Microphthalmia-associated Transcription Factor and Tyrosinase as Markers of Melanoma Cells in Blood of Patients with Melanoma

Ivan Šamija, Josip Lukač, Jasmina Marić-Brozić, Zvonko Kusič

Department of Oncology and Nuclear Medicine, Sisters of Mercy University Hospital, Zagreb, Croatia

Aim. To investigate whether analysis of microphthalmia-associated transcription factor (MITF) as an additional marker to tyrosinase in melanoma patients can improve the detection of circulating melanoma cells by reverse-transcription-polymerase chain reaction (RT-PCR).

Methods. Blood samples were taken from 33 patients with metastatic melanoma. RNA was isolated from mononuclear cell fraction of the blood and reversely transcribed into the complementary DNA (cDNA). The cDNA was assayed by PCR for the expression of tyrosinase and MITF. Peripheral blood samples from 15 healthy subjects were used as controls.

Results. The threshold for detection of both tyrosinase and MITF was set low enough to detect 50 melanoma cells in 10 mL of healthy volunteer blood in the relative ratio of one melanoma cell in 0.82x10^6 peripheral blood leukocytes. Out of 33 blood samples from metastatic melanoma patients, 5 were positive for both tyrosinase and MITF, 7 for tyrosinase only, and 5 for MITF only. All samples from healthy volunteers were negative for both tyrosinase and MITF.

Conclusion. Determination of MITF marker in addition to tyrosinase improved the detection of circulating melanoma cells in patients with metastatic melanoma.

Key words: melanoma; monophenol monooxygenase; neoplasm circulating cells; neoplasm metastasis; reverse transcriptase polymerase chain reaction; tumor markers, biological

Malignant melanoma is a tumor with an increasing incidence (1,2). In Eastern and Southern Europe, the estimated annual change of melanoma incidence rate is 2.3-8.9% (2). The prognosis for melanoma patients with distant metastases is very poor, their estimated 5-year-survival rate being only 5.5% (1,3). Therefore, it is important to find biological factors to predict the clinical behavior of melanoma, especially the spread of metastases, and to improve the management of the patients.

The presence of circulating tumor cells may indicate a high risk of metastases (4-6). Today, we are able to detect a single tumor cell in peripheral blood sample due to the development of highly sensitive reverse transcription-polymerase chain reaction (RT-PCR) (7). The most widely used melanoma-specific marker in RT-PCR detection of metastatic melanoma cells is the expression of tyrosinase gene (7). Tyrosinase, or monophenol monooxygenase, is an enzyme that plays a role in the biosynthesis of melanin and is specifically expressed by melanocytes and melanoma cells (8). Since melanocytes do not normally circulate, the detection of tyrosinase transcripts in the peripheral blood is considered an evidence of circulating melanoma cells. Smith et al (9) were first to use tyrosinase RT-PCR to detect circulating melanoma cells and confirmed it in 4 out of 7 melanoma patients tested. There have been many studies of this method thereafter and in most of them, none of healthy volunteers or patients with non-melanoma cancer tested was positive for tyrosinase (4-6,9-18). Also, a very low threshold set in most studies allowed the detection of a single melanoma cell in one million peripheral blood mononuclear cells (4-6,9-18).

The major limitation in clinical use of tyrosinase as a prognostic marker is a relatively high number of melanoma patients with distant metastases being tyrosinase negative (10). In 23 different studies analyzed by Tsao et al (10), 45% of melanoma patients with distant metastases were positive for tyrosinase on average. It has been shown that two-marker RT-PCR with tyrosinase and Melan-A/MART-1 melanoma markers enables detection of circulating melanoma cells in a higher percentage of melanoma patients (5,18,19). Other molecules, such as MUC18, p97, and gp100, were also analyzed as potential RT-PCR melanoma markers but were also found positive in healthy subjects, which made them unreliable as melanoma markers (18,20,21).
Microphthalmia-associated transcription factor (MITF) is a transcription factor essential for the development and survival of melanocytes (22). There are at least five different isoforms of MITF protein with different amino-terminal regions, MITF-A, MITF-B, MITF-C, MITF-H, and MITF-M (22). MITF has shown high specificity and sensitivity as an immunohistochemical melanoma marker (23-25). RT-PCR analysis has shown high expression of MITF-M isoform of MITF in majority of the melanoma and normal melanocytic cell lines, and no expression in other tumor and normal cell lines analyzed (26,27).

Aim of our study was to determine whether analysis of MITF as an additional marker to tyrosinase could improve the RT-PCR detection of circulating melanoma cells in melanoma patients.

Subjects and Methods

Subjects

We analyzed peripheral blood samples from 33 metastatic melanoma patients (16 men and 17 women) aged 25-73 years. All patients had histologically confirmed malignant melanoma and were treated and followed-up at the Department of Oncology and Nuclear Medicine, Sisters of Mercy University Hospital, Zagreb, Croatia. Clinical stage was defined according to the American Joint Committee on Cancer (AJCC) guidelines (28). Twenty patients had metastases of regional lymph nodes (stage III) and 13 patients had distant metastases (stage IV). Blood samples from 15 healthy volunteers (7 men and 8 women, aged 27-65 years) were taken as controls. Informed consent according to the World Medical Association Declaration of Helsinki was obtained from all the patients and healthy volunteers before their inclusion in the study (29).

Blood Samples Collection and Processing

Ten milliliters of peripheral blood from each subject was collected in tubes containing ethylenediamine tetraacetic acid (EDTA) as anticoagulant. Blood samples were processed within two hours after collection. The mononuclear cell fraction of blood was isolated on Ficoll gradient (1.077 g/cm³; Axis-Shield PoC AS, Oslo, Norway) as described by Boyum (30). Three milliliters of Ficoll was overlayed with 5 mL of blood diluted with 5 mL of phosphate-buffer saline (PBS). After centrifugation for 15 minutes at room temperature at 500 g, the layer between the plasma and the Ficoll was transferred into another tube and washed twice with PBS by centrifugation for 15 minutes at 4°C at 800 g. The cell pellet was used immediately for RNA isolation.

RNA Isolation and Reverse Transcription

RNA was isolated from mononuclear cell fraction with the TriPure Isolation Reagent (Roche, Manheim, Germany), 0.2 mmol/L of each dNTPs (Sigma), 0.2 μmol/L sense primer, 0.2 μmol/L antisense primer, and 0.02 U/μL Taq DNA polymerase (Eppendorf) in a total volume of 25 μL. PCR reaction conditions were as follows: one cycle of 5 minutes at 95°C followed by 35 cycles of 1 minute at 95°C for denaturation, one minute at 55°C for primer annealing, and 45 s at 72°C for polymerase extension, followed with a final 7-minute extension at 72°C.

For the first round of PCR analysis of MITF expression, 2 μL of cDNA were added to the reaction mixture containing final concentrations of 1× Taq buffer with Mg²⁺ (50 mMol/L KCl, 1.5 mMol/L MgCl₂, 10 mMol/L Tris-HCl, 1×PCR, Enhancer Solution (Invitrogen), 0.3 mMol/L of each dNTPs (Sigma), 100 μMol/L MgSO₄ (Invitrogen, Carlsbad, USA), 0.3 μMol/L sense primer, 0.3 μMol/L antisense primer, and 0.025 U/μL Platinum PfX DNA polymerase (Invitrogen) in a total volume of 25 μL. PCR reaction conditions were as follows: one cycle of 2 minutes at 94°C followed by 35 cycles of 1 minute at 94°C for denaturation, 30 s at 60°C for primer annealing, and 50 s at 68°C for polymerase extension, followed with a final 7-minute extension at 68°C.

For the second round of PCR analysis both tyrosinase and MITF expression, 2 μL of the first-round PCR product were used. Cycling conditions and composition of PCR reaction mixture were the same as for the first-round PCR.

For all samples, a housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified to determine the integrity of the RNA. Thirty cycles of PCR were performed. The cycling conditions and composition of PCR reaction mixture were the same as for tyrosinase PCR.

RT-PCR reaction for both tyrosinase and MITF was performed twice for each blood sample, with the same RNA sample. If the results of the second assay did not confirm the results of the first one, the blood sample was excluded from the study.

All PCR products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide and directly visualized under UV light at 302 nm. DNA molecular weight markers VIII or IX (Roche, Manheim, Germany) were included in all gels. A sample was considered positive if a band of expected size (207 bp for tyrosinase, 623 bp for GAPDH, and 421 bp for MITF) was present.

Oligonucleotide Primers

For GAPDH cDNA amplification previously described primer sequences were used (ref 12, Table 1). For tyrosinase

| Table 1. Sequences of oligonucleotide primers used for detection of target genes by reverse transcription-polymerase chain reaction (RT-PCR)* |
|---|---|---|
| Target gene | Primer | Sequence |
| GAPDH | GAPDH1 (sense) | 5'-AAC GGA TTT GCT GGT ATT GGG C-3' |
| Tyrosinase | GAPDH2 (antisense) | 5'-AGG CAT GAT GAT CCT GAG ACC C-3' |
| | HTYR1 (outer sense) | 5'-TTG GCA ATG TCT CGT TAG TTC CC-3' |
| | HTYR2 (outer antisense) | 5'-ACT CAT TGT GCA TGC TTG TT-3' |
| | HTYR3 (inner sense) | 5'-GTC TTT ATG CAA TGG AAC GC-3' |
| | HTYR4 (inner antisense) | 5'-GCT ATC CCA GTA AGT GCA CT-3' |
| MITF | MITF1 (outer sense) | 5'-AGG GAG GAA TAG TCT ACC GTC TCT CC-3' |
| | MITF2 (outer antisense) | 5'-GCA GGG AGG ATT CGC TAA CAA GTG CT-3' |
| | MITF3 (inner sense) | 5'-TAT CAG GTG CAG ACC CAC CTC-3' |
| | MITF4 (inner antisense) | 5'-CAG GCA ACG TAT TTT CCA TT-3' |

*GAPDH – glyceraldehyde-3-phosphate dehydrogenase; MITF – microphthalmia-associated transcription factor.
cDNA amplification primer sequences following the original design by Smith et al (9) were used. The primer sequences used for MITF cDNA amplification were devised from the published sequence for human MITF gene (32). The primers were designed in the way that they span an intron, allowing the differentiation of cDNA products from potentially contaminating genomic DNA. For PCR amplification of MITF, primers that amplify only MITF-M isoform and not the other ones were designed, because only MITF-M is specifically expressed in melanoma cells (22,26,27).

**Setting the Threshold for Detection of RT-PCR Assay**

To set the threshold for melanoma cell detection of the assay, we spiked healthy volunteer peripheral blood samples with serially diluted melanoma cells.

Established human melanoma cell line (HBL) was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (33). The cells for the experiment were collected after trypsinization with 0.25% trypsin and 0.01% EDTA, washed with PBS, and counted. Serial dilutions of $10^5, 10^4, 10^3, 10^2, 50, 10, 1$ cells were prepared each in 1 mL of PBS. The serially diluted cells were added to 10 mL healthy volunteer blood. The number of peripheral blood leukocytes was determined in each blood sample. This allowed us to express the threshold for detection of the assay as the number of melanoma cells with respect to the blood volume and to the number of peripheral blood leukocytes. The blood samples were processed and analyzed by RT-PCR as described above.

The spiking experiment was repeated three times by using three independently cultured cells and one blood sample for each dilution each time.

**Results**

**Threshold for Detection of the RT-PCR Assay**

To set the assay threshold for melanoma cell detection, we added serially diluted cells from the established HBL melanoma cell line to the peripheral blood samples obtained from healthy volunteers. Previous studies have revealed high expression of both tyrosinase and MITF in this cell line (26,34). After two rounds of 35 cycles nested PCR with primers for tyrosinase, we were able to detect 50 HBL melanoma cells in 10 mL of healthy volunteer blood in a relative ratio of one HBL melanoma cell in $0.82 \times 10^6$ peripheral blood leukocytes (Fig. 1). After two rounds of 35 cycles nested PCR with primers for MITF, we were also able to detect 50 HBL melanoma cells in 10 mL of healthy volunteer blood in the relative ratio of one HBL melanoma cell in $0.82 \times 10^6$ peripheral blood leukocytes (Fig. 2).

**Analysis of Blood Samples from Melanoma Patients**

Blood samples from 33 patients with metastatic melanoma were analyzed for the expression of tyrosinase and MITF by RT-PCR. GAPDH mRNA was detected in all metastatic melanoma and healthy volunteer blood samples (data not shown), indicating that both RNA preparation and cDNA synthesis were successful.
Tyrosinase mRNA was detected in blood samples from 12 of 33 tested patients (Fig. 3). Five of 13 patients with distant metastases (stage IV) and 7 of 20 patients with regional lymph node metastases (stage III) were positive for tyrosinase (Table 2). Tyrosinase mRNA was not detected in any of 15 healthy volunteer samples.

MITF mRNA was detected in blood samples from 10 of 33 tested patients (Fig. 4). Five of 13 patients with distant metastases (stage IV) and 5 of 20 patients with regional lymph node metastases (stage III) were positive for MITF (Table 2). MITF mRNA was not detected in any of 15 healthy volunteer samples found positive for MITF (data not shown). The bands obtained were of the size expected for MITF.

### Table 2.
The positive expression of tyrosinase and microphthalmia-associated transcription factor (MITF) in patients with melanoma stages III (regional lymph node metastases) and IV (distant metastases) according to the American Joint Committee on Cancer (AJCC)

<table>
<thead>
<tr>
<th>Expression of tyrosinase and MITF</th>
<th>AJCC stage (No. of patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosinase positive</td>
<td>III (n = 20) IV (n = 13)</td>
</tr>
<tr>
<td>MITF positive</td>
<td>7</td>
</tr>
<tr>
<td>Tyrosinase and MITF positive</td>
<td>2</td>
</tr>
</tbody>
</table>

Both tyrosinase and MITF mRNA were detected in blood samples from 5 of 33 tested patients. Three of 13 patients with distant metastases (stage IV) and 2 of 20 patients with regional lymph node metastases (stage III) were positive for both tyrosinase and MITF (Table 2). MITF mRNA was detected in 5 patients (3 of them with stage III, and 2 with stage IV disease) negative for tyrosinase. Thus, in comparison with tyrosinase analysis alone, additional analysis of MITF allowed for detection of circulating melanoma cells in a larger number of patients (17 vs 12 positive out of 33 patients).

### Discussion

In this study, MITF was investigated for the first time as a marker for RT-PCR detection of circulating melanoma cells. MITF was investigated as an additional marker to tyrosinase, the most widely used melanoma marker for detection of circulating melanoma cells.

None of the 15 healthy volunteer samples analyzed in our study was positive either for tyrosinase or for MITF. These results are in agreement with all previous studies (4-6,9-21). Only two of 521 negative control persons (healthy volunteers or patients with non-melanoma cancer) in 23 different studies analyzed in a meta-analysis by Tsao et al (10) were positive for tyrosinase.

The threshold for detection of both tyrosinase and MITF in our study was low enough to detect 50 HBL melanoma cells in 10 mL of healthy volunteer blood in the relative ratio of one HBL melanoma cell in 0.82 × 10⁶ peripheral blood leukocytes. In most other studies, the threshold for detection between 1 and 10 melanoma cell in 10 mL of blood was reported with all markers tested (4-6,9-21). In our study, higher threshold for detection may be the result of cell line we used. It has been shown that different melanoma cell lines have different levels of expression of different melanoma antigens, including tyrosinase (14,18). The threshold for RT-PCR detection of circulating melanoma cells is influenced by the method of blood sample preparation (15,35). Density gradient separation, the method we used in our research, has been shown to result in lower threshold for melanoma cell detection than whole blood analysis (15,35).

When we used Taq DNA polymerase for PCR analysis of MITF expression, we often obtained some additional bands together with the band of the expected size (data not shown). These additional bands could have been products of amplification of some other isoform of MITF due to a relatively low specificity of Taq DNA polymerase. Therefore, we used Platinum Pfx DNA polymerase for PCR analysis of MITF expression. Platinum Pfx DNA polymerase possesses a proofreading 3’ to 5’ exonuclease activity, which makes it highly specific. We also modified PCR reaction conditions, by increasing the annealing temperature and decreasing the annealing time and elongation time, to further increase the specificity of PCR reaction. By using Platinum Pfx DNA polymerase and modified PCR reaction conditions, we were able to obtain only distinctive bands of the expected size in positive samples.

To detect RT-PCR products, we used electrophoresis on 2% agarose gel stained with ethidium bromide and visualization under UV light. The same method was used in most of the other studies (5,9,13-15,17-20,35). In some studies, the more sensitive method of Southern blot detection after
agarose gel electrophoresis was used, but the results regarding the threshold for melanoma cell detection and percentage of positive samples did not significantly differ from those obtained by ethidium bromide staining (4,6,11,12,16,21).

When we increased the number of second-round PCR cycles from 35 to 40, in two of 15 healthy volunteer samples we obtained bands of the size expected for MITF. We repeated PCR for these samples 3 times at 35 cycles and they were always negative. Since our primers for MITF amplification were designed to span the intron and to be specific exclusively for MITF-M isoform, and because of the high specificity of nested PCR approach, we excluded the possibility of these bands being products of genomic DNA or other MITF isoform amplification. However, these bands might be the result of a very low level of cross contamination (36) or a very low expression of targeted gene in normal blood cells (37). Since MITF has shown high specificity for melanoma cells only when analyzed at protein level by immunohistochemical methods, it is possible to find it expressed by some normal cells when analyzed at mRNA level by RT-PCR, which is a more sensitive method of gene expression analysis (23-25).

In our group of 33 patients with metastatic melanoma, 12 were positive for tyrosinase (7 of 20 stage III patients and 5 of 13 stage IV patients), which corresponds with the findings by Tsao et al (10), especially where patients with stage III were concerned.

The fact that in most studies a relatively high number of melanoma patients with distant metastases (stage IV) were tyrosinase negative makes clinical relevance of RT-PCR for tyrosinase as a prognostic marker disputable. There are several possible explanations for patients with distant metastases being negative for tyrosinase. A model of metastatic process in which tumor cells are shed in the circulation in a random, discontinuous manner may explain negative results in patients with distant metastases (16). This model is corroborated with results of an experiment in which blood samples taken from the same patient at different time points, which is difficult to carry out in practice because blood samples are usually taken during follow-up visits.

Furthermore, it is possible that in a proportion of circulating melanoma cells the expression of tyrosinase gene is below the detection threshold of the method. Indeed, Chen et al. (38) have found different levels of tyrosinase expression in different melanoma tissue specimens, a small proportion of them being even negative. In that case, detection of melanoma cells can be improved by using multiple markers, which is why we decided to investigate MITF as an additional melanoma marker to tyrosinase.

So far, determination of MITF marker has been investigated only as a sensitive and specific immunohistochemical melanoma marker (23-25). RT-PCR analysis has shown high expression of MITF in most melanoma and normal melanocytic cell lines, and no expression in other tumor and normal cell lines analyzed (26,27). That led us to the hypothesis that MITF could be used as a marker for RT-PCR detection of circulating melanoma cells. In our group of 33 patients with metastatic melanoma, 10 were positive for MITF. Analysis of MITF in addition to tyrosinase allowed the detection of circulating melanoma cells in a larger number of patients than the tyrosinase analysis alone (17 vs 12 positive out of 33 patients). This is in agreement with the hypothesis that circulating melanoma cells are heterogeneous regarding the level of expression of different melanoma markers.

The results of other researches also support the use of multiple markers. Additional analysis of Melan-A/MART-1, in comparison with tyrosinase analysis alone, increased the sensitivity of melanoma cell detection by 31% in one research (19) and by 9% in another (18). Curry et al (5) have also shown that the introduction of Melan-A/MART-1 analysis in addition to tyrosinase allowed for the detection of circulating melanoma cells in a larger percentage of patients. Hoon et al (21,39) analyzed four different melanoma markers (tyrosinase, MAGE-3, MUC18, and p97) and found that the number of positive markers was a significant independent variable for predicting disease recurrence.

In our study, we showed that using MITF in addition to tyrosinase improved the detection of circulating melanoma cells. However, to determine a real clinical significance of MITF as a marker for RT-PCR detection of circulating melanoma cells, a larger follow-up study that would address prognostic value of MITF as RT-PCR marker is needed. That study should include a large and representative population of melanoma patients in all stages of the disease and should be designed to allow multiple blood sampling, which were the main limitations of our study.

Acknowledgment

This work was supported by Research Grant 0134011 from the Ministry of Science and Technology, Republic of Croatia. Established HBL melanoma cell line was kindly provided by Dr Sanja Kapitanovic, Ruđer Boškovic Institute, Zagreb, Croatia.

References


19 Schitteb B, Bodingbauer Y, Ellwanger U, Blaheta HJ, Garbe C. Amplification of MelanA messenger RNA in addition to tyrosinase increases sensitivity of melanoma cell detection in peripheral blood and is associated with the clinical stage and prognosis of malignant melanoma. Br J Dermatol. 1999;141:30-6.


Received: October 7, 2003
Accepted: February 10, 2004

Correspondence to:
Ivan Šamija
Department of Oncology and Nuclear Medicine
Sisters of Mercy University Hospital
Vinogradska cesta 29
10000 Zagreb, Croatia
isamija@kbsm.hr