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 B2-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 malignant 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 (9) 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 study 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.
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 Rnase (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 (AGGCATTGTGCATGCTGACTCCATGATG; Ref. 19) was similar to that used for the PCR profile used for 2-microglobulin primers (sense, AC-TCCCATGAGAGTAGTA; antisense, ATCTTCAACCTCCATGTAG; 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, AC-CCCCACTGAAAAAGATGA; antisense, ATCTTCAACCTCCATGTAG; 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 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 1: Melanoma patients tested for tyrosinase and β₂-microglobulin mRNA via nested RT-PCR

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Race</th>
<th>Stage</th>
<th>Prior treatment</th>
<th>Clinical status</th>
<th>Tyrosinase mRNA</th>
<th>β₂-Microglobulin mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>F</td>
<td>W</td>
<td>I</td>
<td>Wide excision</td>
<td>NED</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>M</td>
<td>W</td>
<td>I</td>
<td>Wide excision</td>
<td>NED</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>M</td>
<td>W</td>
<td>II</td>
<td>Wide excision</td>
<td>NED</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>F</td>
<td>W</td>
<td>II</td>
<td>Wide excision</td>
<td>NED</td>
<td>Detected</td>
<td>Detected</td>
</tr>
<tr>
<td>5</td>
<td>58</td>
<td>M</td>
<td>W</td>
<td>II</td>
<td>Wide excision</td>
<td>NED</td>
<td>Detected</td>
<td>Detected</td>
</tr>
<tr>
<td>6</td>
<td>39</td>
<td>F</td>
<td>W</td>
<td>III</td>
<td>Wide excision and node dissection</td>
<td>NED</td>
<td>Detected</td>
<td>Detected</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>M</td>
<td>W</td>
<td>III</td>
<td>Wide excision</td>
<td>NED</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>8A</td>
<td>68</td>
<td>M</td>
<td>W</td>
<td>III</td>
<td>None</td>
<td>AD</td>
<td>Detected</td>
<td>Detected</td>
</tr>
<tr>
<td>8B</td>
<td>69</td>
<td>M</td>
<td>W</td>
<td>III</td>
<td>Wide excision and nodal dissection</td>
<td>NED</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>9</td>
<td>30</td>
<td>M</td>
<td>W</td>
<td>III</td>
<td>Wide excision and nodal dissection</td>
<td>NED</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>10</td>
<td>61</td>
<td>M</td>
<td>W</td>
<td>IV</td>
<td>Multiple resections, limb perfusion, and chemotherapy</td>
<td>AD</td>
<td>Detected</td>
<td>Detected</td>
</tr>
<tr>
<td>11</td>
<td>39</td>
<td>M</td>
<td>B</td>
<td>IV</td>
<td>Excision and node dissection</td>
<td>NED</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>12</td>
<td>49</td>
<td>F</td>
<td>W</td>
<td>IV</td>
<td>Wide excision and node dissection</td>
<td>AD</td>
<td>Detected</td>
<td>Detected</td>
</tr>
</tbody>
</table>

* F, female; M, male; W, white; B, black; AD, alive with disease.
This stage III patient with tumor

Table I and Fig. I

patients had obvious, biopsy-proven, clinical disease (pa-

patient 8A. The positive control and patient 8B reveal the amplified 207-bp

1.

Fig. 1.

from a normal volunteer. Tyrosinase mRNA was consistently
detected at a level of 1–2 melanoma cells in 5 ml of whole
blood. β2-microglobulin (internal control) mRNA was also
consistently detected, confirming that the samples were suitable
for RT-PCR.

The 12 melanoma patients (Table 1), stages I–IV (15),
ranged in age between 30 and 69 years. Melanoma patients
were treated with surgical resection, with only one patient
having undergone chemotherapy. These melanoma patients
consisted of four females and eight males, eleven Caucasians
and one African American. Of the 12 melanoma patients, 9
patients had no clinical evidence of disease (Table 1). Three of
the patients had obvious, biopsy-proven, clinical disease (pa-
tients 8A, 10, and 12), and all of these samples were positive for
tyrosinase mRNA. The two samples that were positive for
tyrosinase mRNA in patients with NED both had high-risk
lesions and/or previously resected diseased lymph nodes. Ty-
rosinase mRNA was detected in five melanoma patients (see
Table 1 and Fig. 1). Of the positive samples, one stage II
patient, one stage III patient, and two stage IV patients had prior
surgery, whereas one stage III patient had no prior treatment.
This stage III patient with tumor in situ had tyrosinase mRNA
detected. It was not found in this patient 4 months after com-
plete 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 experimen-
tal 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 addi-
tion 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, be-
cause 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 mel-
anoma 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 tyrosi-
nase 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 ther-
apy. 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 (23). 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
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clinchoodle 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


Table 2 Cancer patients tested for tyrosinase and β-microglobulin mRNA via nested RT-PCR

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Race</th>
<th>Cancer, stage</th>
<th>Prior treatment</th>
<th>Tyrosinase mRNA</th>
<th>β-Microglobulin mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>71</td>
<td>M</td>
<td>B</td>
<td>Colon, II</td>
<td>Colectomy</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>2</td>
<td>44</td>
<td>F</td>
<td>W</td>
<td>Breast, III</td>
<td>None</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>3</td>
<td>41</td>
<td>F</td>
<td>W</td>
<td>Neurofibrosarcoma, II</td>
<td>Lesion excision</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>4</td>
<td>41</td>
<td>M</td>
<td>B</td>
<td>Rectal, II</td>
<td>None</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>5</td>
<td>34</td>
<td>M</td>
<td>B</td>
<td>Rectal, I</td>
<td>None</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>6</td>
<td>58</td>
<td>F</td>
<td>W</td>
<td>T-cell lymphoma</td>
<td>None</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>7</td>
<td>65</td>
<td>F</td>
<td>B</td>
<td>Breast, IV</td>
<td>None</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>8</td>
<td>39</td>
<td>M</td>
<td>B</td>
<td>Metastatic adenocarcinoma, IV</td>
<td>None</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>9</td>
<td>28</td>
<td>F</td>
<td>B</td>
<td>Leukemysarcoma, IV</td>
<td>Surgery, radiation, and chemotherapy</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>10</td>
<td>67</td>
<td>M</td>
<td>W</td>
<td>NSCLC, II</td>
<td>Pneumonectomy</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>11</td>
<td>61</td>
<td>F</td>
<td>B</td>
<td>SCLC, IV</td>
<td>Chemotherapy</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>12</td>
<td>59</td>
<td>F</td>
<td>W</td>
<td>Breast, IV</td>
<td>None</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>13</td>
<td>52</td>
<td>F</td>
<td>W</td>
<td>Basal cell, II</td>
<td>Wide excision</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>14</td>
<td>60</td>
<td>M</td>
<td>W</td>
<td>Colon, IV</td>
<td>Chemotherapy</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>15</td>
<td>54</td>
<td>F</td>
<td>W</td>
<td>Rectal, IV</td>
<td>None</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
</tbody>
</table>

* M, male; F, female; B, black; W, white.
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