Molecular Detection of Cancer Cells in the Peripheral Blood of Patients with Breast Cancer: Comparison of CK-19, CEA and Maspin as Detection Markers

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Abstract. *Purpose: To investigate and compare the diagnostic value of the detection of cytokeratin 19 (CK-19), carcinoembryonic antigen (CEA) and maspin mRNA by nested RT-PCR in the peripheral blood of women with breast cancer. Materials and Methods: The tumor cell lines MCF-7 and LOVO were used in an experimental tumor cell dilution model to determine the sensitivity of the nested RT-PCR for the 3 detection markers. RT-PCR analysis was performed in the peripheral blood of 54 healthy female blood donors, 28 patients with hematological malignancies, 31 with metastatic colorectal cancer, 75 with operable and 50 with metastatic breast cancer before receiving any cytotoxic chemotherapy, as well as in the bone marrow aspirates of 61 breast cancer patients. Results: Nested RT-PCR for CK-19 mRNA presented the highest sensitivity by detecting 1 tumor cell amongst 10^6 PBMC in 4 out of 5 experiments. CK-19 mRNA was detected in the peripheral blood of 3.7% of female blood donors, 14.3% of hematological malignancies, 32% of operable and 42% of metastatic breast cancer patients. CEA mRNA was undetectable in the blood of female blood donors but was detected in blood samples of 3.5% of hematological malignancies, 19.3% of colorectal cancer and 10% of breast cancer patients. Maspin mRNA was undetectable in the blood of female blood donors, patients with hematological malignancies and colorectal cancer but was detected in 9.3% of operable and 14% of metastatic breast cancer patients. Maspin mRNA positivity correlated with tumor size in patients with early stage breast cancer ($p = 0.057$). The detection rates of CK-19 and maspin mRNA in bone marrow aspirates were 33% and 11% for operable and 62% and 9% for metastatic breast cancer, respectively. During follow-up, 27.4% of blood samples were positive for CK-19 mRNA versus 10.7% for maspin mRNA in patients with operable breast cancer with a concordance rate of only 12.7% for positives and 86% for negatives. Conclusion: RT-PCR positivity for CK-19 mRNA is the most sensitive detection marker for occult tumor cells in operable and metastatic breast cancer, although nested RT-PCR for maspin mRNA appears to be more specific.*

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Despite important advances in the early diagnosis and treatment of breast cancer, about 20%-30% of patients with node-negative disease will develop distant metastases after a 10-year follow-up whereas only about 40% of the patients with node-positive disease will remain disease-free for 10 years or more (1-3). This is due to the fact that breast cancer cells may disseminate from the original tumor early during the natural history of the disease through hematogenous or lymphatic pathways. Indeed, several studies have shown that epithelial tumor cells can be detected in the bone marrow of patients with operable breast cancer and therefore may contribute to the failure of adjuvant chemotherapy and the development of distant metastases; prospective studies including a large number of patients have shown that the detection of occult tumor cells in the bone marrow is an independent prognostic and predictive factor for the overall survival and disease-free interval, respectively (reviewed in ref. 4, 5).

Although most studies have used immunocytochemistry to identify isolated tumor cells in bone marrow aspirates with a very good sensitivity and specificity (4, 5), there is an increasing interest for using molecular techniques such as the reverse transcriptase-polymerase chain reaction (RT-PCR) because of its reproducibility, high sensitivity and convenience in analyzing simultaneously multiple samples. However, because of the high sensitivity of the RT-PCR method, the utilized primer combinations are required to specifically recognize tumor and not bone marrow RNA. Therefore, for epithelial tumors the detection markers should be encoded by genes, which are tissue- or tumor-specific. Indeed, for the

detection of occult breast cancer cells in the bone marrow or the peripheral blood, several genes such as cytokeratin-19 (CK-19) (6, 7), cytokeratin-20 (CK-20) (8), CEA (9), EGF-R (10) and maspin (11) have been used.

Nevertheless, the finding of positive signals in peripheral blood or bone marrow of healthy individuals used as controls in most of the above mentioned studies seems to be a major problem of RNA-based assays designed to detect a small number of epithelial tumor cells. This phenomenon may be due to the amplification of a sequence that is similar but not identical to the target sequence, due to the expression of target genes in a minority of normal cells or, finally, due to the expression of the target gene in all or even some peripheral blood or bone marrow cells at a very low level (illegitimate expression) (reviewed in 4). Therefore, it is important to comparatively evaluate the sensitivity and the specificity of different target sequences which are used for the detection of micrometastatic disease.

In the present study we investigated the sensitivity and specificity of CK-19, CEA and maspin mRNA RT-PCR assays for detecting occult tumor cells. Furthermore we evaluated the diagnostic value of these assays in detecting the presence of occult breast cancer cells in the peripheral blood and bone marrow of patients with operable (stage I and II) and metastatic breast cancer.

Materials and Methods

Cell samples. The human mammary carcinoma cell line MCF-7 (obtained from the American Type Culture Collection; ATCC), which expresses CK-19 and maspin mRNAs, was used as positive control for both PCR reactions. MCF-7 cells were grown in Dulbecco's modified Eagles medium (DMEM) (Gibco Life Sciences, BRL, USA) supplemented with 10% bovine fetal serum (FBS) (Gibco, BRL), 2 mM L-glutamine (Sigma Chemical Company, Ltd, England) and 1 mM pyruvate (Sigma). The human colon adenocarcinoma cell line LOVO (obtained from the ATCC), which expresses CEA mRNA, was cultured in RPMI-1640 medium (Gibco, BRL) supplemented with 10% FBS, 10 μg/mL insulin (Sigma) and gentamicin sulphate (2‰, v/v) (Sigma) and was used as PCR-positive control. Both cell cultures were maintained in 5% CO_2 in air and cells grown in monolayer were harvested by washing the dishes once with phosphate-buffered saline (PBS) pH 7.3. The cells were then incubated with PBS containing 0.53 mM EDTA and 0.05% trypsin (Gibco, BRL) for 10-15 minutes at 37˚C. The cells were washed in PBS and then they were passed through 25G 5/8 needles to dissociate them. The cells were counted and viability assessed by trypan blue exclusion.

Patients and clinical samples. Peripheral blood in EDTA (10ml) was obtained from 28 patients with hematological malignacies (14 with myelodysplastic syndrome with excess of blasts, 4 with chronic myelogenous leukemia and 10 with chronic lymphocytic leukemia) at diagnosis and 31 patients with metastatic colorectal cancer before the initiation of any cytotoxic treatment. Peripheral blood was also obtained from 75 and 50 patients with operable (stage I and II) and metastatic (stage IV) breast cancer, before the initiation of adjuvant or front-line chemotherapy, respectively. Finally peripheral blood was also obtained from 54 healthy female blood donors. All samples were obtained at the mid of vein puncture after the first 5ml of blood were discarded. In 61 patients with breast cancer 2 ml of bone marrow in EDTA was also aspirated from the posterior iliac crest, under local anesthesia. All patients gave their informed consent to participate in the study which had been approved by the Ethics and Scientific Committees of our Institution.

Peripheral blood and bone marrow samples were diluted with PBS (v/v for peripheral blood and 1vol bone marrow / 4-5 volumes PBS for bone marrow samples) and then cells were dissociated by passing them through 25G 5/8 needles. Peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells (BMMC) were obtained by gradient centrifugation with Ficoll Hypaque-1077 (Sigma) at 1200 g for 30 minutes, at 4˚C. The interface cells were removed, washed twice with 50 mL of sterile PBS, pH 7.3, pelleted and resuspended in 1 mL of PBS. The cells were pelleted again at 1200 g for 2 minutes. The cell pellets were kept at -80˚C until RNA extraction. Total RNA isolation was performed by using Trizol LS reagent (Gibco, BRL) according to the manufacturer's instructions. All preparation and handling steps of RNA took place in a laminar flow hood, under RNase-free conditions. The isolated RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at -80˚C until used. The RNA concentration was determined by absorbance readings at 260 nm with the HITACHI UV-VIS (U-2000) spectrophotometer. RNA integrity was tested by PCR amplification of the β-actin housekeeping gene. As positive controls, RNA samples were also prepared from the human cell lines MCF-7 (CK-19, maspin) and LOVO (CEA).

Reverse transcription polymerase chain reaction. Reverse transcription of RNA was carried out with the THERMOCRIPT RT-PCR System (Gibco BRL). cDNA was synthesized from 5 μg total RNA in a final volume of 20 μL, according to the manufacturer's instructions. Four different PCR reactions, with the respective positive and negative controls (DEPC-H_2O instead of cDNA template was used as negative control) were performed with each sample in order to amplify fragments of CK-I9, CEA, maspin and β-actin. The sequences of primers utilised (synthesized by Genset, Paris, France) are shown in the Table I. These primers extend across at least an intron, thus an eventual DNA contamination would not pose a significant problem. PCR reaction mixtures and thermal cycler protocols for each marker are shown in Table II and Table III, respectively. CK-l9, CEA and maspin mRNAs were detected by nested PCR based on modified protocols reported by Datta *et al*. (6), Gerhard *et al.* (12) and Luppi *et al.* (11), respectively. The conditions for β-actin PCR were 1 cycle at 94˚C for 2 minutes, followed by 35 cycles at 94˚C for 30 seconds, 60˚C for 30 seconds and 72˚C for 45 seconds, with a final extension at 72˚C for 4 minutes. Ten μL of all PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide.

Statistical analysis. The associations between RT-PCR assays in the peripheral blood or bone marrow and other prognostic factors were analyzed by Chi-square tests or Fisher's exact test, where appropriate. The McNemar test and the kappa coefficient were used to test the null hypothesis of marginal homogeneity and agreement of outcomes, in 2x2 tables, respectively (13).

Results

Sensitivity and specificity of CK-19, CEA and maspin RT-PCR. To define the sensitivity of CK-19 and maspin RT-PCR, serial dilutions of MCF-7 cells were mixed with various concentrations of normal PBMC. Since MCF-7 cells did not express the CEA gene, for the evaluation of the CEA RT-PCR assay, the LOVO tumor cells were used. The obtained tumor cell/hematopoietic cell ratio ranged from 1:10 to $1:10^6$, thus mimicking the clinical setting for detection of mammary cells in patient's peripheral blood or bone marrow.

Table I. *Primer sequences.*

Gene	Name	5'-3' sequence	Size of PCR product (bp)
CK-19[1]			
forward	P1	AAGCTAACCATGCAGAACCTCAACGACCGC	
reverse	P2	TTATTGGCAGGTCAGGAGAAGAGCC	1069
forward	P3	TCCCGCGACTACAGCCACTACTACACGACC	
reverse	P4	CGCGACTTGATGTCCATGAGCCGCTGGTAC	745
CEA[2]			
forward	C1	TCTGGAACTTCTCCTGGTCTCTCAGGTGG	
reverse	C2	TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC	160
forward	C2	TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC	
reverse	C3	GGGCCACTGTCGGCATCATGATTGG	131
Maspin[3]			
forward	M1	TCAAGCGGCTCTACGTAGAC	
reverse	M2	CCTCCACATCCTTGGGTAGT	447
forward	M3	GATCTCACAGATGGCCACTT	
reverse	M4	GCACTGGTTTGGTGTCTGTC	175
β-actin[4]			
forward	A1	CATCCTGTCGGCAATGCCAGG	
reverse	A2	CTTCTTGGGCATGGAGTCCTG	154

[1]CK-19 mRNA, GeneBank Accession Number Y00503
[2]CEA mRNA, GeneBank Accession Number 29540
[3]Maspin mRNA, GeneBank Accession Number U04313
[4]β-actin DNA, GeneBank Accession Number E00829

Representative results of a RT-PCR-positive assay for each mRNA are shown in Figure 1. The results of the comparison of the three RT-PCR assays evaluated for the detection of MCF-7 or LOVO tumor cells, performed in a series of five experiments, are shown in Table IV. RT-PCR for CK-19 mRNA presented the highest sensitivity since it could detect 1 tumor cell amongst 10^6 peripheral blood mononuclear cells in 4 out of the 5 experiments. The corresponding sensitivities for CEA and maspin were 1 and 0 out of 5 experiments, respectively.

Peripheral blood mononuclear cells from 54 healthy female individuals, 28 patients with hematological malignancies, 31 patients with metastatic colorectal cancer and 50 patients with metastatic breast cancer before the initiation of any cytotoxic treatment, were used to determine the specificity of each RT-PCR assay. Table V shows the number of positive samples detected by each RT-PCR assay in the 4 different groups of samples. RT-PCR for CK-19 had the highest detection rate among breast cancer patients while RT-PCR for CEA had the highest detection rate among colorectal cancer patients. Among the four CK-19 mRNA-positive patients with hematological malignancies, two suffered from chronic myelogenous leukemia, one from myelodysplastic syndrome with excess of blasts and one from chronic lymphocytic leukemia. Maspin mRNA was detected only in patients with breast cancer and therefore showed the highest specificity. All maspin mRNA-positive samples were also CK-19 mRNA-positive whereas 14 CK-19 mRNA-positive samples were maspin mRNA-negative. Moreover, all the samples were positive for β-actin mRNA, indicating the presence of intact RNA and successful first-strand cDNA preparation. Based on these data, RT-PCR assays for CK-19 and maspin mRNAs were chosen for the subsequent evaluation of clinical samples from breast cancer patients.

Analysis of bone marrow samples. Twenty-seven and 34 bone marrow samples were obtained from patients with operable and metastatic breast cancer before the administration of any cytotoxic treatment, respectively; all samples were analysed for the detection of CK-19 and maspin mRNA-positive cells, using the nested RT-PCR assay. All samples were also analysed for the detection of β-actin using the RT-PCR assay; strong β-actin signals were detected in all cases. Table VI shows the number of positive BM samples for CK-19 and maspin mRNA by RT-PCR in patients with operable or metastatic breast cancer. The detection rate of CK-19

Table II. *PCR reaction mixture components for CK-19, CEA, maspin and actin.*

PCR reaction mixture	Volume per reaction (μL)						
	ACTIN	CK-19 1st PCR	CK-19 2nd PCR	CEA 1st PCR	MASPIN 2nd PCR	MASPIN 1st PCR	2nd PCR
Forward primer, 10 μM	1	1	1	1	1	5	5
Reverse primer, 10 μM	1	1	1	1	1	5	5
dNTPs, 5 mM	2	2	2	2	2	2	2
MgCl2, 50 mM	1.5	1.5	1.5	2.5	1.5	2.5	1.5
10x PCR buffer (without $MgCl_2$)	5	5	5	5	5	5	5
cDNA or 1st PCR product	3	5	3	5	3	5	3
Taq polymerase, 5 U/μL	0.2	0.2	0.2	0.5	0.5	0.5	0.5
DEPC-treated H_2O	36.3	34.3	36.3	33	36	25	28
Total volume	50	50	50	50	50	50	50

Table III. *PCR protocols for CK-19, CEA and maspin.*

PCR step	CK-19 1st PCR	CK-19 2nd PCR	CEA 1st PCR	CEA 2nd PCR	MASPIN 1st PCR	MASPIN 2nd PCR
Denaturation	94˚C/6 min	95˚C/5 min	94˚C/5 min	94˚C/5 min	94˚C/5 min	94˚C/5 min
Amplification	94˚C/50 sec	94˚C/50 sec	95˚C/20 sec	95˚C/1 min	95˚C/1 min	95˚C/1 min
			65˚C C/25 sec	69˚C C/1 min	55˚C C/1 min	55˚C C/1 min
	72˚C/2:30 min	72˚C/2 min				
			72˚C/20 sec	72˚C/1 min	72˚C/1 min	72˚C/1 min
Final extension	72˚C/10 min	72˚C/10 min	72˚C/5 min	72˚C/10 min	72˚C/7 min	72˚C/7 min
Number of cycles	35	35	35	30	35	35

mRNA-positive samples was higher than that of maspin mRNA-positive samples in each group. Moreover, the detection rate of CK-19 mRNA-positive samples was almost two times higher in metastatic than operable disease (62% *versus* 33%, respectively). In contrast, the detection rate of maspin mRNA-positive samples was similar in operable and metastatic disease.

Analysis of blood samples in patients with operable breast cancer. In order to further determine the validity of nested RT-PCR for the detection of CK-19 and maspin mRNAs, peripheral blood from 75 patients with operable (stage I and II) breast cancer were analysed for CK-19 and maspin mRNAs before the initiation of adjuvant chemotherapy. As shown in Table VII, 24 (32%) and 7 (9.3%) of the samples were CK-19 and maspin mRNA-positive, respectively. Only four (16.7%) of the 24 CK-19 mRNA-positive samples were also maspin mRNA-positive. The incidence of the detection of CK-19 mRNA-positive samples was not different in patients with stage I *versus* stage II disease, tumors < 5 cm

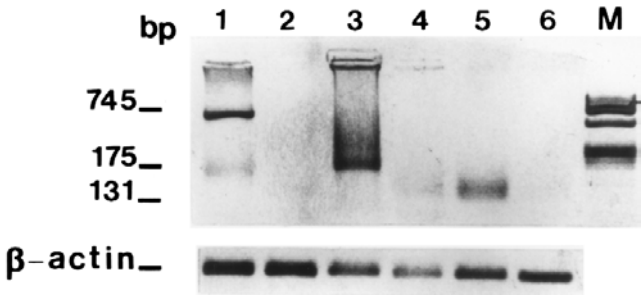

Figure 1. *Nested RT-PCR for the detection of CK-19, maspin and CEA mRNAs. mRNA was isolated from the MCF-7 and the LOVO tumor cell lines and amplified using the appropriate oligoprimers as described in Materials and Methods. Lanes 1, 3 and 5 correspond to the CK-19, Maspin and CEA amplified mRNA, respectively; lanes 2, 4 and 6 correspond to the negative controls (amplification in the absence of cDNA template) of CK-19, maspin and CEA mRNA, respectively. M = molecular markers.*

versus ≥ 5 cm, and negative *versus* 1-3 or ≥ 4 positive axillary lymph nodes. Conversely, the incidence of detection of maspin mRNA-positive samples seemed to increase with the stage of disease, the size of the primary tumor, and the number of involved lymph nodes (Table VII). Indeed, the incidence of maspin mRNA positivity was markedly increased (p = 0.057; Fisher's exact test), in patients with tumors measuring more than 5 cm in comparison with less than 5 cm.

Sequential peripheral blood samples were taken from 75 patients (a total of 430 samples) with operable breast cancer during the period of adjuvant hormone treatment. The median follow-up time was 20 months (range, 2-37) and a median of 6 samples/patient (range, 2-23) were evaluated for the detection of both CK-19 and maspin mRNA-positive cells. The proportion of CK-19 mRNA-positive samples was 27.4% and that of maspin mRNA-positive samples 10.7%. The corresponding McNemar test for the null hypothesis of equal proportions of positive outcomes for CK-19 and maspin mRNA, is highly significant ($p < 0.0001$), demonstrating that CK-19 and maspin mRNA have different proportions of positive outcomes. In 18 (24%) out of 75 patients, RT-PCR for both CK-19 and maspin mRNAs was negative in all tested samples [a total of 88 (20%) out of 430 blood samples]. In the remaining 57 patients with at least one positive test for CK-19 and/or maspin, a total of 342 samples were analyzed and 118 (34.5%) were found to be CK-19 mRNA-positive whereas 46 (13.5%) were maspin mRNA-positive. Table VIII demonstrates that only 15 (12.7%) of 118 CK-19 mRNA-positive samples were maspin mRNA-positive. Conversely 31 (13.8%) of the 224 CK-19 mRNA-negative samples were maspin mRNA-positive. The kappa coefficient was 0.014 (95% C.I. 0.012-0.074) indicating that the observed agreement between CK-19 and maspin was no better than chance alone.

Table IV. *Comparison between the different RT-PCR assays for the detection of MCF-7 (CK-19, maspin) and LOVO (CEA) tumor cells.*

Sample composition			*RT-PCR*		
Tumor cells	PBMC	Tumor cells/PBMC ratio	CK-19	CEA	Maspin
10^6	-	10^6/0	5/5	5/5	5/5
10^5	10^6	1/10	5/5	5/5	5/5
10^4	10^6	1/100	5/5	5/5	5/5
10^3	10^6	1/1.000	5/5	5/5	5/5
10^2	10^6	1/10.000	5/5	5/5	5/5
10	10^6	1/100.000	4/5	3/5	2/5
10	10^7	1/1.000.000	4/5	1/5	0/5
-	10^6	0/1.000.000	0/5	0/5	0/5

Table V. *Comparison of CK-19, maspin and CEA RT-PCR mRNA detection in the peripheral blood of healthy individuals, patients with hematological malignancies and advanced epithelial cancer.*

		Number of positive samples (%)		
Group tested	n	CK-19	CEA	Maspin
Healthy individuals	54	2 (3.7)	0 (0)	0 (0)
Hematologic malignancies	28	4 (14.3)	1 (3.5)	0 (0)
Metastatic colorectal cancer	31	1 (3.2)	6 (19.3)	(0)
Metastatic breast cancer	50	21 (42)	5 (10)	7 (14)

Table VI. *Detection of CK-19 and maspin mRNA-positive cells in the bone marrow of patients with operable and metastatic breast cancer.*

	Number of positive samples (%)	
	CK-19 mRNA	Maspin mRNA
Operable breast cancer (n=27)	9 (33)	3 (11)
Metastatic breast cancer (n=34)	21 (62)	3 (9)

Table VII. *Detection of CK-19 and maspin mRNAs in the peripheral blood of patients with operable breast cancer.*

Patient characteristics	n	Number of positive samples (%)			
		CK-19 mRNA	*p*-value	Maspin mRNA	*p*-value
All patients	75	24 (32.0)		7 (9.3)	
Stage					
I	21	7 (33.3)		0 (0.0)	
II	54	17 (31.5)	0.877	7 (13.0)	0.180*
Tumor size (cm)					
<5	63	20 (31.7)		4 (6.3)	
≥5	12	4 (33.3)	0.914	3 (25.0)	0.057*
Nodal status					
Negative	21	7 (33.3)		0 (0.0)	
1-3	24	6 (25.0)		3 (12.5)	
≥4 (4-19)	30	11 (36.7)	0.651	4 (13.3)	0.222

*Fisher's exact test

Table VIII. *Detection of CK-19 and maspin mRNA-positive cells in consecutive follow-up blood samples from patients with operable breast cancer.*

CK-19 mRNA	n	Number of samples (%)	
		Maspin mRNA Positive	Maspin mRNA Negative
Positive	118	15 (4.4)	103 (30.1)
Negative	224	31 (9.1)	193 (56.4)

Discussion

Tumor cell detection by RT-PCR relies on the selective amplification of mRNA transcripts of genes presumably expressed only in tumor cells and not in normal tissues. Only chimeric gene-transcripts, resulting from a chromosomal translocation, are truly tumor-specific. Such transcripts are not known for the majority of common solid tumors and therefore tumor cell detection usually relies on the amplification of epithelial genes involved in tissue differentiation or the malignant transformation. The cytokeratins (CK) are proteins that are stably and abundantly expressed in a majority of epithelial tumors and in most of the cells of these tumors (5). The malignant nature of CK mRNA-positive cells in the bone marrow has been confirmed through genomic analysis using fluorescence *in situ* hybridization where multiple chromosomal aberrations and amplification of the c-erbB-2 gene have been demonstrated in these cells (14). However, ectopic or illegitimate CK mRNA expression can theoretically occur and CK antigens have rarely been detected in hematopoietic cells (4, 5). On the contrary, CEA, a commonly expressed antigen in epithelial tumors, and maspin, a protein related to the family of serpins, have also been used as occult tumor cell detection markers using RT-PCR for mRNA amplification but positive samples have not been found among healthy control subjects (11, 12). A direct comparison of these three occult tumor detection markers is therefore indicated in order to determine their sensitivity and specificity using the RT-PCR technique.

In the present study we evaluated blood and bone marrow samples of breast cancer patients as well as blood from healthy female blood donors, patients with hematological malignancies and colorectal cancer for the presence of CK-19, CEA and maspin mRNA by RT-PCR. The sensitivity of RT-PCR for each marker was initially determined using the tumor cell lines MCF-7 and LOVO in dilution experiments. RT-PCR for CK-19 mRNA was the most sensitive marker by detecting 1 tumor cell among 10^6 PBMC in 4 out of 5 experiments. RT-PCR for maspin mRNA was the least sensitive marker with a positive detection in 2 out of 5 experiments at a dilution of 1 tumor cell in 10^5 PBMC. These sensitivities are in agreement with previous published reports using RT-PCR for these markers (6, 11, 12). When RT-PCR for CK-19 mRNA was performed in the blood of 54 healthy blood donors, 2 (3.7%) were positive although all were negative for CEA or maspin mRNA. Detection of CK-19 mRNA by RT-PCR in healthy subjects has also been reported by others (15) and is thought to be due to the illegitimate transcription of the CK-19 gene in hematopoietic cells (15, 16) or the amplification of a CK-19 pseudogene (17). It should be noted that, in our study, in all tests, purification of extracted total RNA was checked by running control samples without reverse transcriptase in the cDNA synthesis step. By using the described pair of primers, the pseudogene produces a fragment of 743 bp, whereas the CK-19 gene gives a product of more than 4000 bp, because it contains 5 additional introns. We observed no amplification of the CK-19 pseudogene or genomic DNA, which confirmed the absence of DNA contamination. Furthermore, CK-19 mRNA was detected in 4 out of 28 blood samples from patients with hematological malignancies including chronic myelogenous leukemia, chronic lymphocytic leukemia and myelodysplastic

syndrome. This has also been described in other studies (6, 16) and may be due to increased secretion of cytokines, which can induce transcription of "tissue-specific" genes in hemopoietic cells (18, 19).

In our study RT-PCR detected tumor cells by means of CK-19 mRNA in one-third of blood samples from patients with operable breast cancer and 42% of metastatic breast cancer. This was a higher detection rate than either CEA mRNA (10%) or maspin mRNA (14%). Since CEA mRNA RT-PCR showed the lowest detection rate in patients with metastatic breast cancer, only CK-19 and maspin were compared in the subsequent experiments. RT-PCR for CK-19 mRNA was equally sensitive in blood and bone marrow samples from patients with operable disease (33% positive results) but the bone marrow was more commonly positive than the peripheral blood in patients with metastatic disease (62% *versus* 42%, respectively). Conversely, maspin mRNA detection rates in the bone marrow were similar in operable and metastatic disease (11% and 9%, respectively) and similar in the blood and the bone marrow (14% and 9%, respectively). However only the maspin mRNA positivity but not the CK-19 correlated with disease characteristics such as stage, tumor size and nodal status. Analyzing the follow-up blood samples from patients with operable breast cancer, we found that CK-19 and maspin mRNA have significantly different proportions of positive outcomes and that the agreement between CK-19 and maspin mRNA detection is no better than chance alone. This may be due to tumor antigen heterogeneity of disseminated breast cancer cells, as has been shown for other antigens such as c-erbB-2, CO17-1A, MUC-1 and Lewis Y (20). Alternatively, these variations may be due to reduced or enhanced transcription of the cytokeratin genes and perhaps other genes associated with disease progression (21).

While most investigators agree that RT-PCR for CK-19 is a more sensitive method than immunohistochemistry for detecting micrometastases in breast cancer patients (22), the specificity of CK-19 mRNA detection by nested RT-PCR has been a matter of controversy. Several studies have reported that RT-PCR for CK-19 in the blood and/or the bone marrow is a very specific and reliable method for detecting disseminated breast cancer cells (23-25) while in other studies the finding of a high rate of false-positives has led to questioning the credibility of CK-19 as a detection marker (26, 27). According to our findings CK-19 mRNA is the most sensitive detection marker of disseminated tumor cells in operable and metastatic breast cancer and therefore the most appropriate of the three markers for use in clinical studies. The validity of our conclusion is further substantiated by the findings of our recent study where we evaluated the detection of CK-19 mRNA-positive cells by RT-PCR in the peripheral blood of 148 patients with stage I and II breast cancer before the initiation of any adjuvant therapy. We found that the presence of such cells was an independent adverse prognostic factor associated with decreased DFI and overall survival (28).

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References

1 DeVita VT Jr: Breast cancer therapy: Exercising all our options. N. Engl J Med *320:* 527-529, 1989.
2 Overgaard M, Hansen PS, Overgaad J, Rose C, Anderson M, Bach F, Kjaer M, Gadeberg CC, Mouridsen HT, Jensen MB and Zedeler K: Postoperative radiotherapy in high-risk premenopausal women with breast cancer. N Engl J Med *337:* 949-955, 1997.
3 Ragaz J, Jackson SM, Le N, Plenderleith IH, Spinelli JJ, Basco VE, Wilson KS, Knowling MA, Coppin CM, Paradis M, Coldman AJ and Olivotto IA: Adjuvant radiotherapy and chemotherapy in node-positive premenopausal women with breast cancer. N. Engl J Med *337:* 956-962, 1997.
4 Lambrechts AC, van Veer LJ and Rodenhuis S: The detection of minimal numbers of contaminating epithelial tumor cells in blood or bone marrow: Use, limitations and future of RNA-based methods. Ann Oncol *9:* 1269-1276, 1998.
5 Pantel K, Cote RJ and Fodstad O: Detection and clinical importance of micrometastatic disease. J Natl Cancer Inst *91:* 1113-1124, 1999.
6 Datta YH, Adams PT, Drobyski WR, Ethier SP, Terry VH and Roth MS: Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. J Clin Oncol *12:* 475-482, 1994.
7 Fields KK, Elfenbein GJ, Trudeau WL, Perkins JB, Janssen WE and Moscinki LC: Clinical significance of bone marrow metastases as detected using the polymerase chain reaction in patients with breast cancer undergoing high-dose chemotherapy and autologous bone marrow transplation. J Clin Oncol *14:* 1868-1876, 1996.
8 Burchill SA, Bradbury MF, Pittman K, Southgate J, Smith B and Selby P: Detection of epithelial cancer cells in peripheral blood by reverse transcriptase-polymerase chain reaction. Br J Cancer *71:* 278-281, 1995.
9 Mori M, Mimori K, Ueo H, Tsuji K, Shiraishi T, Barnard GF, Sugimachi K and Akiyoshi T: Clinical significance of molecular detection of carcinoma cells in lymph nodes and peripheral blood by reverse transcription-polymerase chain reactions in patients with gastrointestinal or breast cancer. J Clin Oncol *16:* 128-132, 1998.
10 Mapara MY, Korner IJ, Hildebrandst M, Bargou R, Krahl D, Reichardt P and Dorken B: Monitoring of tumor cell purging after highly efficient immunomagnetic selection of CD34 cells from leukapheresis products in breast cancer patients: Comparison of immunocytochemical tumor cell staining and reverse transcriptase-polymerase chain reaction. Blood *89:* 337-344, 1997.
11 Luppi M, Morselli M, Bandieri E, Federico M, Marasca R, Barozzi P, Ferrari MG, Savarino M, Frassoldati A and Torelli G: Sensitive detection of circulating breast cancer cells by reverse-transcriptase polymerase chain reaction of maspin gene. Ann Oncol *7:* 619-624, 1996.
12 Gerhard M, Juhl H, Kalthoff H, Schreiber HW, Wagener C and Neumaier M: Specific detection of carcinoembryonic antigen-expressing tumor cells in bone marrow aspirates by polymerase chain reaction. J Clin Oncol *12:* 725-729, 1994.
13 Altman GD: Practical Statistics for Medical Research. London, U.K., Chapman and Hall, 1991.
14 Muller P, Weckermann D, Riethmuller G and Schlimok G: Detection

of genetic alterations in micrometastatic cells in bone marrow of cancer patients by fluorescence in situ hybridization. Cancer Genet Cytogenet *88:* 8-16, 1996.

15 Novaes M, Bendit I, Garicochea B and del Giglio A: Reverse transcriptase-polymerase chain reaction analysis of cytokeratin 19 expression in the peripheral blood mononuclear cells of normal female blood donors. Mol Pathol *50:* 209-11, 1997.

16 Chelly J, Concordet JP, Kaplan JC and Kahn A: Illegitimate transcription: Transcription of any gene in any cell type. Proc Natl Acad Sci USA *86:* 2617-21, 1989.

17 Ruud P, Fodstad O and Hovig E: Identification of a novel cytokeratin 19 pseudogene that may interfere with reverse transcriptase-polymerase chain reaction assays used to detect micrometastatic tumor cells. Int J Cancer *80:* 119-25, 1999.

18 Grunewald K, Haun M, Urbanek M, Fiegl M, Muller-Holzner E, Gunsilius E, Dunser M, Marth C and Gastl G: Mammaglobin gene expression: a superior marker of breast cancer cells in peripheral blood in comparison to epidermal-growth-factor receptor and cytokeratin-19. Lab Invest *80:* 1071-7, 2000.

19 Jung R, Kruger W, Hosch S, Holweg M, Kroger N, Gutensohn K, Wagener C, Neumaier M and Zander AR: Specificity of reverse transcriptase polymerase chain reaction assays designed for the detection of circulating cancer cells is influenced by cytokines *in vivo* and *in vitro.* Br J Cancer *78:* 1194-8, 1998.

20 Braun S, Hepp F, Sommer HL and Pantel K: Tumor-antigen heterogeneity of disseminated breast cancer cells: implications for immunotherapy of minimal residual disease. Int J Cancer *84:* 1-5, 1999.

21 Brotherick I, Robson CN, Browell DA, Shenfine J, White MD, Cunliffe WJ, Shenton BK, Egan M, Webb LA, Lunt LG, Young JR and Higgs MJ: Cytokeratin expression in breast cancer: phenotypic changes associated with disease progression. Cytometry *32:* 301-8, 1998.

22 Schoenfeld A, Kruger KH, Gomm J, Sinnett HD, Gazet JC, Sacks N, Bender HG, Luqmani Y and Coombes RC: The detection of micrometastases in the peripheral blood and bone marrow of patients with breast cancer using immunohistochemistry and reverse transcriptase chain reaction for keratin 19. Eur J Cancer *33:* 854-61, 1997.

23 Kruger W, Krzizanowski C, Holweg M, Stockschlader M, Kroger N, Jung R, Mross K, Jonat W and Zander AR: Reverse transcriptase/polymerase chain reaction detection of cytokeratin-19 mRNA in bone marrow and blood of breast cancer patients. J Cancer Res Clin Oncol *122:* 679-86, 1996.

24 Zhong XY, Kaul S, Diel I, Eichler A and Bastert G: Analysis of sensitivity and specificity of cytokeratin 19 reverse transcriptase/polymerase chain reaction for detection of occult breast cancer in bone marrow and leukapheresis products. J Cancer Res Clin Oncol *125:* 286-91, 1999.

25 Kahn HJ, Yang LY, Blondal J, Lickley L, Holloway C, Hanna W, Narod S, McCready DR, Seth A and Marks A: RT-PCR amplification of CK19 mRNA in the blood of breast cancer patients: correlation with established prognostic parameters. Breast Cancer Res Treat *60:* 143-51, 2000.

26 Bustin SA, Gyselman VG, Williams NS and Dorudi S: Detection of cytokeratins 19/20 and guanylyl cyclase C in peripheral blood of colorectal cancer patients. Br J Cancer *79:* 1813-20, 1999.

27 Lambrechts AC, Bosma AJ, Klaver SG, Top B, Perebolte L, van't Veer LJ and Rodenhuis S: Comparison of immunocytochemistry, reverse transcriptase polymerase chain reaction, and nucleic acid sequence-based amplification for the detection of circulating breast cancer cells. Breast Cancer Res Treat *56:* 219-31, 1999.

28 Stathopoulou A, Vlachonikolis I, Mavroudis D *et al.*: Molecular detection of cytokeratin-19-positive cells in the peripheral blood of patients with operable breast cancer: evaluation of their prognostic significance. J Clin Oncol *20:* 3404-12, 2002.

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