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QUARTERLY

Detection of circulating breast cancer cells in peripheral blood by a two-marker reverse transcriptase-polymerase chain reaction assay★

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The aim of this study was to use a two-marker assay for the detection of breast cancer cells circulating in patients' blood. We have applied a PCR-based methodology to follow up the possibility of the development of metastatic disease in stage I and II patients who had undergone curative surgery. Since the number of circulating cancer cells in peripheral blood is very low, the technique for their detection needs to be not only highly sensitive, but also very specific. The reverse transcriptase-polymerase chain reaction (RT-PCR) technique may improve the sensitivity of breast cancer cell detection up to only a few cells per one million. The principle of the RT-PCR assay is to amplify a messenger RNA characteristic for breast epithelial cells in a blood sample. Since we do not expect such cells to be circulating in peripheral blood of healthy subjects, detection of the characteristic mRNA should indicate the presence of circulating breast cancer cells.

We analyzed the usefulness of three mRNA markers: cytokeratin 19 (CK19), mammaglobin (hMAM) and β subunit of human chorionic gonadotropin (β-hCG) for this test. Blood samples (112) were obtained from 55 patients, in stages I and II, with or without metastasis to regional lymph nodes (N0 or N1). We found that a two-marker assay increases the sensitivity of detection of breast cancer cells in comparison with a single-marker one. Combination of two tumor-specific mRNA markers, hMAM/CK19 or β-hCG/CK19, allowed the detection of circulating breast cancer

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Abbreviations: AJCC, American Joint Committee on Cancer; β-hCG, β subunit of human chronic gonadotropin; CK19, cytokeratin 19; hMAM, mammaglobin.

cells in 65% of N1 patients and 38% of N0 patients. By comparison, the combination hMAM/β-hCG allowed the detection of circulating breast cancer cells in the blood of 68% of N1 patients and 46% of N0 patients. Addition of the third marker did not significantly increase the detection sensitivity.

Breast cancer is the most common type of cancer among women in Poland and around the world. In Poland 11 000 new cases are diagnosed per year, of which 5000 patients die (Didkowska *et al.*, 2002). The reason for such low recovery is the advanced stage of the disease at the time of diagnosis after distal metastasis to other organs (tissues) had occurred. However, even early diagnosed patients who have undergone potentially curative surgery may suffer from recurrence as a consequence of undetected metastasis that has occurred before initial diagnosis. Therefore, it is very important to detect occult metastasis in early stage patients, which may be of prognostic significance and may provide new treatment options.

mRNA transcribed in disseminated tumor cells from genes encoding specific markers could be detected in blood, bone marrow, or lymph nodes by sensitive RT-PCR assays in various types of cancer (Zippelius & Pantel, 2000). The commonly assessed mRNA markers include cytokeratins 18 and 19, epithelial mucin (MUC1), carcinoembryonic antigen (CEA), CD44 and maspin.

However, recent studies have shown the expression of several of these markers in normal peripheral blood cells, lymph nodes or bone marrow, leading to false-positive results (Bostick *et al.*, 1998). False-positive results could be avoided by reducing the number of PCR cycles (Schoenfeld *et al.*, 1994). Hoon and other authors have shown that tumor markers may be differently expressed in the primary tumor and its metastasis (Sarantou *et al.*, 1997; Bostick *et al.*, 1998; Taback *et al.*, 2001; Bilchik *et al.*, 2002). Some of these markers are frequently expressed in normal epithelial cells. Moreover, breast and other tumors are composed of heterogeneous cells with different levels of individual gene expression; therefore approaches that rely on a single marker are particularly susceptible to the expression loss or down-regulation. Due to the above-mentioned limitations associated with the single-marker technique, many laboratories have decided to focus on a combination of two or more tumor-specific mRNA markers (Bostick *et al.*, 1998; Bilchik *et al.*, 2000). The multimarker system eliminates some of the inherent problems associated with tumor progression and treatment effects, such as enhancement of tumor heterogeneity, clonal selection and variable expression of individual mRNA markers.

In this study we employed three mRNA markers: cytokeratin19, mammaglobin (hMAM) and β human chorionic gonadotropin (β-hCG). CK19 is a candidate for a general marker of epithelial cancers. This intermediate filament protein has been shown to be widely expressed in all simple epithelia (Burchill *et al.*, 1995; Kruger *et al.*, 1996). Low levels of CK19 have been detected in non-epithelial tissues such as endothelial cells and fibroblasts. Cytokeratin19, originally presented as a promising marker for detection of breast cancer cells, was subsequently questioned as such because of documented false-positive results (Bostick *et al.*, 1998). Mammaglobin, a tissue-specific marker expressed in adult mammary tissues and in 80–90% primary breast tumors, was detected in over 60% of lymph nodes of patients with metastatic breast cancer, but not in normal lymph nodes from non-cancer patients (Watson *et al.*, 1996; Watson *et al.*,1999). Human chorionic gonadotropin (hCG) is a hormone produced by the trophoblast and is critical for sustaining pregnancy. hCG and its individual subunits (α and β) are produced not only in gestational trophoblastic and germ-cell cancers but also in a wide range of non-gonadal tumors, such as gastrointestinal, lung and breast cancers (McManus *et al.*, 1976;

Marcillac *et al.*, 1992; Madersbacher *et al.*, 1994). The mRNA sequence of the β subunit of hCG is specific enough to distinguish it from other glycoprotein hormone subunits with molecular biology methods (Bo & Boime, 1992).

This study was undertaken to establish a two-marker assay for the detection of breast cancer cells in patient's blood by RT-PCR. We determined the sensitivity and specificity of CK19, hMAM and β-hCG mRNA markers. These specific mRNA markers were chosen because of their ubiquitous expression in malignant epithelial tissue (Zippelius & Pantel, 2000). The usefulness of these mRNA markers needs to be well defined before their potential diagnostic clinical utility is assessed.

MATERIALS AND METHODS

Cell lines. The MCF-7 and HBT-39 breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and cultured according to instructions. The human fibroblast cell line FLW-133T used as a negative control was established at the Cancer Center-Institute in Warsaw. All cell lines were grown in Eagle's 1959 medium supplemented with 10% fetal calf serum (FCS), 50 μg/ml penicillin G, 50 μg/ml streptomycin and 0.1% L-glutamine. Cells were detached by trypsinization, then centrifuged, washed once with plain medium and two times with phosphate-buffered saline (PBS) and stored at –70°C.

Patients. Breast cancer patients were treated at the Breast Cancer and Reconstruction Surgery Department of the Cancer Center-Institute in Warsaw. The cohort included 55 patients who underwent breast resections. Approximately half of them presented with metastases to regional lymph nodes. For each patient the first blood sample was taken during surgery or immediately after the diagnosis of distant metastases. Subsequent blood samples (three or four) were taken during consecutive control visits, usually once every 2 to 6 months. Most patients were followed up for 2–3 years. Blood samples were obtained with informed consent after approval of the protocol by the local ethical committee. Control blood samples were taken from 10 healthy donors. In addition, two tissue samples from primary breast carcinoma were included as positive controls.

RNA isolation. Blood samples of 5 ml were collected as described previously (Kulik *et al.*, 2001). RNA was isolated according to Chomczynski and Sacchi (1987) with modifications, as previously (Kulik *et al.*, 2001). Trace amounts of DNA were removed with DNase I (Gibco BRL) using four times less enzyme than recommended by the manufacturer. DNase I was inactivated by heat treatment (65°C, 10 min) and phenol extraction. RNA concentration and purity were determined spectrophotometrically, and its integrity checked electrophoretically.

RT-PCR assay. The RT-PCR assay was carried out as previously described (Kulik *et al.*, 2001). The RT reaction was performed on 2 μg of RNA. The cDNA product was further purified by phenol/chloroform extraction. The reaction mixture for the first round of PCR contained in a final volume of 25 μl: 1× PCR buffer, 200 μM of each dNTP, 1.5 μM $MgCl_2$, 50 ng of primers, 2 μl cDNA and 2.5 units of Taq polymerase (Fermentas). The reamplification with nested primers was carried out using 0.5 μl of the first round product in the same reaction mixture. All primers and PCR conditions used in this study are listed in Table 1. The PCR products were electrophoresed on 2% agarose and visualized by ethidium bromide. cDNA quality was controlled by PCR using primers for GAPDH amplification.

To avoid false-positive RT-PCR amplification of CK19 mRNA due to possible occurrence of pseudogenes, all the samples were also subjected to amplification for this marker without previous reverse transcription.

Table 1. Sequences for PCR primers

Primer	5′-sequence-3′	PCR conditions	Reference
GAPDH1 GAPDH2	GGTCGGAGTCAACGGATTTG ATGAGCCCCAGCCTTCTCCAT	94°C /150 s (94°C /45 s, 60°C /45 s, 72°C /45 s) × 30, 72°C /10 min	Bossart *et al.*, 1994
MG1M MG2M	GAAGTTGCTGATGGTCCTCATGCTGGC CTCACCATACCCTGCAGTTCTGTGAGC	95°C /2 min (95°C /15 s, 62°C /15 s, 72°C /20 s) × 30, 72°C /7 min	Zach *et al.*, 1999
MG3M MG4M	CTCCCAGCACTGCTACGCAGGCTC CACCTCAACATTGCTCAGAGTTTCATCCG	95°C /2 min (95°C /15 s, 62°C /15 s, 72°C /20 s) × 30, 72°C /7 min	Zach *et al.*, 1999
CK19A CK19B	AAGCTAACCATGCAGAACCTCAACGACCGC TTATTGGCAGGTCAGGAGAAGAGCC	94°C /6 min (94°C /1 min, 58°C /150 s) × 40, 72°C /10 min	Datta *et al.*, 1994
CK19C CK19D	TCCCGCGACTACAGCCACTACTACACGACC CGCGACTTGATGTCCATGAGCCGCTGGTAC	94°C /6 min (94°C /1 min, 64°C /150 s) × 40, 72°C /10 min	Datta *et al.*, 1994
β-hCG1 β-hCG2	TCGGGTCACGGCCTCCT TCGGGGTGTCCGAGGGC	94°C /2 min (94°C /1 min, 45°C /90 s, 72°C /2 min) × 30, 72°C /7 min	Hautkappe *et al.*, 2000
β-hCG3 β-hCG4	TCTTGCCCCCGAAGGGTTAGTGTC GCACGCGGGTCATGGTGGGG	94°C /2 min (94°C /1 min, 57°C /90 s, 72°C /2 min) × 30, 72°C /7 min	Hautkappe *et al.*, 2000

Assay sensitivity. To determine the sensitivity of the RT-PCR assay, 5 ml samples of peripheral blood obtained from healthy donors were mixed with increasing numbers of MCF-7 cells. RNA was isolated, then digested with DNase I according to the procedure recommended by the producer. Purified RNA (2 μg) was then taken to for cDNA synthesis. One fourth of the cDNA was used as a template for the nested RT-PCR analysis with primers for the hMAM marker.

RESULTS

Marker expression in breast cancer cell lines

Two breast cancer cell lines, MCF-7 and HBT-39, were assessed for tumor mRNA marker expression. All three mRNA markers, CK19, hMAM, and β-hCG were expressed in these cell lines.

Assay specificity and sensitivity

The specificity of the assay was checked in blood samples from 10 healthy donors, FLW-133T fetus fibroblast and DETA cell line (colon cancer). No CK19 or β-hCG expression was detected in RNA isolated from blood samples of healthy donors. hMAM was expressed in 1 of the 10 blood samples of healthy donors. RNAs for β-hCG and hMAM were expressed in the DETA cell line but not in FLW-133T fibroblasts.

The sensitivity of the assay was checked in samples of peripheral blood obtained from healthy donors mixed with increasing numbers of MCF-7 line cells. The amplification

products were seen in blood samples containing 10 or more MCF-7 breast cancer cells. This means that the assay was able to detect circulating breast cancer cells in the whole bloodstream when their number exceeded about 10^4.

Detection of circulating breast cancer cells in peripheral blood of patients

One hundred and twelve blood samples taken from 55 breast cancer patients were examined. All patients had undergone radical mastectomy and all were clinically classified as stage I or II. Twenty-four patients were histopathologically classified as N0 (no metastasis to regional lymph nodes) and 31 patients as N1 (with metastasis to axillary lymph nodes). The results of particular marker expression assays are presented in Table 2.

Comparison of different two-marker assays is shown in Table 3.

Figures 1 and 2 show examples of amplification of a particular marker mRNA obtained from positive (MCF-7) and negative (FLW-133T) cell lines, blood of healthy donors and breast cancer patients.

The result of the test was treated as positive when the expression of one or more markers was observed in at least two samples taken from the same patient. Applying the above-mentioned criteria, we found that at least one tumor marker among the three examined was detected in 46% (11/24) of N0 patients and 71% (22/31) of N1 patients (Table 3).

Addition of a second marker increased the detection of circulating breast cancer cells in the blood; from 55% (one marker) to 68% (two markers) for N1 patients, and from 38% to 46% for N0 patients. Addition of the third marker did not increase the frequency of detection of breast cancer cells.

The most frequently expressed marker detected in the blood of patients was hMAM, which gave 38% positive results in samples taken from N0 patients and 52% for N1 patients (Table 2). However, this marker gave false positive results in our experiments – one of the 10 examined blood samples from healthy donors was positive.

Table 2. Tumor mRNA marker expression in blood from breast cancer patients

	mRNA marker		
	hMAM	β-hCG	CK19
N0, n = 24	9 (38%)	8 (33%)	4 (17%)
N1, n = 31	16 (52%)	13 (42%)	17 (55%)

N0, no metastasis to lymph nodes; N1, metastasis to axillary lymph nodes.

Table 3. Comparison of two- and three-marker assays

	MAM/ β-hCG	hMAM/ CK19	β-hCG/ CK19	hMAM/β-hCG /CK19
N0, n = 24	11 (46%)	9 (38%)	9 (38%)	11 (46%)
N1, n = 31	21 (68%)	20 (65%)	20 (65%)	22 (71%)

N0, no metastasis to lymph nodes; N1, metastasis to axillary lymph nodes.

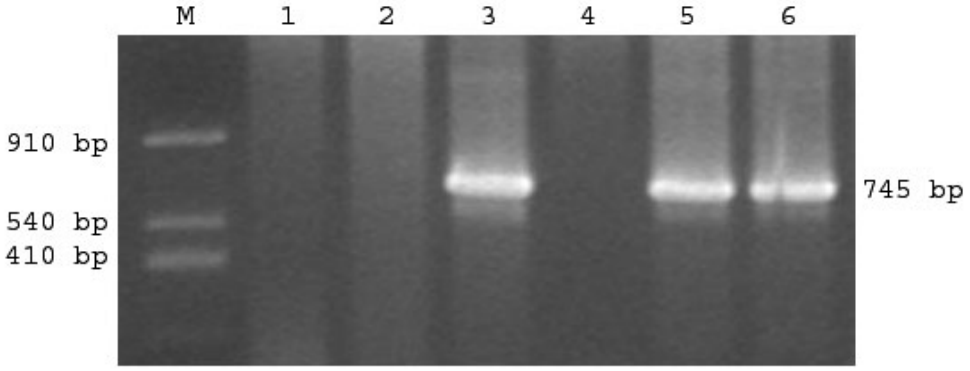

Figure 1. Expression of CK19 in blood of healthy donors and in cell lines.

M, DNA size marker; 1 and 2, healthy donors; 3, placenta; 4, FLW-133T; 5 and 6, MCF-7.

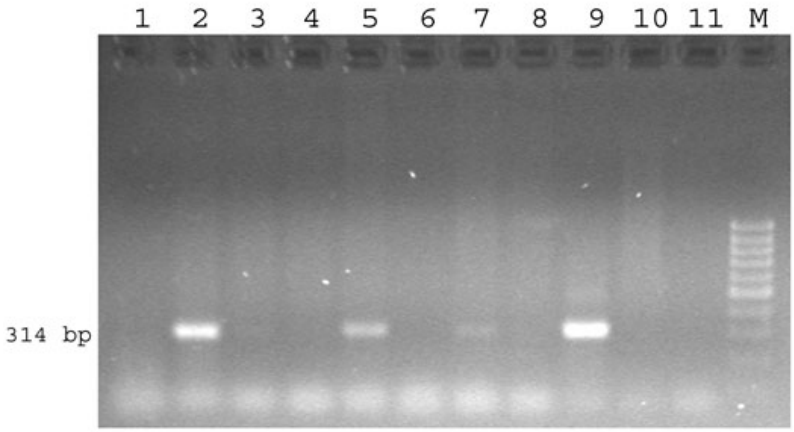

Figure 2. Test for the presence of β-hCG mRNA in blood of breast cancer patients.

M, DNA size marker; 1, 3, 4, 6 and 8, patients negative for β-hCG; 2, 5, 7 and 9, patients positive for β-hCG; 10 and 11, controls without the template.

Combination of the two markers: hMAM/β-hCG was better than other combinations, giving 46% detection for N0 patients and 68% for N1, in comparison to 38% and 65%, respectively for others (Table 3).

DISCUSSION

This study evaluates the usefulness of the three mRNA markers: CK19, hMAM, and β-hCG for detection of breast cancer cells circulating in peripheral blood. Applying these markers we found their frequent expression in breast cancer cell lines and blood from breast cancer patients. No single mRNA could be identified in all samples of patient's blood, with individual marker expression ranging from 17% to 55%. The variable expression in patients' samples may reflect either the absence of breast cancer cells caused by the discontinuous release of metastatic cells into the blood stream or a very low expression of a particular marker mRNA caused by undifferentiation. One of the possible solutions to this problem is to increase the assay frequency (more frequent blood uptake) or to use a combination of markers. Using a combination of two markers in our work improved the assay sensitivity to 38–46% for N0 patients and to 65–68% for N1 patients, which was 13–16% above the highest single-marker detection frequency.

The markers evaluated in this study were previously used by other investigators (Schoenfeld *et al.*, 1994; Burchill *et al.*, 1995; Kruger *et al.*, 1996; Bostick *et al.*, 1998; Leitzel *et al.*, 1998; Watson *et al.*, 1999; Zach *et al.*, 1999; Bilchik *et al* 2000; Hautkappe *et al.*, 2000; Taback *et al.*, 2001). However, conflicting reports have indicated that these specific mRNA markers are also expressed in non-cancer cells from patients with no evidence of malignancy (Bostick *et al.*, 1998). We also found that in our experiments hMAM was expressed in 10% of blood samples from healthy donors. The detection of a target mRNA by RT-PCR can result from pseudogene expression, which in the case of CK19 has a similar nucleotide sequence as the CK19 gene (Datta *et al.*, 1994). Even with primers designed specially to distinguish the true cytokeratin gene from the pseudogene, some reports show the expression of CK19 in blood and bone marrow of healthy donors. These results taken together indicate that CK19 alone is not a good mRNA marker for detection of breast cancer cells in blood. Furthermore, when using such a sensitive technique as RT-PCR, even a few copies of mRNA transcripts may be a problem for any gene that is expressed at low levels in non-cancer cells. The conclusion is that to improve the sensitivity and specificity of the assay, a multiple marker system should be used. Our work showed that a three marker test did not increase the detection of cancer cells in comparison with a two marker test. Therefore, it seems that the two marker test is an adequate approach to this problem.

The patients enrolled in this study were diagnosed with early invasive breast cancer, AJCC stage I and II. Of the N0 patients (without metastasis to lymph nodes), 46% were positive for two markers: hMAM or β-hCG, but for N1 patients (with metastasis to axillary lymph node) this number reached 68%. Comparable results were obtained for other combinations of markers: β-hCG/CK19 and hMAM/CK19 (Table 3). This observation may indicate the potential correlation between the detection of circulating breast cancer cells and metastasis to lymph nodes. However, more cases must be analyzed to obtain statistically significant results.

There is a growing body of evidence that cancer cells infiltrate peripheral blood in very early stages of the disease (Vogelstein & Kinzler, 1993). Only about one per 10^5–10^6 cancer cells will get to and settle in distant organs and a small percentage of those will develop metastasis. Therefore, the detection of breast cancer cells in peripheral blood is not equivalent with the development of metastasis. This fact only indicates the possibility of developing metastasis in distant places of the organism. That may be of prognostic significance and will allow monitoring of disease progression. It may also help the selection of a group of patients who are likely to develop systemic disease.

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