Melanoma-associated Antigens as Messenger RNA Detection Markers for Melanoma

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ABSTRACT

Melanoma is heterogeneous for its biological properties and melanoma-associated antigens (MAAs). This diversity is partially observed in the expression of the MAAs involved with the melanin synthesis pathway. We therefore developed a sensitive multimarker reverse transcription-PCR plus Southern blot assay using five MAAs as molecular markers to detect primary and metastatic melanoma cells. Melanoma cell lines, melanocytes (cultured), primary and metastatic malignant melanoma tissues, and blood from patients with American Joint Committee on Cancer stage I-IV melanoma were assessed for tyrosinase, tyrosinase-related proteins 1 and 2, Pmel 17, and MART-1/Melan-A. All of the MAA mRNA markers were expressed in 100% of melanoma cell lines and cultured melanocytes, 74% of primary and metastatic tumors (excluding tumor-draining lymph nodes), 43% of tumor-involved lymph nodes, and 43% of patients' bloods. Hypomelanotic melanoma tissues expressed a lower frequency of individual mRNA markers. Overall, at least one mRNA marker was expressed in more than 86% of specimens assayed. Normal tissue specimens from patients and blood from normal volunteer donors were negative for MAA mRNA expression. The multimarker MAA reverse transcription-PCR plus Southern blot analysis was more reliable and sensitive than a single-molecular marker assay for the detection of melanoma cells. This molecular assay can also provide information on MAA mRNA expression of metastatic melanoma cells that may assist in monitoring the therapeutic efficacy of active specific immunotherapy toward specific MAA-bearing melanomas.

INTRODUCTION

Malignant melanoma is known to be heterogeneous for its biological, antigenic, and metastatic properties. The enzymes involved in melanin synthesis are of particular interest because many of these derived proteins have been reported to be antigenic in humans (1–3). Tyrosinase (Tys), the most studied melanin enzyme, is involved in the initial steps of the melamyn synthesis pathway and has been investigated as a molecular marker to detect metastatic disease (4). Unfortunately, the heterogeneity of Tys mRNA expression in melanomas contributes to its inconsistency as a reliable mRNA marker for detecting 100% of metastatic melanomas.

The heterogeneity of gene expression in melanoma becomes increasingly evident in advancing stages of tumor progression (5, 6). As melanization declines, progressing toward hypomelanotic characteristics, differences in melanogenesis-related antigen mRNA and respective protein expression have been found in murine models (7). Orlow et al. (7) have shown that Tys was most often absent or undetectable as melanization declined. This suggests that a single mRNA marker assay for detecting occult metastatic melanoma cells will selectively assess more of one clonal population of malignant cells, whereas a multimarker mRNA assay may be more inclusive.

Other aspects of mRNA marker expression must also be considered when devising assays to detect occult malignant disease. Besides the potential for a lack of consistent mRNA marker expression for a particular tumor histology, either low levels of mRNA marker expression or dilution of marker mRNA among normal cell mRNA will limit the usefulness of a single marker assay.

Significant advances in understanding the underlying complexity of the mammalian pigmentation system have been made. Other enzymes besides Tys are known to participate in the melanin synthesis pathway (8, 9). The major melanogenesis pathway enzymes include Tys, TRP-1, and TRP-2 corresponding to the murine albino (10), brown (11), and slaty (12, 13) loci, respectively. These melanogenesis proteins have been found in melanomas and are referred to as MAAs. Other MAAs that are associated with melanomas and possibly with melanin synthesis include Pmel 17 and MART-1/Melan-A. Pmel 17 is related to the murine silver locus and is found on melanosomes; its function is unknown (14). The Pmel 17 gene has a significant nucleic acid sequence homology to gp100, which is a glycoprotein associated with the melanosomal matrix. MART-1/Melan-A is a newly discovered MAA that is found on melanomas and melanocytes, the function of which is also unknown (15).

Tys, TRP-1, TRP-2, Pmel 17, and MART-1/Melan-A are excellent potential molecular markers because their expression is primarily limited to melanocytes and melanomas. It is logical to use these MAAs in combination as mRNA markers to accurately detect as well as identify specific MAA mRNA expression of melanoma cells. Several of the MAAs have also been shown to induce specific T-cell and/or antibody responses in humans. Identifying changes in the MAA mRNA expression profile associated with melanoma progression may be important in the design of active specific immunotherapy toward melanoma.

In this study, we demonstrate the use of Tys, TRP-1, TRP-2, Pmel 17, and MART-1/Melan-A as MAA mRNA markers in an RT-PCR plus Southern blot analysis to detect malignant melanoma cells in primary and metastatic tumors and in the blood of melanoma patients. The heterogeneity of MAA mRNA markers in blood and tissue specimens is shown as well as the efficacy of using combined MAA mRNA markers to detect melanoma.

MATERIALS AND METHODS

Melanoma Cell Lines and Melanocytes. Melanoma cell lines MATT, M101, M10, M12, M14, and M24 were established and characterized at the JWCI as described previously (16, 17). Cells were grown in RPMI 1640 (Gemini Bioproducts, Calabasas, CA) plus 10% FCS (heat-inactivated), penicillin, and streptomycin. M101, M10, M12, M14, and M24 were established and characterized at the JWCI as described previously (16, 17). Cells were grown in RPMI 1640 (Gemini Bioproducts, Calabasas, CA) plus 10% FCS (heat-inactivated), penicillin, and streptomycin. (Life Technologies, Inc., Long Island, NY) in 75 cm² flasks. Adherent cell lines were routinely passaged by trypsinization every 3–4 days (18, 19). When cell lines attained 75% confluency, they were used for RT-PCR analysis. Melanocytes were obtained from Clonetics (San Diego, CA 90404).
CA) and cultured in melanocyte growth medium according to the manufacturer’s instructions.

**Surgical Specimens and Blood Preparation.** Surgical specimens were obtained under written consent in consultation with the surgeon and pathologist. All tissues were collected and dissected under stringent nucleic acid sterile conditions to prevent RNA cross-contamination. Representative tissue samples were obtained from primary lesions (Clark level III–V; Breslow thickness, 1.85–4 mm; n = 4) and metastatic tumors (excluding TDLN, n = 19; TDLN, n = 28) from multiple anatomical sites. These included extremity and axial lesions as well as s.c. lesions, TDLNs, and visceral metastases. Tissues were examined by the Department of Pathology at Saint John’s Hospital and Health Center for the presence of melanoma by conventional H&E staining; if necessary, immunohistochemical staining with anti-S100 and HMB-45 antibodies was performed (18). Hypomelanotic melanoma tissues were obtained by gross intraoperative inspection. These specimens were used to represent relatively extreme examples of nonpigmented melanoma tissue. Operative tissue specimens were processed immediately, and adjacent normal tissue was carefully dissected away from melanoma-involved tissue on ice under sterile conditions in a laminar flow hood.

Normal tissues and lymph nodes from patients without melanoma undergoing elective surgery for other malignancies were assessed by histopathology with H&E as tumor negative. These tissues as well as blood from normal male and female donors were used as negative controls.

Ten ml of blood were collected in sodium citrate-containing tubes as described previously (4). The skin site chosen for venipuncture was cleansed with an alcohol swab. Either the first 5 ml of blood aspirated was discarded or blood for RT-PCR was drawn last when multiple specimens were taken in succession. This lessens the likelihood of potential RNA contamination from the skin plug during venipuncture. Blood was centrifuged using a hypotonic density gradient solution (DOT kit; National Genetics Institute, Los Angeles, CA), and total cells in the blood were collected for RNA isolation (4). This procedure allows recovery of cells at a higher efficiency rate than buffy coat centrifugation or Ficoll-Hypaque gradient centrifugation (4).

All blood and tissues were obtained at the JWCI after obtaining written consent from patients. The protocol for the study was approved by the Saint John’s Hospital and Health Center Research Committee and the JWCI Human Subjects Committee.

**RNA Preparation.** Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) was used to isolate total RNA from the cell lines and surgical specimens, following the manufacturer’s instructions. All specimens were kept on ice during processing. The cells were lysed in 1 ml of Tri-Reagent by surgical dissection and repetitive pipetting and were placed on ice for 5 min. Two hundred μl of chloroform were added. The solution was mixed vigorously, incubated at room temperature for 5 min, and then centrifuged at 12,000 × g at 4°C for 15 min. The upper aqueous phase was transferred into an RNase-free Eppendorf tube, an equal volume of isopropanol was added, and the nucleic acid was allowed to precipitate at −20°C for at least 1 h. The tube was then centrifuged at 12,000 × g for 40 min. The sample was washed once with 75% ethanol, vacuum-dried, and resuspended in 10 ml Tris-HCl with 0.1 mM EDTA solution (pH 8). The concentration, purity, and amount of total RNA were determined by UV spectrophotometry. RNA integrity was confirmed in all specimens by RT-PCR plus Southern blot for β-actin mRNA expression. In specimens with a very high concentration of melanin pigment as noted by visual inspection, nucleic acid was purified with an Elutip minicol density gradient solution (DOT kit; National Genetics Institute, Los Angeles, CA) and cultured in melanocyte growth medium according to the manufacturer’s instructions.

**RT-PCR.** One μg of total RNA was used in the RT-PCR assay to detect each of the molecular markers as described previously (4, 18, 19). Briefly, RNase-free double-distilled water was added to the RNA mixture and then heated to 70°C for 5 min, chilled on ice for 5 min, and added to the RT mixture (total volume, 20 μl), which consisted of 4 μl of Moloney murine leukemia virus 5X RT buffer, 2 μl of 10 mM deoxynucleotide triphosphate, 20 units of RNasin, 0.5 μg of oligo dT15 primer, and 200 units of Moloney murine leukemia virus RT. The reaction mixture was incubated at 37°C for 2 h. All RT reactions were carried out with oligo dT priming to reduce detecting spurious nonpolyadenylated mRNA. Reagents were obtained from Promega Corp. (Madison, WI), U. S. Biochemical Corp. (Cleveland, OH), and Boehringer Mannheim (Indianapolis, IN).

The final PCR mixture consisted of 1X Thermo DNA polymerase reaction buffer (Promega), 200 μM each of dCTP, dATP, dGTP, and dTTP, 2 mM MgCl2, 100 pm of each oligonucleotide primer, 5 units of Taq DNA polymerase (Promega), and RT mixture. RNase-free double-distilled water was added to bring the reaction mixture to 100 μl. Mineral oil (100 μl) was layered on top of the reaction mixture to prevent evaporation. The RT-PCR mixture was incubated in an OmniGene thermal cycler (Hybaid, Middlesex, England) at 95°C for 5 min for 1 cycle; 95°C for 1 min; 55°C for 1 min, 72°C for 1 min for 35 cycles; and 72°C for 10 min. The RT-PCR cDNA products were assessed in a 2% agarose gel containing ethidium bromide. A 100-bp DNA ladder (Life Technologies, Inc., Gaithersburg, MD) was used as a base pair reference marker.

**Automated Southern Blot Analysis.** The RT-PCR cDNA products were run in 2% agarose gel and denatured in alkaline, and automated Southern blotting was performed as described previously (19, 20). Optimally designed cDNA probes for Tys, TRP-1, TRP-2, Pmel 17, MART-1/Melan-A, and β-actin were developed from a RT-PCR cDNA product and labeled with digoxigenin (4). The automated Southern blot procedure allows analysis of multiple specimens, including multiple appropriate controls, under highly stringent, quality-controlled conditions. Southern blot analysis is essential for verification of RT-PCR cDNA product. Ethidium bromide gel analysis is highly subjective and not accurate for low-level RT-PCR cDNA product detection. Specific probe hybridization was detected using an anti-digoxigenin, alkaline phosphatase-conjugated antibody (Boehringer Mannheim) as described by the manufacturer. Southern blot analysis was then performed on the RT-PCR cDNA products. All blots were run with multiple positive and negative RT-PCR cDNA controls as well as Southern blot controls. Positive RT-PCR cDNA controls consisted of MAA mRNA-positive cell lines and melanoma tissue previously tested and confirmed to express a particular mRNA marker. Negative RT-PCR cDNA controls included MAA mRNA-negative cell lines, total reaction without RNA, and total reaction with H2O only. Southern blot controls included a positive cDNA product control and solutions with or without cDNA products not related to the specific cDNA being probed. RT-PCR assays were repeated to confirm both positive and negative results. A computer imager was used to read through the results, which were interpreted by at least two individuals.

**RESULTS**

The melanoma cell lines, melanocytes, primary and metastatic melanoma tissues, and blood from patients with melanoma served to establish the multiple marker RT-PCR assay. Tys, an established molecular marker for melanoma, was assessed along with two other mRNA markers related to the melanin synthesis pathway, TRP-1 and TRP-2. Other MAA mRNA markers studied were Pmel 17 and MART-1/Melan-A.

As a negative control, blood specimens from 15 normal volunteer donors were examined for all individual molecular markers. None of the negative control bloods were positive for MAA mRNA expression.
by RT-PCR assay plus Southern blot. Southern blot results were used as the final verification of detection and specificity of all RT-PCR cDNA products. Consistent interpretation of Pmel 17 by RT-PCR cDNA product expression can sometimes be problematic when evaluating normal bloods. The RT-PCR cDNA product of Pmel 17 can sometimes be misinterpreted with a closely adjacent amplified cDNA band that may be related to the Pmel 17/gp100 gene family.

Detection of MAA in Melanoma Cell Lines and Melanocytes.

Six melanoma cell lines were assessed for the expression of MAA mRNA. All five molecular markers were expressed in every cell line (Table 1). Examples of melanoma cell line MAA mRNA expression are shown in Fig. 1. As expected, cultured melanocytes expressed all five MAA mRNA markers (Table 1). These analyses demonstrate the lack of heterogeneity of MAA mRNA expression in cell lines and melanocytes.

Assessment of Primary and Metastatic Tumor Specimens.

Twenty-three tumor specimens from 21 patients with malignant melanoma were assessed. Tissues were either primary malignant melanoma (n = 4) or metastases (n = 19) from multiple anatomical sites (excluding TDLN). These tissues were obtained from patients categorized as AJCC stages I–IV (predominantly III and IV). All tissues were confirmed to be involved with melanoma by H&E staining.

Of the 23 tumor specimens analyzed by RT-PCR plus Southern blot, 74% (n = 17) expressed all five MAA mRNA markers, 9% (n = 2) expressed four markers, 4% (n = 1) expressed three markers, 9% (n = 2) expressed two markers, and 4% (n = 1) expressed only one marker (Table 1). Tys mRNA was expressed in all tumors, whereas mRNA expression for TRP-1, TRP-2, Pmel 17, and MART-1/Melan-A was 78, 83, 91, and 87%, respectively (Table 2). Interestingly, each of the primary tumors expressed all five markers. A patient with three separate specimens sampled from a single wide excision from a recurrent infraorbital melanoma expressed all markers in two specimens but only mRNA for Tys in a third analyzed concurrently.

Noncancerous tissues from 10 separate patients undergoing elective surgery for malignant tumors other than melanoma (breast tissue, n = 4; colon tissue, n = 3; pancreatic tissue, n = 2; gastric tissue, n = 1) were assessed. Tissue specimens were taken from areas of the malignant operative specimens submitted that appeared as normal, i.e., not involved with the primary malignancy. These tissue specimens were examined with H&E histopathology and found to be free of the respective malignant cells. Of the 10 different tissue specimens from 10 different patients analyzed, MAA mRNA expression was not identified in any.

Twenty-eight TDLNs from 17 patients undergoing regional lymphadenectomy for melanoma were analyzed with RT-PCR plus Southern blot to detect the number of MAA mRNA markers expressed (Tables 1 and 2). All TDLNs except one were confirmed involved with metastatic melanoma by H&E histopathology. Additional immunohistochemical staining with anti-S-100 and HMB-45 antibodies to the node negative for metastasis by H&E staining (multiple sections assessed) did not reveal melanoma metastasis. All MAA mRNA markers except Pmel 17 were expressed in this lymph node.

Of the 28 TDLNs assessed, all five markers were expressed in 43% (n = 12), four markers in 32% (n = 9), three markers in 7% (n = 2), two markers in 11% (n = 3), one marker in 4% (n = 1), and zero markers in 4% (n = 1; Table 1). Tys mRNA was expressed in 93% (n = 26), TRP-1 mRNA in 64% (n = 18), TRP-2 mRNA in 71% (n = 20), Pmel 17 mRNA in 76% (n = 22), and MART-1/Melan-A mRNA in 86% (n = 24) of TDLNs (Table 2).

Five TDLNs from five separate patients undergoing elective surgery for breast cancer were also assessed. These TDLNs did not contain metastatic disease by H&E histopathology. MAA mRNA marker expression was not identified in any of the TDLNs from breast cancer patients.

Hypomelanotic Melanoma Specimens.

Six hypomelanotic melanoma specimens from five patients were evaluated (Table 3). These melanoma tissue specimens were chosen because in addition to the melanotic tissue, there was an apparent hypomelanotic zone that was clearly evident on visual observation. On average, two MAA mRNA markers per specimen were expressed. Four hypomelanotic specimens did not express mRNA for Tys, and two of these expressed mRNA for one of the other MAA mRNA markers. All hypomelanotic melanoma specimens expressed β-actin mRNA.

Detection of Markers in Blood from Melanoma Patients.

Thirty-five blood specimens from 32 patients with AJCC stages I–IV (predominantly III and IV) malignant melanoma were obtained as described in “Materials and Methods.” All five MAA mRNA markers were expressed in 43% (n = 15), four (n = 7) and three (n = 7) markers in 20%, two markers in 6% (n = 2), and one marker in 11% (n = 4) of samples (Table 1). Tys mRNA was expressed in 86%, TRP-1 mRNA 57%, TRP-2 mRNA 74%, Pmel 17 mRNA 86%, and MART-1/Melan-A mRNA in 83% of bloods assayed. None of the normal control bloods (n = 15) were positive for MAA mRNA marker expression by RT-PCR plus Southern blotting.

Three patients had corresponding blood specimens assayed within 8 weeks of each other. One of these patients expressed three of the same MAA mRNA markers at both time intervals. The other two patients expressed three and four markers, respectively, at the initial blood draw. A subsequent blood draw from these same two patients 8 weeks later was positive for four and five MAA mRNA marker expression, respectively. Tys mRNA was expressed in samples from all three patients at both sampling intervals. This suggested the potential for heterogeneity of mRNA expression in metastatic melanoma cells circulating in the blood.

**DISCUSSION**

Multiple MAAs were assessed as RT-PCR markers for detecting and characterizing molecular profiles of melanomas. These MAA mRNA markers are primarily restricted to melanocytes and are, therefore, excellent potential molecular markers for melanoma. They are not expressed in normal tissues, except some neuroectoderm-derived tissues (21). This study has shown that these MAA mRNA markers are expressed in a relatively large number of cells from tumor specimens, TDLNs, and bloods sampled from different patients with melanoma. The heterogeneity in MAA mRNA marker expression exhibited by the primary and metastatic melanomas was also evident.

Melanoma cell lines had a higher frequency of multiple marker mRNA expression than primary or metastatic melanoma-containing tumors. Cultured melanocytes also expressed mRNA from all molec-
MELANOMA-ASSOCIATED ANTIGENS AS mRNA MARKERS

The observation of cell lines expressing a higher frequency of tumor-associated genes or antigens than tumor tissue material is not uncommon (22, 23). Culture conditions often accentuate tumor marker expression through selective growth advantages. Also, tumor cell lines in culture often represent a more clonal population of cells than those of tissue specimens. Therefore, when evaluating the efficacy of new molecular markers for detecting a malignancy, it is essential that mRNA marker assessment be performed from representative tissue specimens from different patients. The discrepancy of individual mRNA marker expression (e.g., Tys) between melanoma cell lines and tissue specimens must also be considered when using cell lines as a standard reference for quantitation.
This was observed when evaluating the hypomelanotic melanoma tumors into specific tumor stages based on MAA mRNA expression. This factor, along with the heterogeneity in MAA mRNA expression heterogeneity and variation in the level of individual mRNA marker detecting circulating melanoma cells in peripheral blood and tissues tumor cells in the blood, lack of expression of specific mRNA in tumor cells, or low copy expression of the mRNA marker diluted among normal cell RNA. These factors all support the concept of using multiple mRNA markers with a highly sensitive detection assay system.

Previous studies have shown the utility of the RT-PCR assay in detecting circulating melanoma cells in peripheral blood and tissues using only Tys (24). These studies do not consider the heterogeneity of individual mRNA marker expression in tumor lesions within individual patients or among patients (25). The continuous production of genetic variant cells by a progressing cancer can contribute to tumor heterogeneity and variation in the level of individual mRNA marker expression (5, 6). MAA mRNA expression can differ among metastatic melanomas from the same patient and between a metastatic lesion and its primary melanoma. The use of single mRNA marker assays may be unreliable because of the heterogeneity and level of single marker mRNA expression in tumor cells. Therefore, using a multiple marker RT-PCR plus Southern blot assay should be more inclusive and avoid potential false-negatives and false-positives. A multiple marker assay assessing mRNA expression for at least two or more markers would be more specific in validating a positive result and improve the sensitivity of detecting metastatic tumor cells.

A panel of MAA mRNA markers may allow the subdivision of tumors into specific tumor stages based on MAA mRNA expression. This was observed when evaluating the hypomelanotic melanoma tissues; only two MAA mRNA markers on average were expressed per specimen, and only one-third of specimens were positive for Tys mRNA expression. This is in contrast to almost 100% mRNA expression for Tys in melanotic tissue. One-half of the hypomelanotic melanoma specimens that did not express Tys mRNA expressed MAA mRNA for another marker. Although only six specimens were evaluated, these results confirm differences observed in mRNA and protein expression observed during tumor progression in murine models (7, 26). A parallel to this process is the potential decrease in expression of antigenic MAA mRNA with the progressive loss of melanization.

The five MAA mRNA markers for detecting melanoma that we have assessed have been demonstrated to be antigenic in humans (1, 27, 28). Identification of MAA mRNA expression profiles in patients with melanoma should be important for directing active specific immunotherapy that can most benefit patients that selectively express certain MAA. This MAA RT-PCR assay may provide a practical approach to assess melanoma cell profiles prior to and after treatment with MAA-specific immunotherapy, thus evaluating the efficacy of treatment. Currently, the use of noninvasive techniques to assess therapeutic interventions in melanoma patients with or without metastases is limited. The MAA RT-PCR plus Southern blot assay should also help in earlier detection of recurrent disease and monitoring tumor progression.

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REFERENCES

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