Available online at www.sciencedirect.com
SCIENCE DIRECT
JNMU
Journal of Nanjing Mecical University, 2007, 21(1): 32–35
www.elsevier.com/locate/jnmu
Research Paper

Detection of micrometastases in bone marrow and sentinel lymph nodes of breast cancer patients

Jia Zhao[a,*], Xiaoan Liu[a], Lijun Ling[a]
[a]*Department of General Surgery, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China*
Received 22 March 2006

Abstract

Objective: To study the sensitivity and clinical significance of HE-staining, IHC and RT-PCR in detecting breast cancer micrometastases in bone marrow and sentinel lymph nodes (SLNs). **Methods:** After general anesthesia, all patients underwent bone marrow puncture and sentinel lymph node biopsy (SLNB) by 1% isosulfan blue, and then HE-staining, IHC and RT-PCR were used to detect micrometastases. **Results:** Of 62 patients with breast cancer whose axillary lymph nodes showed negative HE-staining results, 15 cases presented with positive RT-PCR and 9 cases showed positive IHC results positive in bone marrow micrometastases detection. PT-PCR and IHC showed good uniformity (kappa=0.6945) and there was significant difference in detective rate between these two methods (χ^2=4.1667, P = 0.0412). In SLN samples, 13 showed positive RT-PCR results, while 7 showed positive IHC results. PT-PCR and IHC showed good uniformity (kappa=0.6483) and significant difference was also found in detective rate between these two methods (χ^2=4.1667, P = 0.0412). Both bone marrow and SLN samples were RT-PCR positive in 3 cases, which indicated that bone marrow micrometastases did not always accompany SLN micrometastases (χ^2=0.067, P = 0.796). **Conclusion:** Even if no axillary lymph node involvement or distant metastases are present in routine preoperative examination, micrometastases can still be detected in bone marrow or SLNs. Because the bone marrow micrometastases and axillary node micrometastses are not present simultaneously, combination test of multiple indicators will detect micrometastases more accurately.

Keywords: breast cancer; micrometastases; bone marrow; sentinel lymph nodes; cytokeratin

INTRODUCTION

Breast carcinomas usually spread via lymphatic and venous dissemination. It has been demonstrated that bone marrow with single carcinoma cell may be an important cause for cancer recurrence and metastases [1-5]. With the advent of sentinel lymph node biopsy (SLNB), micrometastases in axillary lymph nodes can also be detected effectively by reducing the number of "objective" nodes. Numerous clinical studies have shown that micrometastses status of bone marrow and SLNs is a potentially powerful factor for prognosis prediction and therapy direction. This study aimed to explore the screening sensitivity and clinical significance of different techniques, including hematoxylin-eosin-staining (HE-staining), immunohistochemistry (IHC) and reverse transcription-polymerase chain reaction (RT-PCR) in detecting micrometastases of breast cancer in bone marrow and sentinel lymph nodes (SLNs).

MATERIALS AND METHODS

Subjects

Bone marrow and SLNs were collected from 62 female patients with breast cancer (clinical stage Ⅰ-Ⅱ), who were confirmed without distant metastases by preoperative examination at Department of General Surgery, the First Affiliated Hospital of Nanjing Medical University, from July 2000 to December 2003. Patients' age was between 29 to 76, and the mean age was 52.47. After general anesthesia, 10

*Corresponding author.
E-mail address: zhaojia8_8@hotmail.com

milliliter bone marrow was drawn from breastbone using heparin for anticoagulation. SLNs were detected by 1% isosulfan blue. Five milliliter 1% isosulfan blue was injected into mammary areola or peritumor in four points at random. Five minutes later, SLNs were dissected and each lymph node was bisected, one half for histological examination, one half for mRNA analysis. Then each patient received modified radical mastectomy. Bone marrow was centrifugated by Ficoll Hypaque to isolate mononuclear cells which were used to detect micrometastases by IHC and RT-PCR.

Immunohistochemistry (IHC)

Strep-avidin-biotin-peroxidase complex method was used to detect micrometastases by cytokeratin 19. All reagents were bought from Dako company (Denmark). The detail operation was conducted according to the instruction in reagent kits. The result was observed by microscopy. Cytokeratin 19 positive cells were dyed in brown color. The cytokeratin was mainly expressed in the cell membrane and/or cytoplasm. The bone marrow and SLNs were diagnosed as positive micrometastases by detecting keratin positive cells among them.

Reverse Transcription-polymerase Chain Reaction (RT-PCR)

Primers

Keratin 19 primers were as follows [4], upstream: 5′-AGG TGG ATT CCG CTC CGG GCA-3′, down stream: 5′-ATC TTC CTG TCC CTC GAG CA-3′. The length of amplification fragment was 460 bp. β-actin primers were as follows, upstream: 5′-CAC TGT GTT GGC GTA CAG GT-3′, downstream: 5′-TCA TCA CCA TTG GCA ATG AG-3′. The length of amplification fragment was 154 bp.

RNA extraction

Total RNA was extracted using the acid guanidium-phenol-chloroform technique, by commercial Trizol (Promega, USA). One milliliter Trizol was put into the mononuclear cells of bone marrow, while one half of SLNs maintained on ice was minced and homogenized manually in the presence of lysis buffer Trizol. RNA fractions were suspended in diethyl pyrocarbonate -treated water and quantitated by the ratio of OD260 nm to OD280 nm.

RNA amplification

First-strand cDNA was synthesized by using AMV reverse transcriptase. Total volume of RT was 20 μl, including:10 × reaction buffer 2 μl,10 mmol/L dNTP 2 μl, 25 mmol/L magnesium chloride 4 μl, AMV reverse transcriptase 1 μl(5U), RNasin 0.5 μl (20U), Oligo(dT) 15 1 μl, RNA3 μl(2 μg), RNAase free water 6.5 μl. Following incubation at 42℃ for 30 min, the mixture was heated to 99℃ for 5min to inactivate AMV reverse transcriptase. Polymerase chain reaction:Specific cDNA sequences were amplified in reaction mix ture (100 μl) composed of 10 μl transcription product, 10 × reaction buffer 8 μl, 25 mmol/L magnesium chloride 4 μl, 5 units of Taq polymerase, 20 pmol/L sequence-specific primers. Forty cycles of amplification were performed with denaturation at 94℃ for 1min, annealing at 60℃ for 1min and extension at 72℃ for 1min with an extra 7 min extension for the last cycle. Gel electrophoresis: PCR products (10 μl) were electrophoresed at 75V for 1-2 h on a 2% agarose gel containing ethidium bromide in Tris-acetate EDTA buffer.

Statistical analysis

The data were analyzed by McNemar-χ^2-test, Kappa-test and χ^2-test. It was performed by statistical software SPSS 10.0.

RESULTS

The amplification product of RT-PCR

The amplification product of RT-PCR was a 460 bp fragment. RNA integrity was confirmed in all samples by the detection of a 154-bp actin product in ethidium bromide-stained gels.

Sensitivity and agreement of RT-PCR and IHC in detection of bone marrow micrometastases

As shown in ***Table 1***, the detective rate of RT-PCR was 24.19% (15/62) while the detective rate of IHC was 14.52%(9/62). There was significant difference in detective rate between these two methods statistically (χ^2= 4.1667, P = 0.0412)and the agreement of the results between PT-PCR and IHC was good (kappa = 0.6945)

Table 1 **Sensitivity and agreement of RT-PCR and IHC testing in bone marrow icrometastases**

IHC	PT-PCR		Total
	(+)	(−)	
(+)	9	0	9
(−)	6	47	53
Total	15	47	62

kappa = 0.6945; χ^2 = 4.1667, P = 0.0412(McNemar-χ^2-test)

Sensitivity and agreement of RT-PCR and IHC in detection of SLNs micrometastases

As shown in ***Table 2***, the detective rate of RT-

Table 2 **Sensitivity and agreement of RT-PCR and IHC testing in SLNs micrometastases**

IHC	PT-PCR		Total
	(+)	(−)	
(+)	7	0	7
(−)	6	49	55
Total	13	49	62

kappa = 0.6483; χ^2 = 4.1667, P = 0.0412(McNemar-χ^2-test)

PCR and IHC was 20.97%(13/62)and 11.29%(7/62) respectively. Significant difference was also found in detective rate between these two methods (χ^2 = 4.1667,P=0.0412) and agreement between PT-PCR and IHC was good(kappa=0.6483).

Relationship between bone marrow micrometastases and SLNs micrometastases

In 62 patients with breast cancer, only 3 patients presented with positive bone marrow and SLNs micrometastases by means of RT-PCR. There was no statistical correlation (χ^2=0.067,P=0.796) between these two kinds of micrometastases. It showed that micrometastases in bone marrow and SLNs did not occue at the same time.

DISCUSSION

Recurrence and metastases are the most important biological behaviors of malignant tumors. Approximately 95% of breast cancer patients are treated according to routine pathologic results when first diagnosed without distant metastases. Nevertheless, 30% of these patients will develop loco-regional recurrence and distant metastases within 5 years after diagnosis[6,7], which indicates that routine pathologic examinations cannot be used as a prognostic factor accurately.

Breast carcinomas usually spread via lymphatic and venous dissemination. Bone marrow metastasis may develop in approximately 80% of patients with recurrent breast cancer [8,9], and has always been the subject of intensive research in recent years. Wiedswang and his group[10] found that bone marrow micrometastases detected by immunocytochemistry were significantly associated with disease-free-survival and distant-disease-free-survival, which will be helpful for clinical direction. Cytokeratin(KT) represents the major structural protein in epithelial cells, which is shown to be only expressed in epithelial-derived tumor cells. KT19 is the most specific and sensitive labels for breast carcinoma cells, in contrast to other tumor label[11]. KT19 has been widely used for micrometastases detection in lymph nodes, bone marrow as well as peripheral blood. It was reported that RT-PCR had superior sensitivity over IHC for evaluation of bone marrow micrometastases[12]. In out study, there was significant difference in detective rate between these two methods statistically (χ^2 = 4.1667,P=0.0412) and RT-PCR was more sensitive than IHC. These findings could not be achieved in our previous study[13], which might be due to limited sample amount and different research subjects.

Axillary lymph node status remains the most significant clinical factor for staging, prognosis predicting and therapy guiding. But routine pathological examinations have two disadvantages: ①unable to tell the pathological status of each axillary lymph node in detail; ②unable to detect micrometastases in lymph nodes. It was reported that node micrometastases, which could not be identified by routine examinations, would be detected by detailed analysis using serial sectioning. The ap plication of IHC increases the detection rate while the use of PCR further improves the detection sensitivity of micrometastases [14,15]. Practically, extensive searching for each axillary lymph node would be luxurious and unnecessary, because approximately 15 nodes would be found in each radical mastectomy specimen in average. The application of SLNB may reduce the labor and cost of the pathologic procedure. In our study, RT-PCR had superior detection rate over IHC (χ^2 =4.1667,P=0.0412) and agreement between PT-PCR and IHC was good (kappa=0.6483). Nevertheless, the clinical significance of SLNs micrometastases remains unclear and needs further study.

In this study, micrometastases was detected both in bone marrow and SLNs in only 3 cases without any statistical relationship, which further supported Fisher's opinion: breast cancer is a general disease even in early stage. In addition, the route and mode of breast cancer micrometastases are complex and lack of rules, which adds more difficulties to clinical research. In conclusion, combining multiple indicators and parameters to assess the metastasis status of breast cancer patients in general will be helpful to predict prognosis and guide treatment strategy.

References

[1] Gebauer G, Fehm T, Merkle E,Beck EP,Lang N,Jager W. Epithelial cells in bone marrow of breast cancer patients at time of primary surgery: clinical outcome during long-term follow-up. *J Clin Oncol* 2001; 19:3669-74.

[2] Zha XM, Fan P, Wang S, *et al*. Detection of breast cancer micrometastases in bone marrow and peripheral blood by means of

reverse transcriptase-polymerase chain reaction. *Chin J Surg* 2001; 39: 382-4.

[3] Traweek ST, Liu J, Battifora H. Keratin gene expression in non-epithelial tissues. Detection with polymerase chain reaction. *Am J Pathol* 1993; 142: 1111-8.

[4] Noguchi S, Aihara T, Motomura K, Inaji H, Imaoka S, Koyama H. Detection of breast cancer micrometastases in axillary lymph nodes by means of reverse transcriptase-polymerase chain reaction. Comparison between Muc1 mRNA and keratin mRNA amplification. *Am J Pathol* 1996; 148: 649-56.

[5] Cai QQ, Huang HQ, Lin TX. Detection and clinical significance of circulating tumor cells in peripheral blood of breast cancer patients. *Chin J Cancer* 2005;24:837-41.

[6] Gardner B, Feldman J. Are positive axillary nodes in breast cancer markers for incurable disease. *Ann Surg* 1993; 218: 270-5.

[7] Sakorafas GH, Geraghty J, Pavlakis G. The clinical significance of axillary lymph node micrometastases in breast cancer. *Eur J Surg Oncol* 2004;30:807-16.

[8] Roodman GD. Mechanisms of bone metastasis. *N Engl J Med* 2004; 350:1655-64.

[9] Choesmel V, Pierga JY, Nos C, Vincent-Salomon A, Sigal-Zafrani B, Thiery JP, *et al.* Enrichment methods to detect bone marrow micrometastases in breast carcinoma patients: clinical relevance. *Breast Cancer Res* 2004;6:R556-70.

[10] Wiedswang G, Borgen E, Schirmer C, Karesen R, Kvalheim G, Nesland JM, *et al.* Comparison of the clinical significance of occult tumor cells in blood and bone marrow in breast cancer. *Int J Cancer* 2006; 118:2013-9.

[11] Stathopoulou A, Mavroudis D, Perraki M, Apostolaki S, Vlachonikolis I, Lianidou E, *et al.* Molecular detection of cancer cells in the peripheral blood of patients with breast cancer: comparison of CK-19, CEA and maspin as detection markers. *Anticancer Res* 2003;23: 1883-90.

[12] Noguchi M. Sentinel lymph node biopsy and breast cancer. *Br J Surg* 2002;89:21-34.

[13] Wang S, Fan P, Liu XA. Clinical study on micrometastases in sentinel lymph node and bone marrow from breast cancer patients. *Chin J Gen Surg* 2004;19(9):570-1.

[14] Zurrida S, Mazzarol G, Galimberti V, Renne G, Bassi F, Iafrate F, *et al.* The problem of the accuracy of intraoperative examination of axillary sentinel nodes in breast cancer. *Ann Surg Oncol* 2001;8:817-20.

[15] Tuttle TM. Technical advances in sentinel lymph node biopsy for breast cancer. *Am surg* 2004;70:407-13.