

# Association between Molecular Detection of *GAGE* and Survival in Patients with Malignant Melanoma: A Retrospective Cohort Study<sup>1</sup>

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## ABSTRACT

We used *GAGE* as a molecular marker to identify melanoma cells with metastatic potential in the peripheral blood and the bone marrow. One hundred thirty-three patients with malignant melanoma (21 clinical stage II, 74 stage III, and 38 stage IV) had a single marrow and/or blood sample drawn immediately prior to surgical resection. Simultaneous bone marrow and blood samples (85 patients), marrow-only samples (35 patients), and blood-only samples (13 patients) were examined for the presence of *GAGE* expression using reverse transcription-PCR. *GAGE* expression was associated with adverse overall patient survival, measured from the time of sampling ( $P = 0.01$ ). When data were stratified for clinical stage, median survival was statistically longer among *GAGE*-negative patients in the stage III cohort only ( $P = 0.01$ ). In a multivariate model, only *GAGE* positivity in blood and/or marrow and clinical stage were significant prognostic variables. It was the detection of *GAGE* in blood but not marrow that was associated with poor survival. The detection of blood *GAGE* by reverse transcription-PCR has significant adverse implications for overall survival of patients with malignant melanoma in this cohort, and it warrants further investigation.

## INTRODUCTION

Molecular markers of MRD<sup>3</sup> may have clinical utility for prognostication, measurement of response to novel adjuvant therapy, and early detection of metastasis or recurrence. Until recently, the methods of measuring MRD were limited. With the availability of powerful molecular tools in signal amplification and the discovery of highly restricted antigens (or cDNA se-

quences), the focus now shifts to narrowing down the list of candidate markers. Issues of sensitivity and specificity of individual markers alone and in combination and, more importantly, their clinical utility require analyses in defined cancer populations. With malignant melanoma, many patients, even after they are rendered clinically free of disease, remain at a high risk of dying from metastasis. Despite the concentration of effort in developing effective immunotherapies and gene therapies for this cancer, their efficacy following surgical resection, although logical, remains difficult to substantiate. Several molecular markers to detect circulating melanoma cells have been developed to identify patients at risk for metastatic disease. These markers include the detection of the melanocytic lineage-specific antigen tyrosinase by RT-PCR (1–4), melanoma antigen recognized by T cells-1 (5), and  $\beta$ -1,4-*N*-acetylgalactosaminyl transferase (6) as well as the use of multiple markers p97, MUC-18, and *MAGE*-3 (7). Similar to *MAGE*-3, *GAGE* belongs to a family of genes that encodes distinct tumor-associated antigenic peptides recognized by autologous CTLs on human melanoma (8). These antigens are expressed in human tumors of diverse histological types but are silent in normal adult tissues, except testis and placenta (9). We have previously demonstrated that *GAGE* can be a sensitive and specific marker of metastatic disease in the bone marrow and peripheral blood among patients with neuroblastoma, a malignancy that is similar to melanoma in its neuroectodermal origin (10). Here, we evaluate the prognostic significance of *GAGE* expression by RT-PCR in the blood and marrow of a cohort of patients with malignant melanoma.

## PATIENTS AND METHODS

### Patients

One hundred thirty-three patients with histologically confirmed malignant melanoma who were treated at Memorial Sloan-Kettering Cancer Center (New York, NY) between 1992 and 1997 were included in this study. Staging evaluation consisted of a complete history and physical examination, blood counts, and a chest X-ray. Imaging studies generally included a head, chest, abdominal, and/or pelvic computed tomography scan, if clinically indicated. Immediately prior to surgery, blood and marrow samples were obtained with informed consent according to Institutional Review Board-approved guidelines. Simultaneous bone marrow and blood samples were obtained from 85 patients, marrow-only samples were obtained from 35 patients, and blood-only samples were obtained from 13 patients. The clinical stage at the time of the marrow and blood collection was defined according to AJCC guidelines (11). Marrows were examined by H&E, and aspirates were examined by Wright-Giemsa staining. All patients underwent wide excision of their primary melanoma (21 AJCC stage II patients), therapeutic lymphadenectomy for gross nodal disease (74 AJCC stage III patients), or resection of distant metastases (38 AJCC stage IV patients). A total of 34 normal volunteers and 14

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<sup>3</sup> The abbreviations used are: MRD, minimal residual disease; RT, reverse transcription; AJCC, American Joint Committee on Cancer.

leukemia patients in remission without clinical melanoma served as controls.

### Sample Preparation and RNA Extraction

Ten to 15 ml of peripheral venous blood and/or bone marrow aspirates were collected immediately before surgery. Whole blood was mixed with an equal volume of PBS and marrow aspirate with 2 volumes of PBS. The samples were then layered over 8 ml of Ficoll (Accurate Chemical and Scientific Corp., Westbury, NY) in a 15-ml tube and then centrifuged at  $200 \times g$  for 30 min at  $4^{\circ}\text{C}$ . The mononuclear layer and one-third of the granulocytic layer were aspirated, rediluted up to 50 ml with PBS, and then centrifuged at  $1800 \times g$  for 20 min at  $4^{\circ}\text{C}$ . After the supernatant was discarded, the pellet was stored at  $-70^{\circ}\text{C}$  or used directly for RNA extraction. After adding 2 ml of RNazol B (Biotech Laboratories, Houston, TX) and 0.2 ml of chloroform to the pellet, the preparation was mixed vigorously and placed on ice for 5 min. The suspension was then centrifuged at  $12,000 \times g$  ( $4^{\circ}\text{C}$ ) for 15 min. The aqueous phase was transferred to a new tube and mixed with an equal volume of isopropanol. The samples were then placed at  $-20^{\circ}\text{C}$  for at least 2 h. This was followed by centrifugation at  $12,000 \times g$  ( $4^{\circ}\text{C}$ ) for 15 min. After the supernatant was discarded, the RNA pellet was washed with 100% ethanol and subsequently centrifuged at  $12,000 \times g$  for 15 min. This washing step was repeated using 75% ethanol. The dry RNA pellet was finally dissolved in 50  $\mu\text{l}$  of diethyl pyrocarbonate-treated water and quantified spectrophotometrically. Samples were coded for the *GAGE* study by investigators with no prior knowledge of patients' clinical status.

### RT-PCR

**RT (cDNA Synthesis).** In a final volume of 20  $\mu\text{l}$ , 1  $\mu\text{g}$  of total RNA was reverse-transcribed in the presence of 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) and oligo(dT)<sub>15</sub> primer (at 2  $\mu\text{M}$  final concentration; Promega, Madison, WI), 1 mM each dNTP (Pharmacia Biotech, Piscataway, NY), 20 units of RNase inhibitor (Promega), and  $1 \times$  RT buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl<sub>2</sub>] as well as 10 mM DTT (Life Technologies, Inc.). The reaction mixture was incubated at  $37^{\circ}\text{C}$  for 60 min and terminated by heating at  $90^{\circ}\text{C}$  for 5 min. Each batch of cDNA synthesis included a melanoma cell line as a positive control.

**PCR.** The oligonucleotides used to detect *GAGE-1* and *GAGE-2* were published previously (8): sense primer, 5'-AGACGCTACGTAGAGCCT-3'; and antisense primer, 5'-CCATCAGGACCATTCTTCA-3'. They recognized *GAGE-1* and *GAGE-2* (GenBank accession nos. U19142 and U19143, respectively) of the *GAGE* family. Both primers were 5' end-labeled with biotin (Integrated DNA Technologies, Coralville, IA). PCR with these *GAGE*-specific primers produced an amplified DNA fragment of 239 bp. The quality of mRNA was verified by PCR using primers specific for a housekeeping gene human  $\beta_2$ -microglobulin with an amplified DNA fragment of 333 bp: sense primer, 5'-CTCGGCTACTCTCTTTCTGG-3'; and antisense primer, 5'-GCTTACATGTCTCGATCCACTTAA-3' (12).

The PCR mixture contained 5  $\mu\text{l}$  of  $10 \times$  PCR buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 15 mM MgCl<sub>2</sub>] from Perkin-Elmer Corp. (Foster City, CA), 0.4  $\mu\text{l}$  of 25 mM dNTP, 0.3  $\mu\text{l}$  of each oligonucleotide primer (20  $\mu\text{M}$ ), 0.2  $\mu\text{l}$  of AmpliTaq DNA polymerase (5 units/ $\mu\text{l}$ ; Perkin-Elmer), and 1/20 of the cDNA synthesis solution as well as nanopure water to achieve a volume of 50  $\mu\text{l}$ . The PCR assay included a positive control (the melanoma cell line SK-MEL-1) and a negative control (H<sub>2</sub>O) to be run in parallel with the patient samples. Both controls were consistent between a total of 48 runs. PCR was performed in an Omnigene Thermal Cycler (Hyaid, Teddington Middlesex, United Kingdom). After the first denaturation step at  $94^{\circ}\text{C}$  for 3 min, 30 cycles of amplification were carried out with denaturation at  $94^{\circ}\text{C}$  for 1 min, annealing at  $55^{\circ}\text{C}$  for 2 min, and extension at  $72^{\circ}\text{C}$  for 3 min. A final extension at  $72^{\circ}\text{C}$  for 8 min completed the PCR. PCR products were electrophoresed on 1.5% agarose gel (Fisher Scientific, Pittsburgh, PA) with BioMarker Low-Biotin DNA molecular weight standards (BioVentures, Murfreesboro, TN) and transferred to Zeta-Probe nylon blotting membrane (Bio-Rad, Hercules, CA) by means of a semi-dry electrophoretic transfer cell from Bio-Rad. DNA was cross-linked to membrane by Stratalinker UV Crosslinker 2400 (Stratagene, La Jolla, CA), and chemiluminescent detection of the membrane-bound DNA was carried out according to the Tropix (Bedford, MA) Southern-light protocol. Identity of select PCR products were confirmed by Southern blot using a digoxigenin-labeled *GAGE* probe, which enhanced specificity but not sensitivity (data not shown). The limit of detection was at least 1 tumor cell in the presence of  $10^6$  normal peripheral blood or marrow mononuclear cells (10).

### Statistical Analysis

Prognostic importance of clinical variables was evaluated by Cox regression using univariate and multivariate analyses. These variables included stage, thickness of lesion, age of patient, and *GAGE*. Clinical stage was analyzed as a discrete variable (stage II, III, and IV). RT-PCR results for *GAGE* were coded as positive if either marrow or blood *GAGE* was positive. However, both blood and marrow samples had to be negative for *GAGE* to be coded negative. There were 85 patients with simultaneous sampling of blood and marrow. Among patients with marrow-only samples (35 patients) or blood-only samples (13 patients), 12 patients were coded *GAGE*-positive because either their marrow or blood *GAGE* was positive. The remaining 36 patients were not evaluable for *GAGE* negativity because either marrow or blood was missing and could be positive. Thus, only 97 (85 plus 12) of 133 patients were included in the univariate and multivariate analyses.

A two-tailed Fisher's exact test was used to assess the association between RT-PCR results and clinical stage. In the overall survival analysis, patients who died were classified as failures, and patients who were still alive at last follow-up were considered censored. Survival from the time of sampling was defined as the interval from the time of blood/marrow sampling for PCR studies to the end point (death or censoring). Patient survival was evaluated by the Kaplan-Meier method and survival comparisons between groups by the log-rank test. Proportional hazards analysis was used to obtain maximum likelihood

Table 1 Patient and control subject characteristics

	No. of patients
Melanoma patients ( <i>n</i> = 133)	
Sex	
Male	54
Female	79
Median age, yr (range)	57 (22–84)
Median follow-up from diagnosis, months	35
Median follow-up from sampling, months	21
Primary site	
Upper extremities	17
Lower extremities	54
Trunk	45
Head and neck	3
Unknown	14
AJCC clinical staging	
Stage II	21
Stage III	74
Stage IV	38
Control subjects, without melanoma ( <i>n</i> = 48)	
Normal volunteer marrow	17
Normal volunteer blood	17
Remission marrow from leukemia patients	14

estimates of relative risk and their 95% confidence intervals in univariate and multivariate analyses and to adjust for potential confounding effects. Parameters significant at the  $P = 0.05$  level in univariate analysis were included in a multiple proportional hazards model.

## RESULTS

**Patient Characteristics.** Among the 133 patients with assessable samples, there were 54 men and 79 women (Table 1). The median age at the time of sample collection was 57 years (range, 22–84 years). There were 21 clinical stage II patients, 74 patients with regional lymph node metastases (clinical stage III), and 38 patients with distant metastases (clinical stage IV). The median follow-up from the time of diagnosis was 35 months (42 months for those who were alive and 27 months for those who died), and the median follow-up from time of sampling was 21 months (28 months for those who were alive and 14 months for those who died). Seventeen marrows from normal volunteers, 14 marrows from leukemia patients in remission, and 17 peripheral blood samples from normal volunteers were used as controls, all with  $\beta_2$ -microglobulin mRNA present. The blood specimen from 1 of these 48 controls was *GAGE* positive.

**Correlation of *GAGE* Detection in Marrow and Blood with Clinical Stage.** Among the 85 patients who had both marrow and blood collected simultaneously prior to surgery, *GAGE* expression in paired samples was 80% concordant ( $P = 0.08$ ; Table 2). Fig. 1 illustrates a representative chemiluminescent film showing *GAGE* expression in eight patients from different clinical stages, including two patients with paired bone marrow and peripheral blood samples. As shown in Table 3, the frequency of *GAGE* positivity in bone marrow was lowest among stage II patients (10%), with twice as many positives in stage III and IV patients (22 and 21%, respectively). In contrast, the frequency of *GAGE* expression in peripheral blood was surprisingly high among stage II patients (29%). When blood and marrow findings were combined (“Patients and Methods”),

Table 2 Concordance in *GAGE* detection between paired bone marrow and peripheral blood samples<sup>a</sup>

Peripheral blood	Bone marrow	
	Positive	Negative
Positive	4	9
Negative	8	64

<sup>a</sup> 85 paired samples with 80% concordance;  $P = 0.08$ .

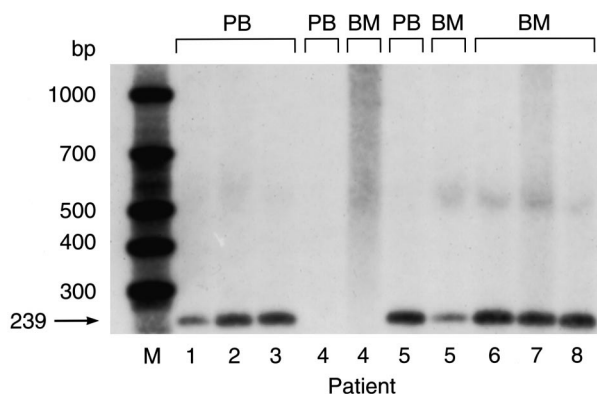


Fig. 1 *GAGE* expression in peripheral blood (PB) and bone marrow (BM) of melanoma patients. RT-PCR was performed on 1  $\mu$ g of total RNA, electrophoresed, transferred to nylon membrane, and detected by chemiluminescence. Lane 1, patient 1, stage IIB; Lanes 2 and 4–8, patients 2 and 4–8, respectively, stage III; Lane 3, patient 3, stage IV; Lane M, biotin-labeled molecular weight marker. Numbers on the left, sizes (in bp). Arrow, diagnostic fragment.

patients with stage II, stage III and stage IV disease had positive percentages of 39, 31, and 37%, respectively, comparable to the positive percentage of 34% among all patients examined. Forty-seven marrows were examined histologically after Wright-Giemsa staining, and no extrinsic cells were detected. Nine of these 47 samples were *GAGE* positive.

***GAGE* Expression and Patient Survival.** Among the 97 patients (18 stage II, 52 stage III, and 27 stage IV) included in the survival analysis, *GAGE* positivity in either bone marrow or peripheral blood was significantly correlated with poor clinical outcome. Median survival from the time of sampling among the *GAGE*-positive cohort was 20.8 months and was significantly shorter than that among patients who were *GAGE* negative ( $P = 0.01$ ; Fig. 2). When data were stratified according to clinical stage, *GAGE* was significant ( $P = 0.01$ ) in its association with survival only among stage III patients (Fig. 3). In a multivariate Cox regression model that also included clinical stage, lesion thickness, and age, only positive *GAGE* (marrow and/or blood) or blood *GAGE* and clinical stage had a statistically adverse influence on patient survival (Table 4).

## DISCUSSION

This report demonstrated the clinical utility of the molecular marker *GAGE* in its association with outcome in a cohort of malignant melanoma patients. When single samples of marrow and/or blood were collected from these patients who had under-

Table 3 GAGE detection according to clinical stage<sup>a</sup>

	Bone marrow		Blood		Bone marrow and/or blood	
	<i>n</i>	No. positive (%)	<i>n</i>	No. positive (%)	<i>n</i>	No. positive (%)
Stage II	21	2 (10)	17	5 (29)	18	7 (39)
Stage III	65	14 (22)	54	4 (7)	52	16 (31)
Stage IV	34	7 (21)	27	4 (15)	27	10 (37)
All stages	120	23 (19)	98	13 (13)	97 <sup>b</sup>	33 (34)

<sup>a</sup> GAGE was scored as described in "Patients and Methods." Patients were staged according to AJCC guidelines.

<sup>b</sup> Total no. of patients included in the survival analysis.

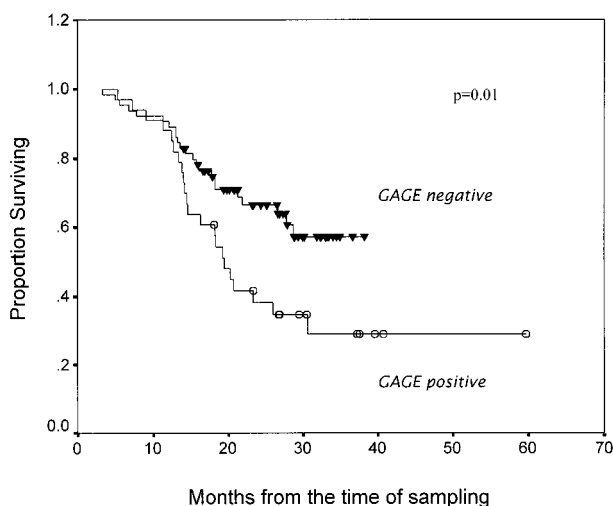


Fig. 2 Kaplan-Meier analysis of survival from the time of sampling according to GAGE (blood and/or marrow) in patients of all stages. ▼, GAGE-negative (64 patients, 41 censored); ○, GAGE-positive (33 patients, 11 censored);  $P = 0.01$ .

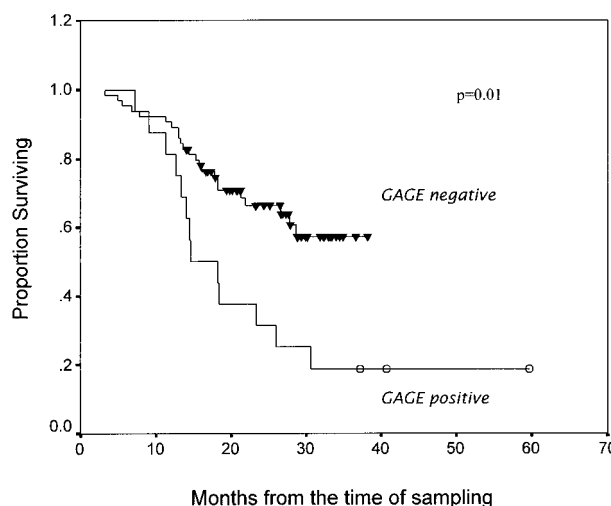


Fig. 3 Kaplan-Meier analysis of survival from the time of sampling according to GAGE (blood and/or marrow) among stage III patients. ▼, GAGE-negative (36 patients, 23 censored); ○, GAGE-positive (16 patients, 3 censored);  $P = 0.01$ .

gone surgery for gross resection, nearly one-third of the patients had detectable GAGE, irrespective of stage, which correlated significantly with patient survival from the time of sampling. In a multivariate model, GAGE and clinical stage were both significant factors in their association with survival after patients were rendered surgically free of disease. When data were stratified by clinical stage, GAGE was correlated with poor survival only among stage III patients. We expect both stage II and IV patients, with a larger sample size, would also reach statistical significance. Our study also differs from most previous reports in the following: our choice of a novel tumor marker, which is generic for many human tumors; the blinding of the sample codes to avoid bias; the simultaneous assay of blood and marrow samples for occult tumor cells; and the use of chemiluminescence to increase sensitivity without the need of nested PCR methods.

With some exceptions, most serological tumor markers measure proteins released by tumor cell turnover and, thus, reflect tumor bulk. They distinguish patients with small locoregional cancer burden from those with widely metastatic disease. Occult tumor cells in blood, marrow, or lymph nodes are signatures of malignancy and reflect a metastatic phenotype. However, they escape detection because of their low numbers. Tumor antigen mRNA is an obvious choice to mark tumor cells

because mRNA is rapidly degraded when released into tissue fluid, usually without leaving misleading footprints, as is often seen with tumor proteins. These signature mRNAs can be reverse-transcribed to cDNA, which can then be exponentially amplified by PCR for detection. Using this technique, numerous markers have been explored. Although most markers of solid tumors in the past were tissue specific or were antigens identified by serological screening, an entirely new class of antigens discovered through screening by specific CTL clones (*e.g.*, GAGE or MAGE) may offer new possibilities. Although tissue-specific markers (*e.g.*, tyrosinase) have been extensively used for following patients with specific solid tumors, generic markers of metastatic disease (*e.g.*, carcinoembryonic antigen; Ref. 13) may have greater potential for general cancer screening in high-risk populations.

A tumor-specific marker like GAGE can be extremely useful (10). In this study, we used 1  $\mu\text{g}$  of RNA in RT and only 1/20 of the cDNA for PCR. It is conceivable that increasing the amount of RNA or cDNA in the RT-PCR will improve the sensitivity of this assay. The sensitivity of chemiluminescence surpassed ethidium bromide visualization by >2000-fold (14). However, false positivity of normal bone marrow and peripheral blood samples can plague ultrasensitive methods. One of the 48

Table 4 Multivariate analysis of prognostic variables on survival from the time of sampling

Analysis	Variable	Relative risk	95% CI <sup>a</sup>	P
1	GAGE <sup>b</sup>	2.2	1.2–4.0	0.01
	Stage III	2.1	1.2–3.8	0.02
	Stage IV	2.3	1.3–4.3	0.01
2	Blood GAGE <sup>c</sup>	1.6	1.1–2.4	0.01
	Stage III	2.0	1.1–3.7	0.03
	Stage IV	2.3	1.2–4.3	0.01

<sup>a</sup> CI, confidence interval.

<sup>b</sup> GAGE in marrow and/or blood.

<sup>c</sup> GAGE in blood.

samples from normal volunteers had detectable GAGE. By increasing the sample size of normal subjects, we should be able to ascertain whether false positivity is a potential concern or whether this particular donor was not a true “normal.” Interestingly, despite the sensitivity of chemiluminescence, only 37% of stage IV patients were positive for GAGE (combined marrow and blood findings), and 15% were positive for blood GAGE. One reason for this low positivity rates could be tumor cell heterogeneity. For tyrosinase, the level of expression varies by >1000-fold among melanoma cell lines (15), whereas some tumors are nonexpressors (16). GAGE expression may be equally variable. A comparison between GAGE and other molecular markers such as tyrosinase transcript (3) in this patient cohort could clarify some of these issues (17). To increase the sensitivity and specificity of detecting circulating tumor, some have proposed serial samplings over time (5, 18) as well as the use of multiple markers (7). After all, tumor cells may not be shedding continuously (19), and not all circulating tumor cells will survive the cell separation procedure. On the other hand, false positivity can result from tumor cell shedding following tumor manipulation or nonclonogenic/dying tumor cells waiting to be scavenged (20).

Because not all circulating tumor cells predict similar biology (20), the clinical significance of marker-positive circulating tumor cells requires an analysis on patient survival. It is possible that, among circulating tumor cells, certain genes may mark those cells with higher malignant potential. Whether the circulating tumor cells detected reflect tumor bulk (tumor shedding) versus tumor biology (metastatic potential) may differ, depending on the specific marker used. Mellado *et al.* (4) found a statistically significant association between tyrosinase blood RT-PCR positivity and clinical stage or recurrence. However, there was no impact on overall survival. In contrast, Ghossein *et al.* (3) reported survival advantage among blood tyrosinase-negative patients, irrespective of stage. Coit *et al.* (21) confirmed this in patients rendered surgically disease free. GAGE positivity has a strong association with poor survival, consistent with the interpretation that blood or marrow GAGE marks circulating cells with a malignant phenotype.

Although marrow sampling is the gold standard for ascertaining marrow disease, there is a significant level of false negativity, as was recently shown for neuroblastoma (22), especially when the extent of disease was minimal. Repeated samplings using invasive procedures such as marrow or tissue

biopsies are not tolerable in most clinical settings. Peripheral blood sampling provides a convenient alternative in tumor surveillance. In melanoma, numerous investigations have focused on circulating tumor cells (2, 4, 5, 7, 19, 23–27). Few have correlated their findings with marker studies on simultaneous marrow samples. The concordance rate of 80% in our study suggests that there is close agreement between marrow and blood sampling. This is comparable to the findings by Ghossein *et al.* (3) in studies of tyrosinase mRNA by RT-PCR and to our previous results of 89% concordance in stage IV neuroblastoma (10). Moreover, in a multivariate model we found that blood GAGE was an independent prognostic variable on survival, whereas marrow GAGE was not. It is possible that marrow GAGE could reach statistical significance if the sample size were larger. An alternative explanation for the superiority of blood GAGE could derive from a fundamental difference in the biology of tumor cells in these two compartments: circulating (blood) versus sequestered (marrow). It is obvious that blood sampling has significantly less morbidity than marrow aspirates or biopsies and is likely to be more conducive to patient compliance. Besides their clinical utility for prognostication, response measurement, and early detection of metastasis or recurrence, molecular markers will likely increase our understanding of tumor control in human cancer. The presence of GAGE in blood could serve as a surrogate end point (rather than survival) to assess the efficacy of systemic adjuvant therapy in patients rendered disease free. In leukemia, rearranged immunoglobulin/T-cell receptor genes (28), or chromosome translocation fusion sequences (29) are useful markers of minimal disease. Many patients in remission have been found recently to carry small numbers of malignant clones (28, 29). This new understanding will demand a reconsideration in our strategy of cancer cure. The goals of therapy may be benign coexistence rather than total eradication, which often requires extreme measures. We believe GAGE can be a useful addition to the panel of molecular markers in monitoring occult melanoma cells, especially among stage III patients who are at high risk for metastasis as well as those undergoing immunotherapy. In addition, it may increase our knowledge base of MRD in patients who are in continual clinical remission.

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