Changes in the Presence of Multiple Markers of Circulating Melanoma Cells Correlate with Clinical Outcome in Patients with Melanoma[1]

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ABSTRACT

Purpose: **Melanoma cells can be found in the circulation of patients with melanoma. The following study was conducted to examine whether changes in their presence could provide an early marker of response to therapy.**

Experimental Design**: We measured the presence of several markers of melanoma cells in the peripheral blood of 118 patients with resected stage IIb, III, or IV melanoma before and after immunotherapy with a polyvalent, shed antigen, melanoma vaccine using reverse transcription-PCR assays for tyrosinase, gp100, MART-1, and MAGE-3. Assays were conducted at baseline and after 3, 5, and 11 months of therapy.**

Results: **Overall, 47% of patients were positive for at least one marker during the study. Before vaccine treatment, circulating melanoma cell markers were present in 23% of patients. After 5 and 7 months of vaccine therapy, the proportion of patients with circulating markers decreased by 27% and 55%, respectively (*P* for trend = 0.02). The recurrence-free survival of patients whose melanoma cell markers disappeared during vaccine treatment was significantly longer than that of patients in whom they increased, *i.e.*, the percentage of patients who were recurrence free at 1 year was 80% *versus* 58% (*P* = 0.03).**

Conclusions**: Therapy with a polyvalent melanoma vaccine was associated with clearance of melanoma cell markers from the circulation, and the clearance was associated with an improved prognosis. These findings suggest that the sequential assay of tumor cells in the circulation by reverse transcription-PCR may provide an early indication of the effectiveness of cancer therapy.**

INTRODUCTION

There is a need for improved procedures to evaluate the clinical effectiveness of new cancer treatments, particularly in the adjuvant setting. The current procedures use as end points disease recurrence and overall survival. Unfortunately, reaching these end points is lengthy and markedly increases the time and expense required to evaluate new therapies.

The rationale for measuring circulating tumor cells as an early marker of treatment effectiveness is based on two considerations: (*a*) tumor cells can be present in the circulation of patients with cancer, even in patients whose tumors have been resected and who are clinically disease free (1, 2); and (*b*) their incidence increases with increasing tumor load. Conceptually, therapies that can clear tumor cells from the circulation may also be able to clear these cells in other metastatic sites. Thus, a reasonable hypothesis is that a decrease in circulating tumor cells may indicate a decreased tumor load and hence may provide an indirect marker of therapy effectiveness. Because circulating tumor cells can be present in resected disease, they may provide a marker that is useful in the adjuvant setting. This is supported by the observations that there are correlations between the presence of circulating melanoma cells and a poorer prognosis (2–5).

To test this hypothesis, we examined the relation between immunotherapy with a melanoma vaccine and the presence of markers of melanoma cells in blood, followed by examination of the relation between changes in the presence of markers and clinical outcome.

MATERIALS AND METHODS

Patients. A prospective study was conducted on 396 blood specimens collected from 118 sequential patients with surgically resected melanoma who were treated with a polyvalent, shed antigen, melanoma vaccine at the New York University Kaplan Comprehensive Cancer. The trial was approved by the New York University School of Medicine Institutional Board of Research Associates.

Vaccine Treatment. All patients were immunized to a polyvalent melanoma vaccine prepared from antigens shed into culture medium by a pool of melanoma cell lines. The preparation of the vaccine has been described previously (6). The vaccine contains multiple melanoma-associated antigens, including the marker antigens used in this study, *i.e.*, tyrosinase, gp100, MART-1, and MAGE-3 (7, 8). Immunizations were

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given intradermally into all four extremities, every 2–3 weeks × 4, monthly × 3, evry 3 months × 2, and then every 6 months for 2 years or to disease progression.

Collection of Blood Specimens. Peripheral blood was collected immediately before initiation of vaccine therapy and 1 week after the fifth, seventh, and ninth immunizations, *i.e.*, at baseline and approximately 3, 5, and 11 months after initiation of treatment. The blood was collected in EDTA tubes, and RNA was extracted within 2 h of collection. The blood sample used for RNA isolation was always the last of several tubes collected concurrently, to avoid contamination with melanocytes that can occur when the needle pierces the skin.

Assay for Markers of Circulating Melanoma Cells by RT-PCR.[3] RT-PCR was used to separately assay for four antigens associated with melanoma cells, *i.e.*, tyrosinase, gp100, MART-1, and MAGE-3, in 0.5-μg aliquots of RNA derived from each blood sample. Peripheral blood lymphocytes and circulating tumor cells were isolated from 4.5 ml of whole blood using a hypotonic gradient solution (DOT kit; National Genetics Institute, Los Angeles CA), as described previously (9). One ml of Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH) was added to cell pellets, followed by addition of 200 μl of chloroform and vigorous mixing. After incubation at room temperature for 5 min, samples were centrifuged at 12,000 × *g* at 4°C for 5 min. Five hundred μl of the aqueous phase were collected, and total RNA was retrieved by isopropanol precipitation. The RNA pellets were resuspended in molecular biology grade water containing 3.33 mM DTT and 0.41 unit/ml RNasin (Promega, Madison, WI). RNA concentrations were measured by UV spectrophotometry.

Four-μg aliquots of total RNA from each sample were converted to cDNA using oligo(dT_{10}) and Moloney murine leukemia virus reverse transcriptase (Promega) following a previously described procedure (9). The final PCR mixtures for each of the melanoma markers consisted of 1:8 (0.5 μg of total RNA) of the reverse transcription mixture, 1× PCR buffer, 0.20 mM deoxynucleotide triphosphates, 2 mM $MgCl_2$, 0.02 unit/μl Taq DNA polymerase (Roche Molecular Systems), and 300 ng/μl of the appropriate oligonucleotide primer pair. PCR assays used primer pairs specific for each of the four antigens (9, 10). Regions amplified by each primer pair were designed to encompass a genomic intron to distinguish PCR products generated from contaminating genomic DNA based on size. Each PCR mixture was incubated initially at 95°C for 5 min followed by 40 amplification cycles. An amplification cycle consisted of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. All reactions were completed by incubation at 72°C for 10 min.

PCR products were detected by automated Southern blot analysis (10). Briefly, PCR products were run on 2% agarose gels, denatured using an alkaline solution, and transferred to membranes. Amplified melanoma molecular markers were hybridized with cDNA digoxigenin-labeled probes (Boehringer Mannheim, Indianapolis, IN) specific for tyrosinase, gp100, MART-1, and MAGE-3 amplification products. Specific probe hybridization was detected using anti-digoxigenin, alkaline phosphatase-conjugated antibody.

As a control, all samples were simultaneously tested by RT-PCR for the presence of β-actin to ensure sample integrity. All blots were run with multiple positive and negative RT-PCR cDNA controls. Negative controls were peripheral blood lymphocytes from normal donors. Dilutions of the M12 melanoma cell line served as the positive control.

Statistical Analysis. The Cox's proportional hazards model was used to evaluate the association between circulating melanoma cell markers at baseline and other potential prognostic factors with recurrence-free survival. As measurements after the fifth, seventh, and ninth immunizations became available, a time-dependent analysis was conducted to assess whether the presence of melanoma cell markers in the circulation at any time before recurrence was associated with recurrence-free survival. Generalized estimating equations were used to determine whether the frequency of circulating melanoma cell markers decreased over time, taking into account the correlation between repeated measurements in the same subjects. In addition, we examined recurrence-free survival according to patterns of presence of melanoma cells at baseline and after the fifth and seventh immunization. This analysis was limited to the 95 patients for whom measurements were available at the three time points, and recurrence-free survival was measured from the date of the seventh immunization to avoid selection bias. Three patterns of change were studied: (*a*) negative at all three time points; (*b*) positive at baseline and a subsequent time point; and (*c*) negative at baseline and positive at a subsequent time point.

RESULTS

Patient Characteristics. The study was conducted on 118 sequential patients with resected melanoma who were participating in melanoma vaccine trials. The characteristics of the patients are provided in Table 1. Fifty-nine percent were male. Fifty percent had AJCC stage IIb (primary melanoma 4 mm or thicker) or early-stage IIIa melanoma (nodes clinically negative and <2 histologically positive), 41% had stage IIIb disease (nodes clinically positive or 2 or more histologically positive), and 9% had resected stage IV melanoma (distant metastases). A total of 396 serial blood samples in these patients were assayed for the presence of gp100, MAGE-3, MART-1, and tyrosinase by RT-PCR. The assays were conducted in all patients at baseline and 1 week after the fifth vaccine immunization (*i.e.*, 3 months after initiation of therapy). Additional assays were conducted 1 week after the seventh immunization (*i.e.*, after approximately 5 months of treatment) in 95 patients and 1 week after the ninth immunization (*i.e.*, after 11 months of treatment) in 51 patients.

Sensitivity of Different Antigenic Markers of Circulating Melanoma Cells. The result of testing 396 blood specimens for markers of circulating melanoma cells are presented in Table 2. One or more of these markers was detected on at least one occasion in 47% of patients. The presence of markers was not influenced by the stage of the disease because they were present on one or more occasion in a similar proportion of patients with early- or late-stage disease, *i.e.*, in 47%, 44%, and

[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; AJCC, American Joint Committee on Cancer; CI, confidence interval.

Table 1 Patient characteristics

	All patients (n = 118)	Marker (+)[a] at baseline (n = 26)	Marker (−) at baseline (n = 92)	
Sex				
Male	70 (59%)	14 (54%)	56 (61%)	n.s.[b]
Age (yrs)				
Mean (SD)		52 (13.7)	50.2 (13.7)	n.s.
Median (range)		54 (18–74)	50 (18–74)	
Site of primary lesion				
Limbs	44 (37%)	11 (44%)	33 (41%)	
Head & neck	21 (18%)	5 (20%)	16 (20%)	
Trunk	41 (35%)	9 (36%)	32 (40%)	n.s.
Nodes clinically (+)	28 (24%)	5 (20%)	23 (26%)	n.s.
Histologically (+) nodes				
0	33 (30%)	9 (36%)	24 (27%)	
1–2	61 (52%)	12 (48%)	49 (56%)	
>3	17 (14%)	2 (8%)	15 (17%)	n.s.
AJCC stage				
IIb, IIIa	59 (50%)	14 (54%)	45 (49%)	
IIIb	48 (41%)	10 (38%)	38 (41%)	
IV	11 (9%)	2 (8%)	9 (10%)	n.s.

[a] Positive for one or more markers at baseline.
[b] n.s., not significant.

Table 2 Relation between extent of melanoma and expression of molecular markers by circulating melanoma cells

AJCC stage	No. of patients	% of patients positive[a]				
		Tyrosinase	gp100	MART-1	MAGE-3	Any marker
IIb or IIIa	59	15	12	17	27	47
IIIb	48	10	8	23	15	44
IV	11	27	9	9	9	54
any	118	14	10	19	20	47

[a] At any time point.

54% of patients with stage IIb or IIIa, stage IIIb, and stage IV melanoma, respectively.

There appeared to be a relation between the stage of melanoma and the marker most likely to be positive. The marker most often positive in patients with stage IIb or IIIa melanoma was MAGE-3, which was positive in one or more blood specimens in 27% of patients, whereas gp100 and tyrosinase were positive in only 12% and 15% of patients, respectively. By contrast, the reverse was true in stage IV disease, where tyrosinase was positive on one or more occasion in 23% of patients, and MAGE-3 was positive in only 8% of patients.

Heterogenicity in Expression of Markers by Circulating Melanoma Cells. The number of individual markers expressed by circulating melanoma cells was variable, with most cells expressing only one marker. Of the 68 blood specimens that were positive for circulating melanoma cells, 90% expressed only one marker, 7% expressed two markers, and only 3% expressed three or all four markers. When two or more markers were expressed, MAGE-3 was most often one of them.

There was considerable heterogeneity in the presence of individual markers in different patients; some patients expressed one marker but not another, whereas the reverse was true for other patients (data not shown). As a consequence, the ability to detect a marker of circulating melanoma cells was greatly improved by measuring multiple markers. As illustrated in Table 2, such cells could be detected in 8–27% of patients if only one marker was studied, but they could be detected in an average of 47% of patients if multiple markers were examined.

Correlation between the Presence of Markers of Circulating Melanoma Cells and Prognosis. A time-dependent Cox's regression analysis showed a statistically significant correlation between the presence of markers of circulating melanoma cells at any time point and a poorer subsequent recurrence-free survival. The risk of recurrence for patients positive for one or more marker was 2.6 times greater (95% CI, 1.35–5.10) than the risk for patients negative for all markers. This difference was significant (P = 0.005) in a Cox model analysis after adjusting for age, the only statistically significant prognostic factor of recurrence-free survival in our data.

In a similar analysis conducted to assess the prognostic role of each individual marker, risk of recurrence was associated with two of the markers, *i.e.*, MART-1 (hazard ratio = 2.51; 95% CI, 1.22–5.21; P = 0.01) and tyrosinase (hazard ratio = 2.27; 95% CI = 1.06–4.86; P = 0.04), but not with the expression of the other two markers.

Correlation between Vaccine Treatment and Clearance of Markers from the Circulation. To examine whether vaccine therapy had an impact on markers of circulating melanoma

Table 3 Relation between melanoma vaccine treatment and clearance of melanoma cells in circulation

Duration of treatment	No. (%) of patients with circulating melanoma cells (n = 95)	% decrease[a]
Baseline	22 (23)	
3 months	16 (17)	27
5 months	10 (11)	55

[a] Percentage decrease in positivity from baseline = [no. baseline positive − no. follow-up positive/no. baseline positive] × 100.

cells, we assayed these before and after vaccine therapy in the same patients. The analysis was initially conducted in a subset of 95 patients in whom assays for melanoma cells were available at baseline immediately before treatment and on at least two time points thereafter, *i.e.*, after 3 and 5 months of treatment. This was done to avoid patient selection bias that might have occurred if patients who were positive at baseline and then dropped out of the study were excluded from analysis. The results are shown in Table 3. Twenty-three percent of patients were positive for one or more marker at baseline, 17% of patients were positive for one or more marker after 3 months of therapy, and 11% of patients were positive for one or more marker after 5 months of therapy. The proportion of positive patients decreased by 27% after 3 months of therapy an by 55% after 5 months of therapy (P for trend = 0.02). The decline was even more pronounced when the analysis was conducted in a subset of 51 of the patients for whom marker assays were available at four time points, *i.e.*, at baseline and after 3, 5, and 11 months of treatment. The percentage of positive patients decreased from 27% at baseline to 16%, 6%, and 8% after 3, 5, and 11 months of therapy, *i.e.*, a decrease of 43%, 79%, and 71%, respectively, in the proportion of positive patients (P for trend = 0.002). The decrease was independent of the stage of melanoma because a decrease of similar magnitude was present when the analysis was restricted to patients with only stage IIB or IIIA melanoma (data not shown).

Because all patients had their disease surgically resected 1.5–5 months before initiation of vaccine treatment, we considered the possibility that the decrease in melanoma marker was a result of the procedure. To examine this possibility, the patients were stratified according to the time interval between surgical resection and the prevaccine treatment marker measurement. As can be seen in Fig. 1, surgery did not appear to have any long-term impact on these markers because there was no correlation between the length of this interval and the proportion of positive patients.

Correlation between Clearance of Markers and Clinical Outcome. We then examined whether there was a relation between changes in markers of circulating melanoma cells and clinical outcome. This analysis was conducted in the subset of 95 patients for whom marker analysis data were available at baseline and after the fifth and seventh immunizations. The results are summarized in Table 4, and the Kaplan-Meier survival curves are presented in Fig. 2. Recurrence-free survival was longest in patients (n = 56) who were negative for all markers at all time points (88% recurrence free at 1 year) and slightly shorter in patients (n = 22) who were positive for one or more marker at baseline but became negative while on treatment (80% recurrence free at 1 year). By contrast, the chances of being recurrence free at 1 year were diminished for patients (n = 17) who were negative at baseline but became positive while on treatment, *i.e.*, only 58% recurrence free at 1 year (P for trend = 0.03, log-rank test).

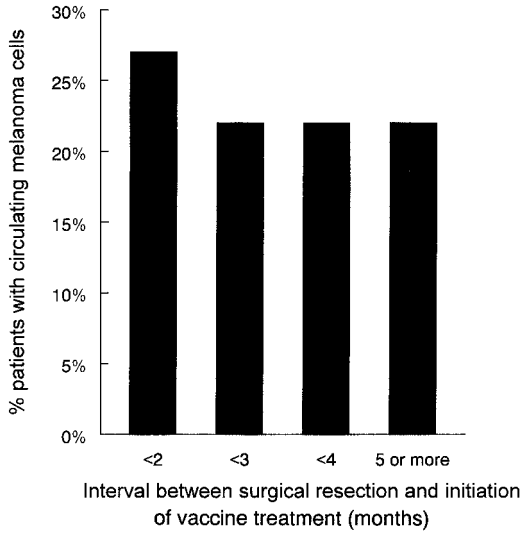

Fig. 1 Relation between surgical excision of melanoma and the presence of melanoma cells in the circulation. The figure illustrates the frequency with which markers for melanoma cells were detected at baseline before vaccine treatment as a function of the interval that elapsed between surgery and the measurement.

Table 4 Relation between treatment-associated clearance in circulating melanoma cells and recurrence-free survival

Change in circulating melanoma cells	Patients (n = 95)	Probability of recurrence-free at 1 year
− to −[a]	56	0.88
+ to −[b]	22	0.80
− to +[c]	17	0.58
P[d]		**0.03**

[a] Negative at all three visits.
[b] Positive at baseline, negative at fifth or seventh visit.
[c] Negative at baseline, positive at fifth or seventh visit.
[d] Log-rank test for trend. Significant value shown in bold.

DISCUSSION

The most interesting finding of this study is that there is a correlation between changes in the presence of markers for melanoma cells in the circulation and prognosis, suggesting that assay of these markers may provide an early indication of response to therapy and/or of disease progression.

The presence of melanoma cells in the circulation of patients with melanoma was first reported by Smith *et al.* (1) in 1991 and was subsequently confirmed by several other investigators (2, 11–13). The cells may be present even though the tumor has been surgically resected. They can be detected using a variety of procedures, the most common being measurement

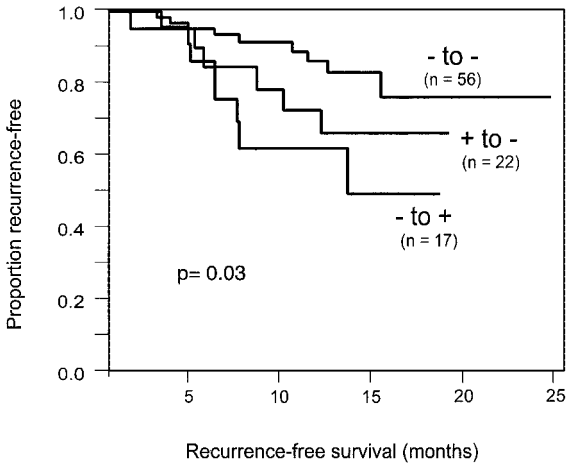

Fig. 2 Kaplan-Meier analysis of recurrence-free survival (in months) in patients with and without vaccine treatment-associated clearance of circulating melanoma cells.

of molecular markers expressed by melanoma cells using RT-PCR. The best marker remains to be determined, but the results of this study indicate that the presence of markers is heterogeneous. One patient may express one marker but not another, whereas another patient may express the second marker but not the first. As a result, the ability to detect markers of circulating melanoma cells is markedly improved by measuring multiple molecular markers (11–13), an observation confirmed in this study. We detected one or more marker of circulating melanoma cells on one or more occasions in 47% of 118 patients when these cells were assayed by RT-PCR for four markers, the melanoma-associated antigens tyrosinase, gp100, MART-1, and MAGE-3. By contrast, only 10–21% of patients were positive for any one marker. The frequency with which any individual marker was present appeared to be influenced by the stage of the melanoma. MAGE-3 was most often present in patients with early melanoma and least frequently found in those with advanced disease, whereas the reverse was true for tyrosinase. The reason for this is not known but may reflect changes to the antigenic phenotype of melanoma associated with disease progression. Lastly, the presence of any marker within the same individual was variable over time. Some patients who were negative at baseline became positive at a subsequent time point and then became negative again at a later time. This change may reflect further boosting of antitumor immunity with continued therapy or that the presence of circulating melanoma cells is transient, and therefore any single measurement may miss the presence of these cells.

Overall, there was a statistically significant inverse correlation between the presence of one or more markers of melanoma cells in the circulation on one or more occasion and a poorer clinical outcome ($P = 0.005$). Others have made similar observations (3–5). The type of antigen marker present appeared to affect prognosis. The presence of MART-1 or tyrosinase was associated with a worse prognosis (*i.e.*, hazard ratios of 2.51 and 2.27, respectively; $P = 0.01$ and 0.04, respectively), whereas the presence of gp100 or MAGE-3 did not appear to influence prognosis.

All patients entered into this study were treated with a polyvalent melanoma vaccine prepared from shed antigens. The vaccine contains multiple melanoma-associated antigens, including the marker antigens measured in this study, *i.e.*, tyrosinase, gp100, MART-1, and MAGE-3. The vaccine can stimulate peptide-specific CD8+ T-cell responses to these antigens (7, 8). The vaccine appears to be clinically effective because in a double-blind and placebo-controlled trial in patients with resected stage III melanoma, the median recurrence-free survival of the melanoma vaccine-treated patients was more than twice as long as that of patients treated with a placebo (human albumin) vaccine [$P = 0.03$ after Cox multivariate analysis (14)].

After initiation of vaccine treatment, there was a progressive diminution in the proportion of patients with detectable markers of melanoma cells in their circulation. Within 3 months of initiating therapy, the percentage of positive patients decreased by 27% from baseline, and after 5 months, the percentage of positive patients decreased by 55% (P for trend $= 0.02$). The decline was even more striking in a subset of 51 patients for whom a fourth time point was available, where the proportion of positive patients decreased by 70% from baseline 11 months after initiation of therapy (P for trend $= 0.002$). Because the decrease occurred gradually with length of treatment, it is unlikely that it resulted from random changes in the results of the assay. Nor was the decline due to a patient selection bias resulting from patients with circulating melanoma cells at baseline having early recurrence and being excluded from subsequent study, because all analyses were restricted to patients for whom all time points were available. The decrease was unrelated to the initial extent of the melanoma because it occurred in patients with primary cutaneous melanoma as well as in those with disseminated disease. Nor was the decrease a result of the prior surgical excision of the melanoma because the time interval between surgery and the initial marker assay did not influence the proportion of patients who were positive. Lastly, the decline was unlikely to be the result of transient changes in the presence of melanoma cells in the circulation because there was no change in the proportion of positive patients tested between 1 and 5 months after surgery while on no vaccine treatment, but a steady decline in these cells was seen once vaccine therapy was initiated. Consequently, the results suggest that the decrease in the markers was a result of vaccine treatment. However, this possibility needs to be confirmed in a randomized trial. The mechanism responsible for the decrease in circulating melanoma cells associated with vaccine treatment is not known. The most likely explanation is that it results from vaccine-induced immune responses clearing the circulating tumor cells. A preliminary observation in our laboratory supports this possibility. We observed that patients who had a vaccine-induced CD8+ T-cell response to a specific melanoma-associated antigen rarely expressed that same antigen on their circulating cells when compared with patients who never developed this type of immune response.

The clinically most interesting result of the study is that there was a correlation between changes in markers for circulating melanoma cells and clinical outcome. Recurrence-free survival was longest in patients without detectable markers at any time and was almost as long in patients who initially were

positive but became negative during treatment. By contrast, recurrence-free survival was significantly worse in patients who were negative at baseline but became positive during treatment, *i.e.*, probability of being recurrence free at 1 year of 0.88, 0.80, and 0.58, respectively; $P = 0.03$.

The clinical implication of these findings is that the sequential assay of markers of tumor cells in the circulation may provide an early marker of the effectiveness of cancer therapy, particularly in patients with no measurable disease. Furthermore, the appearance of the markers in the circulation may prove useful as an early marker of disease recurrence. Lastly, assay of circulating tumor cells may be able to compliment immunological testing as a procedure that can provide early indication of the effectiveness of vaccine therapy.

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