

Featured Article

Real-Time Quantification of CK-19 mRNA-Positive Cells in Peripheral Blood of Breast Cancer Patients Using the Lightcycler System

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Abstract

Purpose: The purpose of this research was to develop a quantitative real-time reverse transcription-PCR (RT-PCR) for CK19-mRNA and evaluate its clinical potential for the molecular detection of occult carcinoma cells in the peripheral blood of breast cancer patients.

Experimental Design: The method is based on real-time monitoring during PCR of fluorescently labeled specific hybridization probes for CK19-mRNA. The breast cancer cell line MCF-7 was used for the development and analytical evaluation of the assay. We analyzed blood samples from 89 healthy blood donors, 77 patients with early breast cancer (stage I-II) postoperatively, and 47 patients with previously untreated metastatic disease (stage IV) before and after chemotherapy. All of the samples were also analyzed by nested RT-PCR.

Results: The method is highly sensitive and specific, because only 2 of 89 (2.2%) of the healthy control subjects had detectable CK19-mRNA+ cells. In 77 patients with early breast cancer, CK19-mRNA+ cells were detected in 24 (31.2%) before and 5 (6.5%) after adjuvant chemotherapy, and their levels differed significantly ($P < 0.001$, Wilcoxon test). In 47 patients with verified metastases 19 (40.4%) and 20 (42.6%) were found positive before and after chemotherapy, and no significant difference in CK19-mRNA+ cell levels was observed ($P = 0.96$, Wilcoxon test). Results obtained by the proposed real-time RT-PCR method corre-

lated well with those obtained for the same samples by nested RT-PCR [concordance in 312 of 337 (92.6%); $P = 0.69$, McNemar test].

Conclusions: The developed method is highly sensitive and specific, and can be used for high-throughput continuous monitoring and quantification of circulating epithelial cells in the peripheral blood of breast cancer patients.

Introduction

Despite the recent advances in early diagnosis and treatment, a substantial proportion of patients with early breast cancer will present distant metastasis and die. The spread of tumor cells, usually undetected by standard diagnostic techniques, has been termed as MRD³ and should be distinguished from clinically evident metastasis (1). The majority of cancer patients have viable tumor cells in their bone marrow at primary tumor diagnosis, and the proliferative potential of these cells determines the clinical outcome (2, 3). It was shown recently that minimal residual cancer cells are highly heterogeneous (4), and the mechanisms determining their migration and invasion into distant organs have begun to be elucidated (5). The clinical importance of the identification of isolated tumor cells in biological fluids of breast cancer patients remains one of the major questions yet to be answered (6).

The increasing use of immunohistochemistry and molecular biology techniques has enabled pathologists to detect microscopic lesions down to the level of isolated tumor cells and has led to an explosion of studies analyzing novel genetic markers as prognostic indicators of breast cancer (3). These molecular markers show great promise for the future and are expected to provide powerful supplemental information to the existing staging system for breast cancer, provided that serious standardization problems in the measurement techniques for many of them are solved (3, 6). Therefore, the development of highly sensitive and specific methods for the early detection of circulating cancer cells is 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 successfully used as a marker for the detection of tumor cells in the bone marrow, lymph nodes, and peripheral blood by immunohistochemistry (7) and RT-PCR (8, 9). Our group has shown recently that the detection of CK-19-positive cells in the peripheral blood of patients with operable breast cancer before the initiation of any adjuvant treatment is an independent prognostic

Received 12/19/02; revised 7/15/03; accepted 7/22/03.

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This work was supported by the Cretan Association for Biomedical Research (CARB) and Roche Diagnostics (Roche Hellas).

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³ The abbreviations used are: MRD, minimal residual disease; RT-PCR, reverse transcription-PCR; Cp, crossing point; GAPDH, glyceraldehyde phosphate dehydrogenase; PBGD, porphobilinogen deaminase; CV, coefficient of variation.

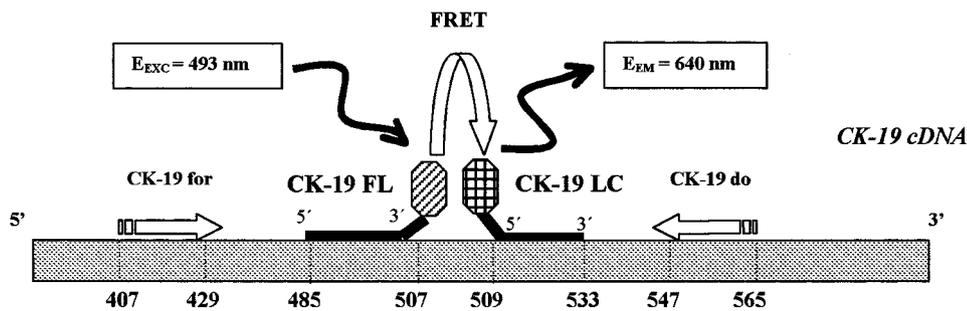


Fig. 1 Principle of the real-time RT-PCR assay for CK-19 mRNA (numbers correspond to the nucleotide position in CK-19 mRNA GenBank accession no: Y00503).

factor associated with an increased risk of disease relapse and shorter survival (10). Quantitative RT-PCR methods for CK19-mRNA that are mainly based on specifically designed competitor cDNA templates as internal controls (11–13) or real-time monitoring of TaqMan probes (14) have been reported. We have described the development of a quantitative RT-PCR luminescent hybridization assay for CK19-mRNA with an RNA internal standard and chemiluminescence detection (15).

Development of real-time PCR technology already has an enormous impact on cancer diagnostics, because it can provide significant and quantitative information about gene expression in an automated, rapid, versatile, and cost-effective way (16). In the field of MRD, real-time PCR has been used recently for the detection of circulating breast tumor cells in the peripheral blood and lymph nodes of metastatic breast cancer patients (stage III/IV) through the differential expression of marker genes, identified by serial analysis of gene expression (17) and through multigene RT-PCR assays (18), respectively.

In the present study, we report the development and analytical validation of a real-time quantitative RT-PCR methodology for CK19-mRNA using the LightCycler system (19). The method was evaluated by comparison to the well-established nested RT-PCR methodology for CK19-mRNA (8) in many peripheral blood samples, obtained from patients with either early or metastatic breast cancer, as well as healthy blood donors.

Materials and Methods

Cell Samples. The human mammary carcinoma cell line MCF-7, which expresses the *CK-19 gene* (obtained from the American Type Culture Collection), was used as positive control and cultured as described previously (15).

Clinical Samples. Peripheral blood in EDTA was obtained from 77 patients with stage I/II breast cancer postoperatively, before the beginning and after the last cycle of adjuvant chemotherapy, 47 patients with previously untreated metastatic (stage IV) breast cancer before the beginning and at the end of the third cycle of first-line chemotherapy, and 89 female healthy volunteers (18–65 years of age). 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. Peripheral blood samples from healthy donors and patients were collected and processed in the same manner. All of the patients and donors gave their informed consent, and the study has been approved by the Ethical and

Scientific Committees of the participating Institutions. The peripheral blood mononuclear cells were isolated within 1 h of venipuncture by gradient centrifugation with Ficoll Hypaque-1077 (Sigma Chemical Company, St. Louis, MO) as described previously (10, 15), and cell pellets were kept at -80°C until RNA extraction.

Total RNA Isolation and cDNA Synthesis. Total RNA isolation was performed by using Trizol LS reagent (Invitrogen) according to the manufacturer's instructions. All of the preparation and handling steps of RNA took place in a laminar flow hood, under RNase-free conditions. The isolated RNA was dissolved in RNA storage buffer (Ambion) and stored at -80°C until used. 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 described previously (10, 15).

Reverse transcription of RNA was carried out with the THERMOSCRIPT RT-PCR System (Invitrogen). Total RNA prepared from the MCF-7 cell line was used as a positive control. cDNA was synthesized from 5 μg of total RNA isolated from peripheral blood mononuclear cells of healthy volunteers and breast cancer patients, according to the manufacturer's instructions.

Nested RT-PCR. Two different PCR reactions, with the respective negative controls, were performed for each sample to amplify fragments of CK-19 and β -actin (8, 10). The CK-19 gene expression was evaluated by nested RT-PCR as described by Datta *et al.* (8). Ten μl of all of the PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide.

Real-Time RT-PCR for CK-19 mRNA. The principle of the proposed real-time RT-PCR assay for CK-19 is shown in Fig. 1. Quantification is based on real-time monitoring during PCR of fluorescently labeled specific hybridization probes for CK-19. The point where the fluorescence rises above background noise (C_p) is best quantified through the LightCycler software as the second derivative maximum of the curve. Real-time RT-PCR for CK-19 mRNA was performed using the LightCycler system (Roche Diagnostics). The primers and the hybridization probes used for CK-19 were designed and synthesized by TIB MOLBIOL (Berlin, Germany; Table 1).

Real-time PCR was performed in a total volume of 20 μl in the LightCycler glass capillaries. For the PCR, 2 μl of cDNA were placed into a 18- μl reaction volume containing 1 μl of the

Table 1 Sequences of primers and hybridization probes used in this study

Gene	Use	Name	Oligonucleotide sequence (5'-3')
CK-19	Forward primer	CK19-for	gCACTACAgCCACTACTACACgA
	Reverse primer	CK19-do	CTCATgCgCAgAgCCTgTT
	Hybridization probe	CK19-FL ^a	TgTCCTgCAgATCgACAAcCCC-FL
GAPDH	Hybridization probe	CK19-LC ^b	LCRed640-CTggCTgCAgATgACTTCCgAACC
	Forward primer	GAPDH F	TTggTATCgTggAAggACTCA
	Reverse primer	GAPDH R	TgTCATCATATTTggCAggTTT
	Hybridization probe	GAPDH 3FL ^a	TgTCCCCACTgCCAACgTgTCAG-FL
	Hybridization probe	GAPDH 705 ^c	LCRed705-ggTggACCTgACCTgCCgTCTAgA

^a Labeled with fluorescein.

^b Labeled with LC Red640 (TIB MOLBIOL).

^c Labeled with LC Red705 (TIB MOLBIOL).

sense primer CK19-for (3 μ M), 1 μ l of the antisense primer CK19-do (3 μ M), 2.4 μ l of the LightCycler Fast Start DNA Master Hybridization Probes reagent (10 \times concentration), 1 μ l of the probe CK19-FL (3 μ M), and 1 μ l of the probe CK19-LC (3 μ M), and DEPC-H₂O was added to the final volume. PCR reaction was initiated with a 10-min denaturation at 95°C and terminated with a 30-s cooling step at 40°C. The cycling protocol consisted of denaturation step at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 20 s, and repeated for 50 times. Fluorescence detection was performed at the end of each annealing step for 0 s.

For quantification, an external calibration curve was obtained by using external standard cDNAs. Total RNA was prepared from 1 \times 10⁶ MCF-7 cells (as verified by a hemocytometer). Serial dilutions of this RNA preparation in DEPC-treated water, corresponding to 1–10,000 MCF-7 cells, were used for cDNA synthesis. These cDNAs were kept in aliquots at –20°C and used throughout the study as external standards. This calibration curve was created by plotting the number of MCF-7 cells corresponding to each external standard cDNA versus the value of its Cp. The number of circulating CK-19 mRNA-positive cells for all of the tested samples was expressed as MCF-7 cell equivalents per 5 μ g of total-RNA, as determined by LightCycler software 3.1, according to the external standard calibration curve.

Real-time PCR for the housekeeping genes GAPDH and PBGD was performed in all of the clinical samples to evaluate the quality of the cDNAs used in the study. The primers and the hybridization probes used for GAPDH were designed and synthesized by TIB MOLBIOL and are shown in Table 1. For PBGD the LightCycler-h-PBGD housekeeping gene set (Roche Molecular Biochemicals) was used.

Statistics. The Mann-Whitney test for unpaired non-normally distributed groups was used to compare the levels of CK-19 mRNA-positive cells in the peripheral blood samples of breast cancer patients with those of the control population. The Wilcoxon test for paired non-normally distributed groups was used to compare the levels of CK-19 mRNA-positive cells in the peripheral blood samples of patients with early breast cancer before and after adjuvant chemotherapy, and patients with metastatic breast cancer before and after chemotherapy. *P*s < 0.05 were considered statistically significant. The McNemar and Fisher's exact test was used to compare nested RT-PCR and

real-time PCR results for CK-19 mRNA detection on the same cDNAs. Data analysis was carried out with the Statmost statistical package (Statmost; DataMost Corp.).

Results

Optimization of the Real-Time RT-PCR. To establish a specific, sensitive, and reproducible quantitative real-time RT-PCR methodology for CK-19 mRNA, we first optimized the primer and probe concentrations, as well as reaction temperatures and times. To determine the analytical sensitivity and linearity of real-time RT-PCR assay, we analyzed the cDNA external standards (prepared as described above) in 17 different experiments. Calibration curves from these data showed linearity over the entire quantification range (1–10⁴ MCF-7 cells) and correlation coefficients >0.99 in all of the cases, indicating a precise log-linear relationship. The mean slope and intercept of the calibration curve was -3.948 ± 0.140 (CV = 3.6%; *n* = 17) and 32.17 ± 0.70 (CV = 2.2%; *n* = 17), respectively, whereas the PCR efficiency expressed as $E = 10^{-1/\text{slope}}$ was 1.793 ± 0.039 (CV = 2.2%; *n* = 17). The analytical detection limit of the method defined as 3.3 times the SD of the Cp of the first external standard (1 MCF-7 cell equivalent) divided by the mean slope of the calibration curve (D.L. = 3.3 SD/slope; Ref. 20) was found to correspond to 0.6 MCF-7 cell equivalents. Real-time monitoring of the PCR reaction in the LightCycler as well as a typical calibration curve, displayed as a linear relationship between Cp values and the logarithm of the initial number of MCF-7 cells, is shown in Fig. 2.

To determine within-run precision of the assay, three cDNA samples corresponding to 1, 10, and 100 MCF-7 cells were assayed in the same run in nine parallel determinations, using the same calibration curve in the LightCycler. As can be seen in Table 2 within-run CVs for MCF-7 cells as determined by the calibration curve ranged from 7.5% to 9.3%, whereas for the corresponding Cp values ranged from 0.9% to 1.5%. To determine between-run precision of the assay, the same cDNA samples were frozen (–20°C) in aliquots and analyzed over a period of 1 month on 17 separate assays performed in 17 different days. As can be seen in Table 2, between-run CVs for MCF-7 cells as determined by the calibration curve ranged from 10.7% to 16.0%, whereas for the corresponding Cp values ranged from 2.2% to 3.2%.

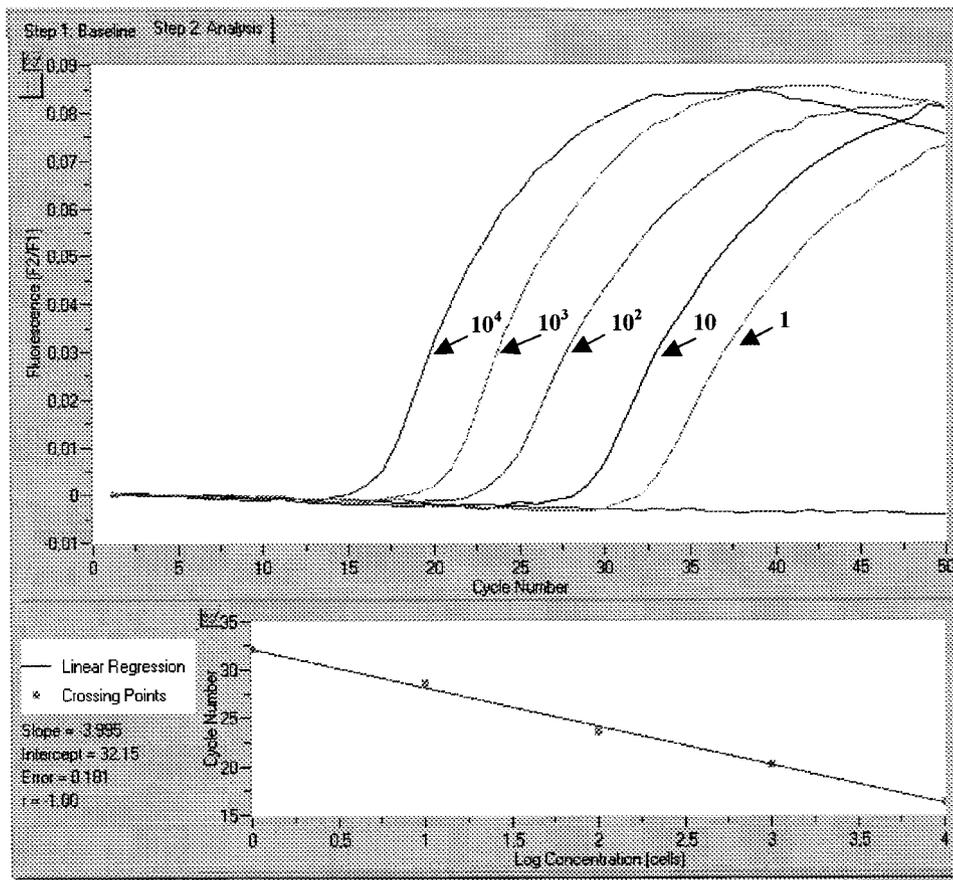


Fig. 2 Quantification of CK-19 mRNA-positive cells by real-time PCR in the LightCycler system. *Top*, logarithmic plot of fluorescence signal (F2/F1) during amplification. Serial dilutions of a total RNA preparation corresponding to a certain amount of MCF-7 cells were prepared, cDNAs were synthesized and used as external standards. The curves from right to left correspond to 1, 10, 10^2 , 10^3 , and 10^4 MCF-7 cells, whereas the flat line represents the negative control. *Bottom*, the graph shows the Cps (Cycle number) plotted versus the log of the number of MCF-7 cells measured in triplicate.

Table 2 Within-run and between-run precision of the real-time RT-PCR for CK-19 mRNA

MCF-7 cell equivalents	Within-run precision ($n = 9$)				Between-run precision ($n = 17$)			
	Cp		MCF-7 cells		Cp		MCF-7 cells	
	Mean (SD)	CV%	Mean (SD)	CV%	Mean (SD)	CV%	Mean (SD)	CV%
1	32.02 (0.50)	1.5	0.90 (0.07)	7.7	32.41 (0.72)	2.2	0.97 (0.15)	15.5
10	27.96 (0.24)	0.9	9.4 (0.87)	9.3	28.44 (0.69)	2.4	9.07 (0.97)	10.7
100	23.44 (0.29)	1.2	128 (9.5)	7.5	23.94 (0.78)	3.2	117 (18.7)	16.0

Real-Time Quantification of CK-19 mRNA-Positive Cells in Peripheral Blood of Breast Cancer Patients. The proposed real-time quantitative PCR methodology was applied in a total of 337 peripheral blood samples obtained from 89 healthy female blood donors, 77 patients with early breast cancer (stage I/II) before the beginning and after the last cycle of adjuvant chemotherapy, and 47 patients with verified metastasis (stage IV) before the beginning and after the end of the third cycle of first-line chemotherapy.

To ensure that amplifiable material was present in all of the

specimens and to avoid false-negative results, real-time amplification of both housekeeping genes *GAPDH* and *PBGD* was performed for all of the samples. The amount of total RNA in each sample was used to normalize our quantitative RT-PCR data (21).

On the basis of the analytical detection limit of our method, the presence of >0.6 MCF-7 cell equivalents/ $5 \mu\text{g}$ RNA in the patient blood sample was considered as a positive result. According to this cutoff, the specificity of the proposed method was evaluated by analyzing peripheral blood samples from 89

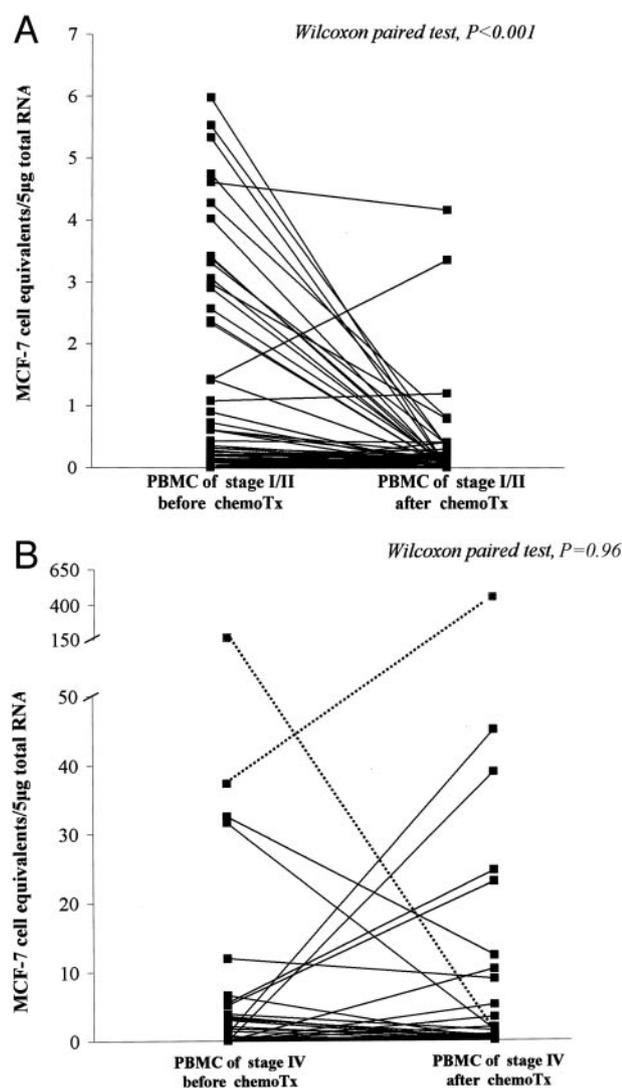


Fig. 3 Real-time RT-PCR quantification of CK-19-positive cells (MCF-7 equivalents/5 μ g total RNA) in the peripheral blood of breast cancer patients. A, stage I/II before and after adjuvant chemotherapy ($n = 77$ pairs); B, stage IV before and after first-line treatment ($n = 47$ pairs).

female healthy blood donors: only 2 samples (2.2%) were found positive by real-time PCR, whereas in the same group of patients 5 samples (5.6%) were found positive by nested PCR. In the group of 77 patients with early breast cancer, 24 (31.3%) and 5 (6.5%) were found positive before and after adjuvant chemotherapy, respectively. The CK-19 mRNA-positive cell levels expressed as MCF-7 cell equivalents/5 μ g RNA in the 24 positive samples before adjuvant chemotherapy ranged from 0.6 to 6.0 (mean \pm SD: 2.8 ± 1.7 , median: 2.9), whereas in the 5 positive samples after adjuvant chemotherapy ranged from 0.8 to 4.2 (mean \pm SD: 2.1 ± 1.6 , median: 1.2). As can be seen in Fig. 3A, CK-19 mRNA-positive cell levels differed significantly in the early breast cancer patients before and after adjuvant chemotherapy ($P < 0.001$, Wilcoxon test analysis; $n = 77$). Furthermore, there was a significant difference between healthy

Table 3 Comparison of nested PCR with real-time PCR for the detection of CK-19 positive cells in peripheral blood samples. Concordance: 92.6% (312/337), $P = 0.69$ (McNemar and Fischer exact test)

Nested PCR	Real-time PCR (LightCycler)		Total
	+	-	
+	59	14	73
-	11	253	264
Total	70	267	337

donors and early breast cancer patients tested before adjuvant chemotherapy ($P < 0.01$, Mann Whitney test) but not after ($P = 0.81$, Mann Whitney test). Among the 47 patients with metastatic disease 19 (40.4%) and 20 (42.6%) were found positive before and after the completion of first-line chemotherapy, respectively. The CK-19 mRNA-positive cell levels expressed as MCF-7 cell equivalents/5 μ g RNA in the 19 positive samples before chemotherapy ranged from 0.6 to 160 (mean \pm SD: 16.6 ± 36.6 , median: 3.5) whereas in the 20 positive samples after chemotherapy ranged from 0.6 to 438 (mean \pm SD: 31.0 ± 96.7 , median: 2.6). As can be seen in Fig. 3B, CK-19 mRNA-positive cell levels did not differ significantly in advanced breast cancer patients ($P = 0.96$, Wilcoxon test analysis; $n = 47$) before and after first-line treatment. On the contrary, there was a statistically significant difference when this group was compared with healthy controls ($P < 0.05$, Mann Whitney test), but no difference was observed when compared with the early breast cancer group before adjuvant chemotherapy ($P = 0.80$, Mann Whitney test). The administration of first-line chemotherapy to patients with metastatic disease resulted in clinical complete response in 8, partial response in 19, stable disease in 12, and progressive disease in 8 patients. Comparison of pre- and postchemotherapy CK-19 mRNA-positive cell levels in the four groups of patients as defined by the clinical response yielded significant differences only for patients who achieved a complete clinical response (prechemotherapy ranged from 0 to 160; mean \pm SD: 25.0 ± 55.0 , median: 2.5 *versus* postchemotherapy that ranged from 0 to 1.4; mean \pm SD: 0.6 ± 0.6 , median: 0.65; Wilcoxon paired t test, $P < 0.05$).

Comparison of Real-Time RT-PCR with Nested RT-PCR. The same cDNA samples were also analyzed for CK-19 mRNA by an established nested RT-PCR assay (8). There was a 92.6% concordance (312 of 337) of positivity and negativity between the two methodologies ($P = 0.69$, McNemar and Fisher's exact test; $n = 337$) as can be seen in Table 3.

Discussion

We have shown recently that the detection of CK-19 mRNA-positive cells in the peripheral blood of patients with operable breast cancer is an independent predictive and prognostic factor (10). Most of the studies reported thus far for CK-19 mRNA expression as an MRD molecular marker are based on qualitative approaches (8–10). However, in recent years, the development and clinical evaluation of quantitative RT-PCR methodology for CK-19 mRNA has been the focus of several studies, and different quantitative methods based either

on relative or absolute quantification of CK-19 transcripts in peripheral blood have been proposed (11–15, 17). Most of these studies are too complicated and not so easily adaptable for standardized clinical applications. Another major limit is the fact that it is difficult to accurately quantify the number of circulating tumor cells corresponding to the mRNA levels found, because these cells are highly heterogeneous and may express different levels of marker gene cDNAs (4).

Moreover, normalization of the results by comparison to the expression of a reference gene introduces an unpredictable variability for comparison among different patients. In a recent study (21) it was clearly shown that single housekeeping gene expression should not be used to normalize real-time PCR results. This approach is inappropriate for quantitative RT-PCR studies, because a significant variation in the expression levels of 10 commonly used housekeeping genes was shown between individuals and samples taken from the same individual. On the contrary, it was shown that normalization to total RNA concentration is an acceptable alternative (21).

The real-time quantitative RT-PCR assay described herein provides a specific and sensitive method for quantifying circulating CK-19 mRNA-positive cells in peripheral blood samples of breast cancer patients. In this assay we express our results as MCF-7 cell equivalents corresponding to 5 μ g of total RNA (amount used for the preparation of sample cDNA), because we have used the breast cancer cell line MCF-7 for the preparation of our external standards calibration curve. The main advantage of this assay, as in all of the real-time PCR assays, is that quantitation is based on the exponential phase of the PCR instead of using the end point accumulation of PCR product at the end of the stationary phase of the PCR. Moreover, both amplification and analysis steps are automated, and there is no need for slab gels and complicated sample manipulation after PCR.

The analytical performance of this assay was validated in detail through a series of experiments. Detection of CK-19 mRNA-positive cells was linear over a range of 4 logs (1–10⁴ MCF-7 cell equivalents/5 μ g of total RNA), with a low detection limit of 0.6 MCF-7 cell equivalents/5 μ g of total RNA. The absolute values of the slopes of the calibration curve as well as of the PCR efficiency did not change significantly from day-to-day as evaluated in a set of 17 independent experiments. The interassay CV of the assay (within-run CV%) was very satisfactory (7.5–9.3%). The assay showed a good performance over a 1-month period with an intra-assay CV (between-run CV%) between 10.7% and 16%.

The developed method showed high specificity, as only 2 in 89 healthy blood donor samples were found positive for CK-19. This was made possible by avoiding contamination of skin epithelial cells during venipuncture, as well as by carefully designing the primers and hybridization probes used, so that amplification of known CK-19 pseudogenes and genomic DNA would be avoided. According to our results, the proposed real-time quantification assay for CK-19 mRNA-positive cells in peripheral blood samples of breast cancer patients has at least the same detection limit as the luminometric hybridization assay (15) and conventional nested RT-PCR (8). The method has shown a good concordance with nested RT-PCR in respect to positivity and negativity in many samples analyzed by both

methods (312 of 337; 92.6%). Moreover, this real-time RT-PCR for CK-19-positive cells offers two major advantages: it avoids the typical cross-contamination problems, common in nested-PCR, and allows rapid quantitative evaluation of the results.

Thus far, real-time RT-PCR methods for CK-19 in peripheral blood have been applied in a limited number of advanced breast cancer patients (14, 17). In the present study, the levels of circulating CK-19-positive cells in peripheral blood of early and advanced breast cancer patients are evaluated before and after chemotherapy for the first time. Our results show that quantification of circulating CK-19 mRNA-positive cells by real-time RT-PCR with the LightCycler system is a promising marker for breast cancer patients, reflecting the response to adjuvant chemotherapy which cannot be assessed with other studies. However, long-term follow-up of these patients is necessary to evaluate whether patients with higher levels of circulating CK-19-positive cells in their peripheral blood have an increased risk of disease recurrence. On the contrary, for patients with metastatic disease CK-19 mRNA measurements reflected the effect of chemotherapy only for patients achieving a complete clinical response. A possible explanation for the lack of significant differences in CK-19 mRNA levels before and after chemotherapy for patients with persistent metastatic disease could be the continuous “seeding” of the peripheral blood with CK-19 mRNA-positive cells from the metastatic tumors. However, because of the few metastatic patients analyzed, our results should be interpreted with caution until verified from an ongoing subsequent analysis including a larger group of patients.

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Clinical Cancer Research

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Clin Cancer Res 2003;9:5145-5151.

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