Isolation by size of epithelial tumor cells in peripheral blood of patients with breast cancer: correlation with real-time reverse transcriptase–polymerase chain reaction results and feasibility of molecular analysis by laser microdissection

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Summary The aim of this study is the counting and the immunomorphological and molecular characterization of circulating tumor cells (CTCs) by the isolation by size of epithelial tumor cells (ISET) method in the peripheral blood of patients with breast cancer. An evaluation of the method's ability to reveal the presence of occult carcinoma cells in blood of a patient with breast cancer was performed and the results compared with those obtained by quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay for the evaluation of cytokeratin-19 (CK-19) mRNA expression. The feasibility of molecular analysis of CTCs after laser microdissection of filters used in ISET was illustrated, referring to HER-2 amplification. Blood samples drawn from 44 patients with breast cancer were preoperatively analyzed by ISET. From the same samples, total RNA was extracted and submitted to quantitative real-time RT-PCR for the detection of CK-19 mRNA-positive cells using TaqMan technology. HER-2 amplification was measured by real-time RT-PCR on DNA extracted from cells recovered by laser microdissection from 7 selected ISET-positive filters. Of 44 samples, 12 (27%) showed the presence of epithelial cells on the filter (mean ± SE: 8.5 ± 2.4 cells per milliliter of blood). A statistically significant agreement ($P = .001$) was observed between real-time RT-PCR results and those obtained by ISET. With regard to HER-2 amplification, a good correspondence was found between the results obtained from microdissected CTCs and those obtained using DNA extracted from the primary tumor ($R = 0.918$; $P < .01$), as well as the immunohistochemistry results. The ISET method allows for the collection of breast carcinoma cells by filtration despite their smaller dimension relative to

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other carcinoma cell types. The sensitivity and specificity of the method is comparable with those obtained using the quantitative real-time RT-PCR assay for the evaluation of CK-19 mRNA expression. Moreover, the laser microdissection technique allows for the recovery of nucleic acids for further molecular analysis and CTC characterization.

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1. Introduction

Ongoing surgical and medical advances have made the optimal care of patients with breast cancer increasingly complex. Accurate staging has become a critical determinant in the clinical treatment plan. Currently, the presence of metastatic cancer in the regional lymph nodes remains the most valuable prognostic indicator for this cancer, with an inverse relationship existing between clinical outcome and the number of positive lymph nodes [1,2].

The opportunity to establish more effective methods to detect metastatic disease is now possible with the combination of breast cancer associate gene identification and recent advances in biotechnology [3-13]. One such technology, the real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay, is highly sensitive, accurate, and reproducible and provides quantitative results [14]. These attributes address some of the most significant problems facing polymerase chain reaction (PCR)-based molecular diagnostics. As a result, numerous investigators are attempting to apply real-time PCR-based diagnostics to a wide variety of tissue types, such as primary tumor, axillary lymph nodes, bone marrow, and peripheral blood [15]. To date, the reliable and sensitive identification of occult metastatic disease in the peripheral blood remains problematic owing to the infrequency of circulating tumor cells (CTCs) and background expression of cancer-associated marker genes in normal peripheral blood. Despite these obstacles, the concept of a *blood test* capable of diagnosing, staging, or determining treatment response remains compelling [16]. Such an assay could potentially replace invasive procedures, such as axillary lymph node dissection, and/or be used for breast cancer screening and monitoring treatment responses [15].

On the other hand, real-time RT-PCR does not permit analysis of cell morphology, counting of tumor cells and demonstration of tumor microemboli that has spread into peripheral blood during surgery. This goal can be reached only by using a relatively novel approach to study circulating tumor cells, the isolation by size of epithelial tumor cells (ISET) method [17-19]. This methodology provides morphological, immunocytologic, and genetic characterization of individual CTCs. The test collects carcinoma cells by filtration, as they are larger in size compared with peripheral blood leukocytes. Furthermore, selected individual cells or groups of cells can be recovered by laser microdissection, and their DNA amplified and screened for genetic abnormalities in target sequences [17]. Additional advantages of this technique are high sensitivity and suitability for application to a broad range of carcinomas.

We applied this experimental approach to the study of patients affected by breast carcinoma. Comparison of ISET results with those obtained by real-time RT-PCR for cytokeratin-19 (CK-19) mRNA expression was performed. Moreover, the collection of CTCs from positive filters was achieved by laser microdissection and DNA was extracted and used to determine HER-2 amplification.

2. Materials and methods

2.1. Cell lines

The MCF-7 cell line (ATCC, HTB 22; H. D. Soule, Bethesda, Md), derived from a human breast adenocarcinoma, was cultured in Dulbecco's modified eagle medium containing 10% fetal bovine serum and used to generate an RNA standard for the TaqMan assay and to evaluate the sensitivity of both the real-time PCR assay and the ISET technology.

The SKBR3 cell line (ATCC, HTB 30; G. Trempe and L. J. Old, 1970), derived from a human breast adenocarcinoma, was cultured in Dulbecco's modified eagle medium containing 15% fetal bovine serum. The cell line was used to generate a positive control for the TaqMan assay as well as for the ISET procedure.

2.2. Patients and samples

Samples from 40 healthy female volunteers were used as the negative control. Forty-four patients with breast cancer (19 stage I, 20 stage II, and 5 stage III) undergoing surgery for breast cancer were involved in this study after receiving their informed consent. Median age was 64 years (range, 29-81 years). Patients did not receive systemic therapy before surgery. For the total of the patients, 10 mL of peripheral blood were collected in buffered EDTA, maintained at 4°C, and processed within 1 hour. For the same patients, a fragment of breast cancer tissue was collected for DNA extraction. Comparable sections were used for immunochemical detection of HER-2 with the DAKO kit (HercepTest, Dako, Glostrup, Denmark).

2.3. Isolation by size of epithelial tumor cells

Isolation by size of epithelial tumor cells was carried out using a previously described [17] module of filtration (licenses EP513139, US5606351, and JO5504405) kindly provided by the Biocom Company (Les Ulis, France) and a

polycarbonate Track-Etch-type membrane (Cyclotron Technology, Saint Paul lez Durance, France) with calibrated, 8-μm-diameter, cylindrical pores. The module of filtration has 12 wells, making it possible to load and filter 12 individual samples in parallel. Each sample is filtered through a 0.6-cm-diameter surface area in the membrane. Peripheral blood (5 mL) from patients with carcinoma or from healthy volunteers was collected on buffered EDTA, diluted 1:10 with the filtration buffer containing 0.175% saponin, 0.2% paraformaldehyde, 0.0372% EDTA, and 0.1% bovine serum albumin, left for 10 minutes at room temperature and filtered. Ten milliliters of diluted solution, corresponding to 1 mL of undiluted blood, were loaded on each well and filtered by gentle aspiration under vacuum (created by a vacuum pump). The membrane was then washed once by aspiration with phosphate-buffered saline, disassembled from the filtration module and allowed to air-dry. The spots, each one corresponding to 1 mL of filtered blood, were then immunostained, washed once with phosphate-buffered saline, and dried. Counting of tumor cells was performed by a single operator. Value of KL1-immunostained cells for each positive patient was the mean of the cells counted on up to 5 different positive filters.

2.4. Isolation by size of epithelial tumor cells sensitivity test

Sensitivity tests were performed on artificial samples created by adding known amounts of MCF-7 cells to peripheral blood samples. One and 3 cultured MCF-7 cells, in 5-fold replicates, were individually collected under a microscope equipped with a magnifying glass and mixed to 1 mL of peripheral blood. These 1-mL samples were then analyzed by ISET. Isolation by size of epithelial tumor cells was carried out by counting tumor cells after KL1 immunostaining.

2.5. Cell immunostaining

The cells were permeabilized with 0.2% Triton for 10 minutes before immunostaining. The primary antibody, diluted 1:100 in 10% fetal calf serum and then applied to the spot for 1 hour at room temperature, was KL1 (cytokeratin gp 56 kd; Immunotech SA, Marseille, France), a cytokeratin, broad-spectrum monoclonal antibody. The spots were then treated with a biotinylated mixture of antimouse and antirabbit secondary antibodies for 30 minutes (peroxidase/diaminobenzidine ChemMate Detection Kit; Dako), followed by a complex, streptavidin-peroxidase, for 30 minutes and diaminobenzidine for 15 minutes. Cell nuclei were counterstained with Mayer's hematoxylin (Dako).

2.6. Total RNA isolation from blood and cDNA synthesis

Blood specimens and cell lines were subjected to RNA extraction using Trizol reagent (Life Technologies, Rock-ville, Md) according to the manufacturer's instructions. Briefly, nucleated cells were isolated by density gradient separation: 5 mL of blood was added to 10 mL Ficoll, centrifuged at 900g for 15 minutes at 4°C, and the supernatant was collected and mixed with 10-mL NaCl solution (0.9%). After centrifugation at 2500g for 10 minutes at 4°C, the supernatant was discarded, and the cells were resuspended in 500 μL of Trizol solution (Life Technologies). RNA was extracted according to the protocol supplied and resuspended in 30 μL of Rnase-free water. The concentration and purity of the RNA were detected spectrophotometrically after Dnase treatment. Three micrograms of each sample was reverse-transcribed using the TaqMan RT-PCR kit (Applied Biosystems, Foster City, Calif) in a final volume of 60 μL.

2.7. Real-time RT-PCR evaluation of CK-19–positive cells in blood

2.7.1. Primers and probe used in RT-PCR assays

Using the Primer Express software (Applied Biosystems) in combination with the universal amplicon design guidelines (Applied Biosystems), the following oligonucleotides were developed to establish real-time quantitative PCR for gene coding of CK-19 (National Center for Biotechnology Information sequence: BC010409): forward primer: 5′-TACAGCCACTACTACACGACCAT CC-3′ (region 432-456), reverse primer: 5′-GGACA ATCCTGGAGTTCTCAATG-3′ (region 488-510); TaqMan probe: 5′-ACCTGCGGGACAAGATTCTTGGTGC-3′ (region 460-484).

Study of the homology of the amplicon sequence with any other human sequence was performed by the standard nucleotide-nucleotide BLAST (blast n) of the National Center for Biotechnology Information (http://www.ncbi. nlm.nih.gov). The results of the BLAST search showed the presence of the complete homology of the amplicon sequence only with the sequence related to the CK-19 gene. In particular, the forward primer does not recognize any CK-19 pseudogene sequences. The specificity of the assay is confirmed by the lack of any positive result among the healthy control subjects.

The fluorogenic probe was labeled at its 5′ end with the fluorescent reporter dye VIC, whereas its 3′ end was modified with the universal quencher dye TAMRA using a linker arm nucleotide. These chemical modifications and the chemical oligonucleotide synthesis were provided by Applied Biosystems.

2.8. Real-time PCR measurements

To calculate the expression of CK-19 mRNA in each sample, we referred to an external reference curve generated with total RNA extracted from the human breast carcinoma cell line (MCF-7). The reference curves ranged from 0.14 to 1.4 $\times$ 10^5 cells per 20 μL of the reverse-transcribed total MCF-7 RNA. One microgram of cDNA in a volume of

20 μL was used for each sample measurement of CK-19 mRNA expression. The PCR mix (30 μL per tube) contains 25 μL of TaqMan Universal Master Mix 2×, 300 nM of each primer, and 200 nM of the TaqMan probe. The CK-19 mRNA concentration was expressed as MCF-7 cell equivalents per microgram of total RNA.

For quantification, an external calibration curve was obtained by using external standard RNA. Total RNA was prepared from 1.4 × 10^6 MCF-7 cells (as verified by a hemocytometer). Serial dilutions of this RNA preparation in DEPC-treated water, corresponding to 0.14-140 000 MCF-7 cells, were used for cDNA synthesis to be performed in each experiment. The calibration curve was created by plotting the number of MCF-7 cells corresponding to each external standard RNA versus the value of its threshold cycle (C_T). The number of circulating CK-19 mRNA-positive cells for all of the tested samples was expressed as MCF-7 cell equivalents per microgram of total-RNA, as determined by SDS software 1.9 (Applied Biosystems), according to the external standard calibration curve. Each sample was run in triplicate, and each experiment included a positive control (20 ng of reverse-transcribed RNA of SKBR-3 cells) and a negative control (with no template). Moreover, for each positive sample, a further control was performed by reverse-transcribing the RNA sample without the addition of reverse transcriptase; these samples were then submitted to the real-time PCR amplification reaction. The imprecision value of the assay (coefficient of variation [CV] for C_T in the TaqMan assay), evaluated based on the standard curve points performed in triplicate, was always less than 2%.

2.9. Laser capture microdissection of tumor cells on filters

2.9.1. Laser pressure catapulting, DNA extraction, and HER-2 real-time PCR assay

Laser microdissection of epithelial tumor cells was performed on KL1 immunostained filters. Single spots were cut from the membrane and mounted with nail polish on a 0.17-mm-thick slide. Slides were then placed on a robot-stage microscope equipped with a 337-nm pulsed laser microbeam (PALM, Munich, Germany). Groups of tumor cells were dissected with the laser microbeam and then catapulted with a single laser shot into the lid of a microfuge tube [10]. The collected cell was then recovered in 20 μL of lysis buffer (100 mmol/L Tris-HCl [pH 8] and 400 mg/mL proteinase K). After lysis at 37°C for 16 hours, the sample was spun down by centrifugation, and proteinase K was inactivated at 90°C for 10 minutes. Of 20 μL of cell lysate, 5 μL was used for determining the HER-2 degree of amplification by real-time PCR, as previously reported by Lehmann et al [20]. Briefly, the primer and probes sequences for the target gene HER-2 and the reference gene amyloid precursor protein (APP, chromosome 4q11-q13) were as follows: (1) HER-2 forward primer

5′-AGCCTCTGCATTTAGGGATTCTC-3′; reverse primer 5′-CTAGCGCCGGGACGC-3′; probe 5′[TET]-TGA-GAACGGCTGCAGGCAACCC-3′[BHQ TET] and (2) APP forward primer 5′-TCAGGTTGACGCCGCTGT-3′; reverse primer 5′-TTCGTAGCCGTTCTGCTGC-3′; probe 5′[FAM]-ACCCCAGAGGAGCGCCACCTG-3′[BHQ FAM]. The PCR amplification for both HER-2 and APP genes was performed using 96-well tray and optical caps (Applied Biosystems) with a 25-μL reaction mixture containing 300 nmol/L of each primer, 200 a nmol/L probe, and 12.5 μL of Universal Master Mix (Applied Biosystems). Six standard DNA solutions (5-fold dilutions ranging from 50 to 0.016 ng per tube) and 2 control DNA (MCF-7 HER-2 unamplified DNA and SKBR-3 HER-2 amplified DNA) were processed in each run. Relative copy number was evaluated as a ratio of the absolute quantities of HER-2 versus the APP gene.

2.9.2. Statistical analysis

The χ^2 and student t tests were used to compare ISET and real-time PCR results for CK-19 mRNA detection. Linear regression analysis and evaluation of the Pearson coefficient was applied to establish the correlation between HER-2 amplification data obtained using DNA extract from CTCs and from the primary tumor. Data analysis was carried out with the SPSS statistical package, version 11.5 (SPSS Inc, Chicago, Ill).

3. Results

3.1. Morphological analysis of CTCs using the ISET method

Morphological analysis of epithelial cells, after KL1 immunostaining, showed that there is no damage to cell morphology after ISET. Leukocytes are also retained on the membrane in a low percentage, often trapped in the pore lumen. However, they are easy to recognize because of their smaller size and nuclear morphology. All carcinoma cells were positive for cytokeratin immunostaining. The staining was particularly intense in MCF-7 cells but weaker on the patients' circulating tumor cells (Fig. 1A and B).

3.2. Sensitivity of ISET method

The sensitivity test was performed adding different amounts of MCF-7 breast carcinoma cells to 1 mL of a normal blood pool. The results demonstrated a sensitivity threshold for ISET of 1 carcinoma cell per milliliter of blood (data not shown), as previously reported by Vona et al [17].

3.3. Isolation by size of epithelial tumor cells evaluation of breast carcinoma blood samples

Blood samples from the 44 patients with breast carcinoma were submitted to ISET. Of 44 samples, 12 showed the presence of epithelial cells on the filter ranging in number
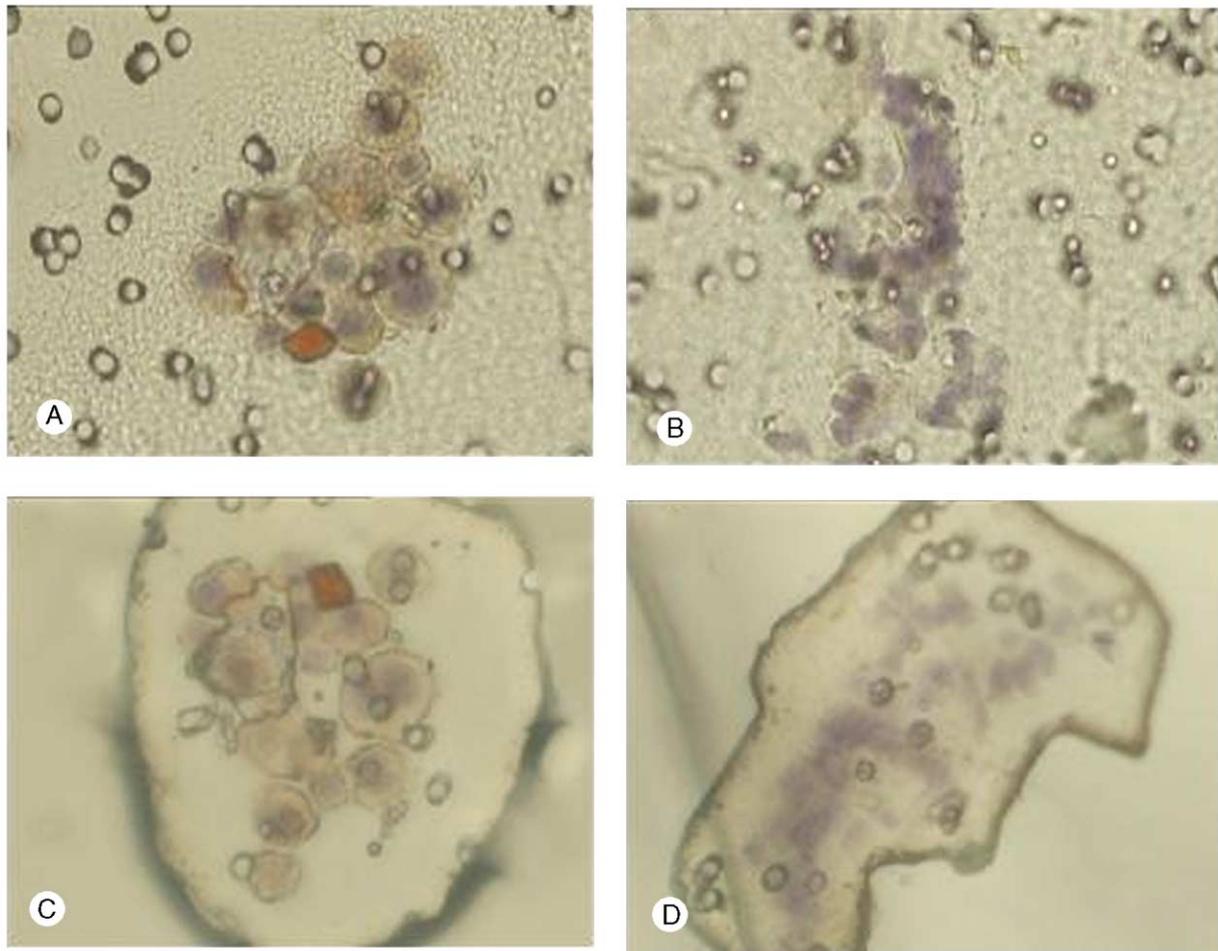
Fig. 1 MCF-7 cells (A) and epithelial cells from a patient with breast cancer (B) on a filter after KL1 cytokeratin immunostaining. After laser pressure catapulting, the microdissected cells can be recovered on the lid of the collection tube (C and D, respectively).

from 1 to 30 cells per milliliter of blood (mean $\pm$ SE: 8.5 $\pm$ 2.4; n = 12). An example of a positive filter is reported in Fig. 1B. None of the control patients gave positive results on the filter.

3.4. Standard curve for CK-19 RT-PCR-positive cell quantification

Calibration curves from these data showed linearity over the entire quantification range and correlation coefficients of 0.99 in all of the cases, indicating a precise log-linear relationship. The mean slope and intercept of the CK-19 calibration curve was 3.15 $\pm$ 0.08 (CV = 2.65%; n = 5) and 36.94 $\pm$ 1.15 (CV = 3.1%; n = 5), respectively, whereas the PCR efficiency expressed as E = $10^{\wedge}(-1$ per slope) was 2.078 $\pm$ 0.04 (CV = 1.9%; n = 5).

To verify sensitivity, linearity, and extraction efficiency of our CK-19 real-time RT-PCR method, we created 10 samples by adding different numbers of MCF-7 cells (from 5.0×10^4 to 1.0 cells) to 1 mL of a blood pool. Each sample was extracted singularly and 1 μg of the total RNA was measured

by real-time RT-PCR method. Linear regression analysis obtained by plotting log (number of cells per milliliter) versus C_T gave the following result: $y = -3.1281x + 37.543$, $r = 0.945$, showing a good parallelism with those obtained with our calibration curve (data not shown). The sample containing 1 cell per milliliter of blood corresponded to 0.1 MCF cell equivalents per microgram of total RNA, and this was considered the detection limit of the CK-19 method.

3.5. Real-time quantification of CK-19 mRNA-positive cells in peripheral blood from patients with breast cancer

The proposed method was applied in a total of 84 peripheral blood samples obtained from 40 healthy female volunteers, 39 patients with early breast cancer (stage I/II), and 5 patients with breast cancer (stage III). No positive samples were found among the group of healthy female volunteers.

On the contrary, 27% (12/44) patients with breast cancer showed the presence of CK-19 mRNA-positive cells in

blood. The mean CK-19 mRNA level in positive samples was 0.55 ± 0.39 MCF-7 equivalents per microgram of total RNA (range, 0.13-1.34). As expected, none of the reactions that used a reverse transcription template obtained in the absence of reverse transcriptase gave a positive result.

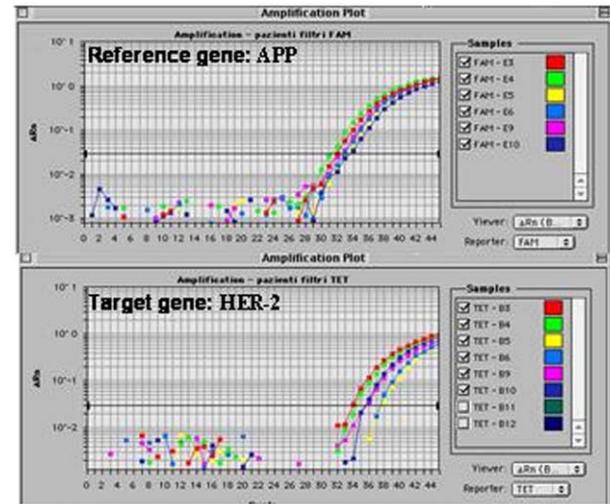## 3.6. Correlation between real-time RT-PCR and ISET results on patients with breast cancer

A statistically significant correlation was found when analyzing samples obtained by CK-19 mRNA expression in real-time RT-PCR and ISET on the 44 patients with breast cancer under investigation. Ten patients were positive and 32 were negative with both methods, whereas in 2 samples circulating tumor cells could be detected only by real-time RT-PCR and 2 samples only by ISET ($P < .001$) (Table 1). Cytokeratin-19 mRNA expression of the 2 ISET-negative samples resulted very close to the detection limit of the CK-19 method (0.13 and 0.28 MCF cell equivalents per microgram of total RNA). On the other hand, with regard to the 2 ISET-positive/CK-19–negative samples, 1 showed no signal for CK-19 mRNA after 50 PCR cycles, whereas the second sample could be amplified even if the CK-19 value did not reach the established cut-off for sensitivity (mean $C_T = 44.03$, corresponding to 0.0083 MCF cell equivalents per microgram of total RNA).

3.7. Feasibility of HER-2 amplification in microdissected epithelial cells

Laser microdissection of KL1 immunostained cells on the filter is illustrated in Fig. 1. The membrane around a cell group was precisely cut by the laser microbeam and catapulted by laser pressure. Inversion of the optical device allowed a check that the cell had been collected into the microfuge lid (Fig. 1C and D).

Amplification plots for both the APP reference gene and HER-2 gene are reported for 2 exemplificative samples (Fig. 2). In the same figure, example of the calculation of the degree of HER-2 amplification of the 2 samples is reported. Patient 1 shows no amplification of the oncogene, whereas in patient 2, results are indicative of an amplified sample.

In Table 2, data of HER-2 amplification, as evaluated in DNA extracted from CTCs obtained by laser pressure catapulting from 7 ISET-positive filters, are reported. Selection of patients was made based on immunohistochemistry performed on the primary tumors (DAKO test) and the level of HER-2 amplification in the paraffin-embedded

Table 1 Correlation between the real-time RT-PCR method for CK-19 mRNA detection and ISET (χ^2 test)

	CK-19–positive (n = 12)	CK-19–negative (n = 32)	P
ISET-positive (n = 12)	10	2	<.001
ISET-negative (n = 32)	2	30	

Fig. 2 Amplification plot generated by real-time PCR on DNA extracted by CTCs recovered from ISET-positive filters. The upper panel shows results for the reference gene APP; the lower panel reports data for the target gene HER-2. The table summarizes the calculation to obtain HER-2 amplification.

section of the primary tumor to test different nonamplified (n = 3) and amplified (n = 4) samples. Good correspondence between results obtained from microdissected CTCs and those obtained using DNA extracted from the primary tumor was highlighted by linear regression analysis of the data ($R = 0.918$, $P < .01$). Moreover, no significant difference was found when comparing the results of HER-2 amplification

Table 2 HER-2 amplification as determined by real-time PCR on DNA extracted from CTCs collected by laser microdissection from selected ISET-positive filters

Patient	HER-2 amplification in filter microdissected CTCs	HER-2 amplification in the primary tumor	DAKO test
5	3.8	2.1	3+
8	4.5	4.3	2+
9	3.0	2.6	2+
10	3.8	3.3	3+
14	1.2	1.2	0
30	1.1	0.9	0
41	0.8	1.0	0

NOTE. Results of HER-2 amplification and immunohistochemistry (DAKO test) performed on the primary tumors are also reported for comparison.

obtained on microdissected CTCs and on primary tumor, as divided based on the DAKO test results.

4. Discussion

The present study was directed to investigate patients with breast cancer (mainly early breast cancer) to demonstrate the feasibility of the detection and isolation of CTCs from peripheral blood samples. Thus, a cytometric approach, the ISET method [17,20-22], was chosen for the isolation and the immunomorphological and molecular characterization of circulating tumor cells. Moreover, a new real-time RT-PCR method was developed, the analytical characteristics (specifically referring to sensitivity, specificity, and accuracy) of which seem to fulfill the criteria for its application in the detection of CTCs in peripheral blood of patients with early breast cancer. The significant correlation of ISET results with those obtained by the real-time RT-PCR method based on the identification of CK-19–positive cells in peripheral blood confirms the suitability of the cytometric technique to study these kinds of cancer cells in patients with early breast cancer. The sensitivity and specificity of the 2 methods are comparable: in 2 of 44 samples, circulating tumor cells could be detected only by real-time RT-PCR and, in 2 of 44 samples, only by ISET. With regard to the 4 discrepant results: (1) CK-19 levels in ISET-negative samples were very close to the detection limit of the real-time RT-PCR method; (2) of the 2 ISET-positive/CK-19–negative samples, 1 effectively showed no signal for CK-19 mRNA after 50 PCR cycles, whereas the second sample could be amplified even if the CK-19 value did not reach the established cut-off for sensitivity. However, it is difficult to find an exact correspondence between the number of CTCs and the mRNA levels found because these cells are highly heterogeneous and may express different levels of marker gene cDNAs [23].

On this basis, the performance of the ISET method could be considered equivalent to the real-time RT-PCR method for CK-19–positive cell detection. Recently, Ring et al [24] published an invaluable evaluation of 3 different techniques (including cell filtration) for the detection of circulating epithelial cells in the blood of patients with breast metastatic cancer. Although they indicate a multimarker real-time RT-PCR assay as the most sensitive technique for this purpose, they themselves affirm that ascertaining a circulating, individual cell phenotype could significantly enhance our understanding of the mechanism of resistance and facilitate target therapy, but this is likely to be possible only when using cytometric technique [24].

Indeed, ISET allows us to isolate CTCs on a filter and to subsequently characterize them by means of cell immunostaining (Fig 1A and B). Furthermore, CTCs can be recovered by laser microdissection (Fig 1C and D) and their DNA amplified and screened for genetic abnormalities in target sequences.

Thus, the main advantage of ISET is the possibility to have a preoperative evaluation of prognostic markers, such as HER-2/neu amplification, just by collecting a blood sample and, thus, avoiding invasive approaches. In some instances, this test could be used to assess presurgery tailored therapy in patients carrying specific genetic abnormalities (ie, Herceptin treatment).

Our results on HER-2 amplification support the feasibility of this approach and represent a tool to compare the genetic features of CTCs and cells constituting the tumor mass in the primary site and/or metastatic ones. Our results, although taken from a limited number of samples, demonstrate a significant correlation between HER-2 amplification, as measured in CTCs and in the primary tumor, and is in agreement with the DAKO test results. The study is ongoing in the attempt to extract RNA from laser-microdissected CTCs from ISET-positive filters to thus proceed to the study of the expression pattern of these tumor-deriving cells and to highlight the expression features that determine their metastatic potential. Overall, ISET constitutes a novel approach that should open up new perspectives in molecular medicine.

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