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A new nested primer pair improves the specificity of CK-19 mRNA detection by RT-PCR in occult breast cancer cells

E. Balducci[1], G. Azzarello[1], L. Valori[1], L. Toffolatti[1], L. Bolgan[1], M.T. Valenti[1], M. Bari[1], G.L. Pappagallo[1], S. Ausoni[2], O. Vinante[1]

[1]Department of Oncology and Hemato-Oncology, P.F. Calvi Hospital, Noale, Venice
[2]Department of Biomedical Sciences, University of Padua, Padua - Italy

ABSTRACT: Reverse transcription polymerase chain reaction (RT-PCR) of cytokeratin-19 (CK-19) has been widely used to detect small numbers of circulating malignant epithelial cells in the bone marrow or the peripheral blood of patients with breast cancer. However, a high percentage of false positive results has been recorded and conflicting reports question the clinical relevance of this technical approach. We demonstrate that the use of a new nested primer pair for CK-19 RT-PCR avoids false positive results without affecting the sensitivity of the assay. Our experiments were carried out using MCF-7 cells alone or mixed with peripheral blood mononuclear cells (PBMNC) of healthy donors. The results were also validated in a large series of healthy donors and in a preliminary study on a limited number of patients with breast cancer, thus suggesting that our assay is feasible for application in the clinical evaluation of occult malignant epithelial cells. (Int J Biol Markers 2005; 20: 28-33)

Key words: CK-19, MCF-7 cell line, RT-PCR, Occult breast carcinoma

INTRODUCTION

The intermediate filament CK-19 is a tissue-specific marker expressed in epithelial cells and in most tumor cells of epithelial origin (1-7). Two major properties make CK-19 a good marker for detecting occult tumor cells of epithelial origin in bone marrow or peripheral blood: 1) CK-19 is stably and abundantly expressed in these cells; 2) CK-19 is not expressed in hematopoietic cells or in tumor cells of different histological origin. The sensitivity and specificity of the detection of CK-19 in occult tumor cells either in bone marrow or peripheral blood may be improved by the use of reverse transcription polymerase chain reaction (RT-PCR) (8). In reconstruction experiments, detection levels were as high as 1 to 10 tumor cells for 10^6 to 10^7 normal peripheral blood mononuclear cells (PBMNC) (6, 9-12). Several groups supported the clinical application of the RT-PCR assay for the detection of circulating epithelial cells in early stages of disease, relapse or minimal residual disease monitoring, and in detection of microcontamination in leukapheresis products (13-16). Moreover, prospective studies revealed a correlation between detection of CK-19-positive cells in peripheral blood or bone marrow of patients with breast cancer and their clinical outcome (15-17). There are, however, some caveats. Several investigators reported detection of CK-19 mRNA in healthy blood donors and in patients with hematological malignancies (6, 10, 11, 13, 18, 19), whereas other groups obtained opposite results, though using the same PCR primers and the same experimental conditions (9, 15). There are indeed multiple causes of false positives. For example, the amount of RNA used for reverse transcription, the number of amplification cycles and the hybridization temperature may affect the results, particularly in the case of nested PCR assays. In addition, specific technical problems for CK-19 amplification need to be considered. These are 1) amplification of low-level, illegitimately transcribed CK-19 from hematopoietic stem cells (20) and 2) amplification of *CK-19a* and *CK-19b* pseudogenes from contaminating genomic DNA (21, 22). On the other hand, false negative results may occur due to problems of sensitivity or because of deficient expression of the marker gene in micrometastatic tumor cells. These conflicting results raise the issue of the specificity of this approach and underline the importance of developing more appropriate experimental conditions to avoid misinterpretations and conflicting results.

The aim of this study was to optimize a CK-19 RT-PCR assay to detect circulating carcinoma cells and reduce false positives. We designed a new nested primer pair that amplifies CK-19 transcripts using MCF-7 cells as positive control mixed with PBMNC. We never detected CK-19 amplification products in a negative cell line (K-

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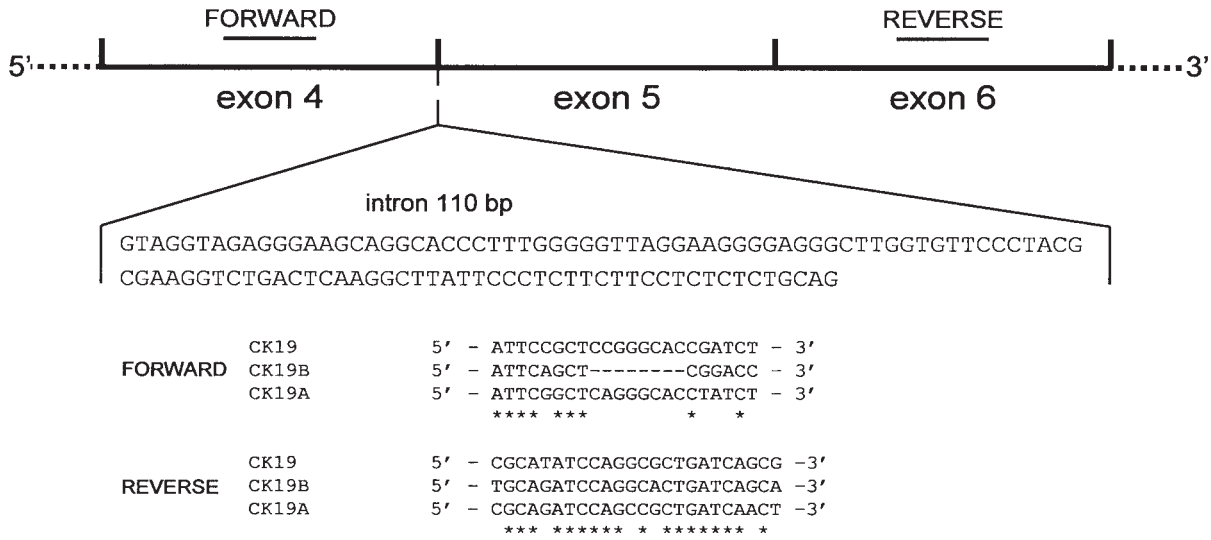

Fig. 1 - *Schematic representation of the CK-19 RT-PCR amplification strategy. Exon 4, 5 and 6 of the CK-19 gene are indicated in the upper panel. F and P indicate the position of the forward and reverse primers used for nested amplification. The 110-bp sequence of intron 4, which was previously unknown, is shown in the inset. The sequences of the CK-19 gene used to design the forward and reverse primers are shown in the lower part of the figure and are aligned with the CK-19 pseudogenes to show identities (*) and mismatches.*

562) or in the cells of healthy controls. Moreover, our new primers gave positive results in a preliminary group of patients affected by breast cancer. Overall, our data support the conclusion that the technical modification described here improves the specificity of the assay without affecting its sensitivity and may be applied in the molecular diagnosis of the disease.

PATIENTS AND METHODS

Cell lines and clinical samples

The human breast carcinoma cell line MCF-7 and the human leukemia cell line K-562 were used as positive and negative controls, respectively, and were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS) and antibiotics.

To determine the sensitivity of the RT-PCR assay and to exclude possible false positives, PBMNC were obtained from a healthy volunteer with no clinical history of malignancy or breast disease. After discarding the first 5 mL of blood to avoid contamination with epidermal epithelial cells, the mononuclear cells were isolated from peripheral blood (10 mL in EDTA) by density centrifugation through Ficoll-Hypaque (Biochrom, Berlin, Germany). Cells of the interphase were mixed with decreasing numbers of MCF-7 cells (10^{-1} to 10^{-6}), thus mimicking the clinical setting for detection of mammary cells in the peripheral blood of patients. After informed consent was obtained, 55 consecutive patients with histologically documented invasive breast cancer (31 at stage I, II and III after surgery and 24 at stage IV as assessed before front-line chemotherapy or hormone treatment) were enrolled for a preliminary survey and underwent the same sample collection procedure. Blood samples from 50 healthy donors, including the one used for the sensitivity assay, were tested in parallel.

RNA extraction, cDNA synthesis and RT-PCR

Total RNA was extracted from mixed cell preparations using a Quigen Rneasy total RNA kit (Hilden, Germany) and treated with RNase-free DNase I (Gibco BRL) to digest possible contaminating genomic DNA. One microgram of total RNA was retrotranscribed using RT-Superscript II enzyme (Gibco BRL).

The first amplification was carried out using the primers published by Datta et al (9). For the nested amplification we used a new primer pair mapping within exon 4 and exon 6 of the CK-19 gene. Sequences were designed using the Primer 3 program. The primer sequences were: forward (F) 5'-ATTCCGCTCCGGGCACCGATCT-3'; reverse (R) 5'-CGCTGATCAGCGCCTGGATATGCG-3'. The first round of amplification was performed as described by Datta et al (9); 1 µL of amplified product was reamplified with the inner primers for 30 cycles under the following conditions: 10X reaction buffer (10mM Tris pH 8.3, 25 mM $MgCl_2$), dNTPs (2mM each), Taq DNA polymerase (1.25 unit/reaction) and 50 mM/primer/reaction. The housekeeping gene β_2-microglobulin (β_2-*MG*) was amplified as internal control using the following primers: 5'-primer: 5'-AGCAGAGAATGGAAAGTCAA-3', 3'-primer 5'-TGTTGATGTTG-

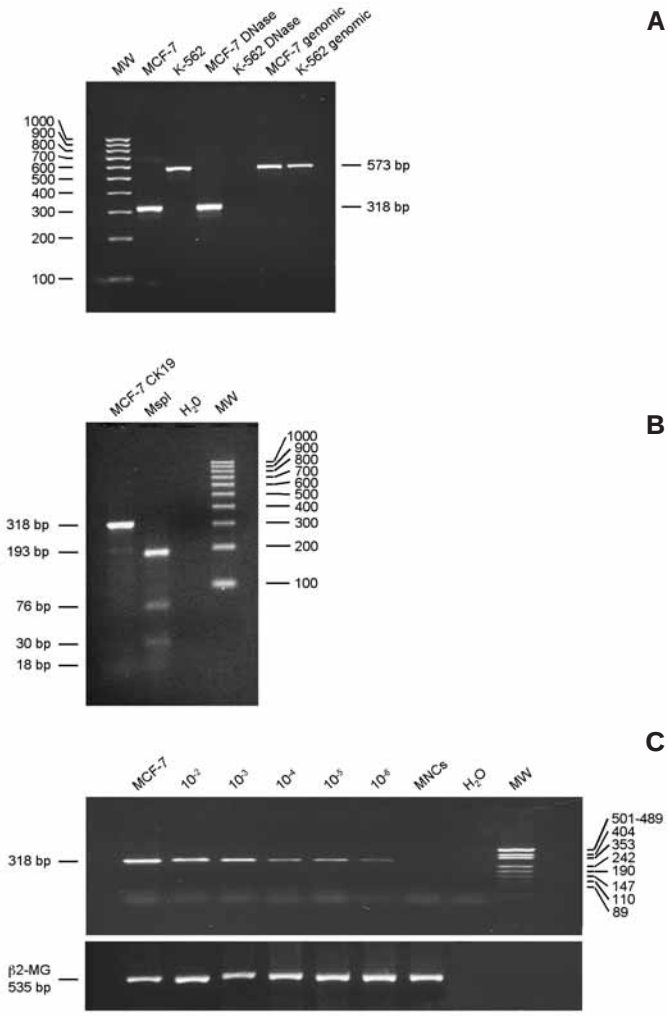

Fig. 2 - *Specificity and sensitivity of the CK-19 RT-PCR assay in MCF-7 and K-562 cell lines. (A) Amplification products from different templates were analyzed on 1.5% agarose gels. MW, DNA molecular weight standard (λ HindIII digest); MCF-7 and K-562, amplifications with no RNA DNase treatment; MCF-7 DNase and K-562 DNase, amplifications after RNA DNase treatment; MCF-7 genomic and K-562 genomic, amplification of genomic DNA obtained from each cell line. The specific CK-19 318-bp fragment and the 573-bp genomic fragment are indicated at the right end. (B) MspI restriction pattern of the 318-bp amplification fragment. (C) Amplification products obtained from serial dilutions (10^{-1} to 10^{-6}) of MCF-7 cells mixed with PBMNC from a healthy donor. MW, DNA molecular weight standard (MspI 2-MG amplification indicates that comparable amounts of total RNA were used).*

GATAAGAGAAT-3'. Genomic DNA was extracted by means of the QIAamp DNA blood kit (Qiagen, Chatsworth, CA, USA) and was used to detect possible contaminating genomic DNA in the RNA preparations.

RESULTS

In a preliminary set of experiments the mRNA of the MCF-7 and K-562 cell lines was amplified, after reverse transcription, using two outer primers as described by Datta et al (9). As expected, the MCF-7 cell line gave a PCR fragment of 1096 bp whereas no specific amplification was present in the K-562 cell line (not shown). For the nested amplification we designed a new pair of inner primers that mapped within exon 4 (forward primer) and exon 6 (reverse primer) of the *CK-19* gene (Fig. 1). To avoid amplification of the pseudogenes *CK-19a* and *CK-19b* numerous mismatches were introduced at the 3' end of the primer sequences (Fig. 1) and several preliminary experiments were carried out before the optimal sequences were obtained. Nested amplification of the MCF-7 cDNA resulted in a single 318-bp fragment (Fig. 2A). No specific amplification was observed with the K-562 cDNA, consistent with the specificity of *CK-19* gene expression in epithelial cells. However, in the K-562 cell line we did observe a 573-bp fragment that was eliminated by DNase treatment of total RNA before cDNA synthesis, probably corresponding to contaminating genomic DNA in the RNA preparation. This conclusion was supported by the evidence that the 573-bp amplification product was obtained from both genomic preparations (Fig. 2A) and from total RNA in the absence of any RNase treatment (not shown). Genomic amplification was never observed in the MCF-7 cell line cDNA, presumably due to the competition for primer utilization by the large amount of CK-19 cDNA present in these cells. PCR products were analyzed using the *Msp I* restriction enzyme that generates distinct fragment profiles in the *CK-19* gene and pseudogenes. Identification of four fragments of 193, 76, 30, and 18-bp products confirmed specific amplification of the *CK-19* gene and excluded pseudogene amplification (Fig. 2B). Direct sequencing confirmed the identity of the amplification product. Furthermore, sequencing of the 573-bp genomic product confirmed that this fragment spans from exon 4 to exon 6 of the gene, including the corresponding introns. The sequence of intron 4, which was previously unknown, is shown in Figure 1.

To evaluate the sensitivity of our assay, known amounts of MCF-7 cells were mixed with PBMNC from a healthy donor (1-10^5 tumor cells among 1x10^7 normal cells), a condition that mimics the clinical setting for detection of mammary cells in the peripheral blood or bone marrow of affected patients. A representative result from multiple independent experiments is shown in Figure 2C. The 318-bp amplification product was detectable up to a dilution of 1x10^{-6} MCF-7 cells in normal hematopoietic cells. Every sample gave specific amplification of the β_2-*MG* housekeeping gene, indicating the presence of similar amounts of total RNA and good quality of the cDNA synthesis.

Detection of occult CK-19 positive cells was verified in a preliminary set of patients. Peripheral blood samples were obtained from 55 patients with histologically documented invasive breast cancer (median age 57 years, range 32-79). CK-19 mRNA was detected in the blood of

TABLE I - PATIENT CHARACTERISTICS

	Early breast cancer		Metastatic breast cancer	
	No.	**%**	**No.**	**%**
Patients enrolled	31	56.4	24	43.6
Age				
Median	56		59	
Range	32-79		33-78	
Stage				
I	9	16.4	-	-
II	17	30.9	-	-
III	5	9.1	-	-
IV	-	-	24	43.6
Grade at diagnosis				
1	6	19.3	1	4.2-
2/3	25	80.7	23	95.8
Lymph nodes				
0	14	45.2	na	na
1-3	10	32.3	na	na
≥4	7	22.5	na	na
Receptor status				
ER+	18	58.1	18	75.0
ER-	13	41.9	5	20.8
PR+	12	38.7	17	70.8
PR-	19	61.3	6	25.0
Unknown	0	0	1	4.2
No. of metastatic sites				
1	na	na	13	54.2
2-3	na	na	11	45.8
CK-19 mRNA cells (+)	3	9.7	11	45.8
(–)	28	90.3	13	54.2

p=0.002; na=not applicable

3/31 (10%) patients with operable breast cancer and in 11/24 (46%) patients with distant metastases (p=0.002) (Tab. I). Interestingly, the number of metastatic sites involved (1 vs 2+3) affected the CK-19 mRNA detection rate (69% CK-19 negative for one site vs 64% positive for 2+3 sites), although the size of type-I error was 0.10. In a preliminary univariate analysis no association between the detection of CK-19 mRNA positive cells and any other prognostic factors was seen. No illegitimate transcription was observed. All RNA samples were uniformly positive for β_2-MG expression. Samples from the 50 healthy donors gave no amplification, which confirmed the specificity of the assay.

DISCUSSION

We improved the specific detection of CK-19 mRNA as a marker of occult malignant epithelial cells both in an experimental setting and in a preliminary selection of patients affected by breast cancer. In our approach, based on conventional RT-PCR as described by others (9, 11, 12), a new set of inner primers for nested amplification was used. The results demonstrated CK-19 amplification in MCF-7 cells with a sensitivity up to $1x10^{-6}$ cells, in line with previous reports (8, 9, 12, 21). Our results were easily reproducible by different operators, they were obtained using small amounts of total RNA (1 µg instead of 5 µg used in most studies) and a limited number (30) of amplification cycles, and they always gave a unique PCR product of 318 bp. Of paramount importance, CK-19 RT-PCR never gave false positive results, neither in the K-562 cell line nor in blood samples from healthy donors. To avoid false positives we took into consideration three potential problems. First, contamination of blood samples by skin cells was avoided by discarding the first 5 mL of blood collected. Second, amplification of pseudogenes was avoided by introducing in the new primers multiple mismatches with the *CK-19a* and *CK-19b* pseudogenes. In addition, DNase treatment of total

RNA was always performed to avoid amplification of the genomic DNA, an important requirement to exclude not only amplification of the pseudogenes but also possible reduction of sensitivity in an assay aimed at detecting very low amounts of mRNA copy numbers. Third, illegitimate transcription, defined as low-level transcription of genes in non-specific cells (23), was carefully checked with slightly different variants of primers before choosing the appropriate oligonucleotides.

Several authors reported variable percentages of false positive results in the detection of CK-19 mRNA and conflicting data on this matter have been found. A number of authors obtained false positives (10, 11, 18, 19) using either the primers described by Datta et al (9) or other primers (12). This is likely due to the amplification of *CK-19* pseudogenes, a possibility that was largely underestimated in the first studies. On the other hand, the PCR conditions and primers used by Ruud et al (19) to avoid *CK-19b* pseudogene amplification were never tested in human samples from patients affected by tumors of epithelial origin, and therefore their potential for clinical application remains unknown. A recent clinical study by Stathopoulou et al (15) reported successful use of CK-19 RT-PCR in a large study group of patients with early or metastatic breast cancer. Using the primers described by Datta et al (9) without any precautions to avoid genomic DNA contamination, these authors observed only a 3.7% false positive rate. Since they reported very few technical details about the amplification conditions, their results are difficult to reconcile with previous reports, at least from a technical point of view.

Having demonstrated the sensitivity and specificity of our experimental conditions for CK-19 amplification, we wondered whether we could validate these data in a preliminary test on patients. The technique was applied to 55 blood samples from women with different stages of breast cancer. Overall, CK-19 mRNA was detected in 25.5% of blood samples. Blood samples from patients with non-metastatic disease were positive in nearly 10% (3/31) of cases while in metastatic disease positivity was observed in nearly 46% (11/24) of cases, reflecting the larger tumor burden. The detection frequency of occult carcinoma cells in our study was similar to that observed by others (15, 18). Although the number of samples examined in our study is too small for any statistical consideration, this preliminary survey indicates that the method presented here should be applied to larger numbers of newly diagnosed breast cancer patients. We will soon be embarking on a new study aimed at prospectively evaluating the prognostic significance of the molecular detection of CK-19-positive cells in early stages of the disease and assessing the usefulness of this new, promising tool in monitoring the response to chemotherapy at the molecular level in advanced breast cancer patients. Interestingly, quantitative RT-PCR has been recently used to study the prognostic significance of CK-19 detection in circulating malignant epithelial cells (24), to determine the normal levels of CK-19 expression and to discriminate tumor-specific CK-19 gene expression from background transcription (25-27). Because the *CK-19* gene remains a marker of choice for this analysis, a methodological improvement of its detection is highly recommendable.

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Address for correspondence:
Giuseppe Azzarello, MD
Dept. of Oncology and Hemato-Oncology
P.F. Calvi Hospital
Largo S. Giorgio, 3
30033 Noale (VE), Italy
e-mail: oncnoale@inwind.it

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