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Quantitative RT-PCR luminometric hybridization assay with an RNA internal standard for cytokeratin-19 mRNA in peripheral blood of patients with breast cancer

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Abstract

Objectives: To develop a highly sensitive quantitative RT-PCR hybridization assay for the determination of CK-19 mRNA in peripheral blood of patients with breast cancer.

Patients and methods: Quantification of CK-19 mRNA was based on the coamplification of CK-19 mRNA with a recombinant CK-19 RNA internal standard (CK-19 RNA-IS) through RT-PCR. The biotinylated amplification products were immobilized on steptavidin coated wells, hybridized with digoxigenin labeled probes and determined through an antidigoxigenin antibody conjugated to alkaline phosphatase by luminometric detection. The developed luminometric hybridization assay was validated with samples containing total RNA of known amounts from CK-19 expressing cells (MCF-7) in the presence of 1 μg total RNA isolated from peripheral blood mononuclear cells (PBMC) of healthy controls and a constant amount of CK-19 RNA-IS. The method was applied for the quantitative determination of CK-19 mRNA in the peripheral blood of 26 healthy volunteers, 14 patients with stage IV breast cancer and 37 patients with stage I/II breast cancer before chemotherapy.

Results: Luminescence ratios for CK-19 mRNA and CK-19 RNA-IS were linearly related to the number of MCF-7 cells within the range of 1 to 2000 cells. The overall reproducibility of the assay (between-run) varied between 8.9% and 13.4%. The method can clearly detect CK-19 mRNA from 1 MCF-7 cell in the presence of 10^6 normal PBMC and is highly specific as none of the 26 healthy controls tested had detectable CK-19 mRNA levels, while 10 out of 14 (71.4%) and 9 out of 37 (24.3%) patients with stage IV and stage I/II breast cancer, respectively, were tested positive.

Conclusion: The developed quantitative RT-PCR hybridization assay for CK-19 is reproducible, highly sensitive and specific, and can be used for a large-scale prospective evaluation of clinical samples.

Keywords: CK-19; Minimal residual disease; Breast cancer; Quantitative RT-PCR; Hybridization

1. Introduction

A substantial proportion of patients with early (stages I and II) breast cancer will present distant metastasis [1,2] despite the recent advances in early diagnosis and treatment. Recently, the mechanisms determining the migration and invasion of breast tumor cells into distant organs have begun to be elucidated [3].

During the last years there is an increasing body of evidence that detection and characterization of tumor cells in bone marrow or peripheral blood of breast cancer patients may be clinically relevant in terms of disease-free interval and overall survival [4]. Moreover, the prospective evaluation of minimal residual disease (MRD) may give information concerning the effectiveness of adjuvant therapy [2]. Therefore, highly sensitive methods for the early detection of circulating cancer cells are very important for the early diagnosis and more effective treatment of MRD.

CK-19 is stably and abundantly expressed in epithelial tumors, but not in mesenchymal hemopoietic cells, and has been succesfully used as a marker for the detection of tumor cells in the bone marrow by immunocytochemistry [5]. Fur-

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thermore, occult epithelial tumor cells could be identified by RT-PCR mRNA detection in bone marrow and lymph nodes of patients with metastatic breast cancer [6–11]. However, the usefulness of CK-19 mRNA as a marker of MRD is questioned because of a significant number of false positive results [12–14] which can be due to: a) amplification of low level illegitimately transcribed CK-19 from hematopoietic cells [12], b) amplification of CK-19 transcripts from contaminating normal epithelial cells [13], and c) amplification of contaminating genomic DNA of the recently described CK-19*a* and CK-19*b* pseudogenes [13,14].

So far, only a few studies have reported either the semiquantitative [15] or quantitative detection of CK-19 mRNA-positive cells [16–18] based either on a competitive PCR approach and the comparison of the relative band intensity of PCR products on agarose gels [15], or the recently developed real time PCR [19]. Since detection in semiquantitative assays is based only on the visualization of bands, a nested PCR is always required because of increased sensitivity [16,17]. On the other hand, real time PCR of CK-19 mRNA in peripheral blood [19] offers several advantages over classic quantitative PCR methods, such as decreased likelihood of contamination and high sample throughput.

Quantification of RT-PCR products in all of these methods [16–19] is based on the use of housekeeping genes [19] or specifically designed competitor templates [16–18] as internal controls. All competitor templates used so far for CK-19 mRNA determination are irrelevant in respect to size and sequence to the target CK-19 mRNA [16–19] and since they are designed at the cDNA level, they are added to the PCR reaction mixture after reverse transcription. However, for an accurate quantification of RT-PCR products the competitor template and the mRNA target should be ideally of the same size and sequence [20,21], so that they can be coamplified with the same efficiency at both the reverse transcription and PCR steps.

In the present report a quantitative RT-PCR luminometric hybridization assay for the determination of CK-19 mRNA in the peripheral blood of patients with breast cancer is described. For the first time the quantification is based on the coamplification of CK-19 mRNA with a specifically designed recombinant CK-19 RNA internal standard and both amplification products are quantified using especially designed hybridization probes. The proposed method achieves the same sensitivity as nested RT-PCR by using a one step PCR approach while its high specificity is achieved by the use of novel in-silico-designed primer pairs and hybridization probes.

2. Materials and methods

2.1. Cell lines

The human mammary carcinoma cell line MCF-7 that expresses CK-19 was used as the positive control of the assay. MCF-7 cells were grown in Minimum Essential Medium (Eagle) supplemented with 10% fetal bovine serum (FBS). The cells were counted by trypan blue exclusion using a hemocytometer.

2.2. Patients and samples

Peripheral blood in EDTA was obtained from 37 patients with primary breast cancer (stage I/II) before chemotherapy, 14 patients with metastatic (stage IV) breast cancer and 26 female healthy volunteers (aged 18–65 yr). To reduce blood contamination by epithelial cells from the skin, the first 5 mL of blood were discarded and the collection tube was at the end disconnected before withdrawing the needle. All patients gave their written informed consent and the study has been approved from the Ethical and Scientific Committees of the participating Institutions. The peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation with Ficoll Hypaque-1077 (Sigma Chemical Co., LTD, England) at 1200 g for 30 min, at 4°C. The interface cells (PBMC) were removed, washed twice with 50 mL of sterile Phosphate-Buffered Saline (PBS, pH 7.0), and cell pellets were kept at −80°C until RNA extraction.

2.3. Total RNA isolation

Total RNA isolation was performed by using Trizol LS reagent (Gibco BRL, Life Technologies) 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 use. 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 gene.

2.4. Primer/probe design

The oligonucleotide sequences used in this study were first designed and evaluated in silico by using the Primer Premier software (Premier Biosoft International, Palo Alto, CA, USA) to avoid primer-dimer formation, false priming sites and formation of hairpin structures. The hybridization probes were selected to position on an intron-exon junction so that hybridization to genomic DNA was completely avoided. Moreover, the primers and probes were designed to differentiate between the highly homologous CK-19*a* and CK-19*b* pseudogenes according to a search in the BLAST Sequence Similarity Search tool (NCBI, NIH). All primers and hybridization probe sequences are listed in Table 1. All oligonucleotides were synthesized at the Lab of Microchemistry of FORTH, Crete, Greece. Primers AL1 and T7-AL1 were biotinylated during oligonucleotide synthesis. The hybridization probes Pr1 and Pr2 were tailed enzymatically with Dig-11-dUTP (Roche Molecular Biochemicals, USA), by using terminal deoxynucleotidyl transferase

Table 1
Sequences, positions, homology with CK-19 pseudogenes and labels of oligonucleotides used in this study.

Use	Name	Oligonucleotide sequence[a] (5′-3′)	Nucleotide position[a]	Homology with CK-19 Pseud a[b] (%)	Homology with CK-19 Pseud b[c] (%)
PCR					
Forward	P1	AAGCTAACCATGC AGAACCTCAACGA CCGC	273–302	80	23
Reverse	P2	TTATTGGCAGGTC AGGAGAAGAGCC	1318–1342	88	44
Forward	P3	TCCCGCGACTAC AGCCACTACTACAC GACC	402–431	63	25
Reverse	P4	CGCGACTTGATGT CCATGAGCCGCTG GTAC	1118–1148	87	26
Forward	bAL1[d]	ACAGCCACTACTA CACGAC	412–430	53	21
Reverse	AL4	ACTTGATGTCCAT GAGCCG	1125–1143	89	21
Construction of CK-19 RNA-IS					
Forward	bT7-AL1[d]	CTAATACGACTCA CTATAGGGACAGC CACTACTACACGC	412–430		
Reverse	PIS2	AGTTTCTTGACAA AAGGAGGAATGGT TCTTCTTCAG	672–687		
Forward	PIS1	TCCTCCTTTTGTCA AGAAACTAGGGGC CAAGTGGGA	708–722		
Reverse	AL4	ACTTGATGTCCAT GAGCCG	1125–1143		
Hybridization Assay					
Probe 1	Pr1[e]	CAGCGTACTGATT TCCTCCTC	687–707		
Probe 2	Pr2[e]	AGTTTCTTGACAA AAGGAGGA	–		

[a] : CK-19 mRNA, GeneBank Accession Number Y00503
[b] : CK-19 pseudogene *a* mRNA, GeneBank Accession Number M33101
[c] : CK-19 pseudogene *b* mRNA, GeneBank Accession Number U85961
[d] : labeled with biotin
[e] : labeled with digoxigenin

(Roche Molecular Biochemicals, USA), as previously described [20]. These labeled probes were used in the hybridization assays without further purification.

2.5. cDNA synthesis

Reverse transcription of RNA was carried out with the THERMOSCRIPT RT-PCR System (Gibco BRL, Life Technologies). cDNA was synthesized according to the manufacturer's instructions. Total RNA was isolated from PBMC of blood samples from healthy volunteers (5 μg), from breast cancer patients (1 μg) and from MCF-7 cells (1 μg). For quantitation, calibrators were prepared by mixing known amounts of total RNA corresponding to 1 to 2000 MCF-7 cells, with a constant amount of total RNA corresponding to 10^6 normal PBMC cells and a constant amount (0.05 μg) of CK-19 RNA-IS (10-fold dilution of the stock solution) before cDNA synthesis. For the quantitation of the CK-19 mRNA in peripheral blood samples, 1 μg of total RNA was mixed with a constant amount (0.05 μg) of CK-19 RNA-IS, as above, before cDNA synthesis. The downstream primer AL4 (20 pmol per 20 μL reaction) was used for reverse transcription.

2.6. PCR amplification

For the quantitative determination of CK-19 mRNA, 3 μL of cDNA and biotinylated-AL1/AL4 (10 pmol of each), used as forward and reverse primers, respectively, were added in the PCR mixture. PCR reaction was initiated with a 2 min denaturation at 94°C and terminated with a 10 min extension at 72°C. The cycling protocol consisted of denaturation at 94°C for 50 s, annealing at 54°C for 30 s and extension at 72°C for 1 min and repeated for 35 times.

These PCR products were further quantitated by the hybridization assay.

A well established nested PCR approach was followed for the qualitative assessment of CK-19 mRNA in the same samples as above [7] for comparison studies. For the first PCR, primers P1 and P2 (10 pmol of each) and 3 μL cDNA were added in a total volume of 50 μL of PCR mixture. This first PCR yields a 1070 bp product. An aliquot of 3 μL of the first PCR product was used as a template for the nested PCR using as inner primers the pairs P3/P4 and AL1/AL4 (10 pmol of each) giving products of 737 bp and 732 bp, respectively. This PCR mixture was the same as in the first PCR and it was also performed in a final volume of 50 μL.

PCR amplification was performed in a Peltier Thermal Cycler (DNA engine, PTC-200, MJ Research, USA). In both protocols, the PCR reaction mixture (50 μL) consisted of 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 0.2 mmol/L dNTPs, 1.5 mmol/L $MgCl_2$ and 1 U Platinum Taq DNA polymerase (Gibco BRL, Life Technologies).

All PCR mixtures were prepared in a closed room (pre-PCR room) separated from any PCR product. After the PCR mixture was prepared the pipettes used were placed under UV light for 60 min. All other steps of the analyses were performed in another post-PCR room.

2.7. Synthesis of CK-19 RNA-internal standard

A cDNA template was firstly synthesized using mRNA extracted from MCF-7 cells. For the synthesis of CK-19 RNA-IS, two separate PCR reactions were set up to create two short products, A and B, each containing a newly introduced sequence of 21 bp. For PCR-A, we used primers AL1 as forward and PIS2 as reverse. The reverse primer (PIS2) contains a 15-bp sequence at its 3′ end necessary for binding to the CK-19 cDNA and an irrelevant to CK-19 21-bp extension at its 5′ end (Table 1). The final product of PCR-A consists of a 275-bp segment identical to the starting DNA and a 21-bp addition. For PCR-B, PIS1 and AL4 were used as forward and reverse primers, respectively. PIS1 contains a 15 bp sequence complementary to the CK-19 cDNA at its 3′ end and a 21-bp extension complementary to the 21-bp extension of the PIS2 primer at its 5′ end. Thus, the PCR-B product consists of 436 bp segment identical to the cDNA template and a 21-bp extension. The PCR mixture was the same as the one described in "PCR amplification". Primers were used in a final concentration of 0.4-μmol/L and 2 μL of MCF-7 cDNA were added in each 50 μL reaction. Each PCR reaction was initiated with a 5 min denaturation at 94°C and terminated with a 5 min extension at 72°C. The cycling protocol for both PCR reactions, A and B, consisted of denaturation at 94°C for 30 s, annealing at 55°C for 50 s and extension at 72°C for 50 s and repeated for 35 times.

Fifty μL of each PCR product were electrophoresed on a 2% agarose gel and visualized with ethidium bromide (Fig. 1). The bands were excised from the gel and purified (Quick

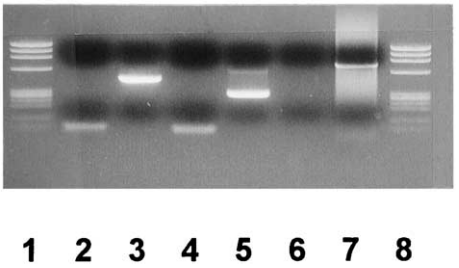

Fig. 1. Recombinant DNA fragments for the preparation of the RNA-IS. Lane 1: ΦX 174 DNA marker, lane 2: PCR blank, lane 3: product B (457 bp), lane 4: PCR blank, lane 5: product A (296 bp), lane 6: PCR blank, lane 7: recombinant DNA-IS (732 bp), lane 8: ΦX 174 DNA marker.

gel extraction kit, QIAGEN, GmbH, Germany). Two μL of each purified PCR product were used in a 10-fold dilution to set up a PCR reaction similar to the one described above, in the absence of primers, to a final volume of 50 μL. PCR conditions were as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. The cycling was repeated 40 times. Three μL of this product were used in a PCR reaction using biotinylated T7-AL1 and AL4 as forward and reverse primers, respectively. PCR conditions were the same as mentioned before. This amplification produced a 753 bp recombinant DNA fragment fused to the T7 promoter. The products of five PCR reactions were pooled and loaded on a 2% agarose gel. After electrophoresis the band was excised from the gel and purified.

The CK-19 RNA-IS was synthesized by *in vitro* transcription of the biotinylated T7-AL1/AL4 purified PCR product. Fifteen μL of the purified biotinylated T7-AL1/AL4 PCR product were immobilized on a 20 μL suspension of streptavidin (SA) coated magnetic beads (Dynabeads M-280, DYNAL AS, Norway), previously washed according to the manufacturer's instructions. SA-Dynabeads were washed again and the transcription reaction was carried out in a total volume of 50 μL, containing 40 mmol/L Tris-HCl (pH 8.0), 15 mmol/L $MgCl_2$, 5 mmol/L dithiothreitol (DTT), 0.5 mg/mL BSA, 1 mmol/L dNTPs mixture and 12.5 U T7 RNA polymerase (Amersham Life Science, USA) at 37°C for 90 min. The produced RNA-IS was purified with Trizol, as previously described, redissolved in 20 μL DEPC water and further diluted in 180 μL of 1 g/L yeast t-RNA containing 37 U RNase inhibitor (Eppedorf Scientific, USA) to ensure stability during storage. This CK-19 RNA-IS solution was treated with RQ1 RNase-free DNase (Promega, USA). The reaction was carried out in a total volume of 20 μL containing 17 μL RNA-IS, 1 U RQ1 DNase, 40 mmol/L Tris-HCl pH 8.0, 10 mmol/L $MgSO_4$ and 1 mmol/L $CaCl_2$. DNase treatment was allowed to proceed at 37 °C for 30 min and the enzyme was inactivated by heating at 65 °C for 10 min. This CK-19 RNA-IS stock solution was kept in aliquots at −80°C until further use.

2.8. Hybridization assay

Opaque polystyrene microtiter wells (Microlyte 2, Dynatech, USA) were coated overnight at room temperature, with 50 μL of streptavidin (1.4 mg/L) diluted in PBS, pH 7.4. During the whole assay the wells were washed three times after each step with wash solution [50 mmol/L Tris (pH 7.4), 0.15 mol/L NaCl and 1 mL/L Tween-20], using a microtiter plate washer (Wellwash x4, Denley). Five μL of PCR products diluted 10-fold in blocking solution [10 g/L blocking reagent (Roche Molecular Biochemicals, USA) in 0.1 mol/L maleic acid and 0.15 mol/L NaCl, pH 7.5] were added in each well and incubated at room temperature with shaking for 30 min. Subsequently, 50 μL of 0.2 mol/L NaOH were added. After a 20 min incubation at room temperature, the non-biotinylated DNA strand was removed by washing the wells. Fifty μL of Dig-labeled hybridization probe (100 μM Pr1 or 100 μM Pr2), 700-fold diluted in blocking solution, were added to each well and incubated at 42°C for 30 min. Fifty μL anti-Digoxigenin antibody conjugated to alkaline phosphatase 750 U/L (Roche Molecular Biochemicals) diluted in blocking solution, were added per well and incubated at room temperature for 30 min. After this step, the wells were washed six times and 50 μL of the chemiluminogenic ALP-substrate LumiPhos (MEDIATORS Diagnostika, GmbH, Austria) were added to each well. The enzymatic reaction was completed after a 30 min incubation at 37°C, in the dark. The chemiluminescence signal integral was measured with a Luminometer (Mediator PhL, MEDIATORS Diagnostika GmbH, Austria).

3. Results

3.1. Specificity

To establish a specific CK-19 amplification assay and eliminate the false positive problem an in silico analysis for known primer pairs, was performed [13], using the established primers P1 and P2 as the outer set [7] and different sets of inner primers so that false positive results due to primer homology to both CK-19 pseudogenes (*a* and *b*), would not be detected. The in silico analysis (Table 1) demonstrated that when primers P3 and P4 were shortened in size (named AL1 and AL4 respectively), exhibited a minimum homology with both CK-19 pseudogenes (*a* and *b*) and did not form hairpins or cross-dimers (data not shown). This was also verified by using the BLAST Sequence Similarity Search tool (NCBI, NIH). The primers AL1/AL4 were further checked by PCR and it was shown that they can distinguish between CK-19 and both CK-19*a* and CK-19*b* pseudogenes, since the presence of 5 μg contaminating genomic DNA did not produce any false positive results (data not shown).

To further establish the specificity of this new set of primers, a total number of 167 cDNAs derived from peripheral blood from patients belonging to four different groups, normal donors ($n = 26$), metastatic colorectal cancer ($n = 31$), metastatic breast cancer ($n = 40$) and early breast cancer ($n = 70$) was analyzed by both sets of primers P3/P4 and AL1/AL4 with a well established nested PCR approach (Table 2). According to the Mc Nemar statistical analysis, performed for each group separately, there was no significant difference between the results obtained by the two sets of primers. However, as can be seen in Table 2 the primer pair AL1/AL4 gave no false positives, while for the same cDNAs the primer pair P3/P4 gave two false positives in 26 normal blood donor samples (7.7%). A CK-19 RT-PCR amplification assay with high specificity was finally obtained by using only one PCR with primers AL1 and AL4 followed by the luminometric hybridization assay.

Table 2 Comparison between the CK-19 primer pairs P3/P4 and AL1/AL4

Patient Group	n	P3/P4 primers CK-19 mRNA positive (%)	AL1/AL4 primers CK-19 mRNA positive (%)
Normal donors	26	2 (7.7%)	0 (0%)
Metastatic CRC	31	1 (7.1%)	0 (0%)
Metastatic Breast Cancer	40	16 (40%)	14 (35%)
Early Breast Cancer	70	26 (37.1%)	24 (34.3%)

3.2. Synthesis of recombinant CK-19 RNA internal standard

The recombinant CK-19 RNA-IS was constructed by PCR, as previously described [20], by replacing a 21 bp sequence of the CK-19 DNA with a new DNA segment of equal size. Primers PIS1 and PIS2 (Table 1) were designed to have a 15 bp sequence complementary to CK-19 cDNA at their 3′ ends and a 21 bp extension complementary to each other at their 5′ ends. By using CK-19 cDNA as a template and primer pairs AL1/PIS2 and PIS1/AL4 two distinct PCR fragments were obtained, A (296 bp) and B (457 bp) respectively (Fig. 1). The 21 bp extension at the 5′ends of primers PIS1 and PIS2 was in silico designed to be completely irrelevant to the whole CK-19 cDNA region amplified by the selected primer pairs AL1 and AL4 and was introduced into the CK-19 target cDNA. This was accomplished by linear amplification of these PCR products (A and B) which join together and produce a recombinant CK-19 fragment of 732 bp, by acting as primers for one another. This recombinant DNA fragment was used as a template for the synthesis of CK-19 RNA-IS after being fused to the biotinylated T7 promoter, through PCR. After transcription, the presence of this new 21 bp sequence in the recombinant CK-19 RNA-IS was confirmed by performing RT-PCR on both the purified CK-19 RNA-IS and the CK-19 mRNA from MCF-7 cells. The two amplification products are identical in agarose gels but clearly distinguishable from one another by the hybridization assay,

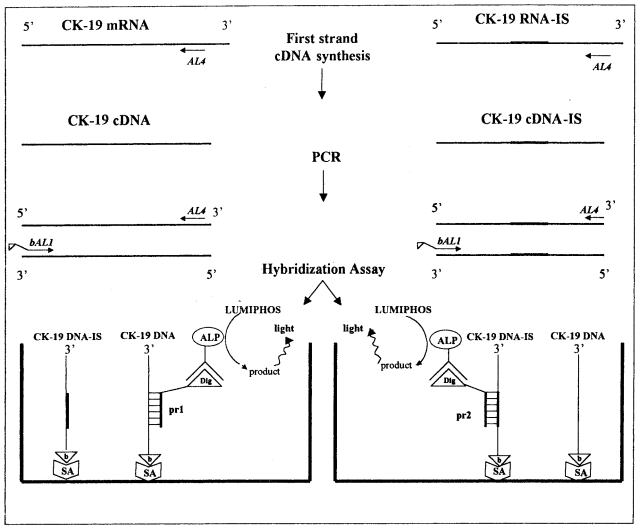

Fig. 2. Principle of the quantitative hybridization assay for CK-19 mRNA.

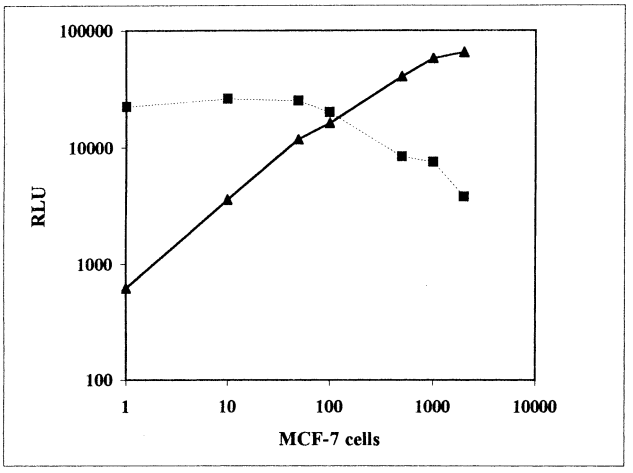

Fig. 3. Variation in relative luminescence units (RLU) for CK-19 mRNA (solid line) corresponding to RNA extracted from 1–2000 MCF-7 cells when coamplified with a constant amount of CK-19 RNA-IS (dashed line), in the presence of 1 g RNA from normal PBMC cells.

through in silico designed hybridization probes, specific for CK-19 (Pr 1) and CK-19 IS (Pr 2) (Table 1).

3.3. Hybridization assay

The principle of the proposed quantitative RT-PCR hybridization assay for CK-19 mRNA is shown in Fig. 2. Quantification is based on the coamplification of CK-19 mRNA with the recombinant CK-19 RNA-IS. Both CK-19 mRNA and CK-19 RNA-IS were reverse transcribed and amplified using the same set of primers. Labeling of the DNA fragments with biotin was achieved during PCR. The biotinylated amplification products were immobilized on sreptavidin coated microtiter wells and detected through hybridization to two different digoxigenin labeled probes, specific either for CK-19 or CK-19 IS. Hybridized amplicons were detected using antidigoxigenin antibodies conjugated to alkaline phosphatase and Lumiphos, which is a very sensitive chemiluminogenic substrate.

Hybridization assay conditions involving all steps, such as reagent concentrations, sample volume and time of incubation periods, were optimized in preliminary experiments in respect to the best signal to background ratios (S/B), sensitivity and rapidity (data not shown). A negative RT-PCR control was used for the estimation of the non specific binding of the hybridization assay throughout the study. The luminescence signals confirm that the Dig labeled hybridization probes, Pr1 and Pr2, bind exclusively to CK-19 DNA target and CK-19 IS, respectively and there is no cross-hybridization between them.

A calibration curve was generated for the RT-PCR assay using known amounts of CK-19 total RNA (corresponding to 1–2000 MCF-7 cells) mixed with a constant amount of total RNA (corresponding to 10^6 normal PBMC cells) and a constant amount of CK-19 RNA-IS (0.05 μg, 10-fold dilution of the stock solution). These preparations were subjected to RT-PCR amplification, in parallel with the unknown samples, in which the same constant amount of CK-19 RNA-IS was added, and the amplification products were analyzed by both hybridization assays. The relative luminescence units obtained for CK-19 RNA ($RLU_{CK\text{-}19}$) and CK-19 RNA-IS (RLU_{IS}) were plotted as a function of the number of MCF-7 cells. Since the same amount of CK-19 RNA-IS is used in every sample, the CK-19 RNA-IS luminescence signal should be the same for every sample and independent of the starting amount of CK-19 RNA. The RLU_{IS} was almost constant for low numbers of MCF-7 cells but decreased as the amount of CK-19 RNA in the sample was increasing (Fig. 3), because of the dose-related competition of the CK-19 RNA-IS with the target CK-19 RNA and the PCR plateau effect. Since the amount of CK-19 RNA-IS added to each sample affects the sensitivity and the analytical range of the assay [20], the constant amount of CK-19 RNA-IS added was optimized to be the smallest one that allowed a wide linear range for quantification of CK-19 mRNA. In Fig. 4 the ratio $RLU_{CK\text{-}19}/RLU_{IS}$ of the luminescence values obtained for CK-19 mRNA and CK-19 RNA-IS is plotted against the number of MCF-7 cells. These data suggest that the quantitative RT-PCR assay for CK-19 mRNA is linear in the range of 1 to 2000 MCF-7 cells.

To evaluate the overall reproducibility of the CK-19 mRNA quantitative RT-PCR, four different CK-19 mRNA concentrations in the presence of 1 μg total RNA isolated from normal PBMC cells were determined in 4 different experiments and all products were analyzed in duplicate by the proposed hybridization assay. The CVs of the $RLU_{CK\text{-}19}/RLU_{IS}$ ratios obtained for the four samples, representative of 1, 10, 100 and 1000 MCF-7 cells, were 13.4%, 11.4%, 8.9% and 10.6%, respectively. Under optimized RT-PCR and hybridization assay conditions, total RNA corresponding to 10^6 normal PBMC cells did not yield any

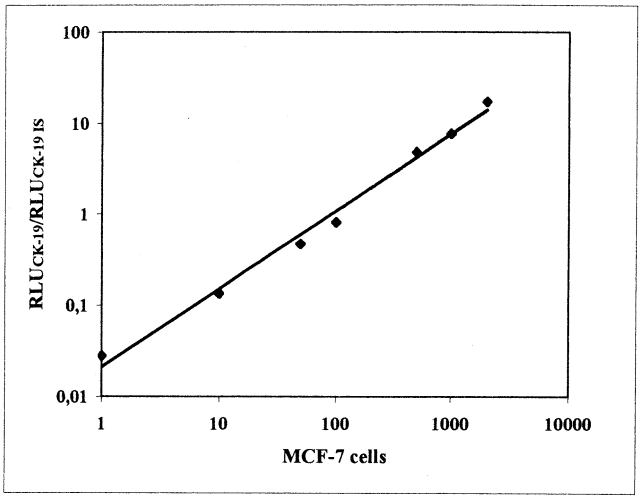

Fig. 4. Calibration curve for quantitative determination of CK-19 mRNA generated by mixing known amounts of CK-19 total RNA from MCF-7 cells with 1 μg RNA from normal PBMC cells and a constant amount of CK-19 RNA IS.

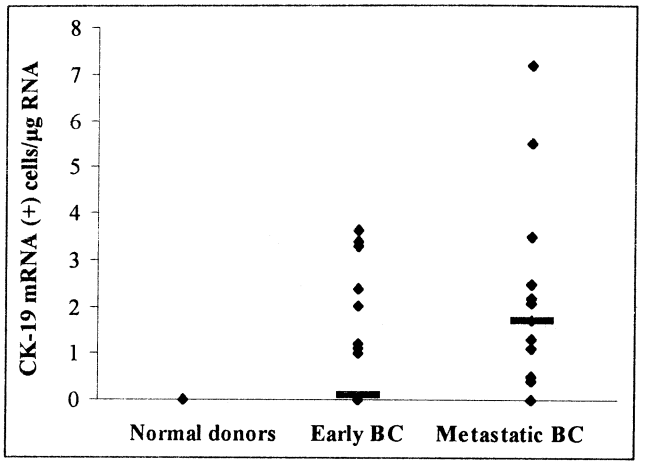

Fig. 5. CK-19 mRNA (+) cells/μg RNA in peripheral blood. a) normal donors, n = 26, not detected, b) early breast cancer (stages I/II, n = 37), range: 0–3.6 MCF-7 cells, c) metastatic breast cancer (stage IV, n = 14), range: 0–7.2 MCF-7 cells.

detectable amplification product, while the S/B ratios for CK-19 mRNA corresponding to 1 and 10 MCF-7 cells were 5.8 and 50, respectively.

The developed hybridization assay associated with the luminometric detection of CK-19 mRNA showed a higher sensitivity than the previously described nested RT-PCR and agarose gel-based detection [6–11]. This was verified since the same RNA samples that could be detected in agarose gels only after a nested RT-PCR were quantified by the developed hybridization assay after only one RT-PCR.

3.4. Quantification of CK-19 mRNA in peripheral blood of patients with breast cancer and control samples

The proposed methodology was applied for the quantitative determination of CK-19 mRNA in a total of 77 peripheral blood samples obtained from 26 healthy controls, 37 patients with stage I/II and 14 patients with stage IV breast cancer. β-actin RT-PCR of all samples revealed a strong positive band on agarose gel electrophoresis indicating the good quality of the extracted RNA. All blood samples were both analyzed for CK-19 mRNA by an established nested RT-PCR assay [7] and quantified by the proposed methodology. There was a complete concordance between the results obtained by the electrophoretic nested PCR approach and the proposed one-step PCR luminometric hybridization assay. None of the 26 peripheral blood samples of the control group was found positive while 9 of 37 (24.3%) and 10 of 14 (71.4%) blood samples from breast cancer patients with stage I/II and IV, respectively, were found positive. However, as can be seen in Fig. 5, CK-19 mRNA levels differed significantly between the two groups of breast cancer patients. The number of CK-19 positive circulating tumor cells in the 14 samples of the advanced breast cancer group (stage IV, verified metastasis) were significantly higher than in the 37 samples of the early breast cancer group (stage I/II), (Mann Whitney, *U* test, $p < 0.001$). More specifically, according to the MCF-7 cells standard curve, the mean number of CK-19 positive circulating tumor cells per μg of total RNA (isolated from 20 mL of peripheral blood) was 0.50 in the early breast cancer group (median:0, range: 0–3.6, $n = 37$) and 2.1 in the advanced breast cancer group (median: 1.7, range: 0–7.2, $n = 14$). Four samples that were found negative were truly false negatives, as this was indicated by the absence of the RNA-IS signal. These samples were not included in the study.

4. Discussion

The quantitative RT-PCR luminometric hybridization assay described here provides a specific and sensitive method for quantifying CK-19 positive circulating tumor cells in peripheral blood samples of breast cancer patients. The present assay differs from all other quantitative CK-19 RT-PCR assays described previously [16–19] by enabling accurate quantification of CK-19 mRNA with the help of a specially designed recombinant CK-19 RNA-IS which is added into the sample before reverse transcription.

So far, housekeeping genes such as GAPDH [19] or especially designed competitor templates [16–18] have been used as internal controls in most quantitative assays for the RT-PCR products of CK-19 mRNA. All competitor templates reported for CK-19 mRNA determination are irrelevant in respect to size and sequence to the target CK-19 mRNA [16–19] and since they are designed at the cDNA level, they are added to the PCR reaction mixture after reverse transcription. However, this approach allows

only comparative quantification, because of the differences in PCR amplification efficiency between the internal control and the target and does not take into account the variations in reverse transcription efficiency between samples. For an accurate quantification the IS and the target should be amplified with the same efficiency. For this reason, they should be ideally of the same size and sequence [20–22], so that they can be coamplified with the same primers. However, a minimal difference in sequence between IS and target is required to distinguish the target from the IS amplification product. The simplest methods described for quantification with a target like IS require the preparation of only one tube per sample. In these methods the sample is quantified using a constant number of IS copies in the sample and an external calibration curve [20,21]. Moreover the use of an RNA internal standard is extremely important for the accuracy of a quantitative RT-PCR assay, since this is the only way that variations in RNA extraction and RT-PCR amplification between samples are precisely controlled [20,21].

In our method, the recombinant RNA internal standard (CK-19 RNA-IS) was specifically designed and synthesized to have the same size and primer binding sequences as the target RNA, differing from the original CK-19 only in a central sequence of 21 bases, in silico designed to be completely irrelevant to the whole CK-19 cDNA region amplified by the selected primer pairs [20]. In this way, an accurate quantification of CK-19 mRNA transcripts is achieved, since the target (CK-19 mRNA) and the CK-19 RNA-IS are reversely transcribed and amplified by PCR with the same efficiency and the same primer pairs. The small (21 bp) difference in their sequence allowed us to design a specific detection probe for the IS. Moreover, the use of CK-19 RNA-IS enabled the analysis of multiple samples with a single set of calibrators over a wide range (1–2000 MCF-7 cells) without the need of multiple assays for the titration of each sample [16].

By performing an in silico analysis first and redesigning the set of primers used, the proposed hybridization assay is highly specific and free of false positive results. The problem of false positive results in RNA-based tests for MRD detection must be addressed effectively before any clinical use of new and sensitive RT-PCR methodologies, since false positives would significantly impair the clinical utility of the test [3,4,12–14]. The recent discovery of CK-19 pseudogene sequences such as CK-19*a* and CK-19*b* [13,14] with virtually identical sequences with CK-19 mRNA has severe implications on the validity of most previous reports regarding the sensitivity and specificity of CK-19 mRNA determination, since false positives may originate from contamination with genomic DNA. By carefully choosing the position of our primers and hybridization probes, this problem has been eliminated. The proposed set of primers AL1/AL4 can distinguish between CK-19, CK-19*a* and CK-19*b* pseudogenes, so that even the presence of 5 μg contaminating genomic DNA did not give any false positive results. Moreover evaluation of the specificity of this new set of primers in comparison to the established set [7] in a total number of 167 cDNAs showed that there was no significant difference between these two sets of primers. However, as can be seen in Table 2 the primer pair AL1/AL4 gave no false positives, while for the same cDNAs the primer pair P3/P4 gave two false positives in 26 normal blood donor samples (7.7%).

The proposed quantitative RT-PCR luminescence hybridization assay for CK-19 mRNA can quantify 1 MCF-7 cell in the presence of 10^6 normal PBMC cells. This was comparable to the detection limit reported for CK-19 RT-PCR qualitative electrophoretic assays in which a nested PCR approach was used [7–9]. The proposed hybridization assay offers many advantages over the established competitive PCR methodologies for CK-19 mRNA. It is highly specific, reliable, sensitive and easy to perform since common instrumentation used for immunoassays in most clinical laboratories is needed. Moreover it is less sensitive to PCR contamination problems, since nested PCR is avoided. The higher sensitivity of the proposed hybridization assay over the well established nested PCR approach for CK-19 detection [6–18], is achieved by the combination of enzymatic amplification and the use of a sensitive chemiluminescent reaction for monitoring the enzymatic activity.

The developed RT-PCR hybridization assay was used for the quantitative determination of CK-19 mRNA in peripheral blood samples from 26 healthy female blood donors, 37 patients with stage I/II and 14 patients with stage IV breast cancer. 10 of 14 (71.4%) patients with advanced breast cancer (stage IV, verified metastasis) and 9 of 37 (24.3%) patients with early breast cancer (stage I/II) had detectable levels of CK-19 mRNA in their peripheral blood. It is important to note that the number of CK-19 positive circulating tumor cells in the advanced breast cancer group were significantly higher than those found in the early breast cancer group, while in addition, all healthy controls showed undetectable levels of CK-19 mRNA in their blood (Fig. 5). The inclusion of the same quantity of the RNA-IS in all of our samples and standards enabled us to identify four false negative results that could be possibly due to the presence of inhibitors of PCR or reverse transcription enzymes in the samples.

In conclusion, we have developed a highly sensitive quantitative RT-PCR luminometric hybridization assay for the determination of CK-19 mRNA in peripheral blood. This is the first quantitative method reported so far for CK-19 mRNA where quantification is based on the use of a recombinant RNA-IS that contains the same primer binding sites and is of the same size as the target CK-19 RNA. In this way the ratio of the luminescence signals reflects the initial ratio of the concentrations of the two RNA samples in the starting mixture [20] since any variations in the efficiency of reverse transcription and PCR would affect both RNA samples equally. This is the main advantage of the proposed hybridization assay overall the other quantitative assays reported so far for CK-19 [16–19]. The inclusion of

the same quantity of the RNA-IS in all of our samples and standards, in addition to the reliable quantification, enabled us to identify false negative results and monitor the whole procedure from the RNA level. The method is free of false positive results, since it is based on the use of in silico modified primers that amplify CK-19 but not CK-19 pseudogenes *a* and *b*. Moreover, the assay is easily applicable for a high number of clinical samples and suitable for routine quantification of CK-19 positive epithelial cells in peripheral blood, since the whole microplate instrumentation needed is already present in many diagnostic laboratories.

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