Detection of Circulating Neoplastic Cells by Reverse-Transcriptase Polymerase Chain Reaction in Malignant Melanoma: Association With Clinical Stage and Prognosis

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Purpose: **Circulating melanoma cells can be detected in peripheral blood by means of tyrosinase mRNA amplification by reverse-transcriptase polymerase chain reaction (RT-PCR). We conducted a prospective study to evaluate the clinical significance of the presence of circulating neoplastic cells in the blood of patients with malignant melanoma (MM).**

Methods: **A sensitive RT-PCR assay was used to detect tyrosinase mRNA in the peripheral blood of patients with stages I to IV melanoma. Healthy subjects or patients with other malignancies were used as negative controls.**

Results: **Ninety-one assessable patients were included in the study. There was a statistically significant association between RT-PCR positivity and clinical stage. Circulating melanoma cells were detected in 36% of patients with localized disease (stages I and II), in 45% of patients with regional nodal involvement (stage III), and in 94% of patients with metastatic disease (stage IV) ($P < .001$). In stage II-III patients who were RT-PCR–positive for mRNA tyrosinase in blood, the recurrence rate and disease-free survival were significantly worse than patients who were RT-PCR–negative. In multivariate analysis, RT-PCR was an independent prognostic factor for recurrence in patients with nonmetastatic disease ($P = .002$).**

Conclusion: **The detection of circulating melanoma cells in peripheral blood by RT-PCR correlated with the clinical stage of patients with melanoma and was an independent prognostic factor for recurrence. Further studies are warranted to better assess the significance of this test in the evaluation of prognosis, early detection of relapse, and in monitoring the effectiveness of systemic therapy.**

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MALIGNANT melanoma (MM) is a tumor with an increasing incidence. Approximately 80% of the patients are diagnosed in early stages of the disease. Primary cutaneous melanoma with a depth less than 0.75 mm is associated with a 95% 10-year survival rate. However, the probability of recurrence is progressively higher as the depth of the primary melanoma increases. Patients with tumors deeper than 4 mm or with regional lymph node invasion have a greater than 50% risk of developing clinically disseminated disease. Patients with metastatic disease have a very poor prognosis, with a 2-year survival rate less than 5%. The role of systemic adjuvant therapy in nonmetastatic melanoma is under investigation.[1-5] Histologic features do not accurately correlate with the risk of developing metastasis in early-stage melanoma. Thus, there is a need to find biologic factors to predict the clinical behavior of melanoma and to improve the management of the patients.

Little is known about the clinical significance of the presence of malignant cells in blood. Based on the rationale that the dissemination of the tumor cells is essential for the formation of hematogenous metastasis, the presence of melanoma cells in the peripheral blood of patients with early-stage disease could indicate a high risk of metastasis. The study of the significance of the presence of circulating melanoma cells in peripheral blood detected by polymerase chain reaction (PCR) might improve our ability to establish prognosis in patients with locoregional melanoma as well as to study the effects of systemic therapy on circulating tumor cells.[6,7] PCR has only recently been introduced for detecting circulating neoplastic cells in patients with solid tumors.[8-20] Melanoma cells in blood can be detected by means of mRNA tyrosinase gene amplification by reverse transcriptase (RT)-PCR.[9-11] Therefore, we conducted a prospective study to establish the patterns of detection of melanoma cells by RT-PCR in peripheral blood in different stages of disease. We also report the evaluation of the clinical outcome of patients according to the presence or absence of circulating melanoma cells.

PATIENTS AND METHODS

Patient Selection

Patients with histologically documented diagnosis of melanoma with either localized or metastatic disease were included in the study. Exclusion criteria was a history of another malignancy or the use

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of systemic therapy during the month previous to the inclusion in the study. Patients were treated and followed-up in the Dermatology and Medical Oncology Departments of the Hospital Clínic, University of Barcelona, Barcelona, Spain, from January 1993 to December 1994. Blood samples were processed in the Biological Hematology Laboratory of the same hospital. Blood samples were drawn at the time of primary tumor diagnosis or at relapse. In patients with nonmetastatic melanoma, blood was obtained perioperatively, between the day before and the day after the surgical treatment. Clinical stage and current or past therapy (if any) were documented at the time of entry onto the study and prospectively followed-up. Clinical staging consisted of medical history, physical examination, cell blood count, blood biochemistry, and thorax x-ray. Other complementary examinations were performed if indicated. Clinical stage was defined based on the American Joint Committee on Cancer (AJCC) guidelines. For further evaluation, patients were considered stages I and II (melanoma affecting only the skin), stage III (regional nodal involvement), and stage IV (distant metastasis), depending on the clinical stage at the time of the inclusion onto the study. Patients were also considered as having no evidence of disease and as being with evidence of disease, depending on the presence or absence of clinical melanoma at the moment of blood extraction. No clinical decisions were made based on the results of the RT-PCR assay. All stage I-III patients were visited every 4 months during the first 2 years after the diagnosis, and every 6 months thereafter. At each follow-up time, a clinical history, physical examination, blood cell count and biochemistry, and chest x-ray were performed. Other tests were performed if clinically indicated.

Negative controls of the study were healthy subjects or patients with other malignancies. The human melanoma–derived cell line SK-mel 28 (American Type Culture Collection) was used as a positive control.

Samples

Between 15 and 20 mL of blood were collected in EDTA anticoagulant from each patient. The mononuclear cell fraction of peripheral blood was isolated by ficoll gradient as described by Boyum.[21] We compared dextran (blood nucleated cells separation) and ficoll (blood mononucleated cells separation) techniques in the first five samples, without observing any difference in the incidence of the RT-PCR positivity (data not shown). All the subsequent samples were processed by Ficoll separation. Total RNA was isolated from the mononuclear cell fraction by guanidinium thiocyanate extraction using the method described by Chomczynski and Sacchi.[22]

RT-PCR Method

RT-PCR was performed following the manufacturers' instructions (Gibco · BRL, Gaithersburg, MD) using 1 mg of total cellular RNA. First strand cDNA was generated with 50 ng of specific primer (HTR2), 0.5 mmol/L deoxynucleotide triphosphate (dNTPs), 1 U RNAsin (Promega, Madison, WI) and 200 U of murine Moloney leukemia virus RT (BRL) in 20 mL final volume. A 10 μL aliquot of this reaction was used in the first round of PCR using 50 ng of each primer (HTR1 and HTR2), 1.6 mmol/L MgCl2, 0.2 mmol/L dNTPs, and 1.5 U Taq Polymerase (BRL) under the following conditions: one cycle of 5 minutes at 95°C for template denaturation, followed by 30 cycles of 65 seconds denaturation at 95°C, 65 seconds at 55°C for primer annealing, and 50 seconds for polymerase extension at 72°C. All PCR reactions were terminated with a 10-minute extension at 70°C. For the second round of PCR, 5 μL of a 1:100 dilution of the first-round PCR product was used in combination with 50 ng of HTR3 and HTR4 primers in a 25 μL final volume. Cycling conditions were the same as the first-round PCR. Final products were electrophoresed on 2% agarose gel and analyzed by direct visualization after ethidium bromide staining. Every RT-PCR reaction was repeated twice to confirm results.

Synthetic oligonucleotides. Primer sequences were devised from published sequences of tyrosinase gene.[9] Outer primers were HTYR1 (sense): TTGGCAGATTGTCTGTAGCC and HTYR2 (antisense): AGGCATTGTGCATGCTGCT. Nested primers were HTRY3 (sense): GTCTTTATGCAATGGAACGC and HTYR4 (antisense): GCTATCCCAGTAAGTGGACT. The outer primers amplified a PCR product of 284 base pairs (bp) and the nested primers amplified a fragment of 207 bp.

Integrity of RNA for RT-PCR assay was determined by performing parallel RT-PCR reactions using primers specific for beta-globin, producing a fragment of 283 bp. Beta-globin primers were devised from published sequences.[9] Samples that failed to amplify products for beta-globin RNA were considered noninformative.

Sensivity testing. To determine the sensitivity of the assay, serial dilutions of SK-mel 28 cells and normal mononucleated peripheral-blood cells were prepared. RT-PCR was performed using 1 μg of total RNA from each dilution.

Specificity of RT-PCR products was determined by Southern blot analysis. Gels were transferred overnight to a nitrocellulose membrane with 20×SSC (3 mol/L sodium chloride, 0.3 mol/L NaCi, pH 7) buffer. Membranes were hybridized using a ^{32}P end-labeled oligonucleotide complementary to a region in the tyrosinase c-DNA (TYRO: CTCTTCAGCTGATGTAGAATTTTGCCTGA) internal to the two inner primers.

Statistic Analysis

Univariate analysis of different variables (RT-PCR status, stage, Breslow or vertical thickness of primary tumor, Clark or level of invasion, number of nodes involved, primary tumor location, growth pattern, systemic therapy, sex, age, relapse) was performed by the χ^2 test. Disease-free survival (DFS) and overall survival (OS) were calculated from the time of inclusion onto the study until relapse or death, respectively. DFS and OS were analyzed by the Kaplan-Meier method. Curves were compared by log-rank test. The proportional hazards model[23] was used to estimate relative risk in multivariate analysis, to adjust potential confounding effects, and to assess possible multiplicative interactions. All parameters significant at the 0.05 level in univariate analysis were included in a proportional hazards model with a backward stepwise selection in multivariate analysis.

RESULTS

Patients

Blood from 93 patients was examined by RT-PCR as previously described. Two patients were excluded from the study because of RNA degradation. Blood from 50 controls (20 healthy subjects, eight patients with other cancers: four lung, two breast, and two colon; and 22 patients with chronic myeloid leukemia) was also examined. Patients' characteristics are listed in Table 1. Thirty nine (42.5%) of 91 patients presented with melanoma localized in the skin (stages I and II), 17 (18.5%) had

Table 1. Patient Characteristics

	No. of Patients
Total	93
Assessable	91
Male/female	49/42
Median age (years)	2
Range	22-78
Clinical stage	
I	17
II	22
III	17
IV	35
Histology (stage I and II)	
Superficial spreading	20
Nodular	10
Acral lentiginous	4
Lentigo maligna	3
Unknown	2
Primary site (stage I and II)	
Lower extremities	11
Upper extremities	6
Trunk	16
Head and neck	6
Disease status (NED/WED)	
Stage I	17/0
Stage II	20/2
Stage III	16/1
Stage IV	2/33

Abbreviations: NED, no evidence of disease; WED, with evidence of disease.

regional nodal involvement (stage III), and 35 (39%) had metastatic disease (stage IV).

RT-PCR Results

Products of RT-PCR analyzed by direct visualization after electrophoresis on a 2% agarose gel with ethidium bromide staining showed a fragment of 284 bp after outer PCR and 207 bp after nested PCR. Samples showing a band of 207 bp after a second round of amplification with nested primers were considered positive (Fig 1). Samples showing no amplification for tyrosinase mRNA and positivity for beta-globin RT-PCR were considered negative. Assay sensitivity was determined by dilution experiment as described previously. A second round of PCR with nested primers detected one SK-mel 28 cell in 10^6 mononucleated cells (Fig 2).

RT-PCR Status and Clinical Stage

Circulating melanoma cells were detected in 32 of 35 patients (94%) with metastatic disease. The two patients who tested negative had a surgically excised isolated metastatic site (nodal in one case and cutaneous in the other) without evidence of clinical disease at the time of blood extraction. One of them remained free of relapse after 14 months of follow-up. Four of 17 (23%) stage I, 10 of 22 (45%) stage II, and seven of 17 (40%) stage III patients showed positivity for the presence of malignant cells in peripheral blood. All negative controls were RT-PCR–negative for tyrosinase mRNA. Table 2 lists the RT-PCR results for each group and subgroup. There was a statistically significant association between the RT-PCR result and clinical status ($P < .0001$).

No statistical correlation between the detection of circulating melanoma cells and Breslow, number of regional lymph nodes involved (Table 2), histology, primary site, treatment, or sex was observed.

RT-PCR Status and Prognosis

Risk of relapse was evaluated in 56 stage I-III patients after a median follow-up of 18 months (range, 8 to 32).

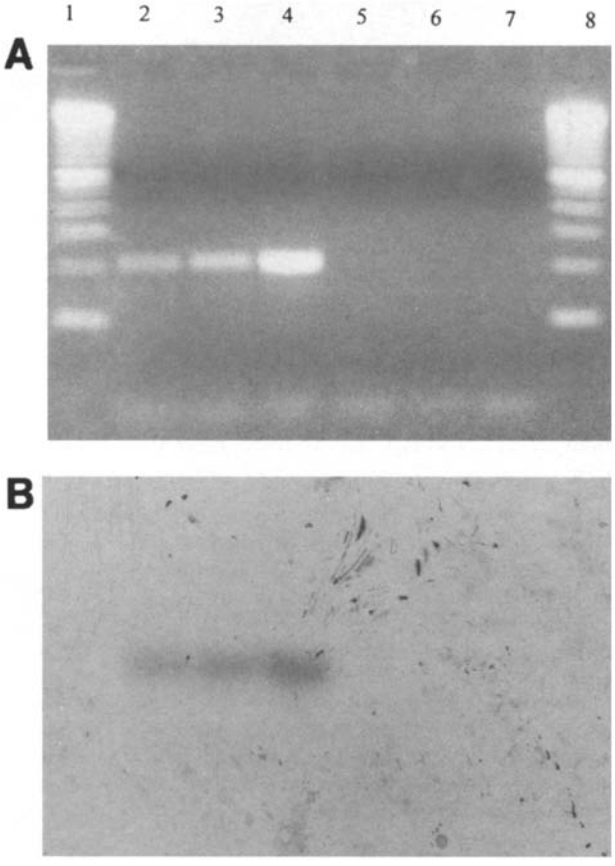

Fig 1. (A) RT-PCR products visualized in a 2% agarose gel, showing a 207-bp band in the positive samples. Lanes 1 and 8, 100 bp DNA ladder; lanes 2 and 3, two melanoma patients; lane 4, positive control (SK-mel 28 cell line); lanes 5 and 6, two melanoma patients; lane 7, healthy donor (negative control). (B) Autoradiograph of Southern blot analysis of the same gel showing hybridization of ^{32}P-labeled tyrosinase probe of the RT-PCR–positive samples (lanes 2-4).

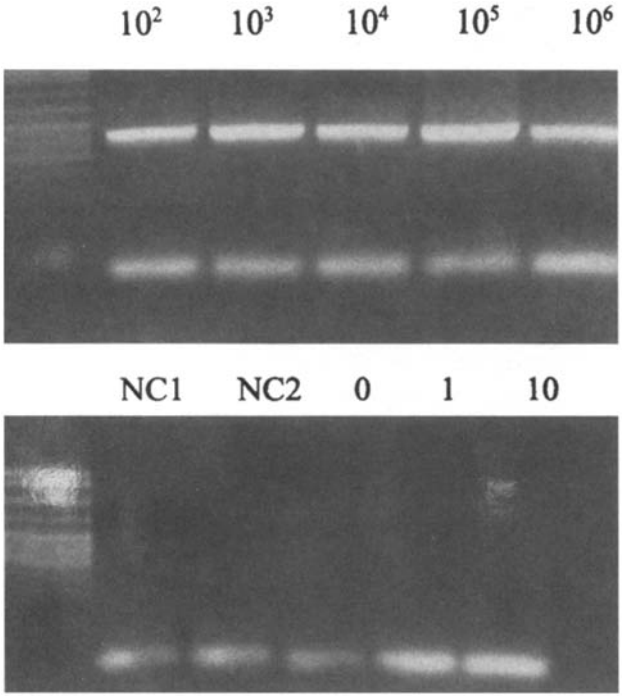

Fig 2. Sensitivity test of the RT-PCR assay. Serial dilutions of SK-mel 28 melanoma cells from 10^6 to 1 melanoma cells/10^7 normal mononucleated cells. One SK-mel 28 cell was detected in 10^6 mononucleated cells. NC, negative control (no RNA was added).

There were no relapses in stage I patients. Only one of 12 stage II patients (9%) with a negative RT-PCR test relapsed versus five of 10 patients (50%) with a positive result. In stage III, four of seven RT-PCR–positive patient (57%) relapsed versus two of 10 patients (20%) with a negative RT-PCR (Table 3). χ^2 univariate analysis showed a statistically significant correlation between recurrence and RT-PCR status ($P < .05$), and clinical stage

Table 2. RT-PCR Results by Stage

	Total	RT-PCR Positive		RT-PCR Negative	
		No.	%	No.	%
Stage I	17	4	23	13	77
B < 0.76 mm	7	1		6	
B 0.76-1.5 mm	10	3		7	
Stage II	22	10	45	12	55
B 1.6-4 mm	16	6		10	
B > 4 mm	6	4		2	
Stage III	17	7	40	10	60
N ≤ 2	10	2		8	
N > 2	7	5		2	
Stage IV	35	33	94	2	6

NOTE. χ^2 test, $P < .001$; χ^2 of linearity, $P = .09$.
Abbreviations: B, Breslow; N, number of nodes involved.

Table 3. RT-PCR Results and Relapse in Stage I-III Patients

	Total	Relapse		No Relapse	
		No.	%	No.	%
Stage I					
RT-PCR$^+$	4	0	0	4	100
RT-PCR$^-$	13	0	0	13	100
Stage II					
RT-PCR$^+$	10	5	50	5	50
RT-PCR$^-$	12	1	9	11	91
Stage III					
RT-PCR$^+$	7	4	57	3	43
RT-PCR$^-$	10	2	20	8	80
Total					
RT-PCR$^+$	21	9	43	12	57
RT-PCR$^-$	35	3	9	32	91

($P < .05$). No correlation between recurrence and other factors analyzed (Breslow, number of nodes involved, histology, primary site, treatment, sex) was observed.

DFS was significantly correlated with the detection of circulating melanoma cells. The 18-month DFS was 91% in RT-PCR–negative stage I-III patients versus 44% in RT-PCR–positive stage I-III patients ($P < .02$). The median DFS was not reached in the negative group, while it was 18 months in the positive group. Since relapse is not a common event in stage I patients within the current follow-up time, the inclusion of these patients in the prognostic analysis of the detection of circulating melanoma cells could bias the results toward a better DFS in the RT-PCR–negative group. Therefore, an independent analysis was performed including only stage II and III tumors (Fig 3). The results confirmed the prognostic value of the

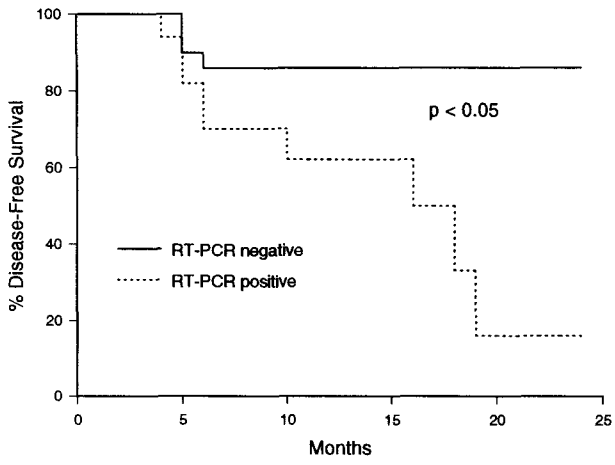

Fig 3. RT-PCR status and DFS in patients with stage II and III melanoma. DFS was calculated by the Kaplan-Meier method. The statistical significance of the difference in DFS in RT-PCR–negative versus RT-PCR–positive patients was calculated by the log-rank test.

detection of melanoma cells in peripheral blood in our series: the 18-month DFS was 86% in the RT-PCR–negative group versus 33% in the positive group ($P < .05$). Median DFS was not reached in the negative group, and in the positive group it was 16 months. There were no significant differences in OS between the RT-PCR–positive versus–negative groups (data not shown).

The two factors, stage and RT-PCR status, found to be significant in univariate analysis were subjected to a multivariate analysis. Only RT-PCR status remained statistically significant ($P = .002$), whereas RT-PCR status was not of independent significance in predicting for survival in locorregional melanoma ($P = .052$). Excluding stage I patients from the multivariate analysis, RT-PCR remained a statistically independent prognostic factor for recurrence ($P = .0022$).

DISCUSSION

In this report, we show that the detection of a molecular marker in blood, the mRNA tyrosinase, correlates with clinical stage and could be useful for establishing prognosis in patients with MM.

The detection of minimal residual disease by amplification of specific tumor cell abnormalities by PCR has been widely used in hematologic malignancies,[24-30] and represents a useful tool to assess treatment response and patient follow-up. RT-PCR has been only recently introduced for detecting malignant cells in peripheral blood in solid tumor patients. The assay used in the present report is based on a tissue-specific marker and not on a tumor-specific genetic abnormality. Tyrosinase is specifically expressed in melanocytes or melanoma cells. Because melanocytes do not circulate, the detection of tyrosinase mRNA in blood indicates the presence of melanoma cells. Similar approaches have been conducted in other tumors: Naito et al[12] and Mattano et al[13] detected circulating neuroblastoma cells by amplification of neuronal-specific mRNA. By RT-PCR amplification of Keratin 19 mRNA it was possible to detect breast cancer cells in the blood and bone marrow of patients with breast cancer.[14] RT-PCR amplification of prostate-specific antigen (PSA) mRNA detected the presence of circulating tumor cells in patients with prostate cancer.[15,16] Amplification of alfa-fetoprotein mRNA[17,18] or albumin mRNA[19] in patients with hepatocarcinoma could indicate the presence of malignant circulating cells. Carcinoembryonic antigen mRNA amplification could be useful in the detection of malignant cells in the blood of patients with gastrointestinal malignancies.[20]

We show that RT-PCR of tyrosinase mRNA is a sensitive and specific method for detecting melanoma cells in the peripheral blood of patients with malignant melanoma. This assay can detect the presence of one melanoma cell in one million normal mononucleated cells. We did not observe mRNA transcription in nonmelanoma controls, confirming the specificity of this test. Smith et al[9] reported the detection of some false-positive assays, reflecting the possibility of a illegitimate transcription process or detection of transcription of specific genes in nonspecific cells. However, the false-positives were reported after 40 cycles of a second round of amplification with nested primers but not by the experimental conditions described here. Moreover, the abundance of transcript in inappropriate cells is very low, estimated at one mRNA molecule per 100 to 1,000 cells.[31,32]

We report a significant association between clinical stage of disease and RT-PCR status. Circulating melanoma cells were detected in a large majority of patients with clinical metastasis, while the percentage of detection progressively decreased in patients with nodal involvement and localized disease. The pivotal study by Smith et al[9] established the usefulness of the mRNA tyrosinase amplification by RT-PCR for detecting circulating melanoma cells. These investigators studied seven patients with metastatic disease, detecting mRNA tyrosinase transcripts in peripheral blood in four of them. In our study, we observed a 94% of RT-PCR positivity in stage IV disease. Since we did not observe any false-positive results in 50 negative controls, we believe that the differences in the percentage of detection of circulating malignant cells in patients with metastatic disease between both studies is the result of random differences among different study populations, rather than false-positive results in our series. In a recently published study[10] using a multiple-marker RT-PCR assay for detecting neoplastic circulating cells in patients with MM, most of the stage II, III, and IV patients presented with circulating malignant cells. In this report, tyrosinase was the only single marker with a significant correlation with disease stage. Brossart et al[11] reported that the amount of circulating cells in patients with MM, evaluated by a semiquantitative tyrosinase RT-PCR assay, correlated with tumor burden and with the response to systemic therapy.

An interesting finding was the detection of circulating malignant cells in a small percentage of stage I patients. The presence of circulating malignant cells in these patients may be a naturally occurring event in a subset of tumors or may be related to contamination of the peripheral blood with tumor cells during the surgical procedure. The first possibility is supported by data showing the shed of neoplastic cells into the systemic circulation in early-stage melanoma in animal models.[33] The second possibil-

ity cannot be ruled out because 50 of 56 stage I-III samples were obtained after surgery.

A novel and significant observation in our series is the association between the presence of circulating melanoma cells and the risk of relapse in nonmetastatic patients. In stages II and III, RT-PCR status defined two groups with different clinical evolution. Recurrence rates in stages II and III were significantly higher in RT-PCR–positive (nine of 17 patients relapsed) than in RT-PCR–negative (three of 22 patients relapsed) patients. In a similar way, actuarial DFS was significantly worse in RT-PCR–positive patients compared with RT-PCR–negative patients. In fact, RT-PCR status was the only independent prognostic factor in multivariate analysis. In stage I patients, the detection of malignant cells in peripheral blood did not affect the very good prognosis of this group of patients. We can hypothesize that the cells shed from these early lesions may have the ability to extravasate but may lack the additional properties required in the multistep process of metastatic dissemination,[34,35] although other reasons may exist such as the number of positive patients and the need of a longer follow-up duration.

In conclusion, our results establish the existence of a statistical and clinically significant association between the stage of malignant melanoma and the detection of circulating neoplastic cells in peripheral blood by RT-PCR. The outcome analysis of our patients showed a poorer prognosis in the group of patients with locoregional disease with circulating melanoma cells. The prognostic value of the detection of circulating melanoma cells in blood may be especially relevant in stage II and III patients, in whom the RT-PCR positivity seems to define a group of patients with a high risk of recurrence. Further studies should be directed to better define the significance of the detection of neoplastic circulating cells by RT-PCR in the evaluation of prognosis, early detection of relapse, and in monitoring the effectiveness of systemic therapy in patients with melanoma.

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