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# Detection of Circulating Melanoma Cells by a Two-marker Polymerase Chain Reaction Assay in Relation to Therapy

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**Malignant melanoma is one of the most rapidly increasing cancer types, and patients with metastatic disease have a very poor prognosis. Detection of metastatic melanoma cells in circulation may aid the clinician in assessing tumor progression, metastatic potential, and response to therapy. Tyrosinase is a key enzyme in melanine biosynthesis. The gene is actively expressed in melanocytes and melanoma cells. Melan A is a differentiation antigen that is expressed in melanocytes. The presence of these molecules in blood is considered a marker for circulating melanoma cells. In this study, we analyzed the usefulness of this marker combination in evaluating the response to therapy in the blood of 30 patients with malignant melanoma. Circulating cells were detected by a reverse-transcriptase-polymerase-chain reaction. The tyrosinase expression was observed in 9 (30%) patients and Melan A in 19 (63.3%) patients before therapy. Following treatment, the tyrosinase mRNA was detected in only one patient, while Melan A transcripts were still present in 14 patients. We suggest that this molecular assay can identify circulating melanoma cells that express melanoma-associated antigens and may provide an early indication of therapy effectiveness.**

**Keywords:** Chemotherapy, Malignant melanoma, Melan A, Tyrosinase

## Introduction

Malignant melanoma is one of the fastest growing cancer forms with an annual increase of about 5% (Mellado *et al*., 1996; Proebstle *et al*., 2000). Patients with metastatic disease have a very poor prognosis, with a 2-year survival rate of less than 5% (Evans and Manson, 1994). In general, malignant melanoma metastasizes via the lymphatics and blood. Unfortunately, the metastatic risk cannot be estimated early enough by routine diagnostic and imaging methods. The presence of melanoma cells in circulation seems to be positively correlated with the rapid progress of the disease (Shivers *et al*., 2001). Therefore, the detection of occult tumor cells in blood may permit a more accurate assessment of the prognosis and have important therapeutic implications (Foss *et al*., 1995).

Tyrosinase is a key enzyme in melanin biosynthesis. The gene codes for a 529 amino acid membrane-bound glycoprotein, and it is only actively expressed in melanocytes (Hoon *et al*., 1995; Lipton *et al*., 1997). The polymerase chain reaction-based detection of the tyrosinase messenger RNA in peripheral blood permits the detection of small numbers of circulating tumor cells. It has been reported that PCR-positivity during the early melanoma stages may indicate an increased risk for the development of hematogenous metastases (Mellado *et al*., 1996; Johansson *et al*., 2000).

Other genes, such as gp100 (Adema *et al*., 1994) or MUC18 and p97 (Curry *et al*., 1998), have also been reported to be expressed in cells of melanocytic origin. However, recent evidence suggests that these proteins are not specific to melanocytic cells, but can also be found in many different cell types (Brouwenstijn *et al*., 1997; Pickl *et al*., 1997; Curry *et al*., 1998). Therefore, these molecules are unsuitable for investigating melanoma cells in peripheral blood.

Another candidate as a marker is Melan A, the Melanoma Antigen that is recognized by the T-cells 1 (MART-1). The gene codes for a 118 amino acid melanocyte-lineage differentiation antigen that is only expressed in melanocytes, melanocytic cells, and in the retina (Coulie *et al*., 1994; Chen *et al*., 1996). The protein is recognized by melanoma-reactive CD8 cytotoxic T-cells (Zarour *et al*., 2000), and a peptide that is derived from the protein binds to the class I major histocompatibility complex HLA A2 molecule (Ouesnel *et al*., 2001). Most melanoma tumors and cell lines express this

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protein. However, its expression is not detected in other tissues and tumors (Goydos *et al*., 1998; Schittek *et al*., 1999).

The detection of circulating melanoma cells in peripheral blood may help in the initial diagnosis and staging by allowing the detection of subclinical metastases. It may also improve our ability to monitor the spreading of metastatic disease and provide a means to study the effects of systemic therapy on circulating tumor cells (Taback *et al*., 2001; Blaheta *et al*., 1998). Although multiple-marker analyses are more accurate than single-assay studies, combining different molecular parameters are rare, and the association between the detection of tumor cells and assessment of responsiveness to therapy and outcome has not been addressed adequately. In this study, we employed a sensitive two-marker RT-PCR assay to investigate the presence of tyrosinase and Melan A mRNAs in the peripheral blood of patients with malignant melanoma before and after therapy, in order to assess their utility in terms of treatment and prognosis.

## Materials and Methods

Peripheral blood samples were collected from 30 patients (13 women and 17 men; mean age, $52.7 \pm 14$) with histologically-confirmed malignant melanoma with either localized or metastatic disease before and after treatment. The first blood sample was taken upon admission when all of the patients were still harboring the tumors. The final blood sample was collected two weeks after the completion of therapy. Blood was collected using EDTA as an anticoagulant, since heparin is known to inhibit both reverse transcriptase and Taq polymerase. Peripheral blood samples from 20 healthy donors were also investigated. To exclude variations in the amount of antigens, the blood samples from each patient were collected on two successive days at different times.

One patient had Stage II, 18 had Stage III, and 11 had Stage IV of the disease. Visceral metastases were present in 11 patients. Patients with the Stage III disease received Interferon ($9 \times 10^6$ IU subcutaneously) 3 times weekly for one year. The Stage IV patients received systemic chemotherapy (DTIC 250 mg/m$^2$ over 5 days at 28-day intervals, 6 cycles). The therapy consisted of 6 cycles of DTIC 200 mg/m$^2$ + Cisplatin 25 mg/m$^2$, for 3 days with 3 week intervals, when solid organ involvement was present.

The median follow-up interval after blood sampling was 17 months (range 11-31 months; mean, $17.9 \pm 5.4$ months) for the patients.

Processing the blood with a density gradient before the RNA isolation resulted in a higher sensitivity than extracting RNA from whole blood (Jung *et al*., 1997; Schittek *et al*., 1999). Therefore, the mononuclear cell fractions were isolated by Ficoll-Histopaque (SIGMA Chemicals Inc., St. Louis, USA) density gradient centrifugation. Total cellular RNA was extracted from mononuclear cells using the Trizol Reagent (Gibco-BRL, USA). RNA quality was investigated electrophoretically on a 1% agarose gel. The integrity of the RNA samples was controlled by the consistent detection of β-actin mRNA to exclude mRNA degradation.

For the PCR analysis of the tyrosinase transcripts, two sets of primers were used from published sequences (Smith *et al.*, 1991);

HTYR1 : 5'-(TTGGCAGATTGTCTGTAGCC)-3'
HTYR2 : 5'-(AGGCATTGTGCATGCTGCTT)-3'
HTYR3 : 5'-(GTCTTTATGCAATGGAACGC)-3'
HTYR4 : 5'-(GCTATCCCAGTAAGTGGACT)-3'.

These primer pairs yielded products of 284 bp and 207 bp, respectively.

Reverse transcription and PCR were performed using an Access RT-PCR kit (Promega, Madison, USA). One μg RNA was heated to 60°C for 5 min, cooled rapidly on ice, and diluted to 50 μl with a 1x PCR buffer, 1 mmol of each dNTP, 1.6 mmol $MgSO_4$, 20 pmol of each primer, 5 U AMV-Reverse transcriptase, and 5 U Tfl Polymerase. A cDNA synthesis was performed for 30 min at 48°C. PCR was initiated for 5 min at 95°C for template denaturation, followed by 35 cycles of denaturation at 95°C for 65 s, annealing at 55°C for 65 s, and extension at 72°C for 65 s. The PCR reaction was terminated by a 10-min extension at 72°C. For the second round, PCR 5 μl of a 1 : 100 dilution of the first round PCR product was used in combination with 20 ng of HTYR 3 and HTYR 4 primers in 50 μl final volume. The cycling conditions were the same.

For the analysis of Melan A, the primers were as follows;

MLA 1 : 5'-CTGACCCTACAAGATGCCAAG-3'
MLA 2 : 5'-GATTAGTACTGCTAGCGGACC-3'
MLA 3 : 5'-ACTGCTCATCGGCTGTTGGT-3'
MLA 4 : 5'-TCAGCATGTCTCAGGTGTCT-3'.

Three μg of RNA was suspended in a 50 μl reaction mixture that contained 1 mmol of each dNTP, 30 pmol of each primer, 5 U AMV Reverse transcriptase, 3.5 mmol $MgSO_4$, 0.2 M DMSO, and 5 U Tfl Polymerase. The PCR conditions were 60 sec at 94°C, 65 sec at 54°C, and 65 sec at 72°C for 35 cycles. For re-amplification, 1 μl PCR product was further processed in a second round of 35 cycles with nested primers (MLA 3 and MLA4) under the same conditions in the presence of 1.5 U Taq polymerase. A product of 266 bp was obtained following the second round of PCR.

The final products were electrophoresed on a 2% agarose gel, and evaluated using a gel documentation system (Vilber Lourmat, Marne-La-Vallee, France) after ethidium bromide staining. Positive RT-PCR results were repeated twice in order to confirm the results. The detection limit of our assay for both transcripts (1 melanoma cell in $10^6$ peripheral blood mononuclear cells) was in line with the studies in literature (Schittek *et al*., 1999; Proebstle *et al.* 2000; Taback *et al.* 2001).

Chi Square tests were used for the statistical calculations; a Kaplan-Meier analysis and logrank test were used for the survival analyses.

## Results

We investigated the presence of tyrosinase and Melan A transcripts in 30 patients with malignant melanoma before and after therapy. The tyrosinase- and Melan A-specific bands in the patient samples are shown in Fig. 1 and 2. Tyrosinase transcripts were detected in 9 of 30 patients (30%) with the metastatic disease before therapy. Five of 9 positive patients (28%) had Stage III; 4 patients (36%) had the Stage IV

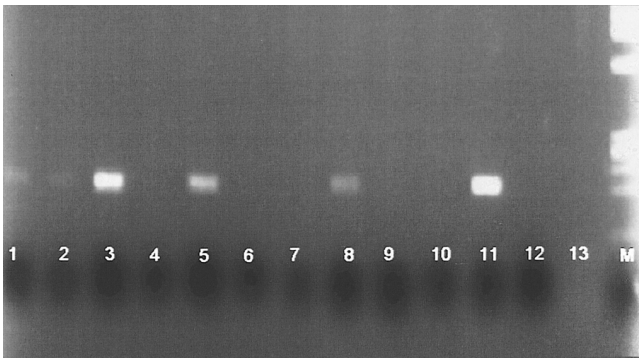


**Fig. 1.** RT-PCR products displaying the tyrosine-specific 207 bp band in the positive samples. Lanes 1, 2, 3, 5, 8, and 11; Tyrosinase-positive melanoma patients. Lanes 4, 6, and 7; Tyrosinase-negative patients. Lanes 9, 10, and 12; Healthy controls. Lane 13, Negative control. Lane M, Molecular weight marker.

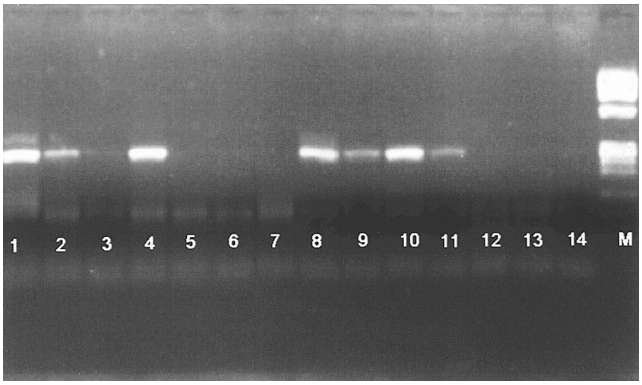


**Fig. 2.** Melan A-specific PCR transcripts. Lanes 1, 2, 4, 8, 9, 10, and 11; Melan A-positive patients. Lanes 3, 5, 6, and 7; Negative samples. Lanes 12 and 13, Healthy controls. Lane M; Molecular weight marker.

disease. The positivity rates for each group are given in Table 1. Following treatment, only one patient remained positive; while no transcript was detected in the remaining patients.

Upon admission, Melan A transcripts were observed in 19 (63.3%) patients. All of the patients with the Stage IV disease were positive for Melan A transcripts. Among the patients at Stage III, 5 were positive for Melan A, 2 were positive for tyrosinase, and 3 patients were positive for both. Following therapy, Melan A was detected in 14 (46.6%) patients. Of these, 8 patients had Stage III and 6 had Stage IV disease.

All of the healthy controls were negative for the tyrosinase and Melan A mRNAs. The difference between the patients and the control group was statistically significant, both for tyrosinase ($p = 0.007$) and Melan A ($p < 0.001$, $\chi^2 = 22.22$, $df = 1$). The specificities of tyrosinase and Melan A for the disease were high (100%); however, the sensitivity of tyrosinase was considerably lower (30%) when compared to Melan A (63.3%). No statistical correlation between the

**Table 1.** Detection of tyrosinase and Melan-A transcripts by stage, and effect of therapy.

| | Stage | n | Tyrosinase | | Melan A | |
|---|---|---|---|---|---|---|
| | | | n | % | n | % |
| Before Therapy | III | 18 | 5 | 28 | 8 | 44 |
| | IV | 11 | 4 | 36 | 11 | 100 |
| After Therapy | III | 14 | - | - | 8 | 57 |
| | IV | 6 | 1 | 9 | 6 | 100 |

presence of PCR products and age or sex was observed. During the entire period four patients were found negative on both parameters. Remarkably, in the negatively-tested group, 2 patients relapsed and were lost during the study.

Following treatment, tyrosinase mRNA was observed in only one patient (9%). This reflects the effect of systemic therapy on circulating tumor cells. On the other hand, Melan A transcripts were still detectable in 14 (70%) patients after therapy. During the follow-up period of the disease, 9 patients (5 patients with the Stage IV disease and 4 patients with the Stage III disease) were lost by disease-related deaths. Of these, two patients were positive for Melan A on admission; one patient was positive for both markers. All of these patients had the Stage IV disease. However, this observation failed to achieve a significant association. Among the patients who were found positive for at least one of the markers on admission, 8 patients are still without relapse, while the remaining patients all had recurrences. Remarkably, among the patients in remission, three were found positive for tyrosinase on admission and turned negative following therapy. However, of the six patients who were positive for Melan A initially, only one turned negative.

The survival analysis revealed no association with PCR positivity for both transcripts.

## Discussion

Routine histologic examinations are inadequate for accurately assessing the diseases spread and monitoring the progression and micrometastatic disease (Goydos *et al*., 1998). A possible alternative is the use of RT-PCR to detect tumor-specific mRNA in circulation. Since subclinical metastasis may remain dormant for many years, monitoring a patients blood for circulating tumor cells may be advantageous for detecting tumor progression before clinically-evident metastases are detected. This information may be useful in identifying patients who may benefit from more intense therapies, and provide an opportunity for therapeutic intervention at an earlier stage. However, the assessment of single markers has limitations in sensitivity and specificity, based on the heterogeneity of the marker expression among tumors (Kulik *et al*., 2001). Furthermore, although specific molecular techniques have been utilized for diagnostic purposes, the

correlation between molecular detection of circulating tumor cells and the response to therapy has not been addressed satisfactorily.

In this study, our goal was to improve the sensitivity to detect micrometastases in the circulation of melanoma patients by the simultaneous analysis of tyrosinase and Melan A transcripts. Also, we investigated to see if these markers are predictive of the therapeutic response in a clinical setting.

In our study, tyrosinase transcripts were observed in 30% of the patients. The positivity rate for tyrosinase transcripts is in accordance with literature (Foss *et al*., 1995; Kunter *et al*., 1996; Pittman *et al*., 1996; Jung *et al*., 1997; Proebstle *et al*., 2000), but higher than two reports in which only a small fraction of the patients were found to have detectable numbers of cells in blood with this technique (Hoon *et al*., 1995; Johansson *et al*., 2000). However, considerable variations in the rate of detection have also been noted (Hoon *et al*., 1995; Jung *et al*., 1997). This may indicate that although tyrosinase transcripts are commonly found in patients with disseminated disease, they may also be absent in patients during the earlier stages of the disease (Berking *et al*., 1999).

All of the patients that were positive for tyrosinase mRNA in our study had Stage III and IV of the disease. This agrees with previous studies (Brossart *et al*., 1995; O'Connell *et al*., 1998), which reported a high sensitivity for patients with stage IV melanoma. A correlation between the presence of tyrosinase mRNA in blood and stage of the disease was reported in several studies (Mellado *et al*., 1996; Forthmann *et al*., 1998; Proebstle *et al*., 2000).

Following chemotherapy, tyrosinase mRNA was found in only one of the patients. This probably reflects the effects of systemic therapy on circulating tumor cells. The continued presence of circulating cells after treatment is likely to be associated with a poor outcome. Our observations agree with reports that tyrosinase alone may not always provide a useful marker in detecting circulating melanoma cells (Hoon *et al*., 1995; Hasselmann *et al*., 2001). This finding supports the view that the use of more than one marker is required in order to verify the presence of occult melanoma cells in peripheral blood (Hoon *et al*., 1995; Schittek *et al*., 1999a; Schittek *et al*., 1999b).

Therefore, another melanocytic-lineage marker, Melan A, was included. Melan A is specifically expressed in melanocytes, melanoma cells, and in the retina (Coulie *et al*., 1994; Chen *et al*., 1996). We observed the expression of the Melan A transcript in 63% of the patients. This ratio is consistent with an earlier report (Curry *et al*., 1999), as well as the hypothesis that most melanoma tumors and cell lines express this gene (Schittek *et al*., 1999). In agreement with previous reports (Kulik *et al*., 2001; Taback *et al*., 2001), the frequency of melanoma cell detection in a patients blood was higher in our study when the two-marker assay was used. However, Melan A transcripts were still detectable in 43% of the patients following chemotherapy. This may indicate that Melan A may be more specific to the disease and not completely eradicated by chemotherapy. It has been reported that positive tyrosinase results are less frequently observed in the treated-Stage IV patients than in the untreated patients (De Vries *et al*., 1999). Alternatively, it may also be due to the fact that the down-regulation of melanin synthesis is more easily achieved by treatment, while the antigen-expressing pathways may still remain functional.

Although the investigation of tyrosinase seems to better reflect the effect of systemic therapy, this may be due to the low sensitivity of tyrosinase. Reports, which suggest that tyrosinase transcripts are commonly found in disseminated disease and may be absent in earlier stages, support this notion (Glaser *et al*., 1997; Schittek *et al*., 1999). Although it was reported that circulating-tumor cells can be detected in melanoma patients without clinical evidence of the disease (Brossart *et al*., 1994), this was not the case in our study group. However, our findings are in agreement with a more recent report (Kunter *et al*., 1996). We observed no association between the presence of transcripts and survival.

Our study supports the view that tyrosinase-negative samples may contain Melan A transcripts (Schittek *et al*., 1999), and that the simultaneous detection of two independent markers may increase sensitivity that enables the detection of micrometastases, particularly in patients in whom the expression of one marker is down-regulated. This finding is consistent with other studies (Hoon *et al*., 1995; Kulik *et al*., 2001; Taback *et al*., 2001). Since detection limits are roughly the same for both transcripts, the higher detection rate for Melan A may be associated with the characteristics of the tumor cell.

The specificity of tyrosinase and the Melan A gene expression makes this approach ideal for detecting melanoma cells in blood. We conclude that this molecular assay can provide information on the persistence of metastatic melanoma cells, and may assist in monitoring the efficacy of treatment. Further studies are needed to better assess the significance of this test in the evaluation of prognosis and early detection of relapse.

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