

Original article

Sensitive detection of circulating breast cancer cells by reverse-transcriptase polymerase chain reaction of maspin gene

M. Luppi,¹ M. Morselli,¹ E. Bandieri,¹ M. Federico,² R. Marasca,¹ P. Barozzi,¹ M. G. Ferrari,¹ M. Savarino,¹ A. Frassoldati² & G. Torelli¹

Department of Medical Sciences, Section of ¹Hematology and ²Oncology, University of Modena, Modena, Italy

Summary

Background: Maspin, a recently identified protein related to the family of serpins, is believed to play a role in human breast cancer. In an effort to improve the present methods of detection, we have developed a reverse-transcriptase polymerase chain reaction (RT-PCR) assay for maspin transcript to identify small numbers of mammary carcinoma cells in the peripheral blood and bone marrow of patients with breast cancer.

Patients and methods: Five non-neoplastic mammary tissue samples, 13 breast cancer specimens as well as 17 peripheral blood and 4 bone marrow samples from normal subjects were screened for the presence of maspin mRNA by RT-PCR. The same assay was applied to peripheral blood or bone marrow samples obtained from 29 patients with stages I to IV breast cancer.

Results: By RT-PCR it was possible to amplify maspin mRNA in all of the primary and metastatic breast cancer specimens, but in none of the normal hemopoietic samples from healthy donors. Thus, detection of maspin transcript in

the peripheral blood or marrow of a patient known to have breast cancer is indicative of the presence of mammary carcinoma cells. In reconstitution experiments, maspin RT-PCR reliably detected 10 mammary carcinoma cells in 1 million normal peripheral-blood mononuclear cells (PBMCs). None of the 9 patients with stages I, II, or III breast cancer had maspin transcript in peripheral blood. Of note, 3 of 9 patients with stage IV breast cancer receiving systemic therapy at the time of sample collection, but only 1 of 11 patients with stage IV not receiving therapy, had detectable maspin transcript in peripheral blood. Moreover, 3 marrow specimens from stage IV patients tested positive by this assay.

Conclusions: This pilot study suggests that maspin RT-PCR assay is a sensitive, specific and sufficiently rapid method for detection of small numbers of circulating cells and marrow micrometastases in breast cancer patients. The possibility of applying this assay in the detection of tumor cell contamination of both marrow and stem-cell apheresis harvests of breast cancer patients merits further investigation.

Key words: breast cancer, maspin, metastasis, RT-PCR

Introduction

Sensitive detection of occult carcinoma in peripheral blood and bone marrow of patients with breast cancer may have important prognostic and therapeutic implications [1, 2]. Immunohistochemical staining [3] and flow cytometry [4] with one or a panel of monoclonal antibodies against cell surface glycoproteins or cytokeratins are often highly sensitive but may be subject to false-positive results when antibodies cross-react with normal peripheral blood cells [1], as well as to false-negative results when an inadequate number of cells is evaluated [5]. Thus, in the detection of a very low number of tumor cells, immunohistochemical analysis should be combined with clonogenic assays [5], which, however, are laborious and require several weeks of tissue culture. To circumvent the problems inherent in immunohistochemical and cell culture techniques, polymerase chain reaction (PCR)-based assays were recently introduced [6]. The effectiveness of PCR in the detection of human tumor cells depends upon a qualitative difference in gene structure (DNA) or expression

(RNA) between the malignant and the normal background cells. Studies of PCR amplification of tumor-specific DNA sequences are possible mainly in hematological malignancies which often show consistent and well-characterized molecular abnormalities, but are of limited importance in solid tumors where such abnormalities are uncommon [6]. Thus, the best approach for the molecular detection of circulating solid tumor cells is based on the reverse transcription and amplification of tissue-specific RNA transcripts which are not expressed in normal hemopoietic tissues and may be used as markers of tumor cells in the blood, thereby avoiding the requirement for a DNA sequence abnormality. Reverse-transcriptase polymerase chain reaction (RT-PCR)-based assays for prostate-specific antigen in prostate cancer [7, 8], for carcinoembryonic antigen in colorectal cancer [9], for tyrosinase in melanoma [10] and for tyrosine hydroxylase mRNA in neuroblastoma [11] are some of the most successful applications of this approach, allowing the sensitive detection of circulating tumor cells in patients with solid cancers. At present, however, the application of PCR in breast cancer tumor

detection is limited because of the lack of relevant molecular markers. In fact, the value of RT-PCR-based assays for cytokeratin 19 (CK19) [12] as well as for the MUC1 [13] gene as markers of mammary carcinoma cells was recently questioned on the basis of the documented expression of both of these genes in either the peripheral blood mononuclear cells (PBMCs) [14] or the lymph nodes [15] of subjects without cancer.

Thus, in order to improve existing molecular methods of detection of small numbers of circulating cells in breast cancer patients, we have evaluated the RNA transcript encoding for maspin as a specific marker for mammary carcinoma cells. Maspin is a recently identified protein apparently related to the serpin family of protease inhibitors [16]. The function of maspin is still uncharacterized, but initial studies on its expression in human mammary tissues suggest a possible biological role of alteration in maspin expression during the development and progression of breast cancer [16]. In this report, we describe the development of a new, sensitive and specific RT-PCR-based assay, first documenting the presence of maspin mRNA in all primary and metastatic breast cancer specimens examined and its absence in a series of normal hemopoietic tissues, thereby providing evidence that the detection of maspin transcript in the peripheral blood and/or marrow of a patient with known breast cancer invariably indicates the presence of mammary carcinoma cells. We finally describe the application of this method in investigating the presence of circulating malignant cells in the peripheral blood specimens of a well-characterized series of breast cancer patients at different stages of the disease.

Patients and methods

Patient samples and RNA preparation

The study population consisted of 29 patients with histologic diagnoses of breast cancer at different stages: 4 had stage I, 3 had stage II, 2 stage III, and 20 stage IV, and 9 were receiving systemic therapy at the time of sample collection. After procuring their informed consent, 5–10-ml samples of heparinized venous blood or bone marrow aspirate were taken from patients at various time points during treatment and processed as described below. Seventeen peripheral blood and 4 bone marrow samples were also collected from healthy donors. Peripheral blood mononuclear cells (PBMCs) were prepared by centrifugation on Ficoll-Hypaque density gradient (Ficoll-Hypaque; Pharmacia, Uppsala, Sweden). Thirteen fresh breast cancer specimens (10 primary tumors and 3 skin metastases) and 5 non-neoplastic mammary tissue biopsies were obtained from surgical resection specimens, snap-frozen in liquid nitrogen within 10 min of their arrival in the pathology specimen reception area and kept frozen until tissue homogenization prior to extraction. Total RNA was extracted according to the acid guanidium thiocyanate-phenol-chloroform standard method [17].

cDNA synthesis and PCR

One microgram of total RNA was reverse transcribed according to the manufacturer's instructions in 1× RT buffer (Gibco BRL Life Technologies, Gaithersburg, MD, USA) supplemented with 0.01 M

dithiothreitol (DTT), 200 units of cloned Moloney murine leukemia virus reverse transcriptase (Gibco BRL), 1 mM of each dNTP (Pharmacia) and 200 units of RNase inhibitor (Boehringer, Mannheim, Germany) in a final volume of 30 µl incubated at 42 °C for 60 min. For amplification, primers designed from the coding sequence of the human maspin cDNA (Gene Bank accession number U04313) were used. The sequences of oligonucleotide primers were as follows: 5' TCAAGCGGCTCTACGTAGAC 3' sense and 5' CCTCCACATCCTTGGGTAGT 3' antisense. PCR was carried out in a 50 µl mixture containing 1× PCR buffer (10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 0.01% gelatin), 125 µmol/l of each dNTP, 50 pM of each external primer, 1 unit of Taq DNA polymerase (Boehringer, Mannheim) and one-sixth of the cDNA sample. After 35 cycles of amplification (1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C), one-fifth of the amplified product was separated electrophoretically in a 1.5% agarose gel, stained with ethidium bromide and observed under UV light. The length of the primary amplified product in the positive samples was 446 base pairs (bp). In order to assess the specificity of the amplified band, one-fifth of the RT-PCR product was subjected to electrophoresis on a 1.5% agarose gel blotted on Zeta-Probe GT Membrane (Bio-Rad Laboratories, Hercules, CA, USA) and hybridized with an internal oligonucleotide probe (5' GCCTCCATGTTTCATCATCT-GCACTGGTTTGGTGTCTGTCT 3') end-labelled with -32 P-ATP (Amersham-USB, Cleveland, OH, USA). To increase the sensitivity of the RT-PCR assay 2.5 µl of the primary amplified product was subjected to a second PCR under the same conditions for 35 cycles with nested oligonucleotide primers. The expected length of the re-amplification product was 175 bp. The sequences of the nested oligonucleotide primers were as follows: 5' GATCTCACAGATG-GCCACTT 3' internal sense and 5' GCACTGGTTTGGTGTCTGTCT-GTC 3' internal antisense. Negative controls, including distilled water and the reaction mixture without the reverse transcriptase enzyme, were also present during the RNA extraction procedure and equalled or exceeded the number of the assayed samples. All standard recommended procedures were performed to avoid false-positive results: 1) the various steps of the procedure (RNA purification, PCR and gel electrophoresis) were physically separated; 2) RNA extraction and PCR were performed in duplicate for each sample and a result was considered definitive when confirmed in different experiments. In all of the specimens tested for maspin expression the presence of intact RNA, an adequate cDNA synthesis as well as the absence of inhibitors were confirmed by a single round of RT-PCR using ABL sequence-specific PCR primers [18]. The nested RT-PCR procedure for the detection of CK19 transcript was previously described by Datta et al. [12]. To determine the sensitivity of the maspin assay and to compare it with the sensitivity of the CK19 RT-PCR assay, PBMCs obtained from a normal donor were mixed with decreasing numbers of MCF7 cells. There was a total of 1 million cells in each sample. Cells were mixed before the RNA preparation, thereby mimicking the clinical setting for detection of mammary cells in the peripheral blood or bone marrow of patients.

Results

Using a conventional RT-PCR-based assay a single 446 bp band was identifiable on ethidium bromide-stained agarose gels in non-neoplastic mammary tissue samples and, more importantly, in all of the primary and metastatic breast cancer specimens examined as well as in the MCF7 mammary tumor cell line (Table 1; Figure 1). This fragment was confirmed by Southern blot analysis to be the fragment of the maspin gene which lies between the two primers used for PCR (see 'Patients and methods'). The 17 peripheral blood mononuclear cell (PBMC) and 4 bone marrow samples obtained

Table 1. Detection of maspin mRNA in a series of mammary tissues and haemopoietic specimens by RT-PCR.

Sample source	No. cases	No. maspin positive
Breast cancer (primary tumor)	10	10
Breast cancer (metastases)	3	3
Non-neoplastic mammary tissue	5	5
Blood (healthy donors)	17	0
Normal marrow	4	0

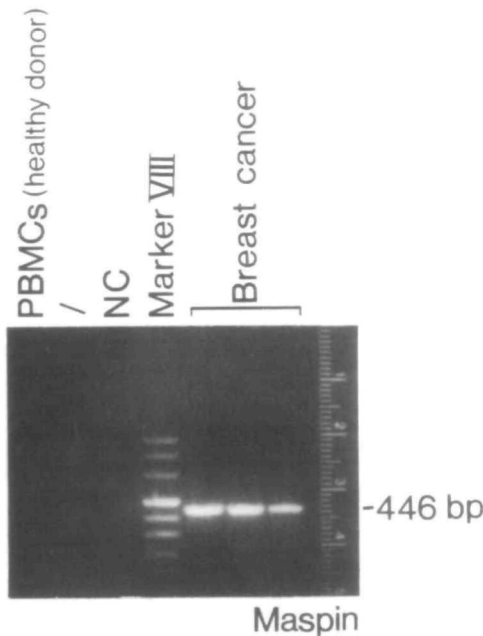


Figure 1. Detection of maspin transcript in three representative breast cancer specimens, after 35 cycles of RT-PCR assay, and visualization of the 446 base pair (bp) amplified product on ethidium bromide. Results of a representative PBMC sample from a healthy donor were negative. NC: negative control (reaction mixture without the reverse transcriptase enzyme). Marker VIII: molecular weight marker (Boehringer, Mannheim).

from healthy donors were negative for maspin transcript even after re-amplification with nested primers and direct visualization on ethidium bromide, as well as by hybridization of the primary PCR product with a specific oligonucleotide probe (Table 1; Figure 1). In contrast, 5 of these normal peripheral blood samples tested positive for the CK19 transcript, after a nested RT-PCR assay (data not shown). In order to assess the sensitivity of the maspin RT-PCR assay, a mixing study was performed using serial dilutions of the mammary carcinoma cell line MCF7. By use of this approach, our assay routinely detected 10 maspin-expressing mammary carcinoma cells mixed with one million normal PBMCs (Figure 2). This sensitivity was comparable to that obtained of the CK19 RT-PCR assay, as evaluated after an identical dilution experiment on the same tumor cell line.

RNA samples derived from the peripheral blood of all 29 patients with breast cancer exhibited successful

amplification of ABL sequence, and were further evaluated for the presence of maspin mRNA by RT-PCR. Neither the 4 patients with stage I, the 3 with stage II, nor the 2 with stage III breast cancer showed detectable maspin transcript in their peripheral blood specimens (Table 2). Of particular interest was the fact that 3 of 9 patients with stage IV breast cancer receiving systemic therapy at the time of sample collection had detectable maspin transcript in peripheral blood as detected after nested PCR and direct visualization on ethidium bromide (Figure 3), as well as after hybridization of the first PCR product with a specific internal oligonucleotide probe (Table 2; Figure 4A). One of these patients showed no detectable maspin transcript when the peripheral blood sample was collected two months after the end of the chemotherapy courses. Furthermore, 1 of 11 patients with stage IV breast cancer not receiving therapy also had positive maspin RT-PCR assay results (Table 2). In addition, 3 bone marrow biopsies from patients with stage IV breast cancer, showing very few tumor cells at standard histologic examination were clearly positive by this RT-PCR assay (Figure 4B). Only one of these three patients with a positive marrow specimen showed maspin-expressing cells in the peripheral blood.

CK19 transcript was detected in the PBMC samples of 8 breast cancer patients of our series and, in particular, in 1 patient with stage I, in 2 patients with stage II,

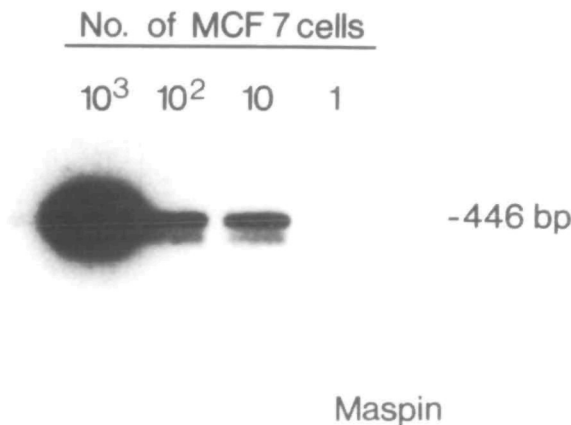


Figure 2. Sensitivity of the maspin RT-PCR assay, evaluated after hybridization of the first amplification product with an internal specific oligonucleotide probe. The number of MCF7 breast carcinoma cells mixed with 1 million normal PBMCs is shown.

Table 2. Detection of maspin mRNA in the PBMCs of breast cancer patients by RT-PCR.

Breast cancer	No. patients (PBMCs)	No. maspin positive
Stage I	4	0
Stage II	3	0
Stage III	2	0
Stage IV (no therapy)	11	1
Stage IV (therapy)	9	3

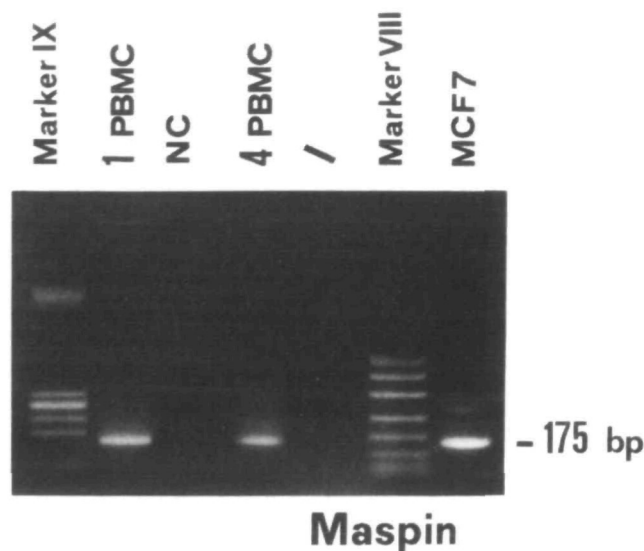


Figure 3. Detection of circulating breast cancer cells in representative peripheral blood samples from stage IV patients (1 PBMC and 4 PBMC), after a nested RT-PCR assay and visualization of the 175 bp amplified product on ethidium bromide. MCF7: breast carcinoma cell line used as positive control. NC: negative control (reaction mixture without the reverse transcriptase enzyme). Marker VIII and Marker IX: molecular weight markers (Boehringer, Mannheim).

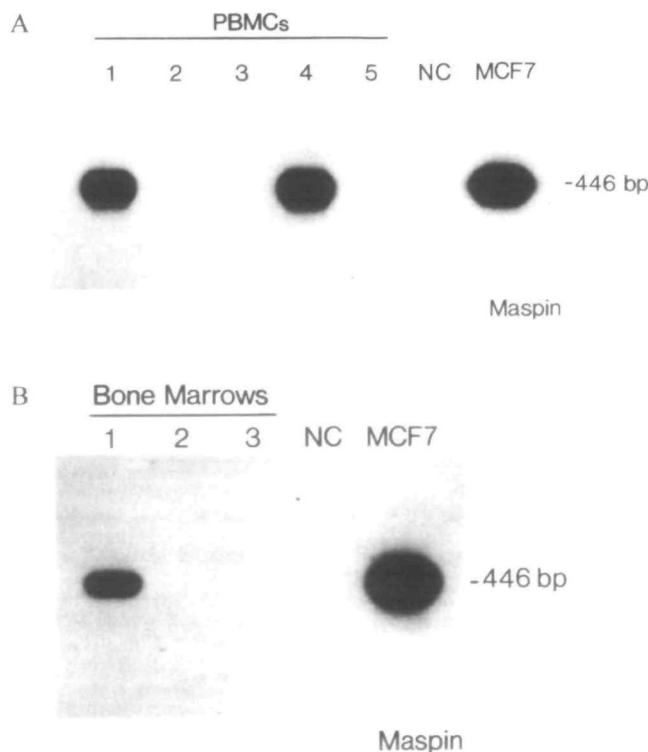


Figure 4. Detection of breast cancer cells in representative peripheral blood samples (1–5) (A) and bone marrow specimens (1–3) (B) from stage IV breast cancer patients. After 35 cycles of amplification and hybridization with a specific oligonucleotide probe, a positive maspin signal was detectable in the PBMC samples from patients 1 and 4 (A) and in the bone marrow sample from patient 1 (B). MCF7: breast carcinoma cell line used as positive control. NC: negative control (reaction mixture without the reverse transcriptase enzyme).

in 3 patients with stage IV not receiving chemotherapy, and in 2 patients with stage IV treated with chemotherapy at the time of sample collection (data not shown).

Discussion

We have looked at the possible use of the maspin gene as a target for RT-PCR detection of metastasising breast cancer cells. On the basis of initial sequence homology studies, Zou et al. proposed that maspin is related to the serpin family of protease inhibitors [16]. This putative function also seemed consistent with the fact that the maspin gene was shown to be expressed in normal mammary epithelial cells while a reduction or even a loss of maspin expression was documented in mammary carcinoma cell lines and in advanced breast cancer specimens examined by Northern blot and immunohistochemical analysis. However, using a RT-PCR assay, we unequivocally demonstrated the expression of maspin in breast cancer *in vivo*, in both primary and metastatic tumor specimens in advanced stages of the disease, as well as in the MCF7 mammary carcinoma cell line. Our result may be explained by the high sensitivity of the technique used, although it is apparently in conflict with the proposed function of maspin, since high levels of maspin would be expected to act protectively by blocking the enzymatic activity of a protease, and thus be unlikely to be detectable in advanced breast cancers specimens. However, the balance of functions of proteases and their inhibitors in the context of breast cancer is complex and high levels of protease inhibitors may be of importance for reimplantation of circulating tumor cells at distant loci. For example, high levels of a proteolytic factor, the urokinase-type plasminogen activator, and its inhibitor, PAI-1, are both involved in breast cancer and have also been claimed to be of independent prognostic value for disease-free and overall survival [19]. Generation and growth of metastases require the formation of a new tumor stroma, which occurs via prevention of protease-mediated degradation of the extracellular matrix, so that the expression of a protease inhibitor like maspin in metastatic breast cancer specimens *in vivo* is not totally unexpected. On the other hand, on the basis of the most recent studies of the primary structure of maspin, it has been proposed that, unlike most serpins, maspin is probably not an inhibitor but may be a ligand-binding serpin, possibly related to thymosin β 4 [20], meaning that a role for this newly identified protein in breast cancer is still far from established. In any case, since the aim of our study was to evaluate mRNA maspin as a target for RT-PCR detection of a very small number of mammary carcinoma cells, we chose to assess the sensitivity of our assay in the MCF7 mammary carcinoma cell line, previously found to be maspin-negative on Northern blot analysis by Zou et al. [16]. Thus, it is of particular importance that the ability to detect 10 maspin-expressing mammary carcinoma

cells mixed into one million normal PBMCs was closely comparable, in the same cell line, to the sensitivity of the RT-PCR assay for CK19 transcript, which is commonly expressed at high levels in epithelial tissues.

Given the paucity of information about the expression of this newly identified gene in human tissues and the possible occurrence of an illegitimate transcription phenomenon [21], we carefully ruled out the presence of maspin transcripts by the same RT-PCR assay in a series of 21 normal hemopoietic specimens collected from healthy individuals. Thus, the specificity of our assay, as a marker of circulating mammary carcinoma cells, is definitely higher than that of the RT-PCR in detection of K19 and MUC1 transcripts. In fact, the results for CK19 are contradictory, with two studies showing no transcription in normal blood samples [12, 22], and a third documenting illegitimate transcription of this cytokeratine gene in about half of the controls examined [14]. In our hands, the presence of CK19 transcripts was detectable in about the 30% of normal PBMC samples examined by RT-PCR, suggesting that the use of the CK19 marker is highly questionable and that it ultimately prevents interpretation of the positive findings in breast cancer patients. Furthermore, a study of CK19 expression in lymph nodes found detectable transcription in nodes from patients without cancer using nested primers, although lowering the sensitivity by using single-round PCR made it possible to distinguish malignant from normal specimens [23]. Similarly, the RT-PCR assay for the MUC1 gene, initially proposed to be a good marker for detecting breast cancer micrometastases, has recently been found to generate a high level of false-positive results in normal blood and lymph nodes, when sensitivity is enhanced with subsequently nested amplification and/or hybridization [15]. Thus, it is possible that the RT-PCR assay for maspin may also be flawed by the false-positive problem, if the sensitivity of the assay is increased. However, it should be borne in mind that, as with most medical testing, sensitivity and specificity need to be balanced. This caveat will likely hold true for all attempts to define micrometastases using the molecular detection of tissue-specific gene transcripts, so that the value of these methods, including the RT-PCR for maspin, will be assessed only after their application in specific clinical settings.

In order to test the feasibility of our assay *in vivo*, we examined a series of patients with breast cancer at different stages to look for the presence of circulating malignant cells in peripheral blood, and, whenever possible, in the bone marrow. The RT-PCR assay for maspin was able to detect circulating breast carcinoma cells in the peripheral blood of patients with stage IV disease, and more frequently in those patients (3 of 9) receiving systemic therapy at the time of the PBMC sample collection. Similarly, Datta et al. showed the presence of CK19-expressing cells by RT-PCR in only 4 of 19 patients with stage IV breast cancer receiving chemotherapy, but they could not document malignant

cells in stages I, II or III patients, nor in stage IV patients not receiving systemic therapy near the time of sample collection [12]. Using a highly sensitive immunocytochemical assay, Brugger et al. documented concomitant tumor cell recruitment upon mobilization of peripheral blood progenitor cells (PBPCs) with multi-agent chemotherapy and a growth factor, in stage IV breast cancer patients [25]. Lack of maspin PCR positivity in the blood of the other stage IV patients examined may have been due to consistently low numbers of circulating carcinoma cells below the threshold of detection by the RT-PCR assay, to sequestration of tumor cells at other sites and/or to intermittent shedding of tumor cells in the circulation, possibly leading to sampling errors. The RT-PCR assay is rapid, with results available in 24 to 48 hours, and may be combined with standard immunocytochemical methods to improve the detection of tumor cells. This assay is potentially useful for evaluation of tumor cell contamination of marrow as well as of stem-cell apheresis harvests of stage IV patients following chemotherapy plus a growth factor administration. However, only the application of the maspin RT-PCR assay in a large series of breast cancer patients with a long-term follow-up will clarify whether this tool may ultimately be clinically useful. Finally, we also obtained preliminary evidence of maspin expression in epithelial tissues other than mammary tissues, namely, liver and colon mucosa, which could broaden the usefulness of this RT-PCR method as a detection assay for a number of common malignancies.

Acknowledgements

This work was funded by grants from Associazione Italiana per la Ricerca sul Cancro, Rome, Italy, and from Consiglio Nazionale per le Ricerche (C.N.R.), Rome, Italy.

References

1. Diel IJ, Kaufmann M, Goerner R et al. Detection of tumor cells in bone marrow of patients with primary breast cancer: A prognostic factor for distant metastasis. *J Clin Oncol* 1992; 10: 1534-9.
2. Shpall EJ, Jones RB, Bearman SI et al. Transplantation of enriched CD34-positive autologous marrow into breast cancer patients following high-dose chemotherapy: Influence of CD34-positive peripheral-blood progenitors and growth factors on engraftment. *J Clin Oncol* 1994; 12: 28-36.
3. Osborne MP, Asina S, Wong GY et al. Immunofluorescent monoclonal antibody detection of breast cancer in bone marrow: Sensitivity in a model system. *Cancer Res* 1989; 49: 2510-3.
4. Simpson SJ, Vachula M, Kennedy MJ et al. Detection of tumor cells in the bone marrow, peripheral blood, and apheresis products of breast cancer patients using flow cytometry. *Exp Hematol* 1995; 23: 1062-8.
5. Ross AA, Cooper BW, Lazarus HM et al. Detection and viability of tumor cells in peripheral blood stem cell collections

- from breast cancer patients using immunocytochemical and clonogenic assay techniques. *Blood* 1993; 82: 2605-10.
6. Johnson PWM, Burchill SA, Selby PJ. The molecular detection of circulating tumour cells. *Br J Cancer* 1995; 72: 268-76.
 7. Moreno JG, Croce CM, Fischer R et al. Detection of haematogenous micrometastasis in patients with prostate cancer. *Cancer Res* 1992; 52: 6110-2.
 8. Israeli RS, Miller Jr WH, Su Sai L et al. Sensitive nested reverse transcription polymerase chain reaction detection of circulating prostatic tumor cells: Comparison of prostate-specific membrane antigen and prostate-specific antigen-based assays. *Cancer Res* 1994; 54: 6306-10.
 9. Gerhard M, Juhl H, Kalthoff H et al. Specific detection of carcinoembryonic antigen-expressing tumor cells in bone marrow aspirates by polymerase chain reaction. *J Clin Oncol* 1994; 12: 725-9.
 10. Smith B, Selby P, Southgate J et al. Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. *Lancet* 1991; 338: 1227-9.
 11. Mattano Jr LA, Moss TJ, Emerson SG. Sensitive detection of rare circulating neuroblastoma cells by the reverse transcriptase-polymerase chain reaction. *Cancer Res* 1992; 52: 4701-5.
 12. Datta YH, Adams PT, Drobyski WR et al. Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. *J Clin Oncol* 1994; 12: 475-82.
 13. Noguchi S, Aihara T, Nakamori S et al. The detection of breast carcinoma micrometastases in axillary lymph nodes by means of reverse-transcriptase polymerase chain reaction. *Cancer* 1994; 74: 1595-600.
 14. Burchill SA, Bradbury MF, Pittman K et al. Detection of epithelial cancer cells in peripheral blood by reverse transcriptase-polymerase chain reaction. *Br J Cancer* 1995; 71: 278-81.
 15. Hoon DSB, Doi F, Giuliano AE et al. The detection of breast carcinoma micrometastases in axillary lymph nodes by means of reverse transcriptase-polymerase chain reaction. *Cancer* 1995; 76: 533-4 (Letter).
 16. Zou Z, Anisowicz A, Hendrix MJC et al. Maspin, a serpin with tumor-suppressing activity in human epithelial cells. *Science* 1994; 263: 526-9.
 17. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156-9.
 18. Roth MS, Antin JH, Bingham EL et al. Detection of Philadelphia chromosome-positive cells by the polymerase chain reaction following bone marrow transplant for chronic myelogenous leukemia. *Blood* 1989; 74: 882-5.
 19. Jänicke F, Scmitt M, Pache L et al. Urokinase (uPA) and its inhibitor PAI-1 are strong and independent prognostic factors in node-negative breast cancer. *Breast Cancer Res Treat* 1993; 24: 195-208.
 20. Hopkins PCR, Whisstock J. Function of maspin. *Science* 1994; 265: 1893-4 (Technical comments).
 21. Chelly J, Concordet J-P, Kaplan J-C et al. Illegitimate transcription: Transcription of any gene in any cell type. *Proc Natl Acad Sci USA* 1989; 86: 2617-21.
 22. Traweek ST, Liu J, Battifora H. Keratin gene expression in nonepithelial tissue-detection with polymerase chain reaction. *Am J Pathol* 1993; 142: 1111-8.
 23. Schoenfeld A, Luqmani E, Smith D et al. Detection of breast cancer micrometastases in axillary lymph nodes by using polymerase chain reaction. *Cancer Res* 1994; 54: 2986-90.
 24. Shpall E, Jones RB. Release of tumor cells from bone marrow. *Blood* 1994; 83: 623-5.
 25. Brugger W, Bross KJ, Glatt M et al. Mobilization of tumor cells and hematopoietic progenitor cells into peripheral blood of patients with solid tumor. *Blood* 1994; 83: 636-40.

Received 11 January 1996; accepted 6 May 1996.

Correspondence to:

Giuseppe Torelli, MD
Dipartimento di Scienze Mediche, Sezione di Ematologia
Policlinico
Via del Pozzo 71
41100, Modena
Italy