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Prognostic significance of a single positive result of the RT-PCR tyrosinase assay for cutaneous melanoma patients without distant metastases

Piotr Rutkowski[1], Janusz A. Siedlecki[2], Zbigniew I. Nowecki[1], Jadwiga Kulig[2], Włodzimierz Ruka[1], Anna Wilczyńska[2], Zbigniew Żurawski[1]

Purpose. The aim of the study was to evaluate the prognostic value of a single assay of tyrosinase mRNA presence in peripheral blood with the use of the polymerase chain reaction (RT-PCR) in patients with cutaneous melanoma without distant metastases. We also attempted to analyze the tyrosinase test results in relationship to classic prognostic factors in malignant melanoma.
Patients and methods. We performed prospective studies on 119 patients with malignant melanoma in clinical stages I-III (according to AJCC-UICC 1992) treated in the Soft Tissue/Bone Sarcomas Department of The M. Skłodowska-Curie Memorial Cancer Center-Institute of Oncology in Warsaw. We analyzed the presence of tyrosinase mRNA in peripheral blood, collected during the first follow-up visit using the RT-PCR method. Blood samples of 47 healthy volunteers and 10 patients with malignancies other than melanoma were taken as negative controls.
Results. Positive results of the tyrosinase RT-PCR assay were observed in 25/119 patients (21%). We failed to find any statistical relationship between the results of tyrosinase RT-PCR assay and: Breslow's primary tumor thickness, Clark's level of invasion, clinical stages I/II or III, pathological subtype of the primary lesion, patients' sex. Median overall survival (OS) was 36.5 months. We were not able to reveal any differences in OS between the TYR/+/ and TYR /-/ groups.
Conclusion. The results of the present study indicate that a single tyrosinase RT-PCR assay is not useful as a prognostic test in patients with cutaneous malignant melanoma without distant metastases.

Wartość prognostyczna pojedynczego oznaczenia tyrozynazy metodą RT-PCR we krwi obwodowej u chorych na czerniaka skóry bez przerzutów odległych

Cel pracy. Celem badania była ocena wartości prognostycznej pojedynczego oznaczenia obecności mRNA tyrozynazy we krwi obwodowej metodą reakcji łańcuchowej polimerazy (RT-PCR) u chorych na czerniaka skóry bez przerzutów odległych oraz porównanie wyników testów na tyrozynazę z klasycznymi czynnikami prognostycznymi w czerniaku.
Materiał i metody. Analizie poddano 119 chorych w stopniach I-III zaawansowania choroby (według AJCC-UICC 1992), leczonych w latach 1994-1999 w Klinice Nowotworów Tkanek Miękkich i Kości Centrum Onkologii-Instytutu w Warszawie. Wszystkim chorym pobrano pojedynczą próbkę krwi obwodowej przy pierwszej kontrolnej wizycie od wykonania zabiegu chirurgicznego. Z pobranej próbki krwi izolowano całkowite RNA, a następnie wykonano pojedyncze oznaczenie metodą RT-PCR, pozwalające na wykrycie mRNA tyrozynazy. Grupę kontrolną stanowiło 47 zdrowych osobników oraz 10 pacjentów chorych na inne niż czerniak nowotwory złośliwe.
Wyniki. Pozytywny wynik testu tyrozynazowego TYR/+/ uzyskano w 25/119 przypadków (21%). We wszystkich przypadkach kontrolnych nie stwierdzono obecności mRNA tyrozynazy. Nie wykazano zależności statystycznej pomiędzy wynikiem testu tyrozynazowego a: grubością zmiany pierwotnej według Breslowa, stopniem nacieku według Clarka, stopniem zaawansowania I, II i III, podtypem histopatologicznym zmiany pierwotnej oraz płcią chorych. Średni czas przeżycia w badanej grupie wyniósł około 36,5 miesiąca. Nie stwierdzono różnicy w długości przeżycia pomiędzy grupami TYR/+/ i TYR/-/.

[1] Department of Soft Tissue/Bone Sarcoma and Melanoma
[2] Department of Molecular Biology
The Maria Skłodowska-Curie Memorial Cancer Center
and Institute of Oncology, Warsaw, Poland

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Wnioski. Wyniki pracy wskazują, że pojedyncze oznaczenie obecności mRNA tyrozynazy we krwi obwodowej w grupie chorych na czerniaka skóry bez przerzutów odległych nie ma wartości prognostycznej.

Key words: cutaneous melanoma, RT-PCR tyrosinase assay, molecular diagnostics
Słowa kluczowe: czerniak skóry, tyrozynaza, RT-PCR, diagnostyka molekularna

Introduction

Malignant melanoma is a neoplasm, which originates from melanocytes derived from the neural crest. The diagnosis of cutaneous malignant melanoma among the white race is being established with rapidly increasing frequency in developed countries. In Poland, in the period from 1983 to 1993, the number of new incidences has increased by 100% [1, 2]. Although the present results of treatment of melanoma are better than some decades ago, in Poland still about 50% of patients die of metastatic disease. The identification of new prognostic features of the tumor has fundamental value, because it helps to choose and design optimal treatment and can be useful in assessing the course of the disease. Classic clinical and pathological variables defining prognosis (tumor thickness, level of invasion, tumor ulceration, primary lesion site, pathologic subtype, patient's sex, involvement of regional lymph nodes) remain the most important factors for predicting disease progression [3-5]. However there is a group of patients, who defy conventional prognostic factors and in these cases the disease, in spite of favorable features, has an unpredictable, aggressive and fatal course [6]. These problems indicate the necessity of developing newer individual tumor prognostic markers. Many efforts have been undertaken to apply very sensitive immunological and molecular methods [4, 5, 7-9]. One of the most promising potential candidates for cell-type--specific marker in melanoma is tyrosinase expression. Tyrosinase (E.C. 1.14.18.1) – a key enzyme in melanin synthesis – is actively transcribed only in melanocytes, melanoma cells and Schwann cells [10]. Therefore, it seems a prefect marker of the presence of circulating melanoma cells in peripheral blood. The application of reverse transcriptase polymerase chain reaction (RT-PCR) assay allows detecting the tyrosinase messenger RNA in peripheral blood with high sensitivity.

RT-PCR for tyrosinase mRNA has been reported as a useful technique for the detection of circulating tumor cells in patients with melanoma. However, the results of different investigations have revealed incoherent results with respect to the clinical and prognostic value of tyrosinase RT-PCR [7, 11-16]. Thus far, published results contained data of patients in all stages of the disease and the percentage of positive results for mRNA tyrosinase (TYR /+/) varies remarkably [7, 13, 17, 18]. It seems that in stage IV patients, when the disease is disseminated, melanoma cells should be present in peripheral blood, in a relatively large number but their detection has practically no significance, because of the refractory of the disease to established therapeutic methods.

The aim of this prospective study, conducted at The M. Skłodowska-Curie Memorial Cancer Center-Institute (MSCMCCI) in Warsaw, was to estimate the prognostic value of a single RT-PCR tyrosinase test in patients with cutaneous melanoma without metastases to distant organs [stage I-III]. Subsequently, we attempted to correlate these results with important classic prognostic factors.

Materials and methods

Patients

All melanoma patients enrolled into the study (119 patients – 58 males, 61 females; mean age: 49.7 ± 12.8 years) with histologically confirmed cutaneous melanoma were treated in The Soft Tissue/Bone Sarcoma Department in MSCMCCI in Warsaw from April 1994 to April 1999. Patients had not undergone any preliminary selection and none presented any features of distant metastases. Peripheral blood samples were taken at the first routine follow-up visit. Oral informed consent was obtained from all patients. All patients were followed up in routine visits at the outpatient clinic. Follow-up time was 14-75 months (median 22 months).

Blood samples were also taken from 47 healthy volunteers and 10 patients with other malignancies as negative controls. A group of 16 patients with disseminated melanoma (6 – stage IVA with *in transit* lower extremity metastases; 10 – stage IVB with visceral metastases) was included for tyrosinase RT-PCR assay as well.

Clinical staging according to UICC-AJCC 1992 classification was determined by physical examination, pathological examination of the primary lesion and/or involved lymph nodes and routine imaging examinations (chest X-ray, ultrasonography of the abdominal cavity) at the time of blood collection. There were 10 patients in stage I, 56 – in stage II, and 50 with regional lymph node involvement (stage III). Mean primary tumor thickness according to Breslow in the group of patients in stage I, II was: 4.2 ± 2.9 mm (median 3.5 mm). 72 patients (60%) had nodular melanoma (NM), 36 (30%) – superficial spreading melanoma (SSM), 8 (7%) – acral lentiginous melanoma and 3 (3%) – lentigo malignant melanoma (LMM).

The patients' clinical status was not known to persons running the RT-PCR assay and RT-PCR results were not known to the surgeons recording disease status.

Synthetic oligonucleotides

All primers were synthesized using Applied Biosystems apparatus type 391. The primer sequences used in this work are presented in Tab. I.

RNA isolation

Blood samples (5 ml) from melanoma patients and healthy donors were collected on EDTA. The samples were centrifuged (1500 g, 10 min) as soon as possible, and the plasma discarded. At this point, the pellet of blood cells can be frozen at -70°C. The RNA was prepared from a fresh or frozen pellet as described previously [16].

Tab. I. Sequences for polymerase chain reaction primers

Name of primer	5'-sequences-3'	Length of the band
GAPDH 1	GGTCGGAGTCAACGGATTTG	
GAPDH 2	ATGAGCCCCAGCCTTCTCCAT	320 bp
HTYR 1	TTGGCAGATTGTCTGTAGCC	
HTYR 2	AGGCATTGTGCATGCTGCTT	284 bp
HTYR 3	GTCTTTATGCAATGGAACGC	
HTYR 4	GCTATCCCAGTAAGTGGACT	207 bp

Reverse PCR methods

For reverse transcription, 2 μg of RNA were dissolved in 12 μl of water containing 1.2 μg oligo$(dT)_{12\text{-}18}$. The solution was heated at 65°C for 10 min, then cooled to room temperature and supplemented to a final volume of 20 μl with 5 x reaction buffer, 0.01 M DTT, 10 mM mixture of dNTPs, HPRI and SuperScript reverse transcriptase according to conditions recommended by the producer (Gibco-BRL). The reaction mixture was incubated at 37°C for 1 h. The cDNA product was further purified by phenol/chloroform extraction and precipitation with 2.5 volumes of ethanol. The precipitated pellet was dissolved in 15 μl of DEPC-treated water. The reaction mixture for the first round of PCR contained, in a final volume of 25 μl: 1 x PCR buffer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 50 ng of primers [Table I], 5 μl of purified cDNA solution and 2.5 u. of Taq polymerase. The samples were heated to 94°C for 2 min prior to amplification. Amplification conditions were as follows: 94°C for 45 sec, 60°C for 45 sec, 72°C for 45 sec; 30 cycles. Amplification was finished by 10-min incubation at 72°C. Reamplification with nested primers was carried out under the same amplification conditions. The resulting reamplification products (10 μl) were analyzed on 2% agarose with pUC 19 digested with Dde I restriction endonuclease as a molecular weight standard.

RNA integrity was checked electrophoretically. The quality of cDNA was controlled by PCR using primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH 1 and GAPDH 2) [Table I].

Statistical Analysis

All statistical analyses were performed using STATISTICA Software [StatSoft] with the χ^2 test for comparison between two groups and Kaplan-Meier method and log-rank test for overall survival (OS) analysis.

Results

Overall, among 119 tested patients there were 25 cases (21%) of positive results of the tyrosinase mRNA RT--PCR test (TYR /+/).

Tyrosinase mRNA RT-PCR assay results did not correlate with Breslow's thickness or Clark's level of invasion of the primary lesion [Tab. II]. Tyrosinase test results did not show any differences between the group of patients with local disease (stage I, II) -12/69 TYR /+/ (17%) and the group of patients with lymph node metastases (stage III) – 13/50 (26%) [Tab. II]. No association between pathological tumor subtype or patients' gender and a positive RT-PCR for tyrosinase mRNA was revealed.

Tyrosinase mRNA was not detected among any negative control subjects (peripheral blood samples of healthy volunteers or patients with malignancies other than melanoma). No tyrosinase mRNA was also detected when mRNA was isolated from fibroblast strains and from other cancer cell lines.

The presence of the mRNA tyrosinase marker (TYR/+/) was found in 8/10 cases in patients in IVB stage (in all cases of IVA, blood samples were negative for tyrosinase TYR/-/). The percentage of TYR /+/ in clinical stage IV was higher than those in patients with locoregio-

Tab. II. RT-PCR tyrosinase test results and selected clinicopathological parameters in melanoma patients

Clinicopathological feature	Number (percentage) of TYR/+/ patients N (%)	Statistical significance; p-value
Breslow's thickness of primary lesion (clinical stage I, II)		
<4,0 mm	5/38 (13%)	p = 0.4790
>4,0 mm	7/31 (23%)	N.S.
Clark's level of invasion of primary lesion (clinical stage I, II)		
Clark II, III	6/40 (15%)	p = 0.7960
Clark IV ,V	6/29 (21%)	N.S.
Clinical stage		
Local disease (I, II)	12/69 (17%)	p = 0.3290
Lymph node metastases (III)	13/50 (26%)	N.S.
Clinical stage		
Locoregional disease (I, II, III)	25/119 (21%)	p = 0.0262
Distant metastases (IV)	8/16 (50%)	(p<0.05)
Pathological subtype of primary melanoma		
NM	8/40 (20%)	p = 0.9368
SSM	4/23 (17%)	N.S.
Gender		
F	17/61 (28%)	p = 0.3332
M	8/58 (14%)	N.S.

N.S. – not significant statistically

nally advanced melanoma – stage I-III (21% vs. 50%) [$p<0.05$].

Kaplan-Meier survival curves are shown in Fig. 1. At the time of this analysis, 46 patients from the studied group had died of the disease (39%). Median OS was 36.5 months. There were no significant differences in OS between TYR /+/ and TYR /-/ groups of patients.

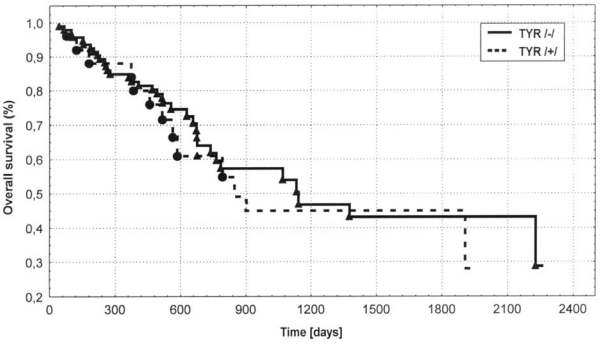

Fig. 1. Overall survival in TYR/-/ and TYR/+/ groups of patients

Discussion

Generally, the presence of circulating tumor cells in peripheral blood can indicate the potential development of secondary disease, which may have a negative influence on patients' survival and predict tumor dissemination. Since the number of circulating melanoma cells in the peripheral blood cell population is very low, the technique for their detection has to be not only highly sensitive, but also tumor-specific. The sensitive molecular method of choice for the detection of circulating tumor cells applied in a number of tumor types is RT-PCR. At first, results of papers analyzing the role of the tyrosinase RT-PCR assay in clinical assessment of patients with cutaneous melanoma seemed very promising [18-20]. However, the small number of investigations were performed on patients with disease limited only to local or lymph nodal disease [7, 12,21], and overall survival was rather rarely analyzed in these groups in correlation to results of tyrosinase RT-PCR tests. Moreover, results of the tyrosinase RT-PCR assay published by different research groups showed some discrepancies [12, 13, 17, 22] and indicated that better sensitivity and reliability of tests can be achieved by searching and adding additional molecular markers, specific to melanoma cells [7, 16, 18].

We have previously reported on the RT-PCR assay for tyrosinase mRNA of melanoma cells in peripheral blood [16, 19]. Using this method, it was possible to detect a single melanoma cell in 5 ml of normal blood. We have also shown that the tyrosinase RT-PCR assay is a highly specific technique. We have not observed any positive results of the assay in total RNA isolated from healthy individuals, patients with other malignancies, nor in total RNA isolated from fibroblast strains, breast, colorectal or lung cancer cell lines [16, 19].

The main purpose of our study was to assess the prognostic value of a single tyrosinase RT-PCR assay in the group of cutaneous melanoma patients without metastases to distant organs (I, II and III in UICC staging). The assay was usually performed on RNA isolated from a single sample of peripheral blood taken a short time (not more than 1-3 months) after removal of the primary lesion. We have found tyrosinase mRNA amplification in only 21% of cases. This percentage value is slightly lower than that observed previously by others [15, 13, 24]. The small number of positives may be caused by false-negative results. These may reflect the discontinuous release of metastatic cells into the blood stream or a very low level of expression or even the absence of tyrosinase mRNA caused by undifferentiation of melanoma cells. On the other hand, taking into account only less advanced cases, compared to those presented by Proebstle et al. [11] and Mellado et al. [21], the number of TYR /+/ is very similar. Positive results of the tyrosinase RT-PCR assay in these papers were obtained in stages I, II in 11-19% of cases and 15-31% in stage III.

Data presented by other authors demonstrated contradicting and questionable results regarding the correlation of tyrosinase RT-PCR assay and survival [12-14, 18]. According to our results, there is no correlation between the results of the tyrosinase RT-PCR assay and treatment outcomes in patients with localized or nodal disease. We did not observe any significant differences between the survival time in the group of TYR/+/ and TYR /-/ (median OS time 36 and 38 months, respectively).

In some reports it has been suggested that the predictive value for the tyrosinase RT-PCR assay can be improved by the use of PCR assays for several different melanoma markers [7, 16, 18]. Palmieri *et al.* [18], in a paper based on 235 melanoma cases demonstrated that a single-marker tyrosinase RT-PCR assay was not reliable for predicting the course of disease. According to this report, a multiple-marker RT-PCR assay using additionally p97, Melan/MART 1 and MUC 18 (this last marker has been recently found in the blood of healthy persons – with over 20% of false-positive results [7, 16]) increased the efficacy of this assay as a prognostic test.

The spreading of disease to the regional lymph nodes significantly affects the prognosis and increases the risk of distant metastases. Despite large variables in percentage of positive tyrosinase RT-PCR, several authors have reported a correlation between positive tyrosinase tests and clinical stages [13, 15, 21, 24]. In the present study no differences were found for positive tyrosinase RT-PCR assay incidences in the group of patients with the lesion limited to the primary site on the skin and group of patients with lymph node involvement. However, it is worth mentioning that the percentage of TYR /+/ for patients with lymph node metastatic disease was slightly higher than that of patients with localized disease (26% vs. 17%; N.S.). Nevertheless the number of TYR /+/ increased significantly among patients with metastatic melanoma (stage IV). These positive results concerned mainly patients with visceral metastases. It is remarkable that

TYR/+/ was not detected in any case of *in transit* dissemination in the lower limb (that is in this very particular manifestation of lymphatic dissemination). Using different molecular markers in their tests, Curly *et al*. [7] demonstrated results, which revealed melanoma circulating cells in peripheral blood samples of all (6/6) patients with *in transit* skin metastases.

Our data also confirmed the suggestions of other authors that there is no correlation between positive results of the tyrosinase RT-PCR assay and the most important conventional prognostic factor, that is Breslow's thickness of the primary lesion for stage I, II. These data indicate that tumor progression may depend not just on primary pathological parameters.

In conclusion, our results reinforce the statement that a single test for the presence of tyrosinase mRNA has no prognostic value in the group of melanoma patients without distant metastases The results of our study also support the hypothesis about great heterogeneity of tyrosinase expression in melanoma cells. The solution of this problem can be either the serial examination of tyrosinase mRNA in peripheral blood of patients during consecutive follow-up visits over a long period of time after initial treatment or using the above-mentioned additional markers to complement tyrosinase [7, 16, 21]. So far, the detection of micrometastases in an anatomical compartment different than blood, that is the sentinel lymph node biopsy technique [2], has the most important clinical relevance in establishing the group of patients with a high risk of developing metastases.

Piotr Rutkowski M.D.
Department of Soft Tissue/Bone Sarcoma and Melanoma
The Maria Skłodowska-Curie Memorial Cancer Center
and Institute of Oncology
Roentgena 5
02-781 Warsaw, Poland
e-mail: rutkowskip@coi.waw.pl

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