Prognostic Significance of the Detection of Circulating Malignant Cells by Reverse Transcriptase-Polymerase Chain Reaction in Long-Term Clinically Disease-free Melanoma Patients

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ABSTRACT

The purpose of this study was to assess the prognostic significance of the detection of circulating melanoma cells by reverse transcriptase-PCR in long-term clinically disease-free melanoma patients. Patients with melanoma who were free of clinical relapse for at least 6 months after primary tumor diagnosis were included and prospectively followed. Tyrosinase mRNA in peripheral blood from these patients was assayed by reverse transcriptase-PCR at the time of their inclusion in the study. One hundred six blood samples from 57 melanoma patients were analyzed. The median time between melanoma diagnosis and inclusion in the study was 24 months (range, 7–51 months). The median follow-up time calculated from the time of inclusion in the study was 27 months (range, 11–36 months). Tyrosinase mRNA in blood was detected in 10 (17.5%) of 57 patients: 2 (18%) of 11 stage I patients, 6 (19%) of 33 stage II patients, and 2 (15%) of 13 stage III patients. Actuarial 2-year DFS was 89% for the tyrosinase-negative patients versus 30% for the positive patients (P = 0.003). Actuarial 2-year OS was 97% for the tyrosinase-negative patients versus 72% for the positive patients (P = 0.001). Tyrosinase mRNA could be detected in the blood of a proportion of long-term disease-free melanoma patients, regardless of their initial clinical stage. The presence of late circulating melanoma cells detected by this assay (11–22)

INTRODUCTION

There is an increasing interest in studying the clinical implications of the detection of tumor cells in the blood from patients with solid tumors. A widely used technique to achieve this purpose is RT-PCR.3 This technique is based on the amplification of tissue or tumor cell-specific mRNAs (1–9).

Smith et al. (10) reported that tyrosinase mRNA could be detected by RT-PCR in the blood from melanoma patients. Tyrosinase mRNA is specifically expressed in melanocytes, Schwann cells, and melanoma cells. Because melanocytes do not circulate, the detection of tyrosinase mRNA in blood indicates the presence of circulating melanoma cells. Subsequent studies confirmed the ability to detect mRNA tyrosinase by RT-PCR in the blood of melanoma patients and investigated the potential clinical implications derived from the presence of circulating melanoma cells detected by this assay (11–22).

Although most of the studies have been focused on the detection of circulating melanoma cells at the time of initial diagnosis, they can also be detected in blood months or years after primary tumor diagnosis (15). However, the biological and clinical implications that may be associated with the presence of circulating malignant cells during the follow-up of melanoma patients are not known. To address the prognostic implications of the presence of late circulating melanoma cells, here we decided to investigate the presence of tyrosinase mRNA by RT-PCR in the peripheral blood from melanoma patients who where clinically free of disease for a median time of 24 months after primary tumor diagnosis. We designate the RT-PCR analysis performed here as late RT-PCR to specifically emphasize that the timing of the RT-PCR test in was different from in our previously published study, in which the blood samples were collected in the perioperative period.

PATIENTS AND METHODS

Patients. Inclusion criteria were histologically documented diagnosis of melanoma and being free of clinical disease for ≥6 months after primary tumor diagnosis and treatment. Exclusion criteria were a history of another malignancy or the use of systemic therapy during the month previous to inclusion in the study. Oral informed consent was obtained from each patient. The study was accepted by the Ethic Committee of the Hospital Clinic. Blood for tyrosinase RT-PCR was drawn at the same time that blood extraction was performed for a previously established follow-up: the first 20 ml of blood collected were used for standard biochemistry and cell blood counts, and the following 15–20 ml were used for RT-PCR studies. Clinical
stage and past therapy were documented at the time of entry into the study. The clinical outcome of the patients was prospectively followed. Clinical staging consisted of medical history, physical exam, cell blood count, blood biochemistry, and chest X-ray. Other complementary exams were performed if they were clinically indicated. Patients with a positive RT-PCR were not subjected to a more intensive follow-up than were those with a negative RT-PCR. Clinical stage was defined based on the American Joint Committee on Cancer guidelines. Patients were visited every 4 months during the first 2 years after the diagnosis, every 6 months up to 5 years after the diagnosis, and yearly thereafter. At each follow-up time, clinical history, physical exam, cell blood count and biochemistry, and chest X-ray were performed. Patients were considered late RT-PCR positive if they had at least one positive result for tyrosinase mRNA in blood. No clinical decisions were made based on the results of the RT-PCR assay.

**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 (23). Total RNA was isolated from the mononuclear cell fraction by guanidinium thiocyanate extraction using the method described by Chomczynski and Sacchi (24). Positive controls were processed separately to avoid contamination.

**RT-PCR Method.** RT-PCR was performed following the manufacturers’ instructions (Life Technologies, Inc.) using 1 μg of total cellular RNA. First strand cDNA was generated with 50 ng of specific primer (HTR2), 0.5 mM dNTPs, 1 unit of RNAsin (Promega), and 200 units of murine Moloney leukemia virus reverse transcriptase (Life Technologies, Inc.) in a 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 mM MgCl₂, 0.2 mM dNTPs, and 1.5 unit of Taq polymerase (Life Technologies, Inc.) under the following conditions: 1 cycle of 5 min at 95°C for template denaturation followed by 30 cycles of 65 s denaturation at 95°C, 65 s at 55°C for primer annealing, and 50 s for polymerase extension at 72°C. All PCRs were terminated with a 10-min 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 was repeated at least twice to confirm results.

For synthetic oligonucleotides, primer sequences were devised from published sequences of tyrosinase gene (9). Outer primers were: HTRY1 (sense), TTGGCAGATTGTCTGTA-GCC; and HTRY2 (antisense), AGGCATGTGCA-AGTGCT. Nested primers were: HTRY3 (sense), GTCTTTATGCAATGAGG- GCC; and HTRY4 (antisense), GCATCCAGTAAAGTGGACT. The outer primers amplified a PCR product of 284 bp, and the nested primers amplified a fragment of 207 bp.

Integrity of RNA for RT-PCR assay was determined by performing parallel RT-PCRs using primers that are specific for β-globin (10) and/or by running RNA in a 1% agarose gel. Samples that failed to show the 28S and 18S ribosomal bands were considered degraded and ineligible for the study.

**Statistical Analysis.** Univariate analysis of different variables (RT-PCR status, stage, Breslow, Clark, number of nodes involved, primary tumor location, growth pattern, systemic therapy, sex, age, and relapse) was performed by the χ² test. DFS and OS were calculated from the time of inclusion into the study until relapse and death, respectively. DFS and OS

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Fig. 1 **A**, sensitivity test of the RT-PCR assay. Serial dilutions of SK-mel 28 melanoma cells from 10⁶ to 0.1 melanoma cells per 10⁷ normal mononucleated cells are shown. One SK-mel 28 cell was detected in 10⁶ mononucleated cells with RT-PCR. **B**, autoradiograph of Southern blot of the same gel transferred to a nitrocellulose membrane showing hybridization of fluorescein-labeled tyrosinase probe of the RT-PCR positive samples. One SK-mel 28 cell was detected in 10⁷ mononucleated cells with RT-PCR plus Southern blot.

Negative controls of the study were blood samples from patients with other malignancies. The human melanoma-derived cell line SK-mel 28 (American Type Culture Collection, Manassas, VA) was used as a positive control. The sensitivity of our RT-PCR was determined by performing serial dilutions of SK-mel 28 cells in 10⁷ normal mononucleated cells. With the described conditions, we were able to detect 1 SK-mel 28 cell in 10⁶ mononucleated cells by RT-PCR and 1 SK-mel 28 cell in 10⁷ mononucleated cells by Southern blot (Fig. 1).

Southern blot was performed to confirm that our RT-PCR amplified products were, indeed, from tyrosinase. After electrophoresis gels were transferred overnight to a nitrocellulose membrane with 20× SSC buffer. Membranes were hybridized with an oligonucleotide complementary to a region in the tyrosinase cDNA, using a using a nonisotopic fluorescein 3'-oligolabeling system (Amersham; Fig. 1B).

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**Integration of RT-PCR and Clinical Staging.** The clinical outcome of the patients was prospectively followed. Clinical staging consisted of medical history, physical exam, cell blood count, blood biochemistry, and chest X-ray. Other complementary exams were performed if they were clinically indicated. Patients with a positive RT-PCR were not subjected to a more intensive follow-up than were those with a negative RT-PCR. Clinical stage was defined based on the American Joint Committee on Cancer guidelines. Patients were visited every 4 months during the first 2 years after the diagnosis, every 6 months up to 5 years after the diagnosis, and yearly thereafter. At each follow-up time, clinical history, physical exam, cell blood count and biochemistry, and chest X-ray were performed. Patients were considered late RT-PCR positive if they had at least one positive result for tyrosinase mRNA in blood. No clinical decisions were made based on the results of the RT-PCR assay.

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were analyzed by the Kaplan-Meier method. Curves were compared by the log-rank test.

RESULTS

Patients. One hundred six blood samples from 57 melanoma patients were tested for tyrosinase mRNA in blood by RT-PCR. The median number of RT-PCR tests per patient was two (range, one to five). For patients with more than one test, the median interval between different blood extractions was 6 months (range, 4–12 months), depending on previously established standard follow-up schedule for each patient. Blood from eight controls (eight patients with breast cancer) was also examined. Patients’ characteristics and prior treatments are described in Table 1. Clinical stages were: stage I, 11 patients; stage II, 33 patients; and stage III, 13 patients. The median interval between tumor diagnosis and late RT-PCR test was 24 months (range, 7–51 months): 23 months (range, 10–51 months) for the positive patients and 25 months (range, 7–48 months) for the negative patients. The patients were then followed for a median time (calculated from the inclusion in the study) of 27 months (range, 11–36 months). Median follow-up time, calculated from primary diagnosis, was 49 months (range, 20–77 months).

Late RT-PCR and Clinical Features. RNA samples that showed the 28S and 18S ribosomal bands were considered eligible for the study (Fig. 2A). Samples showing a band of 207 bp after a second round of amplification with nested primers were considered positive (Fig. 2B). Using the same RT-PCR technique, we previously reported (16) no false-positive results among 50 samples from nonmelanoma control patients (20 healthy subjects and 30 patients with other cancers). In this study, we performed RT-PCR tyrosinase analysis in blood samples from eight breast cancer control patients. All of them tested negative. Hence, no illegitimate transcription in the hematopoietic cells was observed among the nonmelanoma controls with the experimental conditions used.

Here, 18 (16%) of 106 blood samples were positive for tyrosinase. Circulating melanoma cells were detected in 10 (17.5%) of 57 patients: 2 (18%) of 11 stage I patients, 6 (19%) of 33 stage II patients, and 2 (15%) of 13 stage III patients. One stage IV patient, who was rendered disease free after surgical treatment, tested negative. The percentage of late RT-PCR positivity did not correlate with clinical stage (P = 0.9). This finding is in contrast with our previous study, in which the percentage of RT-PCR positivity at the time of initial diagnosis positively correlated with stage (16). There was no correlation between late RT-PCR results and Breslow, Clark, tumor site, histology, sex, or age.

Stage IIB and III patients were candidates for receiving adjuvant chemotherapy following the protocols of our institution during the period of accrual of this study. Thirty-three patients (13 stage III and 20 stage II patients) received adjuvant systemic chemotherapy after surgery. The median interval between the end of chemotherapy and the late RT-PCR test was 16 months (range, 2–57 months). All stage III and 20 of 33 stage II patients received adjuvant chemotherapy before being analyzed by late RT-PCR (Table 2). The possible effect of chemotherapy on the rate of detection of circulating melanoma cells could not be assessed in stage III patients because all of them received adjuvant chemotherapy. In stage IIB, there were no differences between the percentage of positivity between the patients treated or not with chemotherapy: 4 (20%) of 20 patients who were treated with chemotherapy were positive versus 2 (14%) of 13 who were not treated with adjuvant chemotherapy.

Late RT-PCR and Prognosis. At the time of this analysis, 11 (19%) of 57 patients had relapsed [5 (50%) of 10 positive versus 6 (13%) of 47 negative patients, P = 0.01] and 4 (7%) of 57 patients had died from melanoma dissemination [3 (30%) of 10 positive and 1 (2%) of 47 negative patients, P = 0.01]. There were no relapses in stage I patients. In stage II, only
2 (7%) of 27 patients with a negative late RT-PCR test relapsed versus 3 (50%) of 6 patients with a positive result. In Stage III, 4 (36%) of 11 patients with a negative late RT-PCR relapsed versus 2 (100%) of 2 late RT-PCR-positive patients (Table 3). The median interval between the detection of a positive RT-PCR result and the diagnosis of clinical relapse was 8.5 months (range, 1–24 months). For the group with a negative tyrosinase test, the median time between the negative RT-PCR and the diagnosis of recurrence of the disease was 12 months (range, 4–25 months).

The RT-PCR positivity for tyrosinase mRNA in blood during the follow-up of patients with melanoma significantly correlated with a lower DFS and OS, calculated from the time of inclusion in the study. The median DFS was not reached for the group of patients testing negative for tyrosinase in blood, whereas it was 24 months for the positive patients. Actuarial 2-year DFS was 89% for the negative patients versus 30% for the positive patients (P = 0.003; Fig. 3). Median OS was not reached for either the group of patients who tested negative or the positive patients. Actuarial 2-year OSs were 97 and 72% for the negative and positive patients, respectively (P = 0.001; Fig. 4).

**RT-PCR Results at Diagnosis and in Long-Term Disease-free Patients.** In our previous study (16), blood samples from 56 stage I–III melanoma patients were evaluated for tyrosinase RT-PCR during the perioperative period. Twenty-four of these 56 patients were disease free for at least 6 months post-surgery and available for follow-up at Hospital Clinic during the accrual of this study. These 24 patients were included in this study of late RT-PCR and allow to analyze the dynamic changes in individual patients of their RT-PCR results at diagnosis and at late follow-up. In these 24 patients, the median time between the RT-PCR test at diagnosis and late RT-PCR was 24 months (range, 7–36 months). Clinical stage and initial RT-PCR results were the following: eight stage I (three positive and five negative), seven stage II (two positive and five negative), and nine stage III (three positive and six negative). Six of the eight positive patients became negative when tested beyond 6 months of surgery. The two persistently positive tests were observed in two stage I patients and they were free of relapse at 48 and 59 months postsurgery. Two of the negative patients at diagnosis had a positive test during follow-up and subsequently relapsed. Of the 10 patients who had a positive test either at diagnosis and/or during follow-up, 5 (50%) experienced a relapse. In contrast, only 1 (7%) of the 14 persistently negative patients relapsed (P = 0.05).

**DISCUSSION**

In this report, we show that circulating melanoma cells, detected by tyrosinase mRNA amplification by RT-PCR in
Peripheral blood, were present in a proportion of long-term clinically disease-free melanoma patients and that the presence of late circulating melanoma cells predicted a subsequent high risk of relapse and death.

The clinical value of detection of circulating melanoma cells by RT-PCR has been investigated by several groups (11–21). In some studies, the RT-PCR positivity at diagnosis correlated with increased clinical stage (11, 13–17) and other known prognostic factors (17). In patients with metastatic disease, the positivity of the test correlated with lower patient survival (18), the presence of visceral metastasis (19), or higher serum lactate dehydrogenase levels (20). The prognostic significance of the detection of melanoma cells in blood by RT-PCR during the perioperative period has been reported by several groups (14–16, 21). In our previous study, RT-PCR positivity was an independent prognostic factor for recurrence in stage II and III (16). In other two studies, the RT-PCR positivity after surgical treatment (21) or during the 3 first months after diagnosis (15) was an independent prognostic factor for survival.

Here, we specifically studied long-term disease-free melanoma patients to assess the clinical significance of the detection of late circulating melanoma cells. The first finding from our data were that the percentage of positivity for tyrosinase mRNA in peripheral blood among different stages of disease was lower than the observed in our previous series. In our late RT-PCR study, we detected mRNA tyrosinase in 17% of patients, whereas in our initial report, RT-PCR was positive in 40% of stage I–III patients (16). This finding was not unexpected because we assayed blood from a selected group of “good prognosis” patients who were free of clinical disease after a median time after tumor diagnosis of 2 years. In addition, we cannot rule out that, in our initial report, in which blood was drawn in the perioperative period, the surgical procedures caused the shedding of tumor cells into the peripheral blood in some patients, as has been proposed by other groups (15). In contrast with our published perioperative RT-PCR report (16), late RT-PCR positivity did not correlate with tumor stage. This difference might be due to the natural selection process implied by the inclusion criteria of this study.

To assess the impact of late RT-PCR on prognosis, we followed our series of patients for a median of 27 months after inclusion in the study. A positive test for tyrosinase mRNA in blood during the long-term follow-up of patients with melanoma was significantly correlated with a higher risk of relapse and death from melanoma dissemination. In a recent study (15), blood samples from 276 melanoma patients were tested for tyrosinase and MART-1 mRNAs at multiple, sequential time points for at least 2 years from surgery. In agreement with our data, a low incidence of RT-PCR positivity was observed, even up to 2 years after surgery. This occurred both in patients who had been continuously positive and in patients who had developed positive results after several negative tests. Given the short follow-up of the patients, the authors could not assess the prognostic significance of their late RT-PCR-positive results and, consequently, could not exclude that these were false-positive tests. However, the poor prognosis observed in our group of patients with a late RT-PCR positive result presented here suggest that these positivities are due to the presence of melanoma cells in the circulation in long-term disease free patients rather than false-positive tests.

Our study did not address whether the late circulating melanoma cells are shed from occult micrometastases present in secondary organs such as bone marrow. It would have been of interest to have parallel blood and bone marrow samples to study this issue. In this regard, Ghossein et al. (21) reported a significant correlation between blood and bone marrow detection of tyrosinase mRNA in melanoma patients after surgical treatment. On the basis of this observation, it is reasonable to speculate that the late circulating melanoma cells detected in our patients can be shed from secondary organs. If this were true, then the presence of circulating malignant cells in the setting of long-term clinically disease-free melanoma patients would be a marker, rather than a cause, of the existence of micrometastatic disease.

Our clinical findings give further support to the view of melanoma as a systemic disease and that a potential end point of adjuvant systemic immunotherapy could be to maintain patients with undetectable malignant cells in peripheral blood. Because most of our patients received prior adjuvant chemotherapy, we cannot analyze the potential impact of systemic chemotherapy in the RT-PCR results. In addition, the benefit of adjuvant chemotherapy in melanoma remains unproven (25).

Recent publications show that adjuvant treatment with IFN (26) or anti-GM2 antibodies (27) could increase the survival of stage II and III melanoma patients. The development of effective adjuvant treatments, which are associated to significant toxicity and economic cost, highlights the urgent need to find prognostic factors to select those patients who are more likely to benefit from such treatments. Our data suggest that circulating melanoma cells are markers of a high relapse risk, whether detected at the time of diagnosis (16) or during late follow-up. Further studies using mRNA tyrosinase in blood to analyze its prognostic value during patient follow-up and as a surrogate marker of the effectiveness of systemic adjuvant therapy are warranted. Our group is currently investigating if the tyrosinase mRNA amplification by RT-PCR could be useful for monitoring the efficacy of adjuvant IFN therapy in stage II and III melanoma patients.

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Clinical Cancer Research

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