

Molecular Characterization of Circulating Tumor Cells in Breast Cancer by a Liquid Bead Array Hybridization Assay

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BACKGROUND: Molecular characterization of circulating tumor cells (CTCs) is crucial to identify novel diagnostic and therapeutic targets for individualized therapies. We developed a multiplexed PCR-coupled liquid bead array to detect the expression of multiple genes in CTCs.

METHODS: mRNA isolated from immunomagnetically enriched CTCs was subjected to multiplex PCR for *KRT19* (keratin 19; also known as *CK19*), *ERBB2* [v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian); also known as *HER2*], *SCGB2A2* (secretoglobulin, family 2A, member 2; also known as *MGB1*, mammaglobin A), *MAGEA3* (melanoma antigen family A, 3), *TWIST-1* [twist homolog 1 (*Drosophila*)], and *HMBS* (hydroxymethylbilane synthase; also known as *PBGD*). Biotinylated amplicons were hybridized against fluorescent microspheres carrying gene-specific capture probes and incubated with streptavidin-phycoerythrin. We quantified the captured amplicons and decoded the beads by Luminex flow cytometry. The assay was validated for limit of detection, specificity, and comparison with reverse-transcription quantitative PCR (RT-qPCR), and its clinical performance was evaluated in 64 patients with operable breast cancer, 20 patients with metastasis, and 17 healthy individuals.

RESULTS: The assay was specific for each gene in complex multiplexed formats and could detect the expression of each gene at the level of a single SK-BR-3 cell. The assay produced results comparable to those for RT-qPCR for each gene. None of the genes tested was detected in the CTC fraction of healthy donors. We detected *KRT19*, *ERBB2*, *MAGEA3*, *SCGB2A2*, and *TWIST1* in 26.6%, 12.5%, 18.7%, 10.9%, and 31.2% of operable breast cancer patients, respectively, and detected the corresponding

genes in 65%, 20%, 30%, 20%, and 20% of patients with verified metastasis, respectively.

CONCLUSIONS: The expression of 6 genes in CTCs can be measured simultaneously and reliably, thereby saving precious sample and reducing the costs and time of analysis.

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Circulating tumor cells (CTCs)⁴ are well-defined targets for understanding tumor biology and tumor cell dissemination (1). Data from European groups have sustained the prognostic impact of disseminated tumor cells in the bone marrow of breast cancer patients (2). Several clinical studies have established CTC detection and enumeration in breast cancer and have shown a correlation with decreased progression-free survival and overall survival in operable (3–7) and advanced (8) breast cancer. Our group has shown that the detection of CTCs in the peripheral blood of patients with early breast cancer before and after chemotherapy and the detection of postchemotherapy CTCs in breast cancer patients (9) are both of prognostic significance (3–7). Detection of CTCs is associated with prognosis for many human cancers, such as those of the breast, lung, and prostate, and their enumeration and molecular characterization can be used as a liquid biopsy for repeated follow-up examinations (10, 11).

CTCs are highly heterogeneous, and their molecular characterization is important not only to confirm their malignant origin but also to follow immunophenotypic changes with tumor progression for identifying diagnostically and therapeutically relevant targets to help stratify cancer patients for individual therapies (10–13). The production of recognized prognostic factors in CTCs, such as that encoded by *ERBB2*⁵ [v-erb-b2 erythroblastic leukemia viral oncogene homolog 2,

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⁴ Nonstandard abbreviations: CTC, circulating tumor cell; RT-qPCR, reverse-transcription quantitative PCR; FDA, US Food and Drug Administration; PBMC, peripheral blood mononuclear cell; NCBI, National Center for Biotechnology

Information; nt, nucleotides; TMAC buffer, 4.5 mol/L tetramethyl ammonium chloride, 1.5 g/L SDS, 75 mmol/L Tris (pH 8.0), and 3.0 mmol/L EDTA (pH 8.0); MFI, median fluorescence intensity.

⁵ Human genes: *ERBB2*, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (also known as *HER2*); *KRT19*, keratin 19 (also known as *CK19*); *SCGB2A2*, secretoglobulin, family 2A, member 2 (also known as *MGB1*, mammaglobin A); *EPCAM*, epithelial cell adhesion molecule; *MUC1*, mucin 1, cell surface associated; *TWIST1*, twist homolog 1 (*Drosophila*); *MAGEA3*, melanoma antigen family A, 3; *HMBS*, hydroxymethylbilane synthase (also known as *PBGD*).

neuro/glioblastoma derived oncogene homolog (avian); also known as *HER2*] (12–14), as well as such cancer stem cell markers as CD44, CD24, and aldehyde dehydrogenase (15, 16), has also been demonstrated.

Recent progress in cancer research has identified many targets of interest in CTCs, but the fact that CTCs are very rare and the amount of available sample is very limited presents a tremendous analytical and technical challenge (17–19). Recent technical advancements in CTC detection and characterization include reverse-transcription quantitative PCR (RT-qPCR) methods (20–22); image-based approaches, such as the US Food and Drug Administration (FDA)-cleared CellSearch system (Veridex) (8, 23); and a combination of molecular and imaging methods (24). A membrane microfilter device for single-stage capture and electrolysis of CTCs has been introduced (25), and a microchip for CTC isolation and analysis has been developed (26). By using a multimarker assay for CTC in early breast cancer, we have shown that CTCs positive for *KRT19* (keratin 19; also known as *CK19*), *SCGB2A2* (secretoglobin, family 2A, member 2; also known as *MGB1*, mammaglobin A), and *ERBB2* are associated with shorter disease-free survival (6). Recently, *EPCAM* (epithelial cell adhesion molecule), *MUC1* (mucin 1, cell surface associated), and *ERBB2* transcripts were detected in CTCs, and a major proportion of CTCs in metastatic breast cancer patients showed epithelial-to-mesenchymal transition and tumor stem cell characteristics (16).

The most important limitation of all available methodologies for CTC analysis, however, is the amount of sample required and the small number of gene targets that can be analyzed. Liquid bead array hybridization assays have been successfully used in immunoassays and molecular diagnostics (27).

We describe the development and validation of a liquid bead array hybridization assay for studying the expression of 6 genes in a very limited amount of CTC sample. The genes selected are established markers for CTCs: *KRT19*, a specific epithelial marker of prognostic significance (3–7, 9); *SCGB2A2*, a specific marker for mammary gland (6); *ERBB2*, which gives important information about response to therapy (12, 13); *TWIST1* [twist homolog 1 (*Drosophila*)], a marker of epithelial-to-mesenchymal transition; and *MAGEA3* (melanoma antigen family A, 3), the expression of which correlates with metastasis. *HMBS* (hydroxymethylbilane synthase; also known as *PBGD*) was used as a reference gene. With the methodology we have developed, one can measure the expression of these genes simultaneously and reliably in CTCs, thereby saving precious sample and reducing the costs and time of analysis.

Materials and Methods

CELL LINES

We used human mammary carcinoma cell lines SK-BR-3 and MDA-MB-231 to develop the assay and to generate *KRT19*, *ERBB2*, *MAGEA3*, *SCGB2A2*, *TWIST1*, and *HMBS* external calibrators. We counted cells with a hemocytometer and assessed their viability by trypan blue exclusion. We prepared serial dilutions of known numbers of SK-BR-3 cells (1–1000 cells), isolated total RNA from each serial dilution, and synthesized cDNA from each of the RNA preparations. These cDNAs were kept in aliquots at -20°C . We used these aliquots to validate assays before we began the analysis of patient samples.

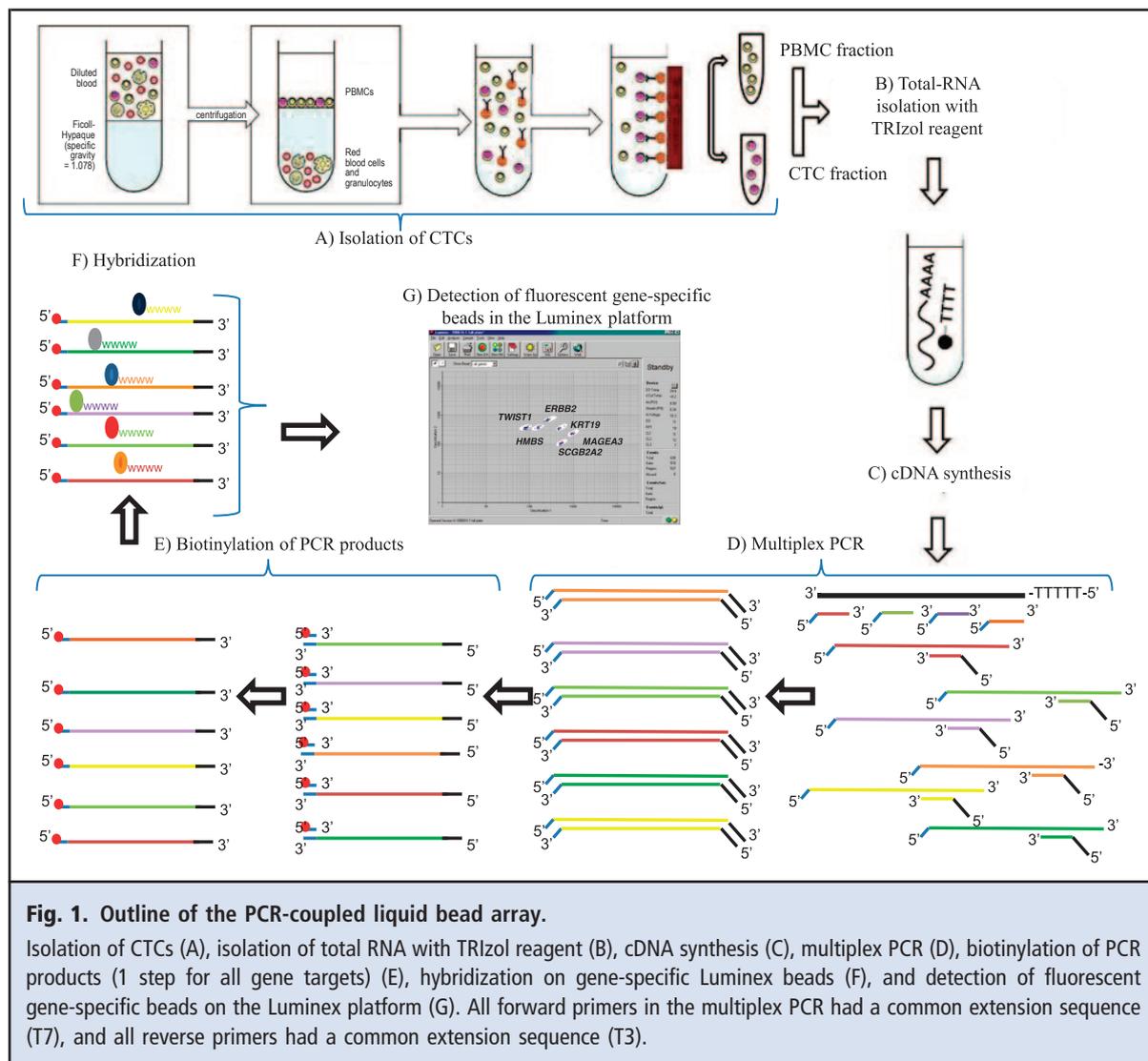
PATIENTS

We studied 84 consecutive patients with breast cancer: (a) 64 patients with operable breast cancer (stage I to III) at least 2 weeks after the removal of the primary tumor and before the initiation of adjuvant chemotherapy; and (b) 20 patients with verified metastasis. Samples from 17 healthy female blood donors were used as controls. Peripheral blood samples (20 mL in EDTA) were obtained as previously described (20, 21). All patients signed an informed-consent form to participate in the study, which was approved by the ethics and scientific committees of our institution.

CTC ISOLATION, RNA EXTRACTION, AND mRNA PURIFICATION

The liquid bead array assay we developed for evaluating gene expression in CTCs is outlined in Fig. 1. After diluting the peripheral blood sample with 20 mL PBS (pH 7.4, Gibco), we obtained peripheral blood mononuclear cells (PBMCs) by gradient density centrifugation with Ficoll-Paque™ PLUS (GE Healthcare BioSciences) at 670g for 30 min at room temperature. The interface cells were removed, washed twice with 40 mL of sterile PBS (pH 7.3, 4 °C) followed by centrifugation at 530g for 10 min, and resuspended in 1 mL PBS. The cells were dyed with trypan blue and counted in a hemocytometer. Immunomagnetic BerEP4-coated Dynabeads® (CELLlection™ Epithelial Enrich; Invitrogen) were used according to the manufacturer's instructions to enrich for epithelial cells. We kept and analyzed both the CTCs and the corresponding PBMC fraction for each sample.

We used TRIzol® reagent (Invitrogen) according to the manufacturer's instructions to isolate total RNA from CTCs and PBMC fractions. All RNA preparation and handling steps took place in a laminar-flow hood under ribonuclease-free conditions. The isolated total RNA was dissolved in 20 μL of RNA Storage Buffer (Ambion) and stored at -70°C . The RNA concentration was measured with a NanoDrop 1000 spectropho-



tometer (Thermo Scientific). For each sample, we used the Dynabeads mRNA Purification Kit (Invitrogen) according to manufacturer's instructions to isolate mRNA from total RNA.

cDNA SYNTHESIS

We used the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) to perform reverse transcription. In all cases, we used 9 μ L of isolated mRNA as template to maximize the detection capability. To test for RNA integrity, we amplified the *HMBS* gene in all samples.

PRIMER DESIGN

Primers and capture probes for *KRT19*, *ERBB2*, *MAGEA3*, *HMBS*, *SCGB2A2*, and *TWIST1* were designed de novo in silico, synthesized by the Foundation

for Research and Technology, and evaluated for their performance (see Table 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol57/issue3>). We designed primers in silico with Primer Premier 5.0 software (Premier Biosoft International) to avoid primer-dimer formation, false priming sites, formation of hairpin structures, and homology with the other genes. All primers and probes were designed to match the assay conditions, such as amplicon sizes and melting temperatures. The specificities of all primer and hybridization probe sequences were first tested by homology searches in the nucleotide database [Nucleotide BLAST; National Center for Biotechnology Information (NCBI)].

Upstream primers consisted of the T7 common extension sequence (5'-TAATACGACTCACTATAGGG-

3') and about 20 nucleotides (nt) of gene-specific sequence. Downstream primers consisted of about 20 nt of gene-specific sequence and the T3 primer site (5'-ATTAACCCTCACTAAAGGGA-3') at the 5' end. Capture probes were designed to match in length a gene-specific sequence (about 30 nt) complementary to the biotinylated strand of the multiplex PCR products, and they were modified with 5' amino modifier C₁₂ to provide a terminal amino group and spacer for coupling to the carboxylated fluorescent microspheres (xMAP Carboxylated Microspheres; Luminex Corporation).

MULTIPLEX (6-PLEX) PCR

We carried out multiplex PCR with 2 μ L cDNA in a final volume of 25 μ L. A PCR negative control containing no target was included in each assay run. The reaction consisted of 12.5 μ L Master Mix (Qiagen Multiplex PCR Kit; Qiagen), 2.5 μ L Q-Solution, and 0.08 μ mol/L of each primer (6 upstream primers and 6 downstream primers). Samples were cycled in an Eppendorf Mastercycler. After we optimized the conditions for all primer pairs, the final PCR conditions were as follows: 95 °C for 15 min and 35 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. Samples were then held at 72 °C for 10 min and kept at 4 °C until use.

BIOTINYLATION OF MULTIPLEX PCR PRODUCTS

We then used a biotinylated T7 primer common for all 6 genes for further biotinylation in a 10-cycle primer-extension reaction in a 20- μ L total reaction volume. Multiplex PCR products (2 μ L) were placed in an 18- μ L reaction volume containing 1 μ L of a 2- μ mol/L solution of the sense biotinylated T7 primer, 0.4 μ L of a solution containing 10 mmol/L of each deoxynucleoside triphosphate, 1 μ L of 50 mmol/L MgCl₂, 0.2 μ L of 5 U/ μ L Platinum *Taq* DNA Polymerase and 2 μ L of 10 \times PCR buffer included with this enzyme (Invitrogen), and 13.4 μ L of diethylpyrocarbonate-treated water. The PCR reaction conditions were as follows: denaturation at 95 °C for 5 min; 10 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s; and a final extension step at 72 °C for 10 min.

COUPLING OF GENE-SPECIFIC CAPTURE PROBES TO THE FLUORESCENT MICROSPHERES

Each gene-specific capture probe was designed in silico to be highly specific for an internal gene-specific sequence of the corresponding amplicon sequence. All capture probes had a reactive amino group with a 12-carbon spacer separating the reactive group from the 5' end of the oligonucleotide for optimum hybridization. Individual gene-specific capture probes were coupled to spectrally distinct fluorescent carboxylated beads that had been internally dyed with a unique spectral address by a modification of the carbodiimide-

coupling method, as has previously been described (27). We stored each probe-bead conjugate separately in the dark at 4 to 8 °C and prepared a fresh bead set containing all conjugates for each run.

BEAD ARRAY HYBRIDIZATION

We prepared a bead set consisting of 2500 beads of each of 6 gene-target conjugates in 1.5 \times TMAC buffer [4.5 mol/L tetramethyl ammonium chloride, 1.5 g/L SDS, 75 mmol/L Tris (pH 8.0), and 3.0 mmol/L EDTA (pH 8.0)] to a final volume of 43 μ L. We added 7 μ L of the amplified PCR product to the bead mix (43 μ L), denatured the PCR products and bead mix at 95 °C for 2 min, and allowed the suspension to hybridize at 60 °C for 60 min. The coupled microspheres were pelleted by microcentrifugation at 11 340g for 4 min, the supernatant was removed, and the microspheres were resuspended in 75 μ L of reporter solution [10 μ g/mL streptavidin-conjugated phycoerythrin in hybridization buffer (1 \times TMAC)] and incubated at room temperature for 15 min.

BEAD ANALYSIS

Resuspended microspheres were placed in 96-well microtiter plates and analyzed with a Luminex 100 instrument (Luminex Corporation). The sample volume was set at 50 μ L, and the flow rate was 60 μ L/min. A minimum of 100 events were recorded for each bead set, median fluorescence intensities (MFIs) were computed, and analysis was completed in 60 s for each sample.

Results

DEVELOPMENT AND COMPARISON OF DIFFERENT LIQUID BEAD ARRAY HYBRIDIZATION PROTOCOLS FOR CTC GENE EXPRESSION

To develop a highly specific method with a low detection limit for gene expression in CTCs, we initially designed 6 different hybridization protocols and compared their performance. The main differences between these protocols were the primer sequences used, the biotinylation approach, and the different designs for hybridization on the Luminex beads.

Protocol A. Multiplex PCR products were biotinylated with biotinylated dCTP in an allele-specific primer extension PCR (27) (see Fig. 1A in the online Data Supplement).

Protocol B. cDNA was synthesized in a solid phase, and single-strand PCR was performed with specific forward primers that contained a common extension at the 5' end (see Fig. 1B in the online Data Supplement).

Protocol C. All forward primers used in the multiplex PCR were biotinylated at the 5' end. All reverse primers

had a specific extension complementary to the coupling sequence of specific commercially available Luminex beads (see Fig. 1C in the online Data Supplement).

Protocol D. In this protocol, forward primers for each gene had a common extension, followed by another specific sequence complementary to the coupling sequence of specific commercially available Luminex beads (see Fig. 1D in the online Data Supplement).

Protocol E. The previously described ligation-based approach was used for liquid bead array hybridization assays (27).

Protocol F. In this protocol, all forward primers in the multiplex PCR had a common extension sequence (T7); all reverse primers also had a common extension sequence (T3). Biotinylation of PCR products took place in 1 step for all gene targets, followed by hybridization on gene-specific Luminex beads and detection of fluorescent gene-specific beads on the Luminex platform (Fig. 1).

After extensive evaluation and comparison experiments, we selected protocol F (Fig. 1) for further optimization and validation because it gave the best results for duration, limit of detection, and simplicity. In this protocol, 6 genes were amplified simultaneously via multiplex PCR with specific primers in which the 5' end contained a gene-specific sequence plus a sequence common to all gene targets (T7 for forward primers and T3 for reverse primers). The PCR was performed for all 6 gene targets with a common set of primers, T7 (biotinylated at the 5' end), and T3. Biotinylated amplicons were hybridized against a pool of 6 sets of optically addressed fluorescent microspheres, with each set carrying immobilized capture probes complementary to a sequence specific for each target gene, and were further incubated with streptavidin-phycoerythrin and fluorescently labeled biotin moieties. Captured labeled amplicons were quantified, and the beads were decoded by flow cytometry on the Luminex platform.

ASSAY OPTIMIZATION

To optimize the assay, we used total RNA from cancer cell lines SK-BR-3 and MDA-MB-231. In all cases, optimized conditions were selected according to the best signal-to-noise ratio. The conditions for multiplex PCR were established in a series of preliminary experiments that evaluated the number of PCR cycles (see Fig. 2A in the online Data Supplement) and the temperature program (data not shown). The hybridization protocol was optimized according to the number of fluorescent microspheres used for each gene target (see Fig. 2B in the online Data Supplement), the tempera-

ture of the hybridization step (see Fig. 2C in the online Data Supplement), and the volume of biotinylated PCR products used in each array (see Fig. 2D in the online Data Supplement). We also studied the effect of the size of the PCR product, after we noticed in our initial experiments that the MFI signals we obtained for the *ERBB2* and *SCGB2A2* gene targets were lower than those for the *KRT19*, *HMBS*, *MAGEA3*, and *TWIST1* genes. We hypothesized that by reducing the length of the target sequence to the range of 100–300 bp, we could minimize steric hindrances that affect hybridization efficiency at the microsphere surface. For this reason, we designed and tested 2 different primer pairs for *ERBB2* and *SCGB2A2*. One pair amplified short sequences, and the other pair amplified longer sequences (see Table 1 in the online Data Supplement). We found that the smaller the size of the PCR product, the stronger the MFI signals (see Fig. 2E in the online Data Supplement).

VALIDATION OF THE LIQUID BEAD ARRAY FOR CTC GENE EXPRESSION

Specificity. We checked the analytical specificities of the primers designed and used for multiplex PCR, as well as those for the capture probes designed and immobilized on the fluorescent microspheres, both in the presence of and in the absence of each gene target.

First, we assessed analytical specificity when only 1 gene target was used as a template. For this experiment, the biotinylated multiplex PCR products of PCRs performed in the presence of only 1 gene target and all primer pairs were hybridized in the presence of all 6 microspheres, in which the capture probes were immobilized for all of the gene targets. We did not observe any of the nonspecific interactions between the 18 oligonucleotides used (3 for each gene target) that theoretically could have occurred (Fig. 2A); therefore, we concluded that the assay was able to discriminate expression specifically for each gene target.

Second, we assessed analytical specificity in the absence of each gene target. For this experiment, biotinylated PCR products of multiplex PCRs that had been performed in the absence of only 1 gene target and in the presence of all primer pairs were hybridized in the presence of all 6 microspheres, in which the capture probes were immobilized for all gene targets. The MFI signal showed high specificity for each of the 6 gene targets (Fig. 2B).

Limit of Detection. A low detection limit is extremely important for CTC analysis. For this reason, before we proceeded to the patient samples, we evaluated the limit of detection of the developed bead array assay with total RNA from known numbers of SK-BR-3 cells. Serial

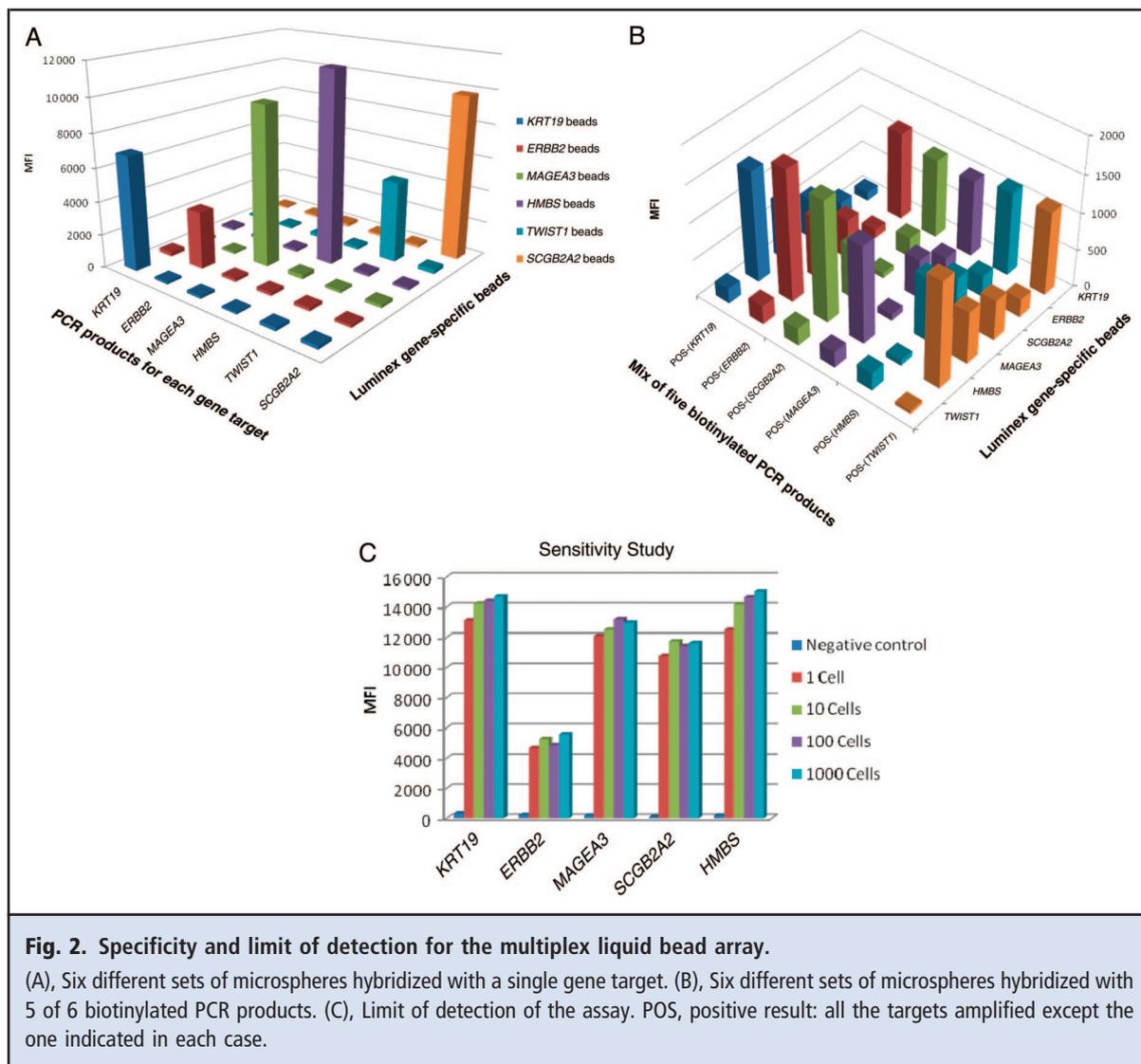


Fig. 2. Specificity and limit of detection for the multiplex liquid bead array.

(A), Six different sets of microspheres hybridized with a single gene target. (B), Six different sets of microspheres hybridized with 5 of 6 biotinylated PCR products. (C), Limit of detection of the assay. POS, positive result: all the targets amplified except the one indicated in each case.

dilutions corresponding to 1, 10, 10^2 , 10^3 , and 10^4 tumor cells were used for cDNA synthesis, kept in aliquots at -20°C , and then run in triplicate. The detection limit was found to correspond to 1 SK-BR-3 cell for *KRT19*, *ERBB2*, *SCGB2A2*, *HMBS*, *TWIST1*, and *MAGEA3* (Fig. 2C; see Table 2 in the online Data Supplement).

Precision. We evaluated intraassay variance (within-run imprecision) by analyzing a sample of total RNA isolated from 100 SK-BR-3 cells and 100 MDA-MB-231 cells. We analyzed the RNA in the same run in 3 parallel determinations and followed the entire analytical procedure. We evaluated interassay variance (between-run imprecision) by analyzing the same cDNA sample (aliquots of which were kept frozen at

-20°C) over a 1-month period. We performed 5 separate assays on 5 different days. Intraassay CVs ranged from 0.5% to 10%, and interassay CVs ranged from 6.8% to 17% (Table 1).

CLINICAL EVALUATION

We analyzed both the CTC fraction and the corresponding PBMC fraction from each patient. We investigated the RNA quality for all samples by evaluating *HMBS* expression. Only samples positive for *HMBS* expression were analyzed further.

We evaluated the diagnostic specificity of the developed protocol by analyzing 17 healthy female volunteers. We observed no expression for *KRT19*, *MAGEA3*, *SCGB2A2*, *TWIST1*, or *ERBB2* in the CTC

Table 1. Precision of the liquid bead array hybridization assay for CTC gene expression.

	<i>KRT19</i>	<i>ERBB2</i>	<i>MAGEA3</i>	<i>SCGB2A2</i>	<i>HMBS</i>	<i>TWIST1</i>
Intraassay precision (n = 3), MFI × 10 ³						
Sample						
1	13.4	5.7	10.7	2.3	14.9	1.30
2	13.6	4.9	10.2	2.5	14.3	1.30
3	12.8	4.9	9.1	2.4	13.5	1.29
Mean	13.3	5.2	10.0	2.4	14.2	1.30
SD	0.42	0.46	0.82	0.10	0.70	0.006
CV	3.0%	8.9%	10%	4.2%	4.9%	0.5%
Interassay precision (n = 5), MFI × 10 ³						
Day						
1	22.3	13.7	22.0	21.7	21.9	2.0
2	23.8	13.4	23.0	22.4	23.7	1.6
3	20.1	10.1	20.2	19.8	20.1	1.7
4	19.8	9.7	19.4	19.7	19.7	1.5
5	19.8	10.0	19.6	19.2	19.7	1.6
Mean	21.2	11.4	20.8	20.6	20.1	1.7
SD	1.8	2.0	1.6	1.4	1.7	0.19
CV	8.5%	17%	7.6%	6.8%	8.5%	11%

fraction of any of the healthy donors. We observed no expression for *KRT19*, *MAGEA3*, or *SCGB2A2* in the PBMC fraction from healthy donors, whereas we did detect *TWIST1* and *ERBB2* expression in all tested samples, albeit at very low levels (i.e., MFI units) (Fig. 3).

When we evaluated the CTC fractions from 64 patients with operable breast cancer after the primary cancer had been removed and before adjuvant chemotherapy had been initiated, we obtained positive results for *KRT19*, *ERBB2*, *MAGEA3*, *TWIST1*, and *SCGB2A2* expression in 17 (26.6%), 8 (12.5%), 12 (18.7%), 20 (31.2%), and 4 (10.9%) of the patients, respectively (Fig. 3A). The MFIs for this group of patients were significantly different from those of the group of healthy donors for all of the genes studied (Fig. 3B). In this group of patients, we found 36 patients (56.2%) positive for at least 1 gene, 16 (25%) positive for at least 2 genes, 7.8% positive for 3 genes, and only 6.2% positive for 4 genes. Finally, only 2 (3.1%) of 64 patients were positive for all genes, whereas no CTCs were detected in 27 (42.2%) of these patients (Fig. 4).

Our evaluation of the CTC fractions from the 20 patients with verified metastasis revealed positive results for *KRT19*, *ERBB2*, *MAGEA3*, *TWIST1*, and *SCGB2A2* expression in 13 (65%), 4 (20%), 6 (30%), 4 (20%), and 4 (20%) of the patients, respectively (Fig. 3A). The MFIs for *KRT19* and *MAGEA3* for this group of patients were significantly different from those of the

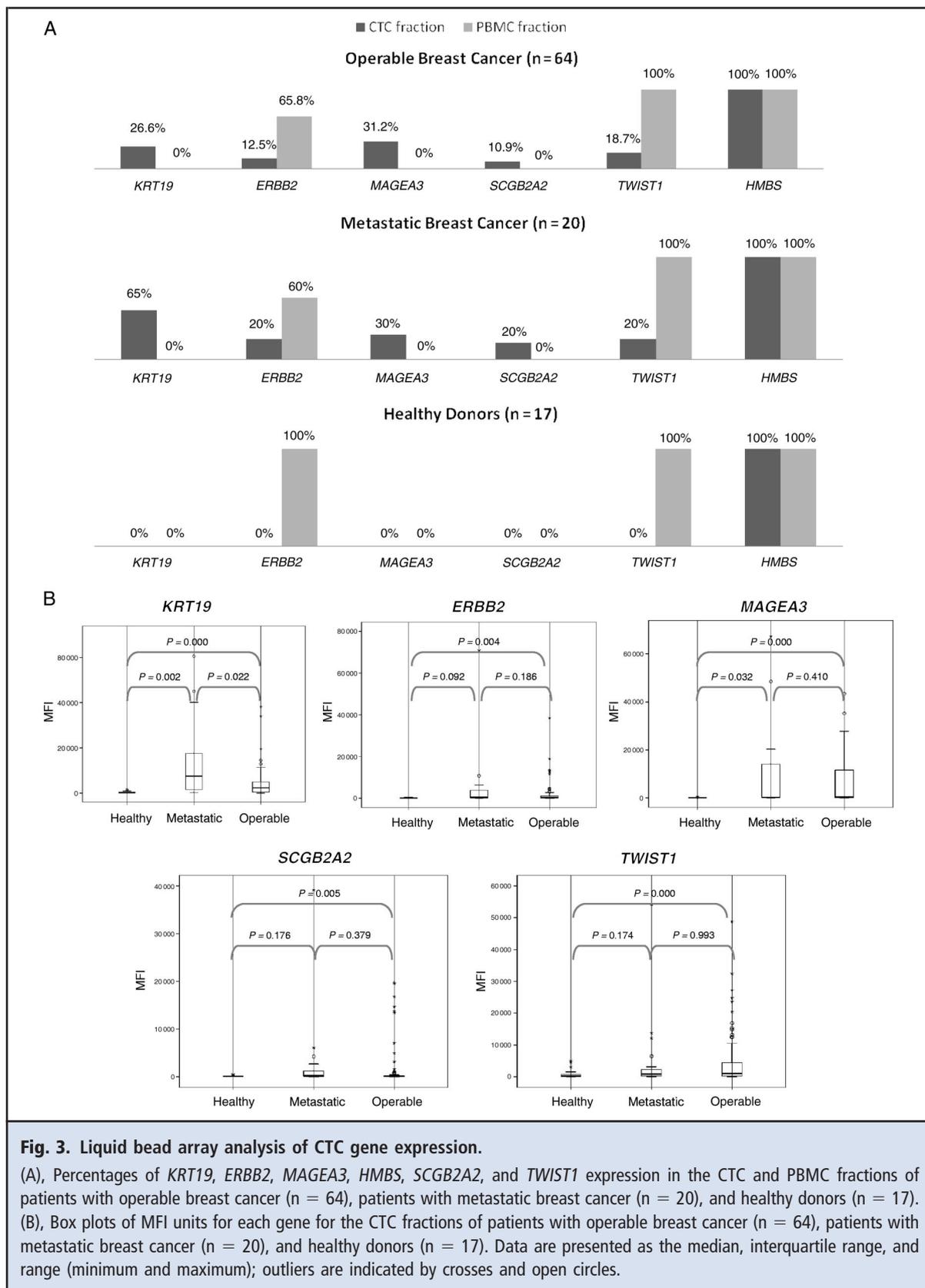
group of healthy donors (Fig. 3B). In this group of patients with metastasis, we found 6 patients (30%) positive for 1 gene, 6 (30%) positive for 2 genes, 3 (15%) positive for 3 genes, and 1 (5%) positive for all 4 of the examined genes; we detected no CTCs in 4 (20%) of these patients (Fig. 4).

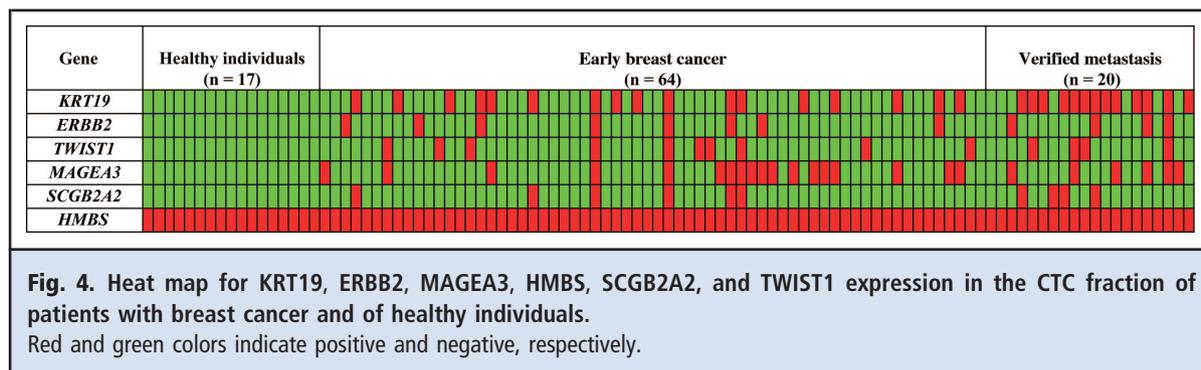
COMPARISON OF LIQUID BEAD ARRAY (LUMINEX) AND RT-qPCR ASSAYS

We evaluated the performance of the liquid bead array assay for measuring CTC gene expression by analyzing the same samples in parallel with both the developed assay and RT-qPCR. We analyzed 60 cDNA samples from operable and metastatic breast cancer patients for *KRT19* and *ERBB2*. For *TWIST1*, we had 53 cDNA samples available for testing. Our analysis of *KRT19* expression in CTCs revealed agreement between the RT-qPCR assay and the CTC gene expression array assay in 49 (82%) of 60 patient samples. For *ERBB2* expression, the 2 assays were in agreement for 51 (85%) of the patient samples. Finally, for *TWIST1* expression, we observed agreement for 42 (79%) of the 53 patient samples studied (Table 2).

Discussion

Molecular characterization of CTCs opens a new avenue for understanding early metastatic spread of tumor





cells and may be able to contribute to the identification of metastatic stem cells, with important implications for the development of improved therapies in the near future (28). Complete genomic profiles and expression

patterns have to be considered to understand the biological properties and molecular characteristics of CTCs, as well as their connection to cancer stem cells (28). Also motivating the interest in CTCs has been the recent development of molecularly targeted cancer therapies that work best on patients whose tumors have a particular mutation (29). Assessing the presence of target antigens on CTCs could be considered a real-time biopsy, allowing the possibility to evaluate the change in tumor phenotype during the clinical course of the disease (10, 11). For all these reasons, molecular characterization of CTCs is a very hot topic in cancer research nowadays (30).

Molecular characterization of CTCs, although important for identifying diagnostically and therapeutically relevant targets that could help in stratifying cancer patients for individual therapies, is difficult to address, however, because these cells are very rare and the amount of available sample is very limited. RT-qPCR methods (20–22) and image-based approaches, such as the FDA-cleared CellSearch system (8, 23), for CTC detection and characterization have the potential for detecting only a small number of targets on CTCs.

The main advantage of our liquid bead array system based on the Luminex platform over both imaging and RT-PCR methods is that it enables the reliable molecular characterization of CTCs for 6 gene targets in parallel with a very limited amount of sample. Our liquid bead array hybridization assay for CTC gene expression is specific for each gene in complex multiplexed formats and is capable of detecting the expression of each gene at the single-cell level, thereby saving precious sample and reducing the costs and time of analysis. The assay produces results comparable to those of RT-qPCR for each individual gene and has the potential to be scaled up to 100 genes.

Table 2. Comparison of CTC gene expression bead array and RT-qPCR results for <i>KRT19</i> , <i>ERBB2</i> , and <i>TWIST1</i> in breast cancer patients. ^a			
	CTC gene expression bead array		
	<i>KRT19</i> (+)	<i>KRT19</i> (-)	Total
RT-qPCR			
<i>KRT19</i> (+)	19	6	25
<i>KRT19</i> (-)	5	30	35
Total	24	36	60
Agreement: 46 of 60 (81.7%), $P < 0.001$			
	CTC gene expression bead array		
	<i>ERBB2</i> (+)	<i>ERBB2</i> (-)	Total
RT-qPCR			
<i>ERBB2</i> (+)	4	4	8
<i>ERBB2</i> (-)	5	47	52
Total	9	51	60
Agreement: 51 of 60 (85%), $P = 0.005$			
	CTC gene expression bead array		
	<i>TWIST1</i> (+)	<i>TWIST1</i> (-)	Total
RT-qPCR			
<i>TWIST1</i> (+)	22	10	32
<i>TWIST1</i> (-)	1	20	21
Total	23	30	53
Agreement: 42 of 53 (79.2%), $P < 0.001$			
^a Chi-square tests.			

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design,

acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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