

# Prognostic value of RT-PCR tyrosinase detection in peripheral blood of melanoma patients

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**Abstract.** Malignant melanoma (MM) prognosis has been related to tumour thickness and clinical stage and metastasis risk has been associated with presence of tumour cells in peripheral blood. The aim of this study was to determine the relationship between presence of tyrosinase in peripheral blood of MM patients and their clinical prognosis. Blood samples from 58 MM patients (stage I–IV) were analysed, using RT-PCR assay to detect tyrosinase mRNA. The results showed that positive RT-PCR assay for tyrosinase were significantly associated with clinical status and tumour thickness. After a median follow-up of 24 months, RT-PCR results were found to be significant correlated with recurrence ( $p < 0.05$ ) and clinical stage III ( $p < 0.05$ ). Separate analysis of stage III tumours to determine the prognostic value of tyrosinase presence in peripheral blood showed an overall 24-month survival rate of 70% in the RT-PCR negative group versus 10% in the positive group ( $p < 0.02$ ). These results suggest that detection of circulating melanoma cells may be especially relevant in stage III patients, in whom RT-PCR positivity defines a subpopulation at high risk of recurrence.

## 1. Introduction

The incidence rate of malignant melanoma (MM) has increased more than that of any other malignancy among white populations over the past 15 years [1]. The development of metastasis is the leading cause of mortality in MM patients [2,3]. Although there is no effective systemic therapy for the treatment of metastatic MM, its early detection can improve clinical assessment of the progression, metastatic potential and response to therapy of the tumour [4]. The identification of a biological factor that could serve to predict the clinical progression of MM has been extensively researched.

Several markers of MM have been proposed to date and the most widely used have been tyrosinase (TYR) and its related proteins (TYRPs). Tyrosinase is a key enzyme in melanin biosynthesis and is exclusively expressed in melanocytes and melanoma cells [5,6]. Detection of tyrosinase enzyme by reverse transcriptase-polymerase chain reaction (RT-PCR) assay has been used to detect occult melanoma cells in blood and lymph nodes of melanoma patients [7–9]. A pivotal study by Smith *et al.* [10], established the usefulness of RT-PCR amplification of tyrosinase mRNA for detecting circulating melanoma cells (CMCs). Our group previously used this method to demonstrate presence of CMCs in patients with melanoma at different clinical stages [11]. There is little information on the clinical significance of tyrosine mRNA detection in peripheral blood of MM patients and conflicting data have been

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reported [12]. In this context, it is accepted that presence of CMCs is correlated with the clinical stage of patients and that detection of these cells in patients with early-stage disease may indicate a high risk of metastasis and a lower probability of overall survival [13]. Recent studies described tyrosinase RT-PCR results as a potentially useful molecular marker that may provide an early indication of therapy effectiveness [14]. However, other authors found no relationship between positive PCR for tyrosinase and disease-free or overall survival [15]. Therefore, the prognostic value of the tyrosinase enzyme test remains controversial.

We report results of tyrosinase detection by RT-PCR in peripheral blood samples from 58 MM patients in all clinical stages (I, II, III or IV). The aim of this study was to determine the clinical relevance of this procedure, exploring the relationship between presence of tyrosinase in peripheral blood of MM patients and their clinical stage and prognosis.

## 2. Materials and methods

### 2.1. Patients

Blood samples were obtained at the Dermatology Service of the University Hospital of Granada from 58 consecutively selected MM patients at time of primary tumour diagnosis or at relapse. Clinical stage was classified according to AJCC recommendations: primary cutaneous tumour (stages I and II), regional skin or lymph node metastasis (stage III) or distant metastatic tumour dissemination (stage IV). All stage I–III patients were visited every 4 months during the first 2 years after the diagnosis and every 6 months thereafter. Follow-up sessions included clinical history, physical examination, blood cell count and biochemistry allowed to carry out the disease control and to determine the presence or the absence of relapse. In each session simple blood was obtained to determine the presence of CMCs by means of RT-PCR. All patients gave written consent after receiving information on the purpose of the blood sampling. The outcome of the PCR analyses had no influence on treatment. Blood samples from 10 healthy subjects or patients without melanoma were used as negative controls and analyzed under identical conditions.

### 2.2. Blood collection and RNA isolation

Samples of peripheral venous blood (10 ml) from each patient were collected in five tubes containing ethylenediamine tetraacetic acid (EDTA), stored at 4°C, and processed on ice within 2–4 h. After centrifugation (650 x g for 5 min) the serum was collected and treated with 45 ml diethyl pyrocarbonate-treated water (H2O-DEP) and 5 ml PBS (x 10), and centrifuged at 650 x g for 5 min several times until a white pellet (PBLC) was obtained. Total RNA was isolated from the cell fraction with the IsoRNA-Fast Plus kit (Master Diagnostica, Granada, Spain) following the manufacturer's instructions. Yield and purity of the RNA were determined by ultraviolet spectroscopy.

### 2.3. Oligonucleotide primers

Oligonucleotide primers specific for tyrosinase were designed according to Smith *et al.* [10]. The primers 5' TTG GCA GAT TGT CTG TAG CC (sense) and 3' AGG CAT TGT GCA TGC TGC TT (antisense) were chosen to amplify a 284-base pair (bp) fragment of the tyrosinase cDNA. Integrity of the RNA was determined using primers 5' ATG GAT GAT GAT ATC GCC GCG (sense) and 3' TCT CCA TGT CGT CCC AGT TG (antisense) that amplified a 248-bp fragment of  $\beta$ -actin [16].

### 2.4. RT-PCR assay

Reverse transcription (RT) was performed using 1  $\mu$ g of total RNA. First-strand cDNA was generated with 0.4  $\mu$ mol/L of random hexamers, 1 mmol/L of each deoxynucleotide triphosphate (dNTP), 24 units of avian myeloblastosis virus RT (Promega, Madison, Wisconsin, USA) and 40 units of RNasin (Promega) in a final volume of 20  $\mu$ L of RT buffer. After incubation at 42°C for 18 min and 94°C for 5 min (Linus Autocycler 32, Cultek S.L. Madrid, Spain), samples were resuspended in 80  $\mu$ L of H2O-DEP. For the first PCR round, 10  $\mu$ L of the RT products were diluted to 100  $\mu$ L (RT buffer) in a mixture with a final concentration of 2.0 mmol/L MgCl<sub>2</sub>, 200  $\mu$ mol/L dNTP and 1  $\mu$ mol/L of each tyrosinase primer. After a hot start (2 min at 94°C), 2 units of Taq polymerase (Boehringer Mannheim, Germany) were added, followed by 30 cycles at 94°C for 1 min, 58°C for 2 min, 72°C for 3 min and 72°C for 7 min. For the analysis, 70  $\mu$ L of the reaction product was run on a 2% agarose gel and stained with ethidium bromide. The gel was viewed and photographed under ul-

traviolet light. Specificity of the amplified product was established by restriction enzyme digestion. Quality of the mRNA was controlled by PCR for  $\beta$ -actin [16]. Lymph node metastasis from an MM patient was used as positive control.

### 2.5. Statistical analysis

Two variables were included in the data analysis: clinical stage and RT-PCR results. Clinical stage (I, II, III and IV) was analyzed as a discrete variable. RT-PCR results for tyrosinase mRNA were classified as positive or negative. A two-tailed Fischer's exact test was used to assess the association between RT-PCR results and clinical stage. The relationship among RT-PCR results, clinical stage and overall survival were evaluated in all patients. The association between RT-PCR and overall survival was also determined within each stage [17]. Survival time was defined as the period from blood extraction for PCR analysis to death or censoring. Patient survival was evaluated using the Kaplan Meier method [18] and survival comparisons among groups were performed using the log rank test [18]. Proportional hazards analysis was used to obtain maximum likelihood estimates of relative risks and their 95% confidence intervals in univariate and multivariate analyses [20,21] and to adjust for potential confounding effects. Parameters significant at the  $P = 0.05$  level in univariate analysis were included in a multiple proportional hazards model. Software for analysis was STAT (STATA Corp, College Station, TX, 1997).

## 3. Results

### 3.1. Patient characteristics

The study included 58 patients with MM at clinical (AJCC) stages I–II in 14 patients, stage III in 28 patients and stage IV in 16 patients. Patients had a median age of 50 years (range, 24–87 years) and 56.9% were female. Patient characteristics are reported in Table 1.

### 3.2. RT-PCR results, clinical stage and thickness

According to RT-PCR findings, tyrosinase was detected in peripheral blood from 14 out of the 16 MM patients (88%) with metastatic disease (stage IV); the two remaining stage IV patients had a surgically excised isolated metastatic site with no evidence of clinical disease at the time of blood extraction. CMCs were

Table 1  
Clinical characteristics of 58 patients with malignant melanoma

Characteristics	No. of patients
Sex	
Male	25
Female	33
Median age, years (range)	50 (24–87)
Primary site	
Upper extremities	9
Lower extremities	24
Trunk	14
Head and neck	11
Clinical stage	
Stage I and II	14
Stage III	28
Stage IV	16

Table 2  
RT-PCR results by stage

	Total	RT-PCR Positive		RT-PCR Negative	
		No	%	No	%
		Stage I–II	14	2	14
< 1.0 mm	10	2		8	
1.1–4 mm	3	0		3	
> 4 mm	1	0		1	
Stage III	28	20	71	8	29
< 1.0 mm	4	1		3	
1.1–4 mm	16	13		3	
> 4 mm	8	6		2	
Stage IV	16	14	88	2	12

also detected in the blood of two of the 14 patients at stage I–II (14%) and in 20 of the 28 patients at stage III (71%). All negative controls were negative for tyrosinase mRNA in the RT-PCR assay. The statistical analysis showed that the RT-PCR results were significantly associated with clinical status ( $p < 0.001$ ) and tumour thickness ( $p < 0.002$ ) (Table 2) but not with primary site or patient gender.

### 3.3. RT-PCR results and prognosis

After a median follow-up of 24 months (range, 3–48 months), in which RT-PCR test were realized, risk of relapse was evaluated in all 58 patients. All stage I–II patients underwent complete resection of primary tumour; 50% (6/12) of stage I–II patients with negative RT-PCR test relapsed compared with 100% of patients (2/2) with positive PCR result. Stage III patients received postsurgical adjuvant chemotherapy; 85% (17/20) of RT-PCR-positive stage III patients relapsed versus 25% (2/8) of RT-PCR-negative patients. Neither of the two RT-PCR-negative stage IV patients had the disease at 24 months, whereas 93% (13/14) of the RT-PCR-positive stage IV patients relapsed (Table 3). X2 univariate anal-

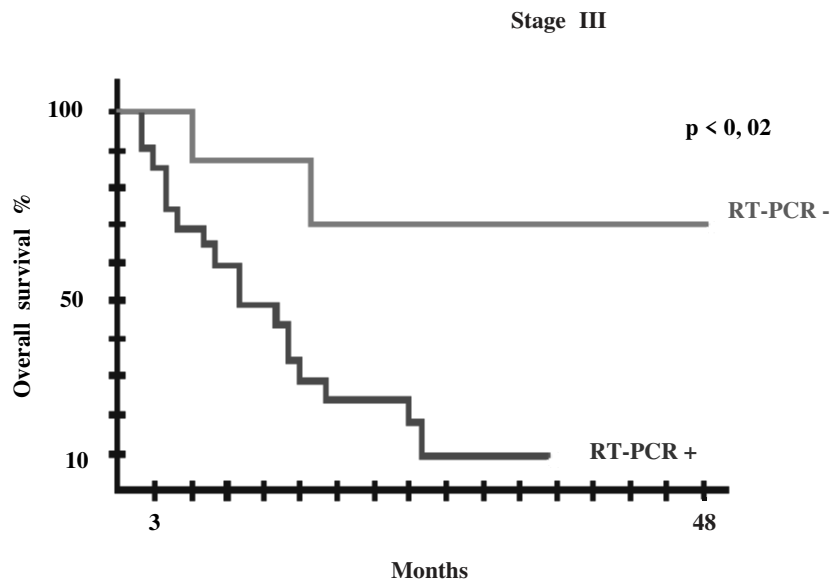


Fig. 1. Overall survival (OS) according to tyrosinase mRNA in blood in stage III patients. OS was calculated by Kaplan-Meier method.

Table 3  
RT-PCR Results and Relapse in stage I–IV patients

	Total	Relapse		Non-Relapse	
		N <sup>o</sup>	%	N <sup>o</sup>	%
Stage I–II					
RT-PCR <sup>+</sup>	2	2	100	0	0
RT-PCR <sup>-</sup>	12	6	50	6	50
Stage III					
RT-PCR <sup>+</sup>	20	17	85	3	15
RT-PCR <sup>-</sup>	8	2	25	6	75
Stage IV					
RT-PCR <sup>+</sup>	14	13	85	3	15
RT-PCR <sup>-</sup>	8	2	25	6	75
Total					
RT-PCR <sup>+</sup>	36	32	89	4	11
RT-PCR <sup>-</sup>	22	8	36	14	64

ysis showed that RT-PCR results were significantly correlated with recurrence ( $p < 0.05$ ) and clinical stage III ( $p < 0.05$ ). No correlation was observed between recurrence and sex, age, histology, Breslow classification or primary site. In a separate analysis of stage III tumours to determine the prognostic value of melanoma cell presence in peripheral blood, overall 24-month survival was 70% in the RT-PCR-negative group versus 10% in the positive group ( $p < 0.02$ ) (Fig. 1). The two significant factors according to the univariate analysis (stage and RT-PCR status) were subjected to a multivariate analysis. A logistic regression model with PCR as dependent variable and stage, histology and thickness as independent variables yielded the following results, taking results for Stage I–III patients as reference: the likelihood that a stage III patient was RT-PCR pos-

itive for tyrosinase mRNA was 15 (95% CI: 2.7–94.6) and that a stage IV patient was positive was 42 (95% CI: 5.1–345.1).

#### 4. Discussion

It has recently been suggested that RT-PCR detection of tyrosinase mRNA in peripheral blood may be useful for establishing the prognosis of patients with MM, although published results have been inconsistent. This hypothesis is based on the fact that detection of CMCs in peripheral blood indicates their mobilization from the site of the primary lesion via the peripheral blood. Some researchers reported a significant correlation between PCR positivity and clinical outcome but others observed no such relationship [13, 22]. The present study demonstrates that tyrosinase detection can be used as a molecular marker in the blood of MM patients. This marker is correlated with clinical stage (the most widely used MM prognostic system) and thickness, and may be useful for establishing the prognosis in these patients.

The detection of minimal residual disease by PCR amplification of specific tumour cell abnormalities has been widely used in haematologic malignancies as a useful tool for treatment response assessment and patient follow-up [23–26]. RT-PCR was recently used to detect malignant cells in peripheral blood of solid tumour patients [9]. Tyrosinase is specifically expressed

in melanocytes or melanoma cells. Since melanocytes do not circulate, detection of tyrosinase mRNA in blood indicates presence of melanoma cells. Numerous authors have determined the percentage of CMC-positive MM patients using tyrosinase as marker, but results have ranged widely from 6% to 59% [27,28]. In the present study, 62% of MM patients showed RT-PCR positivity, a higher percentage than in other studies with similar sample size [29]. We also found a significant association between clinical stage of disease and RT-PCR status. CMCs were detected in a large majority of the patients with clinical metastasis, and in a progressively lower proportion in PCR-detected CMCs with lower stages of the disease. This correlation is of special relevance, because clinical stage is the main clinical datum used for the prognosis [29–31]. Similar findings were published by authors using multiple marker RT-PCR assays [32–35], who found that most stage II, III and IV patients presented CMCs. Among the markers used, tyrosinase showed the strongest correlation with disease stage.

Detection of melanoma cells in peripheral blood has been correlated with clinical disease-free period. In patients with an advanced stage of the disease, a positive test has also been related to lower patient survival [31] and presence of visceral metastasis [36]. However, other authors found no correlation between these variables [37,38]. Unfortunately, the heterogeneity of published series hampers direct comparison among published results. The present study demonstrated a statistically significant correlation between recurrence and RT-PCR results ( $p < 0.05$ ). Moreover, a distinct clinical evolution was observed in stage III patients according to their RT-PCR status, with higher recurrence rates (17/20 vs. 2/8) and worse actuarial DFS in RT-PCR-positive patients. In fact, RT-PCR status was the only independent prognosis factor in the multivariate analysis. In contrast, our results could not show a significant correlation between the patients prognosis of stages I–II and the positive RT-PCR. It has been hypothesised that cells shed from these early lesions may be able to extravasate but lack additional properties required for the multistep process of metastatic dissemination [39]. However, a study of a larger sample of positive patients with a longer follow-up period is required to confirm our findings in these patients.

## 5. Conclusion

In conclusion, these results demonstrate a statistically significant association between malignant

melanoma stage and RT-PCR detection of CMCs in peripheral blood. The outcome analysis showed a poorer prognosis in the group of patients with metastatic disease and detectable CMCs. This detection may be of special relevance in the prognosis of stage III patients, in whom positive RT-PCR detection appears to define a group at high risk of recurrence. Further research is warranted to develop protocols for the use of RT-PCR detection of CMCs in the prognosis of patients with melanoma, in the early detection of recurrence, and in clinical trials on the efficacy of systemic therapies.

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