Sterol receptor status			
ER^+	24	8	33.3
ER^-	24	9	37.5
PR^+	15	5	33.3
PR^-	33	12	36.4
Unclear	6	2	33.3
Pathological type			
Simple carcinoma	16	6	37.5
Invasive ductal carcinoma	13	6	46.2
Medullary carcinoma	6	3	50.0
Invasive lobular carcinoma	5	1	20.0
Mucoid carcinoma	3	0	0
Sclerosing carcinoma	3	2	66.7
Paget's disease	1	0	0
Ductal papillocarcinoma	1	0	0
Unclear	6	1	16.7

* Compare with the group of 0–2.0 cm, $P<0.05$

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 in 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. However, maspin detection outside the breast has been seen in only 20–30% of patients with

metastatic disease.[15] More recently, the mRNA of carcinoembryonic antigen (CEA) and epidermal growth factor receptor (EGFR) were reported as biologic markers,[13,14,16] but they are not representative of specific genes of the breast cancer and are not suitable for micrometastases detection. Therefore, we used an epithelial-specific marker, CK-19, as this has been reported to be presented in most benign and malignant epithelial tissues.[17] This was confirmed by our study in that no CK-19 signal was dedected in the lymph node and bone marrow of patients with non-epithelial malignancies or benign diseases, and this was also proved by other research.[5–8]

Establishing the clinical relevance of staging based on RT-PCR positive bone marrow is the next essential step that will need to be studied to be able to validate this screening method. Several previous studies have found that the biologic behavior of patients with occult histopathologically positive bone marrow, suggesting that RT-PCR positive patients may have a worse prognosis.[18–20] But this still needs further long term follow up of patients with RT-PCR positive and histologically negative specimens to determine the clinical relevance of RT-PCR staging. If it could be validated as a predictor of disease recurrence, the RT-PCR method may provide valuable prognostic information that would allow clinicians to make more adequate therapy decisions.

In conclusion, we have shown in the present study that micrometastases detection in bone marrow by RT-PCR and Southern blotting appears to be a readily available, more comprehensive and highly sensitive method to be used for breast cancer patients. Long-term follow up of patients with RT-PCR and Southern blotting positive specimens will be required to determine its clinical relevance. If it could be validated further as a predictor of disease recurrence, these methods will provide a powerful addition to routine histopathologic analyses.

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