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Molecular Markers in Blood as Surrogate Prognostic Indicators of Melanoma Recurrence[1]

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ABSTRACT

Improvement is needed in the ability to evaluate the prognosis of melanoma patients who are clinically disease-free but likely to develop recurrent metastatic disease. The detection of circulating melanoma cells in blood is a potential surrogate marker of subclinical residual disease. We assessed the prognostic clinical utility of a multimarker melanoma reverse transcriptase-PCR (RT-PCR) assay using blood of 46 patients who were clinically disease-free. All patients were followed up for more than 4 years for disease recurrence. There was a significant correlation between number of RT-PCR markers present in blood and American Joint Committee on Cancer stage ($P = 0.009$). The number of RT-PCR markers detected in blood was an independent prediction factor of disease recurrence in a Cox proportional hazard model ($P = 0.02$). A risk factor model using American Joint Committee on Cancer stage and number of positive RT-PCR markers significantly predicted disease recurrence in 2, 3, and 4 years of follow-up. These studies demonstrate that molecular detection of circulating melanoma cells may be of significant prognostic value in determining early disease recurrence and may be useful for stratifying patients for adjuvant therapy.

INTRODUCTION

The metastasis of melanoma from the primary to regional draining lymph nodes and distant sites correlate often with a very poor prognosis, whereby 5-year survival in these patients is <40%. Surgical treatment of metastatic melanoma is a therapeutic intervention for resectable disease and can improve survival. Although advanced-stage melanoma patients can be made clinically disease-free through surgery, they have a high risk of disease recurrence (1, 2). In recent years, there has been evidence of improved prognosis in advanced-stage melanoma patients after active-specific immunotherapy, cytokine therapy, or bio-chemotherapy (3–6). Recurrence of metastatic disease usually is associated with a very poor prognosis. The detection of systemic "subclinical" disease in clinically disease-free patients would improve patient management and stratification in clinical trials of various adjuvant therapies. Development of better prognostic tests for disease recurrence in patients who have gone through a first line of treatment (surgery) could provide rationale for specific adjuvant treatments. One reason for designing a prognostic molecular assay would be to assess minimal residual disease (subclinical) present in the blood that is undetectable by conventional approaches. The presence of circulating melanoma cells in blood could be a surrogate marker for systemic metastasis.

The detection of metastatic melanoma cells in blood by RT-PCR[3] has been studied by several laboratories (7–14). We first reported the use of a multimarker RT-PCR assay to detect metastatic melanoma cells in blood in 1995 (7). The rationale was based on several known facts of metastatic melanoma. Melanomas are highly heterogeneous for tumor-related protein expression. Individual melanoma cells within a tumor and among different patients' tumors express various levels of tumor marker mRNA transcripts. The heterogeneity of marker expression in these melanoma cells limits the reliability of a single-marker detection assay. In a previous study, blood obtained from 119 melanoma patients with different AJCC stages of disease were assessed using a multimarker RT-PCR assay based on four melanoma mRNA markers: tyrosinase, MAGE-3, p97, and MUC-18 (7). Tyrosinase is an enzyme that plays a role in the initial stages of the melanogenesis pathway and has been well documented in RT-PCR detection studies for metastatic melanoma (7–12). MAGE-3 and p97 are melanoma-associated antigens (7, 15). MUC-18, an adhesion-related glycoprotein found on melanomas, is associated with tumor progression (16). The study demonstrated that the frequency of RT-PCR marker expression and the number of positive markers in blood samples were higher in patients with more advanced stage disease. Using the same RT-PCR markers, these results have recently been further validated in a larger cohort of melanoma patients ($n = 235$ patients) by a European cooperative group (13). Evidence is accumulating that a multiple mRNA marker assay provides a more reliable and informative approach than a single-marker tyrosinase assay for the detection of metastatic melanoma in blood.

Previous studies by our laboratory and others have demonstrated the presence of RT-PCR markers in blood of patients who have no clinical evidence of cancer after disease removal. The clinical relevance of these findings has not been well understood to date. This "systemic subclinical disease" may have the potential to establish distant metastasis. However, the clinical significance of these findings is unknown until appropriate clinical follow-up is carried out. The probability of melanoma progression (disease recurrence and overall survival) in a patient can be predicted to some level in the long-term by established prognostic factors for early and advanced stages of disease based on analysis from melanoma databases (1, 2).

In this study, we assessed the clinical utility of the multimarker RT-PCR assay on blood of melanoma patients who had no evidence of clinically detectable disease and followed up for >4 years. The purpose of the study was to determine whether the multimarker RT-PCR assay could detect subclinical metastatic melanoma in the blood and to evaluate its clinical utility as an independent predictor of early disease recurrence. This study demonstrates that detection of multiple RT-PCR markers can be used as an independent prognostic factor for disease recurrence. A risk factor model was developed with disease stage and number of RT-PCR positive markers to predict early disease recurrence.

MATERIALS AND METHODS

Patients. All melanoma patients entered in the study ($n = 119$; Ref. 7) were from the John Wayne Cancer Institute and had complete documented

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

physical and medical histories at the time of blood draw and during follow-up. All patients had signed an informed consent for a Saint John's Health Center and John Wayne Cancer Institute Institutional Review Board-approved protocol. Clinical disease status was staged at the time of blood draw according to the recent AJCC guidelines (1998). Patients accrued in the study were entered independently of disease status. At the time of the blood draw, clinical status was established by physical and routine examination. All patients were assessed by multimarker RT-PCR assay. Patients who had clinically detectable disease at the time of blood procurement as determined by a later review were not included for further analysis. This report is on patients entered in the study ($n = 46$) who had no evidence of clinically detectable disease at the time of blood draw and who could be followed up at regular postoperative intervals. Recurrence of melanoma or death due to melanoma was considered as disease progression. Recurrence of disease was determined by routine physical examination and conventional imaging techniques during follow-up. One patient in the age category <60 expired without disease recurrence. The study was conducted in a double-blind fashion. The correlative study was performed in a retrospective manner in that the RT-PCR studies were prospectively performed >4 years ago, whereas patient follow-up analysis was more recently determined. The initial study on the correlation of melanoma RT-PCR markers relative to AJCC stage and presence of disease was previously reported (7). In general, AJCC stage II patients were not enrolled in any treatment protocol. AJCC stage III/IV patients received adjuvant melanoma cell vaccine or bio-/chemo-therapy (after disease recurrence) during follow-up. There was no significant pattern of treatment regimen to disease recurrence or skewing of patients with known positive markers to a specific treatment regimen.

Blood Preparation and RT-PCR. Blood was collected and processed, and RNA was extracted as previously described (7). Negative and positive controls were performed for RT-PCR, ethidium bromide gel electrophoresis, and Southern blot analysis. β-Actin gene mRNA analysis was assessed for all specimens for mRNA quality control. Three micrograms of RNA was used in all RT-PCR assays. Blood specimens obtained from normal healthy donors were used as negative controls for the markers. Established melanoma cell lines were used as positive controls for individual markers as previously described (7, 17). A positive result, verified by at least two individuals, was considered only when the RT-PCR Southern blot results are positive.

Oligonucleotide and Probes. Primers and PCR conditions used for the four melanoma-associated markers MAGE-3, MUC-18, p97, and tyrosinase were previously described (7). All procedures were optimized: reagents, primers, PCR conditions, and Southern blot conditions. cDNA probes for Southern blotting were prepared as previously described (7). The specificity of the markers in blood from normal donors ($n = 39$) and in cell lines ($n = 10$) was previously demonstrated (7). MUC-18, as previously described, can be positive in some volunteer donors (approximately 5%) under the assay conditions used. However, in these individuals it has not been "truly" determined if they have cancer or if expression is related to other clinical manifestation.

Statistical Analysis. The Spearmen correlation coefficient was used to assess the association between number of positive markers, age, Clark level, Breslow thickness, and AJCC stage of disease. The correlation between the number of markers expressed to gender and site of the primary were tested using the Wilcoxon rank sum test and Krushal-Wallis test, respectively. The Log rank test was used to assess the number of positive markers as a single prognostic factor for recurrence (18). Disease stage, gender, age, and number of positive markers were tested together using the Cox proportional hazard regression model (19). Log rank test was used to assess the differences among the low, medium, and high-risk groups on recurrences during years 1, 2, 3, and 4, respectively. A 0.05 two-sided significance level was used for determination of statistical significance.

RESULTS

Multimarker Expression. In the initial study of the 119 melanoma patients of different AJCC stages, assessment of blood was performed by multimarker RT-PCR using tyrosinase, p97, MUC-18, and MAGE-3 markers. Analysis of the number of RT-PCR markers detected in blood showed a significant ($P < 0.03$) correlation between the number of markers and disease stage. In patients with AJCC stages III and IV alive with disease, there was a significant probability of bloods being three to four markers positive. This observation supported the rationale for using multimarker expression as a criteria of positive blood specimens. In the present study, our hypothesis was that a multimarker (three or four markers) positive blood specimen would be predictive of early disease recurrence. Our laboratory standardization of data interpretation has been that a multimarker positive sample is more reliable than a single-marker assay, and that it is a more "confident" confirmation of the probability of the presence of tumor cell(s) compared with a single-marker assay.

Patient expression of individual markers and combinations of markers is shown in Table 1. The most commonly expressed marker was MUC-18, followed in decreasing order by tyrosinase, p97, and MAGE-3. In these patients, there were 3 (6.5%), 13 (28%), 13 (28%), 17 (37%) and 1 (2%) patients with zero, one, two, three, and four positive markers, respectively (Table 1). Only three patients expressed no markers. Patient demographic characteristics are shown in Table 2. The majority of the patients were AJCC stage III and had lesions at extremity sites that were Clark level IV with a Breslow 1.51- to 4.00-mm thickness. The analysis of number of positive markers in blood showed a significant ($P = 0.009$) correlation to AJCC stage. There was no correlation between the number of positive markers and age, Clark level, Breslow thickness, site, or gender.

Marker Correlation with Disease Recurrence. To determine the clinical utility of the assay to predict disease recurrence, we assessed patients ($n = 46$) who were clinically disease-free at the time of the blood draw by conventional physical examination and imaging. Data analysis was carried out by subdividing the number of markers into different categories such as zero *versus* one *versus* two *versus* three *versus* four, zero and one *versus* two to four, or zero to two *versus* three and four positive markers for subsequent analysis. The rate of disease recurrence was significantly ($P = 0.02$) lower among patients with zero to two positive markers *versus* those that were three to four markers positive after 4 years of follow-up (Fig. 1). There were recurrences in 7 of 28 (25%) patients having zero to two positive markers *versus* 10 of 18 (56%) patients having three to four positive markers at their blood draw (Table 3). In assessing patients who were zero to one marker positive *versus* two to four markers positive for disease recurrence during a 4-year follow-up, there was a continual difference in the groups; however, this did not reach significance (data not shown). The disease recurrence curves for the zero to one *versus* the two to four marker positive combination during the 4 years of follow-up were separate, particularly at years 1 and 2. There were 27% (4 of 15) recurrences in patients having zero to one marker positive. In comparison, 42% (13 of 31) of patients with two to four markers positive recurred.

Clinical Correlation to Individual Markers. Individual markers were assessed on the 46 patients to determine whether there was any

Table 1 *Expression of mRNA markers in blood*

mRNA markers[a]	Patients expressing
Tyrosinase	24
MAGE-3	10
MUC-18	41
p97	19
Combination of marker expression	
Tyrosinase alone	0
MUC-18 alone	12
MAGE-3 alone	0
p97 alone	1
Tyrosinase, MUC-18	4
Tyrosinase, MUC-18, p97	8
Tyrosinase, p97	2
Tyrosinase, MUC-18, MAGE-3	9
Tyrosinase, MUC-18, MAGE-3, p97	1
MUC-18, p97	7

[a] RT-PCR markers in patients' ($n = 46$) blood was determined as described in "Materials and Methods."

Table 2 *Patient characteristics*

Prognostic factor	Recurrence	Death	Total number
Gender			
Male	11	8	29
Female	6	4	17
Age			
<60	8	9	26
≥60	9	3	20
Breslow depth (primary) mm			
≤0.75	1	1	3
0.76–1.50	6	4	11
1.51–4.00	7	5	17
>4.00	2	1	9
Unknown[a]	1	1	6
Clark level			
II	0	0	2
III	5	3	11
IV	8	6	20
V	3	2	5
Unknown	1	1	8
AJCC stage			
I	0	0	0
II	1	1	7
III	9	6	29
IV	7	5	10
Site			
Extremity	8	5	18
Head/neck	5	2	10
Trunk	4	4	16
Unknown	0	1	2

[a] Unknown Breslow thickness and Clark level.

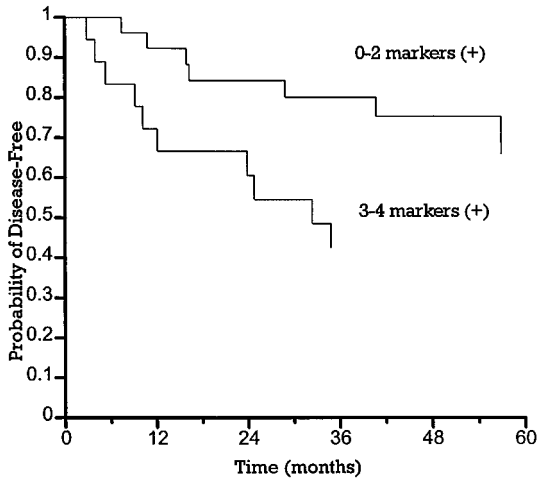

Fig. 1. Use of number of positive markers as a single prognostic factor for disease recurrence in blood of melanoma patients. Correlation of number of positive markers to disease recurrence. Patients' blood with zero to two markers and three to four markers were significantly different by Log rank test ($P = 0.024$).

correlation to clinical characteristics. There was no significant correlation between specific markers and gender, age, Clark level, Breslow thickness, or primary site. However, there was significant correlation (χ^2 test) with tyrosinase ($P = 0.018$) and MAGE-3 ($P = 0.033$) to AJCC stage. The markers MUC-18 and p97 did not correlate with AJCC stage. There was significant correlation between tyrosinase and MAGE-3 expression ($P = 0.003$; Kendall's Tau test). There was no significant correlation of individual markers to recurrence or survival.

Predictive Model for Recurrence. The median follow-up times were 43.2 months for all 46 patients and 51 months for disease-free patients. There were 44, 42, 40, and 37 patients followed at least 1, 2, 3, and 4 years, respectively (Table 4). If patients were stratified into low-risk (AJCC stage I-II, zero to two markers), high-risk (AJCC stage III-IV, three to four markers), and medium-risk (AJCC stage I-II, three to four markers; or AJCC stage III-IV, zero to two markers) categories, there was a significant pattern in disease recurrence (Table 4). The association of risk level and disease recurrence was investigated at years 1, 2, 3, and 4, respectively, using the Log rank test. Overall, there was a significant pattern of disease recurrence in all categories in years 2, 3, and 4 of clinical follow-up. There were no recurrences (zero of five) in the low-risk group, whereas in the medium- and high-risk groups there was 32% (8 of 25) and 66.2% (9 of 16) recurrence, respectively. There were significant ($P = 0.037$) differences in recurrence among the individual risk stratification groups (Fig. 2).

Several standard prognostic factors have been used to predict disease recurrence and survival in patients with malignant melanoma (1–3). Multiple markers were assessed in relation to other known major prognostic factors for predicting disease recurrence. AJCC stage, gender, age, and number of positive markers were assessed together using the Cox proportional hazard regression model. Using this model, there was significant correlation ($P = 0.022$) only between the number of positive markers and disease recurrence (Table 5). For patients with the same disease stage, age, and gender, the hazard rate for patients three to four markers positive compared with patients with zero to two markers was 3.4 times higher (95% confidence interval, 1.19–9.90).

Marker Correlation with Survival. Correlation between the number of markers positive with survival analysis was performed on the 46 patients during follow-up. Overall, 5 of 28 (18%) patients with zero to two markers positive died, whereas 7 of 18 (39%) patients with three to four markers died. Patients were categorized by AJCC stage and number of markers positive. Those patients who were zero to two markers positive with stage II, III, or IV disease had a 0% (0 of 5), 24% (5 of 21), and 0% (0 of 2) death rate, respectively. In patients who were three to four markers positive with stage II, III, or IV disease, there was a 50% (one of two), 12.5% (one of eight), and 62.5% (five of eight) death rate, respectively. A Cox proportional hazard regression model (gender, age, stage, and number of markers positive) was developed to assess the significance of the number of positive markers to death. There was a significant trend correlating an increase ($P = 0.068$; risk ratio of 2.99) in positive markers to death. The power of analysis was limited by the number of patients evaluated.

Table 3 *Patient RT-PCR markers' distribution and characteristics*

RT-PCR marker (+)[a]	Recurrence	Death	Total number
0	0	0	3
1	4	3	12
2	3	2	13
3	10	7	17
4	0	0	1

[a] Study analysis based on 46 patients' blood.

Table 4 *Risk factor model for recurrence*

Risk group	Total patients[a]	Year 1 recurrence	Year 2 recurrence	Year 3 recurrence	Year 4 recurrence
Low	5	0	0	0	0
Medium	25	2	4	6	7
High	16	5	7	9	9
Total	46	7	11	15	16
Log rank test: *P* value		0.081	0.048	0.022	0.034

[a] Patients were classified as with low, medium, or high risk for disease recurrence according to their disease stage and number of positive markers: high risk, if patients had stage III/IV with three to four positive markers; low risk, if they had stage I/II with zero to two positive markers; and medium risk, if they had stage I/II with three to four markers and stage III/IV with zero to two markers. For year 1, recurrence occurring after 1 year was considered disease-free, and follow-up time longer than 1 year was counted as 1 year. The same approach was applied to all other years' recurrence.

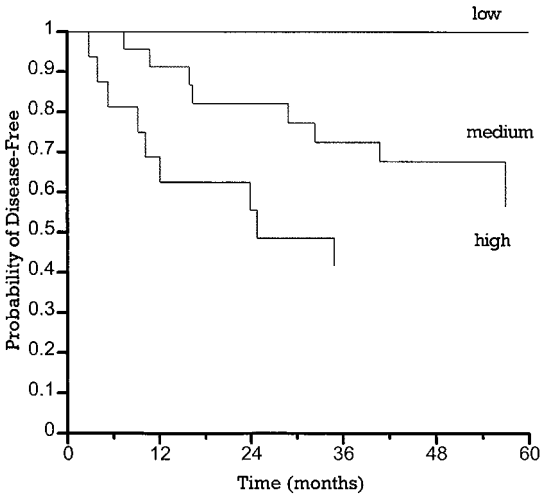

Fig. 2. Stratification of disease risk groups for recurrence. Patients were divided into risk groups based on AJCC stage and number of RT-PCR markers in blood: low risk, AJCC stage I/II and zero to two positive markers; high risk, AJCC III/IV and three to four positive markers; and medium risk, AJCC stage I/II and three to four positive markers or AJCC stage III/IV and zero to two positive markers. There was significant difference between the stratification groups (Log rank test, $P = 0.037$).

DISCUSSION

There are multiple studies documenting the utilization of RT-PCR to detect circulating tumor cells in blood of melanoma patients. Previously, we reported the utility of a multimarker assay for detection of melanoma cells. The use of the multiple markers is based on melanoma cell heterogeneity, tumor antigen heterogeneity, and presence and expression levels of individual marker gene transcripts. Therefore, a multimarker assay would be more sensitive, reliable, and clinically useful than a single-marker assay. To date, there are no major studies examining the clinical utility of molecular detection of occult disease in blood of clinically disease-free melanoma patients with long-term follow-up. The detection of subclinical disease spreading in blood and its prognostic implications are important applications of RT-PCR analysis. To carry out this type of study, rigorous routine follow-up of patients is needed. In this study, we provide evidence that detection of minimal residual disease using the multimarker assay is of clinical utility as an independent prognostic factor to predict disease recurrence.

The number of positive markers in the blood of patients correlated with AJCC stage. This finding was consistent with our hypothesis that more advanced disease would have more tumor cells with greater genetic instability in the blood and therefore a higher probability of expressing multiple melanoma mRNA markers. There are multiple single-marker studies reported for detecting tumor cells in blood; however, the reliability and sensitivity of many single-markers are limited (14, 20). The majority of RT-PCR assays reported involving melanoma patients blood has been based on a single-marker assay using tyrosinase (10, 12, 14, 21). The limitation of tyrosinase mRNA alone as a marker is that its mRNA expression levels can vary considerably from different tumor biopsies of an individual patient and among patients. It is known that amelanotic melanomas are generally more aggressive than highly melanotic tumors (2). Amelanotic tumors are known to have less tyrosinase mRNA transcripts (17). In single-marker assessment of blood by RT-PCR, the dilution factor of a few melanoma cells among millions of leukocytes in blood would reduce the final signal. This dilution factor would be significantly enhanced if the marker mRNA copy level is low. This problem would be further compounded using markers in which the gene expression is variable among tumor cells and is influenced by various host epigenetic factors (22). Our study supports findings by other investigators that tyrosinase as a single RT-PCR marker in blood analysis has limited clinical utility (14, 23, 24). Studies have suggested that detection of melanoma cells in blood using tyrosinase mRNA is not accurately indicative of disease status or stage but may be of value as a predictive progression marker (25). Tyrosinase mRNA alone in our assay did not correlate with disease recurrence or survival. Our study suggests that tyrosinase mRNA is an important RT-PCR marker in blood for increased risk of disease recurrence only when in combination with other melanoma markers. However, one must acknowledge that metastasis is a cascade of events that involves intricate host interactions and inherited properties of the tumor cells. The overall process of melanoma metastasis is inefficient because there are a multitude of factors such as tumor genetic instability, host factors, and host immunity that can play a significant role in determining the success of a tumor cell(s) establishing a metastasis (26–30).

We demonstrated that the number of RT-PCR markers was a significant independent variable for predicting disease recurrence. None of the known prognostic factors, such as Breslow thickness, Clarks level, age, gender, or site, were significant in predicting disease recurrence in the small number of patients studied. A risk factor model was developed using AJCC stage and the number of positive markers. In this risk factor model, a low risk and high risk could be well identified. To our knowledge, this is the first study indicating that a molecular variable as an independent prognostic factor can be used to predict melanoma recurrence. There was significant correlation of prediction of disease recurrence in years 2, 3, and 4 of clinical follow-up using this risk stratification model. One of the major challenges to an oncologist is to be able to determine which patients who are disease-free are at increased risk of recurrence within a couple of years. These high-risk patients would benefit from aggressive treatment and follow-up for clinically detectable disease. In a recent study, we demonstrated the increased sensitivity of detecting micrometastasis in melanoma patients sentinel nodes using a multimarker RT-PCR assay (27). The multimarker assay was very sensitive in significantly upstaging melanoma cell detection over immunohistochemistry and correlated with disease recurrence. This study also demonstrated that tyrosinase as a single marker was not as good in detecting micrometastasis as compared with multimarker analysis.

In a previous report by our group, it was observed that melanoma patients ($n = 1512$) who progressed from AJCC stage I/II to stage IV took an average of 33.8 months (26). Patients progressing from AJCC stage I/II to stage III took an average of 9 months. The data in the present study on disease recurrence prediction with AJCC stage and RT-PCR markers in 2, 3, and 4 years correlate with this previous observation. The detection of subclinical disease in blood should help in improving prediction of disease recurrences. Current melanoma prognostic factors are tumor-based or host demographic-based and do not take into consideration ongoing tumor spreading. Development of methods to measure actual ongoing tumor progression and subclinical

Table 5 *Cox proportional hazard model correlation to disease recurrence*

Covariate[a]	Parameter estimate	Standard error	Wald test	Conditional risk ratio
Gender (0 = female; 1 = male)	0.52	0.53	$P = 0.330$	1.68
Age (0 = <60; 1 = ≥60)	−.20	0.52	$P = 0.691$	0.81
AJCC Stage (0 = stages I & II; 1 = stages III & IV)	1.45	1.07	$P = 0.174$	4.28
Marker positive (0 = 0–2; 1 = 3–4)	1.24	0.54	$P = 0.022$	3.44

[a] Cox proportional hazard regression model included gender, age, AJCC stage, and number of markers positive. Current AJCC staging incorporates Breslow thickness of primary, node status, and distant metastasis status.

disease such as circulating tumor cells in blood will help to improve patient management and to identify disease status.

As more information is learned in treatment responses relative to specific clinicopathological parameters, it is inevitable that molecular detection of residual disease will have an important role in treatment stratification. Although the aim of this study was primarily to assess the predictive value of marker positive bloods and disease recurrence, we also assessed overall survival. Long-term follow-up for analysis of survival requires consideration of other established prognostic factors for each particular cancer. Prediction of melanoma patient overall survival has been well documented in multicenter studies regarding the significance of specific prognostic factors (see review in Ref. 1). Using a Cox proportional hazard model in the correlation of number of positive markers to survival, we found a significant trend. Although studies have reported the significance of melanoma cells in blood detected by tyrosinase marker RT-PCR as a prognostic factor to predict survival outcome (21, 31), these analyses are often univariate and lack consideration of known prognostic factors in the prediction of survival. One must be particularly cautious in interpreting the prognostic significance of an assay using an univariate model.

The clinical utility of the multimarker RT-PCR assay can also be of value in monitoring adjuvant therapy of patients clinically free of disease. The use of RT-PCR analysis of blood can be used as surrogate end points to predict therapy response. In the future, this approach may curtail unnecessary long-term treatment schedules and may predict much earlier which treatments are less likely to be effective. As cancer therapy regimens become more complicated using different agents, sequences of treatment combinations, and dosages, it is becoming increasingly more important to develop better surrogate end-point markers. The permutations of treatment regimens can be exhausting; in reality, the number of patients available for specific clinical criteria for these treatments is decreasing because of competition among treatment protocols. The melanoma patient treatment arena is becoming congested with numerous types of therapies. Unfortunately, patients entered into early clinical phase trials that assess only safety or host responses usually have limitations for long-term patient care benefit. Therefore, there is an increasing need for more and informative surrogate markers of early disease progression. Molecular markers in blood can be very informative surrogate end points of ongoing disease progression and stratification factors for adjuvant therapy of solid tumors (32).

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