Prognostic Value of Circulating Melanoma Cells Detected by Reverse Transcriptase–Polymerase Chain Reaction

By Giuseppe Palmieri, Paolo A. Ascierto, Francesco Perrone, Sabrina M.R. Satriano, Alessandro Ottaiano, Antonio Daponte, Maria Napolitano, Corrado Caracò, Nicola Mozzillo, Maria T. Melucci, Antonio Cossu, Francesco Tanda, Ciro Gallo, Rocco A. Satriano, and Giuseppe Castello

Purpose: **Factors that are predictive of prognosis in patients who are diagnosed with malignant melanoma (MM) are widely awaited. Detection of circulating melanoma cells (CMCs) by reverse transcriptase-polymerase chain reaction (RT-PCR) has recently been postulated as a possible negative prognostic factor. Two main questions were addressed: first, whether the presence of CMCs, defined as the patient being positive for any of the three markers, had a prognostic role; and second, what the predictive value of each individual marker was.**

Patients and Methods: **A consecutive series of 200 melanoma patients observed between January 1997 and December 1997, with stage of disease ranging from I to IV, was analyzed by semiquantitative RT-PCR. Tyrosinase, p97, and MelanA/MART1 were used as markers to CMCs on baseline peripheral blood samples. Progression-free survival (PFS) was used as a unique end point and was described by the product limit method. Multivariable analysis was applied to verify whether the auspicated prognostic value of these markers was independent of the stage of disease, and a subgroup analysis was performed that excluded patients with stage IV disease.**

Results: **Overall, 32% (64 of 200) of patients progressed, and a median PFS of 52 months in the whole series was observed. The presence of CMCs and the markers individually or combined was predictive of prognosis in the univariate analysis but did not provide additional prognostic information to the stage of disease in multivariable models. In the subgroup analysis of stage (ie, I-III subgroup), similar results were observed.**

Conclusion: **Detection of CMCs in peripheral blood samples at the time of MM diagnosis by semiquantitative RT-PCR does not add any significant predictive value to the stage of disease. Thus, this approach should not be used in clinical practice, and further studies are required to determine its usefulness.**

J Clin Oncol 21:767-773. ***© 2003 by American Society of Clinical Oncology.***

THE INCIDENCE and mortality rate of malignant melanoma (MM) are increasing worldwide,[1-2] and there is a generalized need for improved methods to predict the clinical outcome of patients. Stage of the disease,[3] ascertained by accounting for level of invasion,[4] tumor thickness,[5] and presence of lymph node or distant metastases,[3] is the most widely accepted prognostic factor.[6] Melanoma patients have poor prognosis because of frequent distant dissemination of the disease. Although the size of the primary lesion is frequently small, it is obvious that in many patients there has already been metastatic spread at the time of diagnosis. The detection of circulating melanoma cells (CMCs) has been proposed as a potentially effective tool in selecting patients that have a high risk of relapse at the time of the diagnosis.[7]

Reverse transcriptase-polymerase chain reaction (RT-PCR) can detect a single specific mRNA in a mixed cell population; thus, it can be a sensitive method for identification of circulating tumor cells.[8-13] Tyrosinase (TYR), an enzyme that is involved in the melanin biosynthesis pathway,[14] is the marker most frequently used to detect the presence of CMCs; however, its usefulness as a marker is highly debated.[15-21] Because the use of TYR mRNA as a unique marker could be of limited value in the management of MM patients, a multimarker assay, which includes p97 and MelanA/MART1 in addition to TYR, has been proposed to improve sensitivity and specificity of the procedure.[22]

We have previously demonstrated a positive association between clinical stage of MM and the detection of tumor-associated mRNAs in peripheral blood by a multimarker RT-PCR assay.[23] To evaluate the clinical usefulness of such a procedure, we planned the present study to determine whether detecting CMCs by RT-PCR in a consecutive series of patients (with all stages of disease) can improve prognostic prediction, which is commonly based on pathologic and clinical prognostic factors. To explore whether circulating melanoma-associated markers can allow the detection of minimal residual disease in patients who have undergone radical surgery, we also performed analyses limited to the subgroup of patients with stage I to III disease; we also addressed the role of each of the markers individually and combined.

PATIENTS AND METHODS

Patient Selection

The study was conducted with a series of 200 patients referred to the National Cancer Institute (NCI) of Naples between January 14, 1997, and

From the Institute of Genetics of Populations, Consiglio Nazionale delle Ricerche, Alghero (Sassari); Division of Clinical Immunology, Clinical Trials Unit, Division of Medical Oncology A, and Division of Surgical Oncology B, National Cancer Institute of Naples; Institute of Pathology, University of Sassari; and Medical Statistics and Department of Dermatology, Second University, Naples, Italy.

Submitted January 28, 2002; accepted September 25, 2002.

This article was published ahead of print at www.jco.org.

Supported by Italian Ministry of Health, Health Department of Campania Region (Italy), and Regione Autonoma della Sardegna. The Clinical Trials Unit is partially supported by AIRC (Associazione Italiana per la Ricerca sul Cancro).

Both G.P. and P.A. contributed equally to this work.

Address reprint requests to Paolo A. Ascierto, Division of Clinical Immunology, National Cancer Institute, Via M. Semmola, 80131 Naples, Italy; email: pasciert@tin.it.

December 17, 1997. Patients were consecutively collected, and they were considered eligible for participation in the study if they had a histological diagnosis of cutaneous malignant melanoma, which was performed either inside or outside the NCI. In the latter situation, pathologists from the NCI reviewed the patient slides. Patients were eligible for collection of a baseline peripheral blood sample if no more than 4 weeks had passed since surgical treatment for early-stage (ie, 0 to III) disease; patients with stage IV disease (candidate for systemic treatment) had their baseline blood sample collected before starting treatment. Informed consent from each patient was sought in regard to collection of blood samples, and the study was approved by the Ethical Review Board of the National Cancer Institute of Naples. Treatment strategy for patients was not decided on the basis of the RT-PCR findings. Disease stage was coded, a posteriori, according to the American Joint Committee on Cancer (AJCC) guidelines.[6] All early-stage patients were visited every 6 months after diagnosis. At each follow-up visit, a clinical history, physical examination, full cell blood count, RT-PCR assay, and blood biochemistry were performed. Instrumental assessments (ie, computed tomography, ultrasonography, bone nuclear scan) were performed if clinically indicated. Stage IV patients were followed up according to rules dictated by the chemotherapy program.

Sample Preparation and RT-PCR Assay

Nucleated cells from peripheral blood samples were processed to isolate total RNA using standard procedures.[23-25] Primer sequences and protocols for RT-PCR have been previously described.[27] Integrity of RNA was determined by performing parallel RT-PCR assays using primers specific for the housekeeping gene GAPDH.[23] Blood samples that failed to amplify products for GAPDH RNA were considered noninformative and were discarded from further use. In each RT-PCR assay, products were separated by electrophoresis on a 2% agarose gel and were analyzed by direct visualization by ethidium bromide staining. Specificity of the RT-PCR products was assessed by Southern blot analysis, as previously described.[27] Samples were considered positive when RT-PCR products were detected by either direct visualization or Southern blot analysis.

Statistical Analysis

To answer the primary study question of whether the detection of CMCs could have prognostic value, a few assumptions were made. First, it was assumed that the false-positive rate of RT-PCR products was negligible with any of the three markers and that whichever positive marker was observed was considered a signal for the presence of CMCs. Thus, 100% specificity was assumed. Second, because of the lack of an external standard for CMCs, sensitivity of each marker individually or combined could only be assessed using patients with at least one positive marker as a reference. Thus, sensitivity for a particular marker was calculated as the percentage of patients positive for that marker out of the number of patients positive for at least one marker.

Cross-tabulations and a graphic representation were used to describe the associations among the three markers. The number of positive markers for each patient was summed, producing a variable parameter (ie, the number of positive markers for each patient) that had a scoring system from zero to three. Univariate associations between markers and other baseline variables were investigated by χ^2 test.

Time-to-event analyses were performed for PFS, which was defined as the time from the date of enrollment in the study to the date of disease progression or disease-associated death. PFS curves were estimated by the Kaplan-Meier method.[26] Hazard ratios of progression were estimated by the Cox model[28] and are reported with 95% confidence intervals (CIs) that are either unadjusted or adjusted for stage of disease. Presence of CMCs was used as a binary (ie, yes/no) variable, and stage of disease was used as a continuous variable (ie, 0 to IV). The number of positive markers (ie, 0 to 3) was also investigated. Because of the small number of events in this study, overall survival was not considered for analysis. Taking into account that finding a prognostic value of the detection of CMCs could be crucial in the therapeutic planning of patients with localized disease (stages 0 to III), PFS analyses were performed on both the entire patient population (ie, including stage IV disease) and on the subgroup of patients with localized disease (ie, excluding stage IV disease).

Table 1. Characteristics of Patients

	No.	%
Sex		
Female	115	58
Male	85	42
Age, years		
Median, range	50	16-85
Interquartile range		40-60
AJCC/UICC stage		
0	9	4.5
I	93	46.5
II	51	25.5
III	24	12
IV	23	11.5
Site of primary tumor		
Head & neck	18	9
Trunk	90	45
Superior limbs	32	16
Inferior limbs	57	28.5
Unknown	3	1.5
Lymph-node assessment		
Clinical/instrumental	105	52.5
Sentinel-node biopsy		
Nonmetastatic	37	18.5
Metastatic	6	3
Lymphadenectomy		
Nonmetastatic	21	10.5
Metastatic	31	15.5

Abbreviations: AJCC/UICC, American Joint Committee on Cancer/International Union against Cancer.

RESULTS

A consecutive series of 200 patients diagnosed MM was studied. Patients were mostly female (58%), with a median age of 50 years (range, 40 to 60 years). According to the new AJCC/International Union Against Cancer (UICC) stage classification, almost the half of the patients had stage I disease, and approximately one quarter had stage II disease. Overall, patients without distant metastases (stages 0 to III) accounted for 88.5% of patients (Table 1).

A total of 163 of the 200 patients (81.5%) had at least one positive marker; thus, they were considered positive for CMCs. p97 was the most sensitive marker, being positive in 82% (140 of 163) of CMC positive patients. Distribution of the positive RT-PCR markers is detailed in Table 2.

Table 2. Distribution of Positive RT-PCR Markers

	mRNA markers		
No. of Patients	p97	MelanA/MART1	Tyrosinase
37	−	−	−
46	+	−	−
8	−	+	−
17	−	−	+
19	+	+	−
36	+	−	+
4	−	+	+
33	+	+	+

Abbreviations: RT-PCR, reverse transcriptase-polymerase chain reaction.

Table 3. Association Among RT-PCR Markers and Baseline Patient and Tumor Characteristics

	CMC-Positive		p97-Positive		Tyrosinase-Positive		MelanA/MART1-Positive		Number of Positive Markers			
	%	*P*	%	*P*	%	*P*	%	*P*	1+, %	2+, %	3+, %	*P*
Sex		.79		.75		.35		.24				.99
Females, n = 115	81		66		48		29		36	29	17	
Males, n = 85	82		68		41		36		35	31	19	
Age, years		.60		.22		.14		.04				.04
≤ 60, n = 150	81		65		42		28		39	29	13	
> 60, n = 50	84		74		54		44		24	32	28	
Stage, AJCC/UICC		.009		< .0001		.09		.03				.002
0, n = 9	56		22		22		22		44	11	0	
I, n = 93	75		58		39		28		38	26	12	
II, n = 51	84		67		47		27		41	29	14	
III, n = 24	92		87		54		33		33	33	25	
IV, n = 23	100		100		65		61		13	48	39	

Abbreviations: CMC, circulating melanoma cells.

The presence of circulating mRNA markers was significantly associated with the stage of disease. Similarly, each individual marker and the number of positive markers were associated with the stage of disease. However, no association of markers with sex and age of the patient was evident, with the exception of the presence of MelanA/MART1 and an increasing number of positive markers, which were both significantly associated with older age (> 60 years; Table 3).

As of July 2001, 64 patients (32%) had suffered disease progression, with a median PFS of 52 months for the entire patient population; 46 patients (23%) had died, with a median follow-up of 44 months for living patients. In the univariate analysis, the presence of CMCs had a significant predictive value, with a hazard ratio (HR) of progression of 3.15 (95% CI, 1.26 to 7.85; $P = .01$) for patients with at least one positive marker (Table 4, left panel; Fig 1). However, when the Cox model was adjusted by stage of disease, the predictive value of CMCs was not found (HR, 1.44; 95% CI, 0.55 to 3.74; $P = .46$). Similar results were observed when patients with stage IV disease were removed from the analysis (HR, 2.23; 95% CI, 0.88 to 5.67; $P = .09$ for unadjusted and HR = 1.40; 95% CI, 0.54 to 3.63; $P = .49$ for adjusted; Table 4). A similar pattern of results was observed with p97, TYR, and number of positive markers (Table 4; Figs 2 and 3). In contrast, the predictive value of MelanA/MART1 was retained in the model limited to patients with stage 0 to III cancer, even when adding stage into the model (HR, 2.05; 95% CI, 1.11 to 3.79; $P = .02$); however, the addition of patients' age to the latter model did not made the association between MelanA/MART1 and prognosis any more significant (HR, 1.79; 95% CI, 0.95 to 3.37; $P = .07$). Thus, stage of disease was closely associated with prognosis, both when including and excluding patients with stage IV disease.

Table 4. Progression-Free Survival Analyses

	All stages (n = 200; events = 64)			Stages 0 to III (n = 177; events = 43)		
	HR	95% CI	*P*	HR	95% CI	*P*
At least one positive marker						
Unadjusted	3.15	1.26 to 7.85	.01	2.23	0.88 to 5.67	.09
Adjusted by stage	1.44	0.55 to 3.74	.46	1.40	0.54 to 3.63	.49
p97 positive						
Unadjusted	3.24	1.65 to 6.37	.0006	2.22	1.09 to 4.5	.03
Adjusted by stage	1.36	0.65 to 2.87	.42	1.32	0.63 to 2.78	.46
Tyrosinase positive						
Unadjusted	1.63	0.99 to 2.69	.053	1.56	0.85 to 2.85	.15
Adjusted by stage	1.02	0.61 to 1.71	.93	1.20	0.65 to 2.21	.57
MelanA/MART1 positive						
Unadjusted	2.41	1.47 to 3.96	.0005	2.17	1.18 to 4.00	.01
Adjusted by stage	1.39	0.82 to 2.35	.23	2.05	1.11 to 3.79	.02‡
Number of positive markers*						
1 *v* 0	1.87	0.69 to 5.08	.22	1.57	0.56 to 4.35	.39
2 *v* 0	3.65	1.38 to 9.63	.009	2.34	0.83 to 6.59	.11
3 *v* 0	6.00	2.22 to 16.13	.0004	4.53	1.57 to 13.10	.005
Number of positive markers†						
1 *v* 0	1.30	0.47 to 3.56	.61	1.12	0.40 to 3.14	.83
2 *v* 0	1.54	0.55 to 4.30	.41	1.42	0.49 to 4.07	.52
3 *v* 0	1.73	0.59 to 5.07	.32	2.35	0.78 to 7.03	.13

Abbreviations: HR, hazard ratio of progression; CI, confidence interval.

*Unadjusted.

†Adjusted by stage.

‡Level of significance was unchanged after addition of age as a variable.

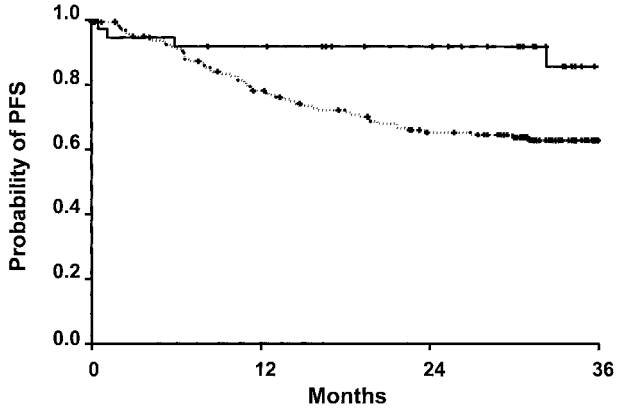

Fig 1. Progression-free survival (PFS) curves according to absence (solid line) or presence (dotted line) of circulating melanoma cells. Vertical lines indicate censored patients.

DISCUSSION

In this article, we show that the presence of CMCs, determined by the detection of mRNAs corresponding to melanoma-associated molecular markers in peripheral blood, is significantly associated with the stage of disease—the most commonly used prognostic system for melanoma patients—but does not play a role as an independent prognostic factor for clinical outcome.

The hypothesis that detection of CMCs could improve prognostic prediction was based on at least two issues. First, it is obvious that mobilization of cells from the site of the primary lesion through the blood stream is necessary (although not sufficient) to produce distant metastases. Thus, detection of CMCs may correspond to the identification of an early and potential step in metastatic spread. Second, many studies have dealt with the possible prognostic value of the presence of CMCs with conflicting, but mostly positive, findings.

In regard to the first point, our data cannot rule out the theoretical assumption that detecting CMCs may be a signal of metastatic spread. However, physical invasion of the blood stream by tumor cells is among the earliest events in the tumor progression cascade, and many other steps are required for metastatic colonization of distant parenchymas. Detection of CMCs can be considered as a surrogate marker of such initial events for the establishment of distant metastases. However, in this case, identification of melanoma-associated transcripts in histologically negative, regional lymph nodes by RT-PCR could represent a more useful marker for staging melanoma patients than detection of CMCs, as previously suggested by our group.[29]

In regard to the second point, to the best of our knowledge, 15 extended papers have been published in recent years, dealing with the possible prognostic value of CMCs detected by RT-PCR on peripheral blood samples (Table 5). First, the percentage of patients found to be positive for CMCs varies, ranging from 6% to 93%.[30-38] This variability is partly caused by the number of markers used to detect CMCs and the higher rates of positive patients being reported in two trials using four different markers.[22,36] Nevertheless, among seven studies using TYR as the sole marker, the range of patients found to be positive for CMCs also varied from 6%[33] to 59%.[30] Four studies included a number of patients greater than or close to the number of patients in the present study.[32,33,35,38] All studies used TYR as a marker: TYR alone in seven studies,[12,15,21,30,33,34,39] combined with MelanA/MART1 in six studies,[31,32,35,37,38,40] and combined with p97 in two studies.[22,36] Two studies focused on patients with stage IV disease only.[37,39] All the other studies included earlier stages of disease in which the clinical relevance of prognostic prediction is higher. A weakness of all these studies, which include mostly patients with early-stage disease (AJCC stage I and II), is the low number of events available for analysis (requiring a longer follow-up evaluation). In this regard, even though the majority of patients in our study presented with localized disease (153 patients [76.5%] with stage 0 to II disease), the median follow-up period was quite long (44 months). Only one other study presented a similar follow-up period (48 months), but with a much lower number of enrolled patients and events.[36] Therefore, most of the studies published to date are focused on PFS, a surrogate end point that, although reliable, cannot completely substitute for the value of overall survival. In addition, all these studies are retrospective, including ours, by being based on the

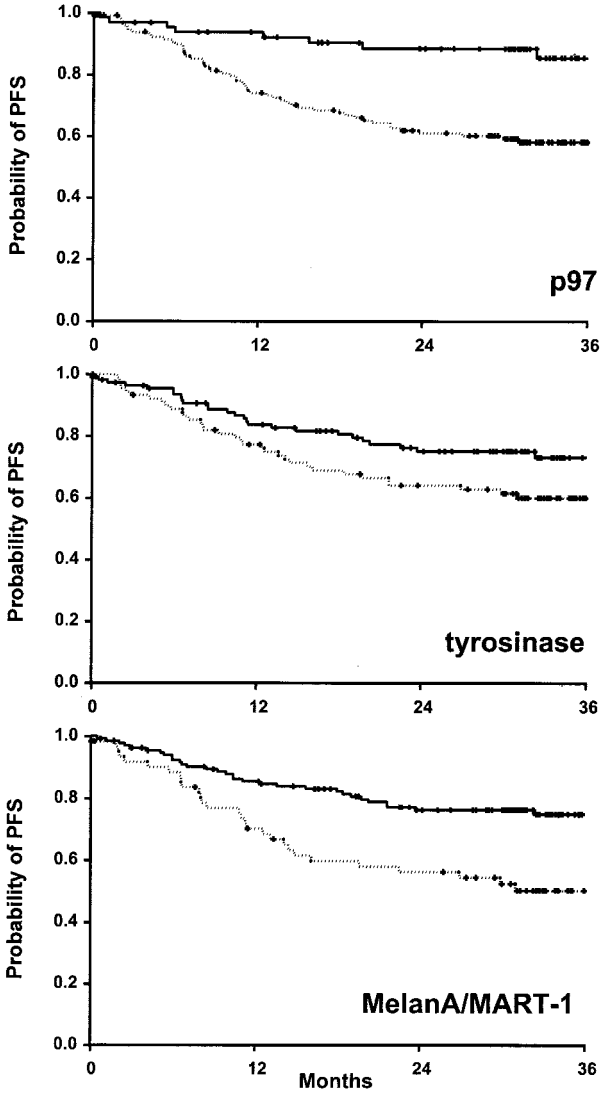

Fig 2. Progression-free survival (PFS) curves according to negative (solid line) or positive (dotted line) RT-PCR for p97, tyrosinase, and melanA/MART1. Vertical lines indicate censored patients.

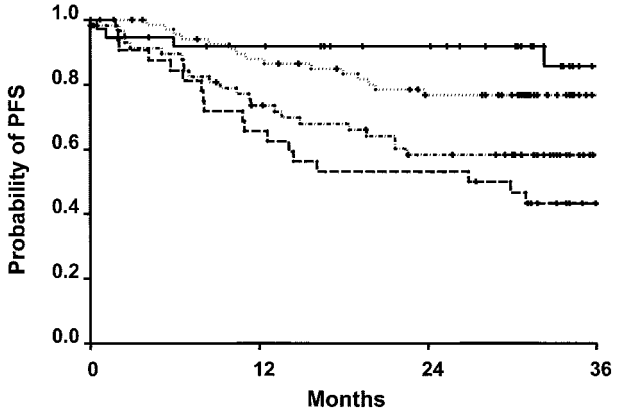

Fig 3. Progression-free survival (PFS) curves according to the number of positive RT-PCR markers (0 = solid line, 1 = dotted line, 2 = dotted/dashed line, 3 = dashed line). Vertical lines indicate censored patients.

analysis of patients for whom CMC assay has been performed. Thus, selection biases cannot be definitively ruled out.

Altogether, there were five negative studies[21,23,38,39,40] that are consistent with our findings. Time-to-event (disease-free or overall survival) analysis was not performed in any of these studies. In the larger of the studies, [38] only the association of CMCs with stage of disease was tested to explore prognostic significance. Ten studies were reported with positive conclusions, in contrast to our findings. Seven of these studies did not use a multivariable statistical approach to analysis;[12,15,22,31,34,35,37] thus, their results, which are consistent with our unadjusted results, cannot definitively prove the prognostic value of RT-PCR detection of CMCs. Three studies applied an analytic approach with multivariable analysis; two of them had fewer than 100 patients (the strength of their conclusions being overcome by our present data),[30,36] and the third study[32] had 186 patients (followed up for at least 24 months), with 73 progressions observed. However, because PFS was limited to small subgroups of patients defined on the basis of site of recurrence, the conclusion of this latter study could be biased and cannot be considered definitive.

Although a strength of our study is the number of patients enrolled, which is higher than most studies dealing with the same issue, it is debatable whether the use of several mRNA markers really improves the chances of the RT-PCR technique being a useful detector of CMCs. Indeed, if the postulate that one positive marker is sufficient to diagnose the presence of CMCs were true, then MelanA/MART1, the least sensitive marker, adds little information to TYR and p97, which has a sensitivity of 39% (64 of 163 CMC positive patients), and its evaluation, therefore, could be useless.

Considering sensitivity alone as the measure to choose markers for detecting metastatic tumor cells in peripheral blood can be misleading, which is confirmed by the fact that p97, the most

Table 5. Published Studies on Prognostic Value of RT-PCR Detected Circulating Melanoma Cells

Author, year[ref.]	No. of patients	Markers	% CMC positive	Stage included	Association with Stage	Outcome descriptor	No. of events	Median FU (months)	Statistical analysis		Interpretation
									TTE	Multivariable	
Hoon, 1995[22]	119	TYR, MAGE3, MUC-18, p97	92%	all	Yes	none	nr	nr	No	No	Optimistic
Battayani, 1995[12]	93	TYR	17%	all	Yes	Progression (33 patients)	nr	nr	No	No	Optimistic
Mellado, 1996[30]	91	TYR	59%	All	Yes	Progression death (56 patients)	12 PD, nr deaths	18	Yes	Yes	Optimistic
Kunter, 1996[15]	64	TYR	14%	all	Yes	Death	nr	20	Yes	No	Optimistic
Curry, 1998[31]	123 (out of 276)	TYR, MART1	46%	I, II, III	Yes	Progression	47	18 (minimum)	Yes	No	Optimistic
Curry, 1999[32]	186	TYR, MART1	49%	I, II, III	Yes	Progression	73	24 (minimum)	Yes	Yes	Optimistic
Hanekom, 1999[33]	181	TYR	6%	all	No	Progression	20	nr	No	No	Negative
Mellado, 1999[34]	57	TYR	18%	I, II, III NED > 6 mos	No	Progression death	11, 4	27	Yes	No	Optimistic
Schittek, 1999[35]	225	TYR, MART1	32%	all	Yes	Progression	87	4	No	No	Optimistic
Aubin, 2000[21]	39	TYR	8%	I, II, III	No	Progression	nr	nr	No	No	Negative
Hoon, 2000[36]	46	TYR, MAGE3, MUC-18, p97	93%	all	Yes	Progression death	17, 12	48 (minimum)	Yes	Yes	Optimistic
Schrader, 2000[37]	31	TYR, MART1, TRP-1, TRP-2, MAGE3	23%	IV	nr	Death	nr	11	Yes	No	Optimistic
Brownbridge, 2001[38]	299	TYR, MART1	51%	all	Yes	Progression	17	nr	No	No	Negative
Waldmann, 2001[39]	20	TYR	40%	IV	Yes	Death	12	19.5	No	No	Negative
Strohal, 2001[40]	76	TYR, MART1	21%	all	Yes	None	nr	nr	No	No	Negative
Present study	200	p97, TYR, MART1	82%	all	Yes	Progression	64	44	Yes	Yes	Negative

Abbreviations: FU, follow-up; TTE, time-to-event; nr, not reported; NED, no evidence of disease.

sensitive marker in our series of patients (82%), has been demonstrated to be among the least specific markers for melanoma cells. Indeed, we found that p97 was detected in the peripheral blood of 19 of 21 (90%) patients with Kaposi's sarcoma, whereas in the same patients, MelanA/MART1 and TYR were less frequently detected (occurring in 11 [52%] and three [14%] patients, respectively).[41] Therefore, it is important to remember that there is as yet no clear explanation for the presence of p97 mRNA in the blood of normal subjects.[42] In addition, our data do not support the hypothesis that any of the markers we tested (ie, p97, TYR, and MelanA/MART1) indicate specific biologically aggressive phenotypes, especially considering that their behavior follows a similar pattern within unadjusted and adjusted analyses. We cannot exclude the possibility that if both the number of positive markers and the probability of developing distant metastases were a function of the number of CMCs, the number of positive markers could be a potentially useful prognostic factor. The linear trend we found when analyzing the risk of progression for patients with positive markers (0, 1, 2, and 3; see Table 3; Fig 3) is consistent with this hypothesis, although this association seems to be weaker than that found in some studies that have a smaller number of patients.[36-37]

In conclusion, this study indicates that detection of melanoma-associated mRNA in peripheral blood of melanoma patients at the time of diagnosis by RT-PCR does not add precision to the predictive power of stage of disease. Although it seems reasonable to wait for more mature and definitive results by assessing overall survival in larger series of patients using the same standardized assays and the most specific mRNA markers, such studies should be limited to clinical trials that can help define the prognostic value of RT-PCR detection of CMCs, and they should not be used in clinical practice or affect treatment decision making.

APPENDIX

The appendix is available online at www.jco.org.

REFERENCES

1. Schuchter LM: Melanoma and other skin neoplasms. Curr Opin Oncol 9:175-177, 1997
2. Berwick M, Halpern AH: Melanoma epidemiology. Curr Opin Oncol 9:178-182, 1997
3. Balch CM, Soong SJ, Gershenwald JE, et al: Prognostic factors analysis of 17,600 melanoma patients: Validation of the American Joint Committee on Cancer Melanoma Staging System. J Clin Oncol 19:3622-3634, 2001
4. Clark WH Jr, Elder DE, Guerry D IV, et al: A study of tumor progression: The precursor lesions of superficial spreading and nodular melanoma. Hum Pathol 15:1147-1165, 1984
5. Breslow A: Thickness, cross-sectional area and depth of invasion in the prognosis of cutaneous melanoma. Ann Surg 172:902-908, 1970
6. Balch CM, Buzaid AC, Soong SJ, et al: Final version of the American Joint Committee on Cancer Staging System for Cutaneous Melanoma. J Clin Oncol 19:3635-3648, 2001
7. Smith B, Selby P, Southgate J, et al: Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. Lancet 338:1227-1229, 1991
8. Brossart P, Keilholz U, Willhauck M, et al: Hematogenous spread of malignant melanoma cells in different stages of disease. J Invest Dermatol 101:887-889, 1993
9. Brossart P, Keilholz U, Scheibenbogen C, et al: Detection of residual tumor cells in patients with malignant melanoma responding to immunotherapy. J Immunother 15:38-41, 1994
10. Brossart P, Schmier J, Kruger S, et al: A PCR-based semiquantitative assessment of malignant melanoma cells in peripheral blood. Cancer Res 55:4065-4068, 1995
11. Foss AJ, Guille MJ, Occleston NL, et al: The detection of melanoma cells in peripheral blood by reverse transcription-polymerase chain reaction. Br J Cancer 72:155-159, 1995
12. Battayani Z, Grob J, Xerri R, et al: PCR detection of circulating melanocytes as a prognostic marker in patients with melanoma. Arch Dermatol 131:443-447, 1995
13. Sarantou T, Chi DDJ, Garrison DA, et al: Melanoma-associated antigens as messenger RNA detection markers for melanoma. Cancer Res 57:1371-1376, 1997
14. Brichard V, Van Pel A, Wolfel T, et al: The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J Exp Med 178:489-495, 1993
15. Kunter U, Buer J, Probst M, et al: Peripheral blood tyrosinase messenger RNA detection and survival in malignant melanoma. J Natl Cancer Inst 88:590-594, 1996
16. Stevens GL, Scheer WD, Levine EA: Detection of tyrosinase mRNA from the blood of melanoma patients. Cancer Epidemiol Biomarkers Prev 5:293-296, 1996
17. Pittman K, Burchill S, Smith B, et al: Reverse transcriptase-polymerase chain reaction for expression of tyrosinase to identify malignant melanoma cells in peripheral blood. Ann Oncol 7:297-301, 1996
18. Jung FA, Buzaid AC, Ross MI, et al: Evaluation of tyrosinase mRNA as a tumor marker in the blood of melanoma patients. J Clin Oncol 15:2826-2831, 1997
19. Glaser R, Rass K, Seiter S, et al: Detection of circulating melanoma cells by specific amplification of tyrosinase complementary DNA is not a reliable tumor marker in melanoma patients: A clinical two-center study. J Clin Oncol 15:2818-2825, 1997
20. Reinhold U, Ludtke Handjery HC, Schnautz S, et al: The analysis of tyrosinase specific mRNA in blood samples of melanoma patients by RT-PCR is not a useful test for metastatic tumor progression. J Invest Dermatol 108:166-169, 1997
21. Aubin F, Chtourou M, Teyssier JR, et al: The detection of tyrosinase mRNA in the peripheral blood of stage I melanoma patients is not of clinical relevance in predicting metastasis risk and survival. Melanoma Res 10:113-118, 2000
22. Hoon DSB, Wang Y, Dale PS, et al: Detection of occult melanoma cells in blood with a multiple-marker polymerase chain reaction assay. J Clin Oncol 13:2109-2116, 1995
23. Keilholz U: Diagnostic PCR in melanoma, methods and quality assurance. Epalinges, Switzerland, 26/27 January 1996. Eur J Cancer 32A:1661-1663, 1996
24. Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156, 1987
25. Maniatis T, Fritsch EF, Sambrook J (eds): Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989
26. Kaplan E, Meier P: Non-parametric estimation from incomplete observations. J Am Stat Assoc 53:457-460, 1958
27. Palmieri G, Strazzullo M, Ascierto PA, et al: Polymerase chain reaction-based detection of circulating melanoma cells as an effective marker of tumor progression. J Clin Oncol 17:304-311, 1999
28. Lin DY, Wei LJ: The robust inference for the Cox proportional hazards model. J Am Stat Assoc 84:1074-1078, 1989
29. Palmieri G, Ascierto PA, Cossu A, et al: Detection of occult melanoma cells in paraffin-embedded histologically negative sentinel lymph

nodes using a riverse transcriptase polymerase chain reaction assay. J Clin Oncol 19:1437-1443, 2001

30. Mellado B, Colomer D, Castel T, et al: Detection of circulating neoplastic cells by reverse-transcriptase polymerase chain reaction in malignant melanoma: association with clinical stage and prognosis. J Clin Oncol 14:2091-2097, 1996

31. Curry BJ, Myers K, Hersey P: Polymerase chain reaction detection of melanoma cells in the circulation: relation to clinical stage, surgical treatment, and recurrence from melanoma. J Clin Oncol 16:1760-1769, 1998

32. Curry BJ, Myers K, Hersey P: MART-1 is expressed less frequently on circulating melanoma cells in patients who develop distant compared with locoregional metastases. J Clin Oncol 17:2562-2571, 1999

33. Hanekom GS, Stubbings HM, Johnson CA, et al: The detection of circulating melanoma cells correlates with tumor thickness and ulceration but is not predictive of metastasis for patients with primary melanoma. Melanoma Res 9:465-473, 1999

34. Mellado B, Gutierrez L, Castel T, et al: Prognostic significance of the detection of circulating malignant cells by reverse transcriptase-polymerase chain reaction in long-term clinically disease-free melanoma patients. Clin Canc Res 5:1843-1848, 1999

35. Schittek B, Bodingbauer Y, Ellwanger U, et al: Amplification of MelanA messenger RNA in addition to tyrosinase increases sensitivity of melanoma cell detection in peripheral blood and is associated with the clinical stage and prognosis of malignant melanoma. Br J Dermatol 141:30-36, 1999

36. Hoon DSB, Bostick P, Kuo C, et al: Molecular markers in blood as surrogate prognostic indicators of melanoma recurrence. Cancer Res 60: 2253-2257, 2000

37. Schrader AJ, Probst-Kepper M, Grosse J, et al: Molecular and prognostic classification of advanced melanoma: A multi-marker microcontamination assay of peripheral blood stem cells. Melanoma Res 10:355-362, 2000

38. Brownbridge GG, Gold J, Edward M, et al: Evaluation of the use of tyrosinase-specific and melanA/MART-1-specific reverse transcriptase-coupled-polymerase chain reaction to detect melanoma cells in peripheral blood samples from 299 patients with malignant melanoma. Br J Dermatol 144:279-287, 2001

39. Waldmann V, Wacker J, Deichmann M, et al: Prognosis of metastatic melanoma: No correlation of tyrosinase mRNA in bone marrow and survival time. Recent Result Cancer Res 158:118-125, 2001

40. Strohal R, Mosser R, Kittler H, et al: MART-1/Melan-A and tyrosinase transcripts in peripheral blood of melanoma patients: PCR analyses and follow-up testing in relation to clinical stage and disease progression. Melanoma Res 11:543-548, 2001

41. Palmieri G, Ascierto PA, Satriano SMR, et al: Circulating melanoma-associated markers detected by RT-PCR in patients with classic Kaposi's sarcoma. Ann Oncol 11:635-636, 2000

42. Ghossein RA, Bhattacharya S, Coit DG: Reverse transcriptase polymerase chain reaction (RT-PCR) detection of melanoma-related transcripts in the peripheral blood and bone marrow of patients with malignant melanoma. What have we learned? Recent Results Cancer Res 158:63-77, 2001