Detection of Tyrosinase mRNA from the Blood of Melanoma Patients

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Abstract

Surgical therapy for localized melanoma is highly successful. However, if melanoma spreads beyond its primary site, the results of treatment are poor. Therefore, early detection of circulating melanoma cells in the blood may be important. Currently, circulating melanoma cells are undetectable. Tyrosinase is an enzyme in the melanin synthetic pathway the expression of which is only found in melanin-producing cells. Because melanocytes are not normally found in the peripheral blood, we hypothesize that melanoma cells circulating in the peripheral blood could be detected by amplifying the tyrosinase mRNA using the reverse transcription-PCR (RT-PCR). The purpose of this study was to determine the sensitivity of a RT-PCR-based assay for tyrosinase mRNA from peripheral blood and evaluate correlations with tumor status in melanoma patients.

RNA was isolated from the peripheral blood or tissue culture cells, and cDNA was prepared. DNA was amplified using RT-PCR with nested primers for tyrosinase and β2-microglobulin. Serial dilution experiments using cells from the SK-MEL-28 cell line were performed in culture media and in whole blood. Twelve patients with melanoma, 10 healthy controls, and 15 patients with nonmelanoma malignancies were tested for tyrosinase expression in peripheral blood.

The sensitivity of this assay was determined to be as low as 1 melanoma cell in 5 ml of whole blood. No tyrosinase was found in healthy subjects or other cancer control patients. Tyrosinase mRNA was detected in the blood of five melanoma patients (one stage II, two stage III, and two stage IV). Three of these tyrosinase-positive patients had biopsy-proven evidence of melanoma, whereas the other two had no clinical evidence of malignant disease after surgical resection. The remaining seven melanoma patients had no evidence of disease and tested negative for tyrosinase mRNA.

This study suggests that a RT-PCR-based assay for the detection of tyrosinase mRNA in peripheral blood is feasible. Moreover, the presence of tyrosinase mRNA in the blood seems to correlate with the stage of melanoma. Further study and follow-up are needed to clarify the role of tyrosinase mRNA as a tumor marker for malignant melanoma.

Introduction

The incidence of melanoma in the United States is increasing sharply, with nearly 32,000 new cases reported every year (1). It has been estimated that 1 in 75 Caucasians will develop melanoma by the year 2000 (2, 3). Melanoma, if treated early, has an excellent long-term survival rate; however, outcomes for more advanced stages have remained poor (4). A method for detection of micrometastatic disease could, therefore, have significant impact on the detection and treatment of melanoma.

Tyrosinase is the key regulatory enzyme in melanin synthesis (5). It is specifically expressed in melanocytes, and seems to be an ideal target for detecting the presence of melanoma cells. In previous studies (6-8) a nested RT-PCR was used to detect tyrosinase mRNA expression in the blood of predominantly advanced stage III and IV melanomas (6, 7). The sensitivity of this assay was determined to be as low as a single melanoma cell in 2-5 ml of blood (6-7). The RT-PCR technique has also been used to detect circulating cells in cancers of the prostate (9), hepatocellular (10) breast carcinomas (11, 12), and other tumor sites (13). Detection of tumor cells in the bloodstream could be a significant prognostic indicator and may aid in determining which patients would be likely to benefit from systemic antineoplastic therapy. The use of RT-PCR for tyrosinase mRNA may also be useful in monitoring the effectiveness of therapy (14).

The purpose of this study is to determine detection limits of a nested RT-PCR for tyrosinase, as well as to evaluate potential clinical utility of using peripheral blood samples from patients with melanoma to establish the presence of circulating melanocytes.

Materials and Methods

Patients were recruited from the Medical Center of Louisiana, New Orleans and Louisiana State University outpatient clinics. Five ml of peripheral blood were obtained from 37 patients. Patients included 10 healthy controls, 15 patients with nonmelanoma malignant disease (cancer controls), and 12 melanoma patients. Clinical data were collected for correlation with the RT-PCR results. All tumor staging was done in accordance with the American Joint Committee on Cancer system (15). This study was approved by the Louisiana State University Medical Center Institutional Review Board.

The analytical sensitivity of the procedure used in this...
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study was established by the detection of tyrosinase mRNA in serially diluted total RNA from a melanoma cell line (SK-MEL-28) in the presence and absence of 5 μg leukocyte RNA from a normal volunteer. One μg of total melanoma RNA was serially diluted to 5 × 10^{-8} μg/μl, followed by the extraction of RNA from serially diluted SK-MEL-28 cultured cells and the performance of RT-PCR. Finally, the analytical sensitivity was further characterized by the addition of known numbers of SK-MEL-28 tissue culture cells to 5 ml of whole blood. The cells from the melanoma cell line were harvested, counted, and serially diluted and then centrifuged at 1000 rpm. The medium was then decanted, and 5 ml of whole blood were added directly to the melanoma cells. These samples were then analyzed by isolation of RNA and RT-PCR protocols.

Total RNA was isolated from whole peripheral blood samples using standard techniques according to the instructions provided in the Trizol LS total RNA isolation kits (Life Technologies, Gaithersburg, MD; Ref. 16). For reverse transcription, 5 μg of total RNA were added to 0.1% (v/v) diethyl pyrocarbonate treated distilled water, 28 units of Rnasin (Promega Biological Research Products, Madison, WI), 10× Taq PCR buffer (Roche Molecular Systems, Inc., Raritan, NJ; containing 1.5 mM magnesium chloride), 10 mM deoxynucleotide triphosphates, 100 units Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD), and 500 ng tyrosinase outer antisense primer (AGGCATTGTGCATGCTGCTCTCCATGATG; Ref. 19) was similar to that used for the 2-microglobulin primers (sense, ACCTCATTGTGCATGCTGCTCTCCATGATG; antisense, ATCTTCAACACTCCATGATG; Ref. 19) was similar to that used for the tyrosinase outer primers except that the annealing temperature was 57°C. For sensitivity testing, whole blood from a normal patient was used as an additional internal control to ensure that each sample had not been contaminated by RNases. Only a single PCR was necessary to detect β₂-microglobulin mRNA expression. The PCR profile used for β₂-microglobulin primers (sense, ACCCCACTGAAAAAGATGA; antisense, ATCTTCAACACTCCATGATG; Ref. 19) was similar to that used for the tyrosinase outer primers except that the annealing temperature was 57°C. For sensitivity testing, whole blood from a normal patient was used as an additional negative control to ensure that no tyrosinase transcripts were detected. All the necessary precautions against contamination of PCRs were rigorously observed (20).

Tyrosinase mRNA detection was classified as positive if a band 207 bp in size was detected after the initial nested RT-PCR and was confirmed by repeat analysis. A sample was considered negative for tyrosinase mRNA expression if no band 207 bp in size was detected. Detection of β₂-microglobulin mRNA was considered positive if a band 123 bp in size was detected on each run.

Results

In an effort to define the sensitivity of the assay RT-PCR performed upon serially diluted SK-MEL-28 cultured cells revealed that tyrosinase mRNA was consistently detected in the dilution containing one melanoma cell. The presence of 5 μg of leukocyte RNA had no effect on the analytical sensitivity. Sensitivity of the assay was further analyzed by the direct

<table>
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<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Race</th>
<th>Race</th>
<th>Stage</th>
<th>Prior treatment</th>
<th>Clinical status</th>
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<th>β-Microglobulin mRNA</th>
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<td>F</td>
<td>W</td>
<td>III</td>
<td>Wide excision and nodal dissection</td>
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<td>Detected</td>
<td>Detected</td>
</tr>
<tr>
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<td>M</td>
<td>W</td>
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<td>Wide excision and node dissection</td>
<td>AD</td>
<td>Detected</td>
<td>Detected</td>
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</tr>
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* F. female; M. male; W, white; B, black; AD, alive with disease.

Table 1 Melanoma patients tested for tyrosinase and β-microglobulin mRNA via nested RT-PCR

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This stage III patient with tumor in situ had tyrosinase mRNA detected. It was not found in this patient 4 months after complete surgical resection.

The cancer control patients (Table 2), stages I-IV (15), ranged in age from 34 to 71 years. Nine were female, and six were male; eight were Caucasian, and seven were African American. The healthy control group consisted of 10 patients with no history or clinical evidence of malignancy, between 25 and 66 years of age. One was African American, seven were Caucasian, and two were Asian; seven were female, and three were male. Both control groups also underwent RT-PCR using 1 and 5 µg of total RNA. No tyrosinase mRNA was detected in the 1 or 5 µg of samples of any healthy or cancer control patient. β2-microglobulin was detected in all of the experimental and control samples.

**Discussion**

This study confirms that a RT-PCR-based assay for tyrosinase mRNA can detect tyrosinase in the blood of patients with cutaneous malignant melanoma. The sensitivity of the system used in this study, analyzed by serial dilution and direct addition of melanoma cells, was found to be as low as 1 melanoma cell in 5 ml of whole blood. This level of detection is better than the one found in the initial description of this technique (6) and equivalent with that found by other groups (7, 8). Furthermore, this study confirms previous reports using RT-PCR techniques as a tumor marker from peripheral blood (6-14).

Because none of the healthy volunteers or cancer control patients had a positive result, it appears that the specificity of this marker is quite high (100% in this study). However, because there is no standard method to determine the presence of circulating melanoma cells, the true specificity of this assay is unknown. However, all three patients with biopsy-proven melanoma had a positive result, suggesting a good sensitivity for gross disease. Clearly, further experience is required to define the precise sensitivity and specificity of RT-PCR based tyrosinase assays.

Four out of five of the tyrosinase-positive samples were advanced (stage III-IV) melanomas. This result correlated well with previous studies that also frequently detected tyrosinase mRNA in more advanced-stage disease (6-8). Furthermore, the positive result in cases 5 and 6 (see Table 1) was found in patients who clinically had no evidence of disease. Should these patients go on to develop clinical recurrence, this would suggest potential utility of this assay to screen for recurrence or to detect occult metastases. The use of other markers in addition to tyrosinase may increase the accuracy of this approach (8). Whether tyrosinase mRNA blood assays will be able to detect subclinical disease before signs or symptoms become manifest will require additional follow-up (21).

Surgical resection of the primary lesion was associated with a change to no detectable mRNA in patient 8. The ability to confirm the success of a surgical resection with tyrosinase has promise as a potential indicator for further adjuvant therapy. However, any correlation between surgery and melanoma cell detection is an area demanding future exploration.

To form clinically detectable metastases, circulating tumor cells must extravasate, survive, and grow (22). The finding of circulating melanoma cells in patients without distant clinical evidence of metastatic disease may refute the premise that most cells extravasate and survive (22). Whether any patient who is found to have circulating melanoma cells by tyrosinase RT-PCR is doomed to distant metastasis remains to be seen (22). Furthermore, it may be possible, using a quantitative RT-PCR technique, to monitor the success of therapy for advanced or
clinically occult melanoma (24). Additionally, this system may also be applicable to ocular and mucosal melanoma (25). Clearly, further follow-up and experience will clarify possible clinical applications of peripheral blood tyrosinase mRNA assays as a tumor marker for melanoma.

References

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