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mRNA Expression and *BRAF* Mutation in Circulating Melanoma Cells Isolated from Peripheral Blood with High Molecular Weight Melanoma-Associated Antigen–Specific Monoclonal Antibody Beads

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Abstract

BACKGROUND—The detection of circulating tumor cells (CTCs) in the peripheral blood of melanoma patients by quantitative real-time reverse-transcription PCR (qRT-PCR) analysis correlates with a poor prognosis. The assessment of CTCs from blood has been difficult because of a lack of a good monoclonal antibody (mAb) directed against surface cell antigens to capture melanoma cells.

METHODS—Blood was collected prospectively from 57 melanoma patients (43 test and 14 test-development cases) and 5 healthy donors. High molecular weight melanoma-associated antigen (HMW-MAA)-specific mAbs bound to immunomagnetic beads were used to isolate CTCs. mRNA and/or DNA were extracted from CTCs. Testing for the expression of a melanoma-associated gene panel (*MLANA*, *MAGEA3*, and *MITF*) with qRT-PCR and for the presence of *BRAF*mt (a *BRAF* gene variant encoding the V600E mutant protein) verified the beads-isolated CTCs to be melanoma cells. A peptide nucleic acid– clamping PCR assay was used for *BRAF*mt analysis.

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RESULTS—Spiking of peripheral blood cells (PBCs) with melanoma cells showed that the beads-based detection assay can detect approximately 1 melanoma cell in 5×10^6 PBCs. qRT-PCR analysis detected *MLANA*, *MAGEA3*, and *MITF* expression in 19 (44%), 29 (67%), and 19 (44%) of the patients, respectively. At least one biomarker of the panel was positive in 40 (93%) of the 43 melanoma patients. *BRAF*mt was detected in 17 (81%) of the 21 assessed stage IV melanoma patients.

CONCLUSION—The assay of bead capture coupled with the PCR has utility for assessing CTCs in melanoma patients, which can then be characterized for both genomic and transcriptome expression.

The metastasis of melanoma cells to distant sites often portends a poor prognosis (1,2), and detection of melanoma cells in the circulation is useful to assess disease progression (3). Previous studies have shown that the detection of melanoma cells in blood by direct isolation of mRNA without isolation of tumor cells is associated with a poor disease outcome (3–9) and is a predictor of subclinical disease. Furthermore, we and others have reported that serial monitoring of circulating tumor cells (CTCs)[5] may be useful for predicting disease outcome for melanoma patients receiving adjuvant therapy (8–10).

To date, investigators have identified a limited number of biomarkers that are expressed by melanoma cells but are not detectable in healthy cells. We have developed a multimarker quantitative real-time reverse- transcription PCR (qRT-PCR) assay that uses specific prognostic biomarkers to detect melanoma cells in the blood and identify lymph node metastasis (9,11,12). This panel of genes includes *MLANA*[6] (melan-A; also known as *MART-1*, melanoma antigen recognized by T cells 1), *MAGEA3* (melanoma antigen family A, 3) (12,13), and *MITF* (microphthalmia-associated transcription factor) (9). MLANA is a melanocyte-differentiation antigen that is frequently synthesized by melanoma cells (12,14). MAGEA3 is commonly synthesized by malignant cells of different embryologic origin and is not detectable in healthy tissues except male germline cells and placenta (12,13). MITF plays an important role in melanocyte development and melanoma growth (9,15). These qRT-PCR markers are able to detect both occult metastatic melanoma cells in sentinel lymph nodes (12) and CTCs in the blood, demonstrating their prognostic utility for melanoma patients (3, 9,10).

A mutant form of the *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) gene (*BRAF*mt) encoding the V600E variant protein occurs at high frequency in melanomas (16). *BRAF* encodes a serine/threonine kinase downstream in the RAS-MAPK pathway that transduces regulatory signals from RAS through MAPKs (mitogen-activated protein kinases) (17,18). *BRAF*mt has been detected in 50%–80% of metastatic lesions (16,19). In addition, we have described the presence of circulating *BRAF*mt DNA in the serum of melanoma patients and demonstrated its clinical utility (19).

Direct RT-PCR analysis of blood permits transcriptome and phenotypic characterization of CTCs. To date, characterization of CTCs for abnormalities in genotypic and mRNA expression has not been well studied in melanoma patients. This situation is due in part to the difficulty of isolating CTCs with monoclonal antibodies (mAbs) capable of recognizing cell surface markers.

[5]Nonstandard abbreviations: CTC, circulating tumor cell; qRT-PCR, quantitative real-time reverse-transcription PCR; *BRAF*mt, *BRAF* gene variant encoding the V600E mutant protein; MAPK, mitogen-activated protein kinase; mAb, monoclonal antibody; HMW-MAA, high molecular weight melanoma-associated antigen; PBC, peripheral blood cell; AJCC, American Joint Committee on Cancer; FRET, fluorescence resonance energy transfer; PNA, peptide nucleic acid; LNA, locked nucleic acid; qPCR, quantitative PCR.
[6]Human genes: *MLANA*, melan-A (also known as *MART-1*, melanoma antigen recognized by T cells 1); *MAGEA3*, melanoma antigen family A, 3; *MITF*, microphthalmia-associated transcription factor; *BRAF*, v-raf murine sarcoma viral oncogene homolog B1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

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Characterizing the genotypic and phenotypic defects in CTCs would assist researchers in understanding the mechanisms that CTCs use to escape immune recognition and may suggest strategies for counteracting them. Therefore, in the present study we used high molecular weight melanoma-associated antigen (HMW-MAA) as a marker to develop a method for isolating CTCs from peripheral blood cells (PBCs). This cell surface antigen is highly expressed on melanoma cells in at least 85% of melanoma patients and is not detectable on healthy PBCs. We were able to obtain high-affinity mAbs that recognize distinct and spatially distant antigenic determinants of HMW-MAA and used a mixture of these HMW-MAA mAbs to capture CTCs.

Materials and Methods

MELANOMA CELL LINES

The human melanoma cell lines MA, MB, MC, MD, ME, MF, MG, MH, MI, MJ, MK, ML, and MM established and characterized at the John Wayne Cancer Institute were used for in vitro studies. Cells were grown in RPMI 1640 medium containing 100 mL/L heat-inactivated fetal calf serum and 10 mL/L penicillin–streptomycin (Gibco/Invitrogen) in a T75 (75-cm^2) culture flask. Cells were harvested for mRNA analysis when they reached 70%–80% confluence, as previously described (20).

PATIENTS

All melanoma patients enrolled in this study signed consent forms for the use of blood samples and physical and medical histories. The study was carried out according to the guidelines set forth by the Saint John's Health Center/John Wayne Cancer Institute Institutional Review Board. Disease stage was determined according to the American Joint Committee on Cancer (AJCC) and recorded at the time of blood draw. Blood was obtained from 14 stage IV melanoma patients for pilot experiments to optimize assays, and blood samples from 43 stage IV melanoma patients were used to validate assays. The study was conducted in a double-blind fashion. The investigators who performed the PCR assays did not know the patients' disease status.

MONOCLONAL AND POLYCLONAL ANTIBODIES

Mouse mAbs 225.28, 763.74, TP61.5, VF1-TP41.2, and VF4-TP108, which recognize distinct and spatially distant determinants of HMW-MAA [as described previously (21,22)], were purified from ascitic fluid by sequential precipitation with ammonium sulfate and caprylic acid (23). The purity of mAb preparations was assessed by SDS-PAGE analysis, and the activity of mAb preparations was monitored by testing with HMW-MAA–bearing melanoma cells in a binding assay. R-phycoerythrin–labeled $F(ab')_2$ fragments of goat antimouse IgG antibodies were purchased from Santa Cruz Biotechnology.

FLOW CYTOMETRIC ANALYSIS

Cells were incubated at 4 °C for 1 h with each HMW-MAA–specific mAb (1μg) or with an isotype-matched control mAb. Cells were washed twice with PBS containing 5 g/L BSA, incubated at 4 °C for an additional 30 min with an optimal amount of R-phycoerythrin–labeled $F(ab')_2$ fragments of goat antimouse IgG Abs, washed twice again, fixed in 40 g/L paraformaldehyde, and analyzed with a flow cytometer (FACSCalibur; BD Biosciences). Melanoma cells (10^4) were acquired for each sample. Debris, cell clusters, and dead cells were gated out by light-scatter assessment before single-parameter histograms were drawn. Data were analyzed with the aid of CellQuest software (BD Biosciences).

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ISOLATION OF MELANOMA CELLS FROM PBCs WITH IMMUNOMAGNETIC BEADS

Blood samples (1 mL) were collected into sodium citrate–containing tubes, and the first several milliliters were discarded (10). Total cells in blood were collected with Purescript PBC lysis solution (Gentra Systems) according to the manufacturer's instructions. CTCs were isolated from peripheral blood samples with immunomagnetic beads (CELLection™ Pan Mouse IgG Kit; Invitrogen) according to the manufacturer's recommendations, with minor modifications. In brief, HMW-MAA–specific mouse mAbs 225.28, 763.74, TP61.5, VF1-TP41.2, and VF4-TP108 (3μg each) were added separately or in combination to cells purified from PBCs and resuspended in 4.5 mL PBS containing 1g/L BSA. Samples were incubated at 4 °C overnight with rotation on an RKDynal rotor (Dynal Biotech/Invitrogen). After 2 washes with this PBS/BSA solution, immunomagnetic beads (antimouse IgG–coated) were added to the samples, and incubation was continued at 4 °C for an additional 40 min with rotation on the RKDynal rotor. Samples were then placed in a Dynal MPC-6 magnetic device (Invitrogen) at 4 °C for 2 min. The fluid was removed by careful pipetting, and the beads were resuspended in the PBS/BSA solution. The beads were washed twice with this washing procedure and then resuspended in RPMI 1640 medium containing 10 mL/L fetal bovine serum. Cells were released from the beads by the addition of Releasing Buffer (Invitrogen) and a 20-min incubation on a mixing device. The mixture was then vigorously pipetted and placed in the Dynal MPC-6 magnet for 2 min. The medium containing the released CTCs was collected with a pipet.

RNA ASSAYS

Total RNA was extracted with Tri Reagent (Molecular Research Center) as described previously (10). RNA was quantified and assessed for purity by ultraviolet spectrophotometry and the RiboGreen assay (Molecular Probes/Invitrogen). Blood processing, RNA extraction, qRT-PCR assay setup, and post-PCR product analysis were carried out in separate designated rooms to prevent cross-contamination. Reverse-transcription reactions were performed with Moloney murine leukemia virus reverse transcriptase (Promega) with oligo(dT) primer. qRT-PCR assays were performed with the ABI 7900HT instrument (Applied Biosystems). Primer and probe sequences designed for qRT-PCR analyses have previously been described (3,9, 11,12). The fluorescence resonance energy transfer (FRET) probe sequences were 5′–FAM-CAGAACGTCACCACCACCT TATT-BHQ-1–3′ for *MLANA*, 5′–FAM-AGCTCCT GCCCACACTCCCGCCTGT-BHQ-1–3′ for *MAGEA3*, 5′–FAM-AGAGCACTGGCCAAAGAGAGGCA-BHQ-1–3′ for *MITF*, and 5′–FAM-CAGCAATGCCTCCTG CACCACCAA-BHQ-1–3′ for *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). Five microliters of cDNA synthesized from 250 ng total RNA were transferred to a well of a 96-well PCR plate (Fisher Scientific), along with each primer, probe, and custom iTaq Supermix (Bio-Rad Laboratories). After a precycling hold at 95 °C for 10 min, samples were amplified with specific numbers of PCR cycles for each marker: denaturation at 95 °C for 1 min; annealing for 1 min at 55 °C for *GAPDH*, at 58 °C for *MAGEA3* and *MITF*, and at 59 °C for *MLANA*; and extension at 72 °C for 1 min. We generated a calibration curve with threshold cycle values for 9 serial dilutions of plasmid templates (10^0 to 10^8 copies) (3). The threshold cycle of each sample was interpolated from the calibration curve, and the number of mRNA copies was calculated by the iCycler iQ Real-Time PCR Detection System software (Bio-Rad). Thirteen melanoma lines and blood samples from 49 healthy donors were used to optimize the assay, as has previously been described (3,9,10). We performed each assay at least twice, and each assay included positive controls (melanoma lines), negative mRNA controls (PBCs), and PCR reagent controls without template. *GAPDH*, a so-called housekeeping gene, was used as an internal control to verify the integrity of the RNA and reverse-transcription efficiency. Any sample with an inadequate quantity of *GAPDH* mRNA was excluded from the study. The mean mRNA copy number calculated was used for analysis (10).

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DNA EXTRACTION AND *BRAF* DNA MUTATION

We collected blood samples for the *BRAF*mt study from a subset of 21 melanoma patients (AJCC stage IV) paired with qRT-PCR and isolated CTCs as described above. Genomic DNA was extracted from CTCs (24), and *BRAF*mt was assessed as previously described (19,25). In brief, primers were designed to amplify exon 15 of the *BRAF* gene, which includes the mutation hot spot that encodes the V600E variant. The peptide nucleic acid (PNA) (Applied Biosystems) was designed to clamp the hot spot on the wild-type template and block the wild-type template from being amplified in the PCR (19,25). A FRET dual-labeled locked nucleic acid (LNA) probe (Proligo/Sigma–Aldrich) was designed to recognize and hybridize specifically to the T-to-A mutation at the sequence encoding V600E, because this mutation is the most frequently seen for the *BRAF* gene at this hot spot. The design of a second FRET DNA probe (BioSource/Invitrogen) was based on using sequences adjacent to the LNA probe and avoiding the hot spot. This probe was used to amplify and quantify the total number of DNA templates, both wild-type *BRAF* and *BRAF*mt, in the PCR. Quantitative PCR (qPCR) with both the PNA clamp and the FRET LNA probe was used for mutation detection.

BRAF qPCR used the following primers and probe: forward, 5′–CCTCACAGTAAAAATAGGTG–3′; reverse, 5′–ATAGCCTCAATTCTTACCA–3′; LNA, 5′–CTACAGAGAAATCTCGAT-BHQ-1–3′; and PNA, 5′–CTACAGTGAAATCTCG–3′. The iCycler iQ Real-Time PCR Detection System (Bio-Rad) was used for the PCR assay. CTC genomic DNA (20 ng) was amplified by qPCR in a 20-μL reaction volume containing each PCR primer, LNA, PNA, deoxynucleoside triphosphates, $MgCl_2$, PCR buffer, and AmpliTaq Gold Polymerase (Applied Biosystems). The PCR conditions were 50 cycles of 94 °C for 1 min, 72 °C for 50 s, 53 °C for 50 s, and 72 °C for 1 min. Each sample was assayed in triplicate; control reactions included PCRs with templates derived from the appropriate positive and negative cell lines and a PCR reagent control without template.

DNA from the MA cell line was established as the reference for measuring units of *BRAF*mt target DNA (heterozygous). The amount of target mutant DNA in 1μg/mL MA genomic DNA was arbitrarily established as 1 U of *BRAF*mt. qPCR results for samples were normalized to this reference to quantify the relative units of *BRAF*mt in all of the samples. All PCRs for mutant-sequence analysis were done in triplicate, and the median was used for data analysis.

Representative *BRAF*mt and wild-type *BRAF* genes from V600E melanoma tumors were sequenced to confirm the accuracy of the PCR assay, as has previously been described (19, 25). The *BRAF* PCR used primers 5′–TGTTTTCCTTTACTTACTACACCTCA–3 (forward) and 5′–AGCATCTCAGGGCCAAAAAT–3′ (reverse). The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and then sequenced directly at 58 °C with the GenomeLab DTCS Quick Start Kit (Beckman Coulter) according to the manufacturer's instructions. Products of dye-termination reactions were assessed by capillary array electrophoresis on a CEQ 8000XL Genetic Analysis System (Beckman Coulter).

Results

HMW-MAA PROTEIN EXPRESSION ON MELANOMA CELLS

The expression of HMW-MAA protein on cells of melanoma lines was initially screened by flow cytometric analysis with HMW-MAA–specific mAbs 225.28, 763.74, TP61.5, VF1-TP41.2, and VF4-TP108 to verify the sensitivity and specificity of each mAb (Fig. 1). HMW-MAA expression was detected on all melanoma lines with all HMW-MAA–specific mAbs tested. The individual mAbs recognize different epitopes on HMW-MAA. We subsequently used a mixture of all 5 HMW-MAA–specific mAbs for this assay.

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ISOLATION OF MELANOMA CELLS MIXED WITH PBCs

We conducted pilot experiments to optimize conditions for isolating CTCs from peripheral blood with HMW-MAA mAbs and immunomagnetic beads (Fig. 2). We used the immunomagnetic beads to assess captured CTCs via both an indirect technique and a direct technique to determine the optimal assay. In brief, the indirect technique entails first incubating melanoma cells with HMW-MAA mAbs; melanoma cells labeled with HMW-MAA mAbs are then bound and captured by the immunomagnetic beads. In the direct technique, HMW-MAA mAbs are first incubated with immunomagnetic beads; melanoma cells are then bound and captured by immunomagnetic beads with HMW-MAA mAbs. Melanoma cells were added to healthy donor PBCs at ratios of $1–10^3$ per 5×10^6 PBCs. qRT-PCR analysis successfully detected mixtures of 1–5 melanoma cells in 5×10^6 healthy donor PBCs by the indirect technique but required at least 100 melanoma cells in 5×10^6 PBCs with the direct technique. Thus, the indirect technique was better than the direct technique for separating melanoma cells from healthy donor PBCs. A mixture of HMW-MAA–specific mAbs (225.28, 763.74, TP61.5, VF1-TP41.2, and VF4-TP108) was used to confirm the improvement in tumor cell capture. qRT-PCR analysis was always successful in detecting approximately 1 melanoma cell mixed with 5×10^6 PBCs in quadruplicate experiments that used the mAb mixture but was not always successful when a single HMW-MAA mAb was used. For in vitro studies, the mixture of HMW-MAA mAbs was better than a single HMW-MAA mAb for isolating melanoma cells from mixtures with healthy donor PBCs. In a pilot study that used samples from 14 melanoma patients, we determined that the optimal and most efficient amount of mAbs to use was 3 μg of each mAb. The optimal incubation time with CTCs and primary antibody was determined to be overnight.

qRT-PCR DETECTION LIMIT FOR MELANOMA CELLS MIXED WITH PBCs

To assess the limit of detection for melanoma cells in blood with the optimized immunomagnetic bead method (i.e., indirect technique, overnight incubation with a mixture of all 5 HMW-MAA–specific mAbs), we performed immunomagnetic bead separation with a mixture of HMW-MAA mAbs and a qRT-PCR analysis of melanoma cells that we serially diluted with healthy donor PBCs. In the assays, we added serial dilutions of melanoma cells (10^4, 10^3, 10^2, 10, 1, and 0 cells) expressing *MAGEA3*, *MLANA*, and *MITF* to 5×10^6 donor-derived PBCs and assessed the qRT-PCR assay's ability to detect each marker. This in vitro assay was performed both with and without the immunomagnetic bead technique, but always with the HMW-MAA–specific mAbs added. The assay was repeated multiple times to validate the reproducibility and robustness of the assay system. This in vitro model system was used to mimic the detection of CTCs in blood. Use of the beads permitted the detection of *MAGEA3*, *MLANA*, and *MITF* mRNAs from approximately 1 melanoma cell mixed with 5×10^6 PBCs (Table 1). The number of mRNA copies detected gradually decreased with serial dilution of the melanoma cells (Fig. 3). The number of mRNA copies for individual markers varied with the cell line, as expected. This heterogeneity in mRNA production is also to be expected in blood samples from patients. All biomarkers were positive for detecting 10 melanoma cells mixed with 5×10^6 PBCs in all 4 experiments (Table 1). Detection frequencies for *MAGEA3*, *MLANA*, and *MITF* transcripts from approximately 1 melanoma cell mixed with 5×10^6 PBCs were 100%, 100%, and 75%, respectively. We performed the same experiments without immunomagnetic beads to assess the detection of melanoma cells in blood via direct isolation of mRNA from blood (Fig. 3). The numbers of mRNA copies obtained for each marker in samples processed with immunomagnetic beads were higher for all serial-dilution experiments than for samples processed without beads. The numbers of copies of *MAGEA3* and *MLANA* transcripts obtained for bead-processed samples were the same as those obtained for samples without the bead-capture method when approximately 100 melanoma cells were mixed with 5×10^6 PBCs.

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ASSESSMENT OF CTCs IN PBCs

We performed immunomagnetic beads– based qRT-PCR assays with samples from healthy blood donors and obtained no positive signals for any of the 3 markers. These results demonstrated the specificity of the assay. In blood samples from 43 melanoma patients, *MAGEA3*, *MLANA*, and *MITF* mRNAs were detected in 67%, 44%, and 44% of the patients, respectively, when we used the assay with optimized CTC isolation (Table 2). At least one of the markers was detected in 40 (93%) of the 43 patients investigated when the panel of all 3 biomarkers was assessed; this detection level was higher than that obtained for any single biomarker. This analysis demonstrated the sensitivity and efficiency of the assay. The combination of the capture of melanoma CTCs with immunomagnetic beads containing HMW-MAA mAbs and qRT-PCR analysis constitutes a unique approach that integrates 2 different techniques to assess CTCs with unparalleled specificity.

ASSESSMENT OF *BRAF* MUTATION IN CTCs

In an in vitro model, the assay detected *BRAF*mt from 1–5 MA cells in mixtures with 5×10^6 healthy donor-derived PBCs. *BRAF*mt was assessed in isolated CTCs from melanoma patients. Genomic DNA was extracted from 21 melanoma patients (AJCC stage IV), and *BRAF*mt was detected in 17 (81%) of these patients. The study demonstrated that HMW-MAA–positive melanoma cells frequently have *BRAF*mt. The analysis also demonstrated that *BRAF*mt is frequently detected in CTCs with this assay.

Discussion

To characterize CTCs in PBCs, we combined immunomagnetic-bead isolation with DNA and RNA PCR assays. We previously reported that qRT-PCR–based methods can detect CTCs and that positive signals for markers are associated with metastatic disease and poor prognosis in melanoma patients (3,9,10). The specificity of these qRT-PCR assays with respect to the cell type being assessed is controversial, however, primarily because it is not clear whether all melanoma-associated biomarkers actually are derived from CTCs in the blood of melanoma patients. The use of immunomagnetic-bead capture of melanoma cells in our approach exploits the selective expression of HMW-MAA on melanoma cells (22).

HMW-MAA has been shown to be heterogeneous in the expression of antigenic determinants recognized by mAbs. To minimize the occurrence of false-negative results caused by differential expression of HMW-MAA determinants on melanoma cells and to increase the detection capability of our isolation procedure, we used a mixture of mAbs that recognize distinct and spatially distant determinants of HMW-MAA to isolate CTCs from the PBCs of melanoma patients. The use of an HMW-MAA mAb mixture enabled us to maximize the exclusion of healthy blood cells and other types of circulating cells and to maximize the isolation of CTCs (i.e., HMW-MAA–positive melanoma cells). The immunomagnetic beads–based assay is capable of capturing intact HMW-MAA–positive melanoma cells. The identity of these CTCs as melanoma cells was established by subsequent analysis of 3 recognized melanoma-associated mRNA biomarkers frequently found in melanomas. The genes encoding these mRNA biomarkers do not have directly correlated functions (3,9,10).

Our in vitro model demonstrated that our assay can detect approximately 1 melanoma cell diluted into 5×10^6 PBCs from healthy donors. This assay provides a new opportunity not only to improve melanoma diagnosis but also to identify patients who have spreading systemic disease. The approach of coupling the capture of CTCs with the PCR permits improved characterization of the CTCs in blood both for the expression of tumor biomarkers and for genomic aberrations.

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Our previous studies found that metastatic melanoma tumors were heterogeneous for the expression of melanoma-associated markers (3,10–12). For the clinical application, we selected 3 mRNA biomarkers for detecting CTCs in the blood of melanoma patients. Use of a combination of melanoma-associated markers in the qRT-PCR assay can circumvent the problem of heterogeneity in mRNA expression of individual markers. We previously demonstrated that melanoma-associated genes are not expressed at 100% in all melanoma cells (3).

The *MAGEA3* transcript had the highest overall detection rate in PBCs from melanoma patients, followed by *MLANA* and *MITF* transcripts. Differences between results obtained for cell lines and blood samples may be related to the physiology of cells in the circulation or to the phenotype of the clone shed into the blood (26). Detection rates of individual biomarkers with qRT-PCR assays after immunomagnetic beads– based capture was slightly higher for some biomarkers in paired patient blood samples than for individual biomarkers assessed by qRT-PCR without bead capture.

We previously reported that the presence of a *BRAF* mutation in circulating DNA in serum may have clinical utility in predicting tumor response and disease outcome (19); however, whether the *BRAF* mutation in serum DNA actually derives from metastatic melanoma, tumors, or CTCs remains to be determined. Our results demonstrated a high frequency of *BRAF*mt in cells captured with HMW-MAA mAbs. *BRAF*mt analysis in the present assay may be useful as a melanoma-associated biomarker. Our description of this assay for a surrogate biomarker of CTCs in the blood of melanoma patients is the first such report. The *BRAF* mutation encoding the V600E variant is specific to tumor cells, particularly in melanoma, although nevi cells have been reported to possess the V600E variant (27). This mutation can be found in up to 80% of patients with metastatic cutaneous melanomas. A recent study found that circulating non-small cell lung cancer cells were captured and that the cells featured epidermal growth factor receptor mutation in 11 of 12 patients (28).

Currently, melanoma prognosis is based on the tumor and demographic and prognostic factors of the host, but ongoing dynamic factors of tumor metastasis are also important. Our findings demonstrate the potential clinical usefulness of an immunomagnetic beads– based assay for detecting CTCs in the blood of melanoma patients. Future studies involving serial blood analysis and long-term follow-up of patients may be needed for a more detailed assessment of the clinical utility of this assay.

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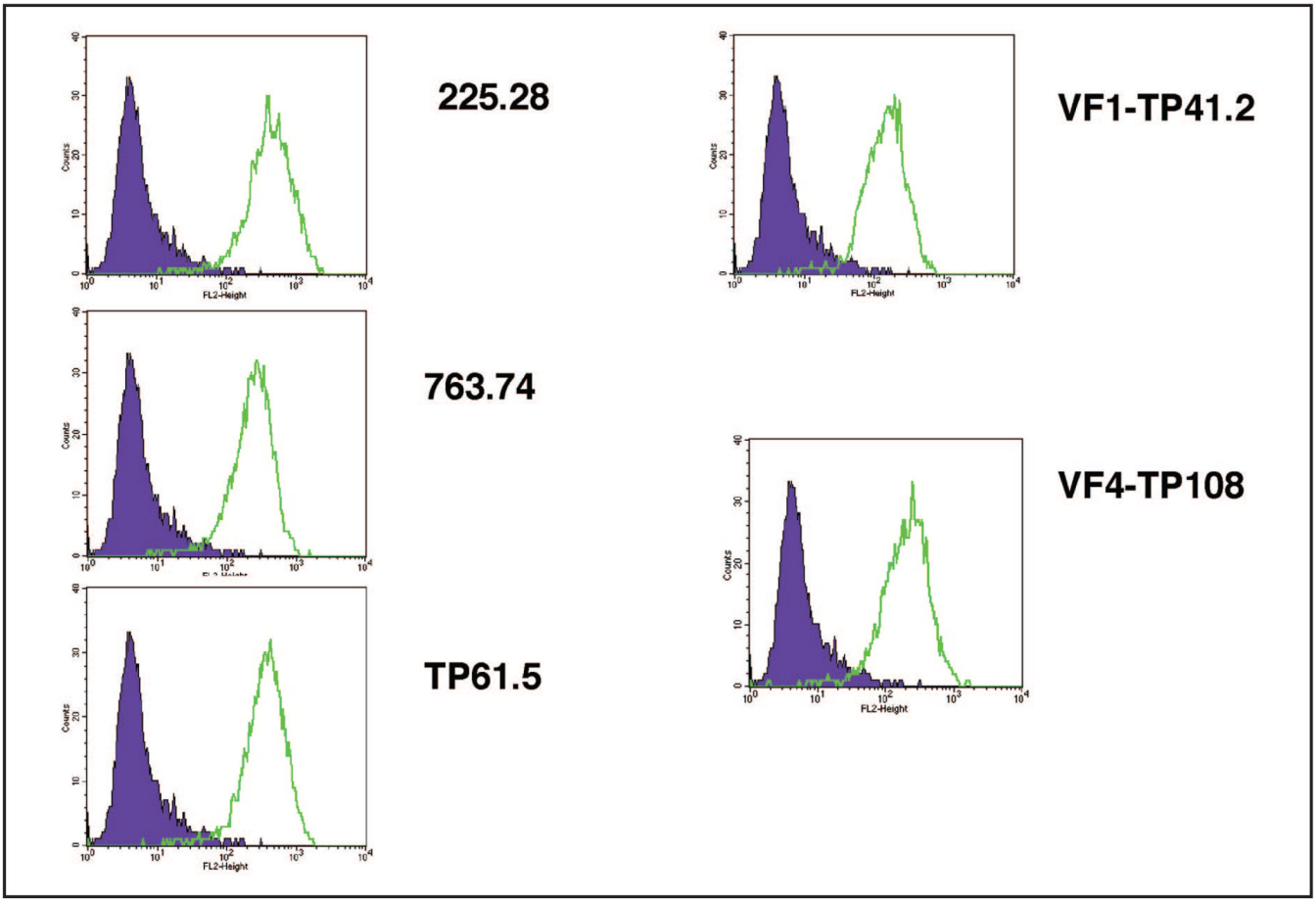

Figure 1.
HMW-MAA protein expression on melanoma cell lines by flow cytometry analysis with each of the HMW-MAA specific mAbs (225.28, 763.74, TP61.5, VF1-TP41.2, and VF4-TP108) and an isotype-matched control Ab.

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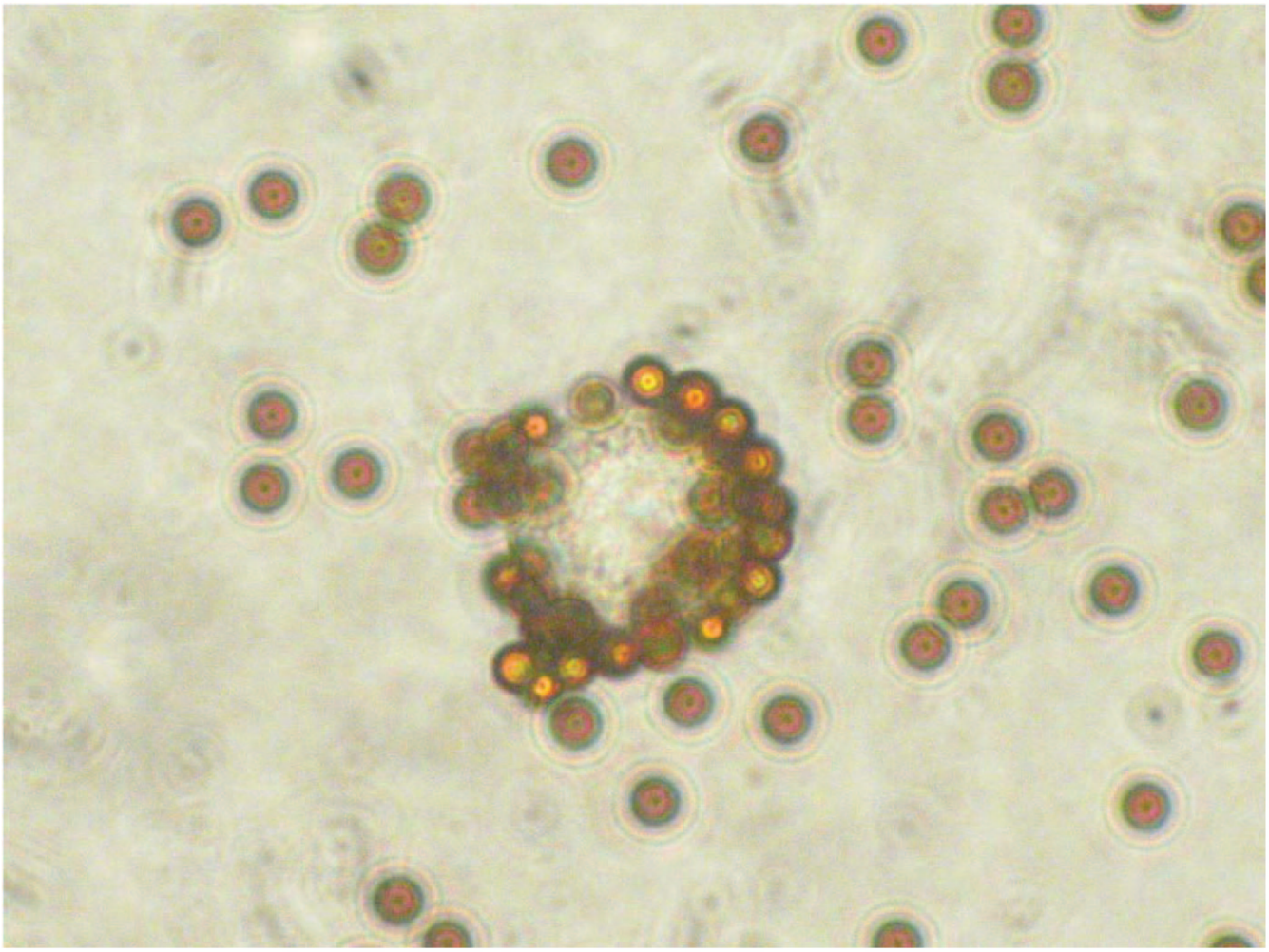

Figure 2.
HMW-MAA–positive cells were isolated from suspensions of healthy donor PBCs and melanoma cells by means of an HMW-MAA mAb mixture and immunomagnetic beads.

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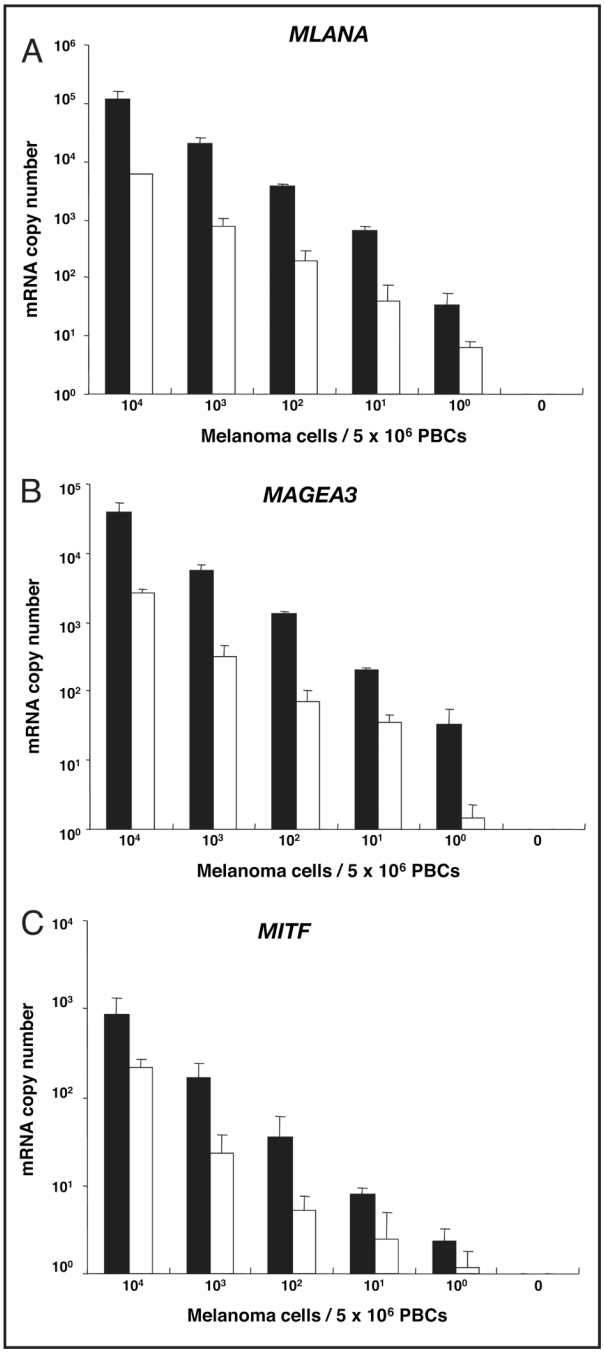

Figure 3.

qRT-PCR detection limit for melanoma cells (10^4, 10^3, 10^2, 10, 1, and 0 cells) mixed with 5 × 10^6 PBCs from healthy donors.

Assay for *MLANA* (A), *MAGEA3* (B), and *MITF* (C) with (filled columns) and without (open columns) the use of immunomagnetic beads with bound anti–HMW-MAA mAbs. Data are presented as the mean (SD).

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Table 1

qRT-PCR detection of melanoma markers in serially diluted melanoma cell lines (n = 4).[a]

	Cell number					
Marker	10^4	10^3	10^2	10^1	10^0	0
GAPDH	4	4	4	4	4	0
MLANA	4	4	4	4	4	0
MAGEA3	4	4	4	4	4	0
MITF	4	4	4	4	3	0

[a]Data represent the number of times the indicated marker was detected (of 4 experiments) at the indicated numbers of melanoma cells mixed with 5×10^6 PBCs. An HMW-MAA mAb mixture was used in an indirect assay with immunomagnetic beads. *GAPDH* is a housekeeping gene used as an internal control.

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Table 2

Multimarker mRNA expression in blood samples from melanoma patients (n = 43).

	Positive expression, n (%)
Melanoma marker	
MLANA	19 (44)
MAGEA3	29 (67)
MITF	19 (44)
No. of markers detected	
0	3 (7)
1	19 (44)
2	15 (35)
3	6 (14)
4	0 (0)
≥1	40 (93)