Prognostic significance of tyrosinase mRNA detected by nested RT-PCR in patients with malignant melanoma*

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The aim of this study was to evaluate the role of tyrosinase mRNA appearance in blood of malignant melanoma (MM) patients, especially with advanced stages, for predicting the disease progression, and consequently the survival. The tyrosinase mRNA was measured by nested RT-PCR in peripheral venous blood samples obtained from 86 patients (53 male and 33 female) with mainly stage III and IV MM. The data were analyzed using standard methods for survival analysis and logistic regression.

Tyrosinase was negative in the MM patients with the disease stage I or II. Tyrosinase was positive in 11/50 patients with stage III and in 5/22 patients with stage IV. Systemic metastases developed in 14/16 patients with positive tyrosinase and in 41/70 with negative tyrosinase. The 3-year survival was 8% and 28% among the patients with positive and the patients with negative tyrosinase, respectively. The log rank test showed statistically significant better survival of tyrosinase negative patients when compared to tyrosinase positive patients (p=0.039). Multivariate analysis using logistic regression indicated tyrosinase to be a statistically significant prognostic factor for the survival of MM patients after controlling for Breslow and ulceration values (p=0.006).

Positive tyrosinase in peripheral venous blood is statistically significant, and more importantly independent negative predictor of survival.

Key words: malignant melanoma, tyrosinase, mRNA, nested RT-PCR

The prognosis and treatment of MM largely depend on the stage of disease [1]. Several efforts have been made to make the staging system more accurate and the last revision of TNM classification added micrometastatic disease in lymph nodes as an independent parameter [2]. As micrometastatic disease in lymph nodes is merely an indirect marker for distant disease recurrence, the more direct approaches by detecting free tumor cells in the blood might better prognosticate disease progression. Recently, S-100 protein, MART, and tyrosinase mRNA have been considered as reliable markers for MM cells. However, those markers are not strongly specific for MM cells because they are also produced in other cells (i.e. cells of the nervous system, monocytes, macrophages, melanocytes). Therefore, the repertoire of their applications in MM patients is quite narrow and depends on the type of marker [3–5]. S100 protein is a stage-dependent serum marker applicable for monitoring the therapy. MART is important in stimulating the immune processes and creating different tumor vaccines. Tyrosinase mRNA could serve as a marker for detection of circulating MM cells in the blood.

Since SMITH et al in 1991 introduced the RT-PCR method for detecting tyrosinase mRNA in peripheral blood [6], there has been growing interest in the procedure. Tyrosinase is the key enzyme in melanin synthesis and is expressed only by normal skin melanocytes or MM cells [7]. Therefore, the source of tyrosinase mRNA in the blood can only be circulating melanoma cells, suggesting increased potential for developing metastases. RT-PCR, compared to the other methods (i.e. flow cytometry), is extremely sensitive. It allows the detection of one melanoma cell in a background of 10^5–10^7 normal peripheral blood cells. By using the nested PCR, which

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means additional amplification of the primary signal with shorter inner primers, the sensitivity of the method is even increased [5, 8].

The presence of tyrosinase mRNA in the blood of MM patients is related more to the prediction of survival than directly to the extent of the disease, as positive tests appear also among the patients with early stages of the disease [9]. Although the positivity rates of tyrosinase mRNA increase with the stage of disease (18% sensitivity for stage I disease, 28% for stage II disease, 30% for stage III disease, and 45% for stage IV disease) in most published series, its impact on the survival in various studies differs [9]. In some studies, a reduced overall survival was observed in the patients who were RT-PCR tyrosinase positive [10–17], while in others this correlation was not observed [18, 19]. BATTAYANI et al found that the stage III patients with positive tyrosinase result have both reduced survival and also rapid progression of disease [10].

In our previous work it was clearly shown that the nested RT-PCR for detection of tyrosinase mRNA is an assay of clinical relevance having the ability of detecting 1 MM cell in a background of 10^8 normal peripheral blood cells [8]. The aim of this study was to test the predictive value of positive RT-PCR tyrosinase result on the survival of MM patients in general, and especially in stage III disease.

Patients and methods

Patient characteristics. In 2002, 86 consecutive MM patients who were referred to the Melanoma Tumor Board at the Institute of Oncology, Ljubljana and classified according to the AJCC 2002 classification (Tab. 1), were included in the study.

The patient's disease status was determined at the time of venous blood sampling. The patients were followed up every 3 months for the first 2 years and every 6 months after the second year, with the mean follow-up time of 21 months (range 1–40 months). Written informed consent was obtained from all subjects. The study was conducted by utilizing the double-blind technique.

Preparation of the blood samples. Ten ml of peripheral venous blood were collected from each subject into an EDTA Vacutainer (BDVS, Plymouth, UK). To avoid contamination of the blood samples with the skin melanocytes, the blood collected in the first tube was used for standard clinical tests. Only the blood collected in the next tube by the same needle was used for RNA isolation. Blood samples were then mixed with TRIzol LS reagent (Gibco BR, Paisley, Scotland) in the ratio 1 to 2, and stored at –70 °C.

RNA isolation, primers and nested RT-PCR. Total RNA was isolated from the cells of the whole peripheral venous blood or from B-16 MM cells, using the modified single-step RNA isolation method described by CHOMCZYNSKI and SACCHI [20].

The outer and inner (nested) primers for tyrosinase (HTYR1-4), and GAPDH as a control housekeeping gene, were ordered at Perkin Elmer Biosystems and MWG, and used as previously described [6].

Reverse transcription of total RNA using random hexameres, first round PCR using outer HTYR1 and HTYR2 primers and nested PCR using inner HTYR3 and HTYR4 primers were done as previously described in a GeneAmp PCR System 2400 incubator (Perkin Elmer) [8]. Murine B-16 clone F1 MM cells were used as a positive control.

Statistical analysis. The survival of the patients was analyzed using the KAPLAN-MEIER method [21]. The log-rank test was used to test the equality of the survival curves. The influence of selected prognostic factors on the survival was evaluated using univariate and multivariate logistic regression analysis. The value of $p<0.05$ was considered as the limit of statistical significance. Statistical analysis was performed using the Stata 7.0 computer programme (College Station, Texas, USA).

Results

Patients. The patients' clinicopathological characteristics (Tab. 1) and the results of tyrosinase mRNA detection (Tab. 2) are shown. Although the data on Breslow thickness were missing in 10 patients, the AJCC stage could still be es-

Table 1. Patients' clinicopathological characteristics

	Number
Number of all patients	86
Male/female	53/33
Age mean (years)	58
Age min./max. (years)	7/84
Follow-up mean (months)	21
Follow-up min./max. (months)	1/40
Primary site	
Trunk	37
Extremities	31
Head and neck	14
Unknown origin	4
Breslow thickness	
Data missing or unknown origin	10
<1.01	3
1.01-2.00	8
2.01-4.00	23
>4.00	42
Clark level of invasion	
Data missing or unknown origin	11
I	0
II	2
III	23
IV	35
V	15
Ulceration	
Data missing or unknown origin	10
Yes	60
No	16

Table 2. Results of tyrosinase mRNA detection, therapy and follow-up

	Number
Stage at sampling/tyrosinase positive	
I	3/0
II	11/0
IIIA	2/0
IIIB	9/2
IIIC	36/9
IIIx	3/0
IV	22/5
Interferon α2b therapy	
No	23
3M/week, 30 weeks	51
3x6M/week, 110 weeks	12
Dead after follow-up	
I	0 (0%)
II	3 (27%)
IIIA	0 (0%)
IIIB	3 (33%)
IIIC	21 (58%)
IIIx	2 (67%)
IV	18 (82%)

tablished at the time of sampling because these patients had advanced disease. Tyrosinase was positive in 16/86 (17%) patients, in 11/50 (22%) patients with stage III, and in 5/22 (23%) patients with stage IV MM.

Patient survival analysis. The median overall survival (OS) of the entire group of patients included in our study was 28 months. The same median OS of 28 months was observed in the group of tyrosinase negative patients. A much lower median OS of 14 months was observed among the tyrosinase positive patients. The Kaplan-Meier OS estimate of all patients included in our study after 3 years was 23%, for tyrosinase negative patients after 3 years it was 28%, while for tyrosinase positive patients after the same period of time it was only 8% (Tab. 3). During the follow-up, 59% (41/70) of tyrosinase negative patients developed systemic metastases, while the percentage of patients with systemic metastases of tyrosinase positive patients was 88% (14/16).

The log rank test of Kaplan-Meier survival curves showed a statistically significant better survival for the tyrosinase negative patients compared to the tyrosinase positive patients (p=0.039) (Fig. 1).

Stage III patient survival analysis. The median OS of stage III patients included in our study was 29 months. The same median OS of 29 months was observed in the group of tyrosinase negative stage III patients. A lower median OS of 26 months was observed among the tyrosinase positive stage III patients. The Kaplan-Meier OS estimate of stage III patients included in our study after 3 years was 28%, for tyrosinase negative stage III patients after 3 years it was 38%, while for tyrosinase positive stage III patients after the same period of time it was only 9% (Tab. 3). During the follow-up, 54% (21/39) of tyrosinase negative stage III patients developed systemic metastases, while the percentage of patients with systemic metastases of the tyrosinase positive stage III patients was 91% (10/11).

Even though the Kaplan-Meier survival curve of tyrosinase negative stage III patients was better than the survival curve of tyrosinase positive stage III patients (Fig. 2), the log rank test of survival curves for these two groups of patients was not significant (p=0.059).

Univariate analysis. Univariate logistic regression showed tyrosinase, primary melanoma thickness according to Breslow grouped into T stages (Breslow thickness), and ulceration to be prognostic factors for overall survival in MM patients (Tab. 4).

Multivariate analysis. Prognostic factors shown to be sta-

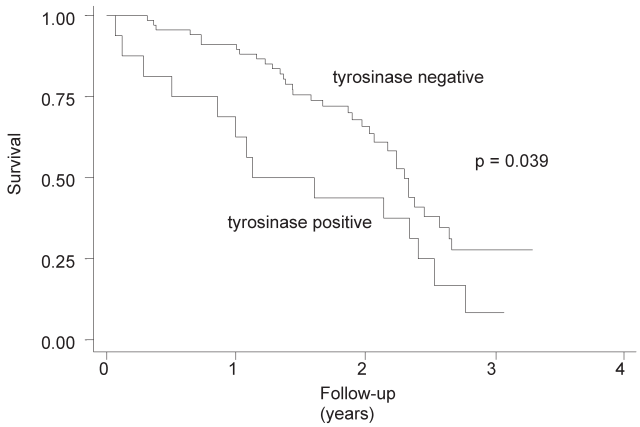

Figure 1. Kaplan-Meier survival curves for all tyrosinase positive and negative MM patients (n=86).

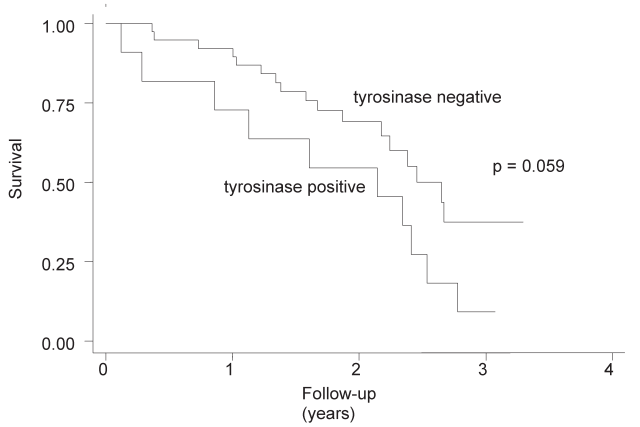

Figure 2. Kaplan-Meier survival curves for tyrosinase positive and negative stage III MM patients (n=50).

Table 3. Comparison of OS between tyrosinase negative and tyrosinase positive patients in all stages, and in particular in stage III

Parameter	All stages	All stages tyrosinase –	All stages tyrosinase +	Stage III	Stage III tyrosinase –	Stage III tyrosinase +
Median OS (months)	28	28	14	29	29	26
Kaplan-Meier OS estimate after 3 years (%)	23	28	8	28	38	9

Table 4. Univariate logistic regression of OS for all 86 patients included in our study

Prognostic factor	Rel. risk	St. error	p value	95% CI
Breslow thickness	1.85	0.56	0.042*	1.02–3.35
Tyrosinase positive	7.41	5.88	0.012*	1.57–35.06
Male gender	1.62	0.72	0.282	0.67–3.89
Clark level IV and V	1.18	0.58	0.743	0.45–3.08
Ulceration present	3.54	2.13	0.036*	1.09–11.50
Interferon therapy	1.98	0.98	0.167	0.75–5.20
Localization trunk	2.09	0.93	0.100	0.87–5.02

*statistically significant

Table 5. Multivariate logistic regression of OS for all 86 patients included in our study

Prognostic factor	Rel. risk	St. error	p value	95% CI
Breslow grouped into T stage	1.62	0.65	0.228	0.74–3.56
Tyrosinase positive	26.46	31.82	0.006*	2.51–279.48
Ulceration present	4.49	4.05	0.096	0.77–26.27

*statistically significant

Table 6. Multivariate logistic regression of OS for stage III and IV patients included in our study

Prognostic factor	Rel. risk	St. error	p value	95% CI
Stage III (against stage IV)	0.24	0.15	0.026*	0.07–0.84
Tyrosinase positive	6.19	5.07	0.026*	1.24–30.83

*statistically significant

tistically significant in univariate analysis were used in the multivariate analysis. Multivariate logistic regression showed that tyrosinase is a statistically significant prognostic factor, even after controlling for Breslow and ulceration values (Tab. 5).

Most importantly, we observed tyrosinase to be a statistically significant prognostic factor in stage III and IV patients, even after controlling for stage effect (Tab. 6).

Discussion

The most important prerequisite for the final success of therapy in MM patients is an early diagnosis. Patients with early stages of disease have a 10-year survival of about 80% [2]. On the other hand, in the patients with an advanced MM, the prognosis is unfavorable and the outcome in these patients predominantly depends upon the early detection of metastases and their treatment. In order to detect the patients that are "high risk" for development of metastases, different MM tumor markers were used in blood, lymph nodes and bone marrow. Even though different serum tumor markers were reported to be independent prognostic parameters in the patients with metastatic MM, not a single one is specific and sensitive enough to predict metastases or be useful in their early detection [3, 7, 12, 22].

Since the original report by SMITH et al in 1991 [6], there has been a growing enthusiasm for tyrosinase as a molecular tumor marker to contribute to the diagnosis and, more importantly, prognosis of patients with MM. Tyrosinase is a key enzyme in melanin synthesis, expressed only by normal skin melanocytes and MM cells [7], and thus, an ideal marker for detection of circulating MM cells that might lead to the development of blood borne distant metastases. The present study was, therefore, conducted to establish whether the presence of tyrosinase mRNA in the peripheral venous blood of patients with MM could serve as the prognostic factor for the survival of these patients. Of special interest was the assessment of the ability of tyrosinase mRNA to predict the progress in the patients with advanced stages (III and IV) who are currently without evident clinical signs of disease.

The results of previous studies about the sensitivity and specificity and, about the prognostic importance of tyrosinase mRNA RT PCR remain slightly contradictory. Few groups of authors have reported the use of tyrosinase mRNA RT PCR to detect circulating melanoma cells in the blood of patients with MM to be of prognostic importance [10–17]. Other groups have declared low sensitivity and specificity of tyrosinase mRNA RT PCR for detection of circulating melanoma cells in the blood of MM patients up to stage IV. Only in the peripheral blood of the patients with systemic metastases did the detection of tyrosinase mRNA correlate with the disease progression [18, 19]. The results of our study are at the same time both in agreement and in disagreement with the results of all groups. Actually, the tyrosinase was always negative in the patients with stage I or II, and positive in

11/50 (22%) patients with stage III and in 5/22 (23%) patients with stage IV MM. In an earlier study of our group, it was clearly shown that the nested tyrosinase mRNA RT PCR is highly sensitive (1 MM cell per 10^8 blood cells) and highly specific (no tyrosinase mRNA detection in the blood of healthy volunteers) for the detection of circulating melanoma cells in the blood [8]. In view of this, we are strongly convinced that negative results in the peripheral blood of patients with earlier stages of the disease reliably indicate the nonappearance of circulating tumor cells. In spite of that, it is not possible to claim an absolute absence of tumor cells from the whole blood in the circulatory system, but this nonappearance is true only for the samples in which the detection was performed. We believe that the differences in the detection limits are frequently the result of strictly methodological variations [12, 23] or the heterogeneity of tyrosinase expression and intermittent shedding of tumor cells [22].

Statistical evaluation of our results obtained during the follow-up period revealed that the survival of tyrosinase positive MM patients is significantly worse compared to that of tyrosinase negative MM patients after 3 years. This is in agreement with the results of the authors who previously reported significantly reduced disease-free survival [12, 14–16] and overall survival [12, 14, 16] of the patients with MM who tested positive for tyrosinase. On the contrary, the results of other authors confirmed only positive correlation between the tyrosinase detection and tumor thickness and ulceration, but direct significance to survival was not confirmed [18, 19].

Another important question in this study was whether the tyrosinase negative patients with the same advanced stage of MM have a longer overall survival than the tyrosinase positive patients. It is evident from Figure 2 that even though the survival of tyrosinase negative stage III patients is evidently better than the survival of tyrosinase positive stage III patients, the difference between the survivals of these two groups does not reach statistical significance ($p=0.059$). Still, our opinion is that the reason for the non-significant difference between the survivals is due to the small number of patients in the groups compared.

As expected, the univariate logistic regression showed Breslow thickness and ulceration to be prognostic factors for the survival in our group of MM patients, which is definitely in accordance with the well-known data and with the AJCC TNM classification [2]. More important in this study was identification of tyrosinase as a prognostic factor for the survival of our patients. These results are in concordance with the results of some, but not all researchers dealing with the issue [10–19]. Furthermore, as an additional confirmation of suitability of the study design, we considered also the observation that the Clark level – previously known to be a prognostic factor [1] – did not prove to be prognostic in our group of patients. This is in agreement with recent AJCC TNM classification which no longer includes the Clark level [2]. Taken together, the study results are consistent with the fact that the tyrosinase appears to be a truly useful prognostic factor in MM patients.

The outcomes of the multivariate analysis using logistic regression surprisingly confirmed only the tyrosinase to be an independent prognostic factor for the overall survival of MM patients even after controlling for Breslow and ulceration values (Tab. 5). This might be due to the non-proportionally larger inclusion of patients with advanced stages of MM (III and IV). It is, indeed, known that in melanoma disease progression, the characteristics of primary tumor play an insignificant role in predicting survival once the regional nodal involvement is observed [2, 24].

We are aware that the tyrosinase detection in stage III and IV patients is currently more experimental than practical in its nature, due to the otherwise extremely poor prognosis of the patients. Although there are no standard effective non-surgical therapies available for the advanced disease at the moment, there are new promising drugs, such as high dose interferon and tumor vaccines [25, 26] that could be useful in the high-risk stage III patients. Considering this, it is important to discover the proper method for distinguishing the patients with higher from those with lower survival probability [27]. The multivariate analysis using logistic regression of overall survival of MM patients with stage III and IV indicates that the tyrosinase has a significant prognostic effect even after we have controlled for stage effect (Tab. 6). Thus, when observing two patients with the same stage of disease, the one with positive tyrosinase has six times higher odds of dying.

Since the treatment of metastatic MM is still unsatisfactory, prevention and early diagnosis is crucial for decreasing mortality rates. In our study, the presence of tyrosinase in peripheral venous blood shows to be a statistically significant and independent negative predictor of MM patients' survival. The assay described is thus of clinical relevance and is potentially a powerful tool for studying different aspects of MM biology, treatment planning, and residual disease testing. Because the series of patients in our study was, from the statistical point, relatively small, resulting sometimes in a non-significant difference between the groups compared or in a wide confidence interval when the difference was statistically significant, it would be necessary to pool the existing series of patients and confirm the results obtained.

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