Annals of Oncology **12**: 1693–1698, 2001.

Original article

Maspin and mammaglobin genes are specific markers for RT-PCR detection of minimal residual disease in patients with breast cancer

P. Corradini,[1,4] C. Voena,[1] M. Astolfi,[3] S. Dell'Oro,[4] S. Pilotti,[5] G. Arrigoni,[2] M. Bregni,[1] A. Pileri[3] & A. M. Gianni[4]

[1]*Department of Hematology, Istituto Scientifico H.S. Raffaele, Milan;* [2]*Department of Pathology, Istituto Scientifico H S. Raffaele, Milan;* [3]*Department of Hematology, University of Torino;* [4]*Medical Oncology and Bone Marrow Transplantation, Unit Istituto Nazionale Tumori, University of Milan,* [5]*Department of Pathology, Istituto Nazionale Tumori, Milan, Italy*

Summary

Background: This study evaluates the specificity of some reverse-transcriptase polymerase chain reaction (RT-PCR) assays for the detection of residual tumor cells in breast cancer patients. The following markers have been analysed: carcinoembryonic antigen (CEA), cytokeratins (CK19 and CK20), polymorphic epithelial mucin (MUC-1), epidermal growth factor receptor (EGFR), maspin, and mammaglobin. RT-PCR was employed to detect breast cancer cells in peripheral blood (PB), bone marrow (BM), and stem cell leukoaphereses (PBPC).

Patients and methods: We evaluated the specificity of our RT-PCR assays on a panel of breast cancer specimens (n = 30), on PBPC in patients undergoing high-dose chemotherapy (n = 38), on BM (n = 7) and PB (n = 5) samples obtained from patients with breast cancer. Marrow cells, PB, and PBPC from normal subjects or hematological tumor patients were tested as negative controls.

Results: Only maspin and mammaglobin met the criteria of sensitivity and specificity required for the detection of residual disease; they were expressed in 80% and 97% of breast cancer specimens, respectively, and not expressed in normal controls. CK19, CK20, EGFR, MUC-1, and CEA were sometimes expressed in normal blood cells and/or hematological tumors.

Conclusions: Our data support the notion that maspin and mammaglobin are useful markers for RT-PCR detection of minimal residual disease (MRD) in breast cancer patients, and that perspective clinical studies are needed to determine wether RT-PCR assays will be useful in assessing prognosis, tailoring therapy, or developing new strategies for *ex vivo* purging.

Key words: breast cancer, mammaglobin, maspin, residual disease

Introduction

The value of high-dose chemotherapy with autologous hematopoietic cell transplantation has been recently questioned as a treatment modality for high-risk or advanced breast cancer, due to early results of randomized studies [1, 2]. However, long-term remissions have been achieved with this approach in advanced disease, and in high-risk patients with more than ten involved axillary nodes [3–5]. It is still not clear if post-transplant relapse is due to persistence of systemic disease, infusion of clonogenic tumor cells, or a combination of both causes. With the aim of investigating the role of minimal residual disease (MRD) in breast cancer patients, a variety of assays have been developed. Immunocytochemical staining and flow cytometry, using a panel of monoclonal antibodies directed against cell surface glycoproteins or cytokeratins, have shown that both bone marrow (BM) and peripheral blood progenitor cells (PBPC) collected after high-dose chemotherapy may contain residual tumor cells. These techniques have a sensitivity detection of one cancer cell in up to 10^3–10^4 normal hematopoietic cells [6–9]. The presence of occult cytokeratin-positive metastatic cells in the bone marrow of patients with stage I–III breast cancer has recently been reported to predict distant relapse rate and death from breast cancer [10]. Thus, the detection of MRD by immunocytochemistry may be relevant for the prognosis of breast cancer.

Recently, several reverse-transcriptase polymerase chain reaction (RT-PCR) assays have been developed to improve the sensitivity of the detection of breast micrometastases in BM, peripheral blood (PB), and PBPC [11–15]. The optimal approach for the RT-PCR detection of circulating breast cancer cells would be the amplification of tissue-specific RNA transcripts not expressed in normal hematopoietic tissues. A number of breast cancer markers have been reported in the literature, and many of them have shown important limitations for their use in the clinical setting. Cytokeratin 19 (CK-19), an intermediate filament protein expressed by all simple epithelia and by a small number of other tissues, has been originally presented as a promising, specific and sensitive marker for the detection of breast cancer cells in axillary lymph nodes, PB and BM [7]. The reported value of the RT-PCR based assay for CK-19 was sub-

sequently questioned because of the documented expression of this gene in either PB or lymph nodes of normal subjects [16]. Mucin-1 (MUC-1), cytokeratin 20 (CK-20), carcinoembryonic antigen (CEA) and maspin have been reported to be sensitive RT-PCR markers for cancer cell detection in patients with breast cancer; however, several studies refer MUC-1, CK-20, and CEA mRNA expression in the lymph nodes, PB and/or BM of patients with no evidence of breast cancer [16–20]. Recently, Hildebrandt et al. reported epidermal growth factor receptor (EGFR) as a reliable mRNA marker of breast cancer with a high specificity and sensitivity [21]. More recently mammaglobin, a tissue specific mRNA marker expressed in adult mammary tissues and in 80%–90% primary breast tumors, has been detected in over 60% of the lymph nodes of patients with metastatic breast cancer, but not in the normal lymph nodes from non-cancer patients [18, 22]. These results indicate mammaglobin as a hypothetical optimal marker for the detection of circulating breast cancer cells [23].

The aim of our study was to determine the sensitivity and specificity of CK-19, CK20, MUC-1, EGFR, CEA, maspin and mammaglobin markers in detecting breast cancer cells by RT-PCR.

Patients and methods

Cell lines and patient samples

Human breast cancer cell lines MCF-7 (estrogen receptor-positive) and SKBR3 (estrogen receptor-negative) obtained from the American Type Culture Collection (Rockville, Maryland) were used in the development of the RT-PCR assays. The tumor samples analyzed consisted of 30 surgical specimens from patients with histologic diagnosis of breast cancer (Table 1). BM, PB, and PBPC were obtained from a study population of 38 breast cancer patients undergoing high-dose chemotherapy as an adjuvant treatment for high-risk disease (i.e., more than ten involved axillary nodes), or as a salvage treatment for advanced disease. The study protocols were approved by the local IRB, and patients gave their informed consent to the study. All breast tissues were obtained from surgical resection specimens snap-frozen in liquid nitrogen within 10 minutes of their arrival in the pathology specimen reception area, and kept frozen until tissue homogenization prior to extraction Ten normal PB, 12 normal BM and 2 normal PBPC were obtained from healthy donors. Four PB, 8 BM and 12 leukapheresis samples were collected from patients with hematological malignancies (lymphoma, multiple myeloma and leukemias). To avoid potential contamination from skin epithelial cells, the first 1 ml of PB and 0.5 ml of BM aspirates were stored, and not used for PCR analysis Samples were processed as described below.

To determine the sensitivity of the assays, peripheral blood mononuclear cells (PB) obtained from a normal donor were mixed with decreasing numbers of MCF-7 cells or SKBR3 cells. Cells were mixed before the RNA preparation, thus mimicking the clinical setting for detection of tumor cells in the PB or BM of patients.

RNA preparation

Cells were separated on a Ficoll-Hypaque density gradient. Ten million mononuclear cells obtained from PB, BM, leukapheresis samples and breast tissues homogenates were lysed in TRIZOL reagent (GIBCO-BRL, Gaithersburg, Maryland) and sheared to homogeneity, following the one-step guanidinium isothiocyanate/phenol RNA preparation as originally described [24]. RNA was precipitated in isopropanol, washed in 70% ethanol, and resuspended in RNAse-free water. To eliminate contaminating DNA, RNA was exposed to RNAse-free DNAse I (1 U/μg RNA; Promega, Madison, Wisconsin) at 37 °C for 10 minutes. After heat inactivation of the enzyme at 95 °C for five minutes, RNA was reprecipitated and resuspended.

Table 1 Histologic specimens analyzed in the study.

Histology	Ductal infiltrating = 21
	Lobular infiltrating = 4
	Ductal-lobular = 3
	Mucinous = 2
Grade	2 = 19
	3 = 11
Stage	I = 6
	IIA = 12
	IIB = 5
	IIIA = 2
	IIIB = 5

cDNA synthesis and PCR amplification

Total RNA was reverse transcribed into total cDNA using an oligo-dT primer (Promega, Madison, Wisconsin). Five μg of total RNA was incubated with 50 ng of oligo-dT primer at 70 °C for 10 minutes. A 50 μl reaction was performed in 10 mM dNTPs (Pharmacia LKB Biotechnology, Uppsala, Sweden), 10 mM DTT, 1 × reverse transcriptase buffer (50 mM Tris Hcl, 6 mM $MgCl_2$,40 mmmol/l Kcl) final concentration, adding 20 U of ribonuclease inhibitor (RNAsin; Promega, Madison, Wisconsin), and 200 U Moloney murine leukemia virus reverse transcriptase (Superscript; GIBCO BRL, Gaithersburg, Maryland). The reaction mixture was incubated for one hour at 37 °C. Reverse-transcriptase was heat inactivated at 95 °C for five minutes. Two μl of each reaction product were analyzed by nested-PCR.

The cDNA was amplified in 200 μmol/l dNTPs, 1 × Taq buffer [50 mmol/l Kcl, 10 mmol/l Tris/HCl pH, 1 mmol/l $MgCl_2$, 1% (wt/vol) gelatin] final concentration, adding 2,5 U of Taq DNA polymerase (Promega) and from 10 to 20 pmol of primers in a final volume of 50 μl Nested RT-PCRs were performed using published primer sequences as described for CK-19, CEA and maspin [12, 13, 17]. MUC-1, EGFR, CK-20 and mammaglobin nested RT-PCR were performed using first round published primer sequences [18, 21, 25, 26] and second-round internal primers designed during the study (for MUC-1: MUC-1c 5'-CAAGCTCTACCCCAGGTG-3' and MUC-1d 5'-GACAATGGCCAGCGGCAAC-3'; for EGFR: EGFR3 5'-TCTCAGCAACATGTCGATGG-3' and EGFR4 5'-TCGCACT-TCTTACACTTGCG-3', for CK-20: CK20a 5'-GCAGATTCGGAGTAACATGGA-3' and CK20d 5'-GCACGACTGTCTTAATCTTGG-3'; for mammaglobin: MG3 5'-GAGTTCATAGACGACAATGCC-3' and MG4 5'-CCGTAGTTGGTTTCTCACCAT-3'. MUC-1 nested RT-PCR was performed at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s. After 30 cycles 2 μl of amplified cDNA were used as template for a second round PCR with internal primers (94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s for 30 cycles). CK19 first round nested RT-PCR reaction was performed at 94 °C for 50 s, 72 °C for 90 s for 35 cycles. After completion of the first round RT-PCR, 2 μl amplified cDNA were used as template for a second round PCR with internal primers (94 °C for 50 s, 72 °C for 90 s for 35 cycles). EGFR first round nested RT-PCR was performed at 94 °C for 40 s, 60 °C for 1 min, 72 °C for 1 min for 30 cycles. After completion of the first round RT-PCR 2 μl of amplified cDNA were used as template for a second round PCR with internal primers (94 °C for 40 s, 60 °C for 1 min, 72 °C for 1 min for 30 cycles). CK-20 first round RT-PCR was performed at 94 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s, for 32 cycles. Two μl of amplified cDNA were used as template for a second round PCR with internal primers (94 °C for

30 s, 58 °C for 1 min, 72 °C for 1 min for 32 cycles). CEA first round nested RT-PCR was performed at 95 °C for 1 min, 72 °C for 2 min for 30 cycles. After completion of the first round RT-PCR 5 µl of amplified cDNA were used as template for a second round PCR with internal primers (95 °C for 1 min, 69 °C for 1 min, 72 °C for 1 min for 30 cycles). Maspin first round RT-PCR was performed at 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, for 30 cycles. Two µl of amplified cDNA were used as template for a second round PCR with internal primers (94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s for 35 cycles). Mammaglobin first round RT-PCR was performed at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, for 32 cycles. Two µl of amplified cDNA were used as template for a second round PCR with internal primers (94 °C for 30 s, 58 °C for 1 min, 72 °C for 1 min for 35 cycles) PCR products were run on 2% agarose gel and visualized by ethidium-bromide staining. Expected size of the second PCR products for each marker was: 450 bp for MUC-1, 745 bp for CK-19, 475 bp for EGFR, 175 bp for maspin, 194 bp for CK-20, 199 bp for mammaglobin and 131 bp for CEA. All the specimens were tested for the presence of intact RNA, adequate cDNA synthesis, and absence of inhibitors by amplifying β_2 microglobulin gene using a single step PCR. All primer pairs were used to amplify genomic DNA and gave no products, confirming that primer choice and DNAse treatment were effective.

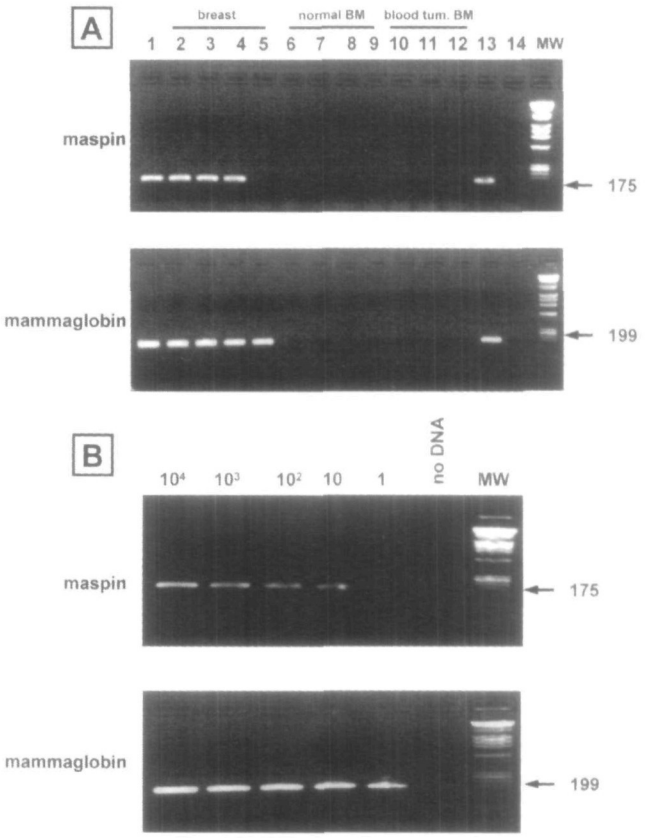

Figure 1. (A) Maspin and mammaglobin expression in breast specimens, normal bone marrow (BM) and blood tumor BM by RT-nested-PCR analysis. Lack of detectable maspin and mammaglobin expression in normal and blood tumor BM. Lane 1, MCF-7 cell line positive control; lane 2–5, breast specimens; lane 6–9, normal BM; lane 10–12; blood tumor BM; lane 13, 10^{-5} MCF-7 cell line dilution for maspin (10 breast cancer cells in 10^6 normal PB cells and 10^{-6} MCF-7 cell line dilution for mammaglobin (1 breast cancer cell in 10^6 normal PB cells); lane 14, no DNA, MW, molecular weight marker. (B) Maspin and mammaglobin sensitivity by RT-nested-PCR performed on 10-fold serial dilutions of the MCF-7 cells in 10^6 normal peripheral blood cells (from 10^4 to 1 breast cancer cells diluted in 10^6 normal peripheral blood cells)

Results

Assay sensitivity and negative controls

To determine the sensitivity of RT-PCR assays, 1 million PB mononuclear cells obtained from a normal donor were mixed with decreasing numbers of MCF-7 and SKBR-3 cell lines (for the last dilution 10 breast cancer cells were diluted in 10 million normal cells). RNA was isolated and RT-PCR analysis performed as described in the methods section. The sensitivity for all assays was satisfactory, and the detection limit ranged between 1 to 10 MCF-7 or SKBR-3 cells in one million PB cells. Results on specificity are shown in detail in Table 2. In our study, 3 out of 10 PB from healthy subjects expressed CK-19 and all the healthy individuals expressed the MUC-1 mRNA. The EGFR, CK-20, CEA, maspin and mammaglobin transcripts were not detected in PB samples from healthy donors. The expression of CK-19, CK-20, MUC-1, EGFR and CEA occurred at variable frequency in the BM cells obtained from healthy donors. Maspin and mammaglobin transcripts were not found in marrow cells from normal subjects or from patients with hematological malignancies. Few

Table 2 Nested RT-PCR analysis of mRNA markers expression in non breast cancer control group.

Specimen	CK-19		MUC-1		EGFR		CK-20		CEA		Maspin		Mammaglobin	
	No. of total	%	No. of total	%	No. of total	%	No. of total	%	No. of total	%	No. of total	%	No. of total	%
Normal BM	1/11	9	5/11	45	4/10	40	1/9	11	0/9	0	0/12	0	0/12	0
Normal PB	3/10	30	10/10	100	0/10	0	0/10	0	0/9	0	0/10	0	0/10	0
Normal PBPC	0/2	0	1/2	50	0/2	0	0/2	0	0/2	0	0/2	0	0/2	0
Blood tumors	1/8	12	6/8	75	5/8	62	0/5	0	1/5	20	0/8	0	0/5	0
Blood tumors PB	1/4	25	3/4	75	2/4	50	0/4	0	2/4	50	0/4	0	0/4	0
Blood tumors PBPC	0/12	0	12/12	100	0/11	0	1/6	16	4/6	67	0/12	0	0/6	0
Solid tumors[a]	2/7	28	3/7	42	1/7	14	3/7	42	3/7	42	0/8	0	0/8	0

Abbreviations: BM – bone marrow; PB – peripheral blood; PBPC – peripheral blood progenitor cells.
[a] Two renal carcinoma, two ovarian cancer, one pancreas carcinoma, one colon carcinoma, one gastric carcinoma

Table 3. Nested RT-PCR analysis of mRNA markers expression in breast cancer patients and breast cell lines.

Specimen	CK-19		MUC-1		EGFR		CK-20		CEA		Maspin		Mammaglobin	
	No. of total	%	No. of total	%	No. of total	%	No. of total	%	No. of total	%	No. of total	%	No. of total	%
Breast cancer BM	0/7	0	3/7	43	1/7	14	0/7	0	5/7	71	0/7	0	3/7	43
Breast cancer PB	0/5	0	2/5	40	0/5	0	0/5	0	2/5	40	0/5	5	0/5	0
Breast specimens	21/30	70	22/30	73	19/30	63	17/30	57	26/30	87	25/30	80	29/30	97
Breast PBPC	0/38	0	5/12	42	0/38	0	2/22	9	6/37	16	2/37	5	3/37	8
Cell lines	2/2	100	2/2	100	2/2	100	2/2	100	2/2	100	2/2	100	2/2	100

Abbreviations: BC – breast cancer; BM – bone marrow; PB – peripheral blood; PBPC – peripheral blood progenitor cells.

cases of solid tumors other than breast were tested: maspin and mammaglobin transcripts were not detected.

To assess whether the treatment with G-CSF could alter the expression of such mRNA markers, we have tested mobilized PBPC from both healthy donors and patients with hematological tumors. All PBPC specimen were found negative for maspin, mammaglobin, CK-19 and EGFR transcripts, while MUC-1 CEA and CK-20 were expressed at variable frequency (Table 2).

Marker expression in breast cancer patients and cell lines

The results of nested RT-PCR assays in breast cancers and cell lines are shown in Table 3. MCF-7 and SKBR3 cell lines expressed all the mRNA markers. The expression of these markers in primary breast cancer cells ranged from 57% (CK-20) to 97% (mammaglobin). BM and PB cells were obtained from seven and five breast cancer patients, respectively; BM samples were negative for CK-19, CK-20 and maspin transcripts, while MUC-1, EGFR, CEA and mammaglobin were expressed at variable frequency (Table 3). The only markers that were expressed in the PB of breast cancer patients were MUC-1 and CEA.

Detection of residual breast cancer cells in PBPC harvests

In order to evaluate tumor cell contamination of PBPC harvests collected after high-dose chemotherapy, we performed RT-PCR on 38 apheresis products. Overall the expression of breast cancer markers was as follows: MUC-1 in 5 of 12 (42%) PBPC samples, maspin in 2 of 38 (5%), CEA in 6 of 37 (16 %), and mammaglobin in 3 of 37 (8%). None of PBPC samples were found positive for CK19 or EGFR transcripts (Table 3). In particular, RT-PCR on PBPC samples from patients with metastatic disease gave the following positive results: MUC-1, one case, CEA four cases, maspin two cases, and mammaglobin one case.

Discussion

Detection of breast cancer cells is possible by conventional immunocytochemistry, cell culture techniques, flow cytometry, and RT-PCR amplification of epithelial-specific mRNA sequences. The major advantage of RT-PCR over the other methods is the operator independency, the possibility of permanent documentation and the sensitivity. Recently published data show that occult breast cancer cells have been detected by RT-PCR in the PB of 15% to 30% of patients, in the BM aspirates of 50% to 60% of patients, in axillary lymph nodes (without overt metastases) of 3% to 55% of patients [6, 7, 13, 14, 21].

A major concern in the use of RT-PCR assays arises from the conflicting data reported in the literature on the specificity of different assays. The specificity of the RT-PCR techniques is basically abrogated when the mRNA marker is expressed by the normal hematopoietic cells. Several studies have reported the expression of CK-19, MUC-1, CK-20 and CEA in PB, BM and lymph nodes of patients with breast cancer, leading to the conclusion that tumor cells were circulating [7, 9, 12, 13, 17]. On the other hand, recent reports have indicated that these molecular markers are also expressed by normal cells [8, 9, 12]. Our study shows that CK-19, MUC-1, CK-20, EGFR and CEA transcripts are detected in PB or BM of healthy subjects, and in patients with hematological malignancies, thus they seem not specific enough to be used for the detection of occult breast cancer cells (Table 2). Maspin, a protein related to the serpin family of protease inhibitors, and mammaglobin, a tissue-specific marker expressed in adult mammary tissue, have been recently reported as specific markers for the detection of occult breast cancer cells [17, 18, 23, 27]. In our study, both these markers were not detected in PB and BM from healthy subjects or from patients with hematological malignancies. Our results confirm that maspin and mammaglobin are sensitive and specific markers for the detection of occult breast cancer cells (Table 2).

In autografting procedure, the presence of residual tumor cells remains a major concern, since some authors have described the presence of contaminating tumor cells in PBPC harvests used to support the hematopoietic recovery after high-dose chemotherapy [7, 9]. It is currently unknown whether the reinfusion of grafts containing occult breast cancer cells contributes to disease relapse. However, the presence of gene-marked

tumor cells at the site of disease relapse has been documented in other malignancies [28]. The developement of specific and sensitive methods for the detection of residual tumor cells would be useful to monitor the response to chemotherapy, the presence of occult cells in autografts and the efficiency of purging techniques.

Lòpez-Guerrero et al. reported CEA and CK-19 to be specific and sensitive markers for detecting the presence of breast cancer cells in leukapheresis samples, and maspin to be a non-specific marker for the detection of tumor cells contamination in PBPC collections [29]. In our study, CK-19, and EGFR mRNA expression was not observed in PBPC harvests of breast cancer patients; while MUC-1, CK-20, maspin and mammaglobin rate mRNA expression ranged from 5% to 40% (Table 3). In order to evaluate the specificity of the RT-PCR assays, marker expression was evaluated in PBPC from blood tumor patients. MUC-1, CK-20 and CEA transcripts were detected in PBPC samples from patients with blood tumors. In the same samples, CK-19, EGFR, maspin and mammaglobin transcripts were not detected (Table 2). We conclude that MUC-1, CK-19, CK-20, EGFR and CEA markers have a limited diagnostic applicability because of their expression in PB, BM and PBPC harvests of normal subjects or blood tumor patients. Hence, our data indicate that RT-PCR detection of breast cancer cells should be based on maspin and mammaglobin markers.

The relevance of low levels of breast cancer cells in PBPC harvests from patients following high-dose chemotherapy remain to be defined in the clinical setting [30]. The role of these cells in causing disease relapse, and the necessity for intervention (i.e., purging of the stem cell product), are issues that are still controversial and require further investigation. Long term follow-up is needed to determine whether RT-PCR assays will be useful in assessing prognosis, guiding clinical decisions, or in developing novel purging strategies.

Acknowledgement

This work was supported in part by CNR grant no. 00.00026.st97 awarded to Marco Bregni.

References

1. Peters WP, Rosner G, Vredenburgh J et al. A Prospective, randomized comparison of combination alkylating agents as consolidation after CAF in high-risk primary breast cancer involving ten or more axillary lymph nodes: Preliminary results of CALGB 9082/SWOG 9114/NCIC MA-13. Proc Am Soc Clin Oncol 1999; 18: 1a.
2. Stadtmauer EA, O'Neill A, Goldstein LJ et al. Phase III randomized trial of high-dose chemotherapy and stem cell support shows no difference in overall survival or severe toxicity compared to maintenance chemotherapy with cyclophosphamide, methotrexate and fluorouracil for women with metastatic breast cancer who are responding to conventional induction chemotherapy. The 'Philadelphia' Intergroup Study (PBT-1). Proc Am Soc Clin Oncol 1999; 18: 1a.
3. Peters WP, Shpall EJ, Jones RB et al. High-dose combination alkylating agents with bone marrow support as initial treatment for metastatic breast cancer J Clin Oncol 1988; 6: 1368–73.
4. Antman K, Ayash L, Elias A et al. A phase II study of high-dose cyclophosphamide, thiotepa, and carboplatin with autologous marrow support in women with measurable advanced breast cancer responding to standard-dose therapy. J Clin Oncol 1992; 83: 920–6.
5. Gianni AM, Siena S, Bregni M et al. Efficacy, toxicity, and applicability of high-dose sequential chemotherapy as adjuvant treatment in operable breast cancer with ten or more involved axillary nodes: Five-year results. J Clin Oncol 1997; 15: 2312–21
6 Cote RJ, Rosen PP, Lesser ML et al. Prediction of early relapse in patients with operable breast cancer by detection of occult bone marrow micrometasases. J Clin Oncol 1991; 9: 1749–52.
7. 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.
8. Debus E, Moll R, Franke WW et al. Immunoistochemical distinction of human carcinomas by cytokeratins typing with monoclonal antibodies. Am J Clin Pathol 1984; 114: 121–9.
9. 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.
10. Braun S, Pantel K, Muller P et al. Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. N Engl J Med 2000; 342: 525–33.
11. Schoenfeld A, Luqmani Y, Sinnet HD et al. Keratin 19 mRNA measurement to detect micrometastases in lymph nodes in breast cancer patients. Br J Cancer 1997; 76: 1639–42.
12 Mori M, Mimori K, Inoue H et al. Detection of cancer micrometastases in lymph nodes by reverse transcriptase polymerase chain reaction. Cancer Res 1995; 55: 3417–20.
13. Datta Y, Adams PT, Drobsyki WR et al. Sensitive detection of occult breast cancer by reverse-transcriptase polymerase chain reaction. J Clin Oncol 1994, 12: 475–82.
14 Noguchi S, Aihara T, Motomura K et al. Detection of breast cancer micrometastases in axillary lymph nodes by means of reverse transcriptase polymerase chain reaction. Comparison between MUC1 mRNA and keratin 19 mRNA amplification. Am J Pathol 1996; 148: 649–56.
15. Burchill SA, Bradbury MF, Pittman K et al. Detection of epithelial cancer cells in peripheral blood by reverse trancriptase-polymerase chain reaction. Br J Cancer 1995; 71: 278–81.
16 Bostick PJ, Chatterjee S, Chi DD et al. Limitations of specific reverse transcriptase polymerase chain reaction markers in the detection of metastases in the lymph nodes and blood of breast cancer patients. J Clin Oncol 1998; 16: 2632–40.
17 Luppi M, Morselli M Bandieri et al. Sensitive detection of circulating breast cancer cells by reverse-transcriptase polymerase chain reaction of maspin gene. Ann Oncol 1996; 7. 619–24.
18 Min J, Tafra L, Verbanac KM. Identification of superior markers for polymerase chain reaction detection of breast cancer metastases in sentinel lymph nodes. Cancer Res 1998; 58: 4581–4.
19. Mori M, Mimori K, Ueo H et al. Clinical significance of molecular detection of carcinoma cells in lymph nodes and peripheral blood by reverse transcription-polymerase chain reaction in patients with gastrointestinal or breast carcinomas. J Clin Oncol 1998; 16: 128–32.
20. Gherard M, Huhl H, Kalthoff H et al. Specific detection of carcinoembyonic antigen expressing tumor cells in bone marrow aspirates by polymerase chain reaction J Clin Oncol 1994; 12: 725–9
21. Hildebrandt M, Mapara MY, Korner IJ et al. Reverse trancriptase-polymerase chain reaction (RT-PCR)-controlled immunomagnetic purging of breast cancer cells using the magnetic cell separation (MACS) system: A sensitive method for monitoring purging efficiency Exp Hematol 1997; 25: 57–65.
22. Watson MA, Fleming T Mammaglobin, a mammary-specific

member of uteroglobin gene family, is overexpressed in human breast cancer. Cancer Res 1996; 56: 860–5.
23. Zach O, Kasparu H, Krieger O, Hehenwarter W et al. Detection of circulating mammary carcinoma cells in the peripheral blood of breast cancer patients via a nested reverse transcriptase polymerase reaction assay for mammaglobin mRNA. J Clin Oncol 1999, 17: 2015–9
24. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162: 156–8.
25. Brugger W, Bross KJ, Glatt M et al. Mobilization of tumor cells and hematopoietic progenitor cells into peripheral blood of patients with solid tumors. Blood 1994; 83: 636–40
26. Zrihan-Licht S, Vos H, Baruch A et al. Characterization and molecular cloning of a novel MUC1 protein, devoid of tandem repeats, expressed in human breast cancer tissue. Eur J Biochem 1994; 224: 787–95.
27. Sabbatini R, Federico M, Morselli M et al. Detection of circulating tumor cells by reverse transcriptase polymerase chain reaction of maspin in patients with breast cancer undergoing conventional-dose chemotherapy. J Clin Oncol 2000; 18: 1914–20.
28. Brenner MK, Rill DR, Moen RC et al. Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. Lancet 1993; 341: 85.
29. Lòpez-Guerrero JA, Bolufer Gilabert P, Gonzàlez EA et al. Use of reverse-transcriptase polymerase chain reaction (RT-PCR) for carcinoembyonic antigen, cytokeratin 19, and maspin in the detection of tumor cells leukapheresis products from patients with breast cancer: Comparison with immunochemistry. J Hematother 1999; 8: 53–61.
30. Gunn J, McCall JL, Yun K et al. Detection of micrometastases in colorectal cancer patients by K19 and K20 reverse trancriptions polymerase chain reaction. Lab Invest 1996; 75: 611–6.

Received 16 February 2001, accepted 27 July 2001.

Correspondence to:
P. Corradini, MD
Bone Marrow Transplantation Unit
Istituto Nazionale per lo Studio e la Cura dei Tumori
Via Venezian 1
20133 Milan
Italy
E-mail: paolo.corradini@unimi.it