Clinical Cancer Research

Development of a Prognostic Genetic Signature to Predict the Metastatic Risk Associated with Cutaneous Melanoma

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Abstract

Purpose: The development of a genetic signature for the identification of high-risk cutaneous melanoma tumors would provide a valuable prognostic tool with value for stage I and II patients who represent a remarkably heterogeneous group with a 3% to 55% chance of disease progression and death 5 years from diagnosis.

Experimental Design: A prognostic 28-gene signature was identified by analysis of microarray expression data. Primary cutaneous melanoma tumor tissue was evaluated by RT-PCR for expression of the signature, and radial basis machine (RBM) modeling was performed to predict risk of metastasis.

Results: RBM analysis of cutaneous melanoma tumor gene expression reports low risk (class 1) or high risk (class 2) of metastasis. Metastatic risk was predicted with high accuracy in development (ROC = 0.93) and validation (ROC = 0.91) cohorts of primary cutaneous melanoma tumor tissue. Kaplan–Meier analysis indicated that the 5-year disease-free survival (DFS) rates in the development set were 100% and 38% for predicted classes 1 and 2 cases, respectively ($P < 0.0001$). DFS rates for the validation set were 97% and 31% for predicted classes 1 and 2 cases, respectively ($P < 0.0001$). Gene expression profile (GEP), American Joint Committee on Cancer stage, Breslow thickness, ulceration, and age were independent predictors of metastatic risk according to Cox regression analysis.

Conclusions: The GEP signature accurately predicts metastasis risk in a multicenter cohort of primary cutaneous melanoma tumors. Preliminary Cox regression analysis indicates that the signature is an independent predictor of metastasis risk in the cohort presented. *Clin Cancer Res; 21(1); 175–83. ©2015 AACR.*

Introduction

Cutaneous melanoma is an aggressive form of skin cancer with more than 75,000 cases of invasive cutaneous melanoma diagnosed in 2012 (1). Patients with early-stage disease can often be cured by surgical excision alone. Specifically, stage I cutaneous melanoma tumors have a 5-year overall survival rate of 91% to 97% (2). The prognosis is more dismal for late-stage patients (stage IV), whereas it is highly variable for intermediate grade patients.

Melanoma staging is characterized by the American Joint Committee on Cancer (AJCC) TNM (T = primary tumor, N = regional lymph nodes, M = distant metastases) system that defines cutaneous melanoma stage s 0–IV (2). While the majority of clinical stage I patients will be disease-free at 5 years, some stage I patients will develop advanced disease. Furthermore, prognosis for clinical stage II and III cases by TNM is highly variable, as evidenced by a 5-year survival rate of 53% to 82% for stage II patients and a 5-year survival rate of 22% to 68% for stage III patients (2, 3).

While thickness of the tumor and sentinel node positivity are the strongest predictors of metastatic spread, the clinical use of each factor has limitations. Gene expression profile (GEP) signatures have been shown to have powerful prognostic capabilities for many tumors (4–8). For example, in uveal melanoma, the GEP signature can accurately distinguish those patients whose tumor has a low risk of metastasis (95% 5-year metastasis-free survival; MFS) from those who have a high risk (20% 5-year MFS; refs. 4, 9). The signature has been shown to provide a significant improvement in prognostic accuracy compared with classification by TNM staging criteria (9, 10). Other examples have demonstrated that molecular characterization of tumors can improve prognostic accuracy compared with traditional staging systems, such as in

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Translational Relevance

Patients with cutaneous melanoma diagnosed with stage I or II disease are considered to have a low risk of metastatic recurrence. However, up to 20% of those patients will die from this disease within 4 years of the initial diagnosis. Accurate identification of patients with early-stage melanoma who harbor the highest risk of recurrence would allow for enhanced surveillance and earlier therapeutic intervention. In an era when therapeutic options for melanoma are rapidly advancing, this is more critical than ever. We have identified a gene expression profile signature in primary cutaneous melanoma tumor tissue that appears to distinguish patients with low risk of metastatic recurrence from patients with high risk of recurrence. The signature represents a tool that could contribute significant clinical information when considered in combination with current AJCC staging criteria.

large B-cell lymphomas by determining if the lymphoma cells have a genetic signature indicative of a specific B-cell subtype or Her-2neu amplification status in breast cancer cells. Today, the TNM classification of melanoma is used to identify stage III patients as those with relatively aggressive disease, whereas those patients with stage I and II disease are viewed as having low-risk disease (11). High-risk patients with stage I and II disease may benefit from adjuvant therapy and/or enhanced imaging protocols to allow for early detection of metastasis. This is particularly important today, with the recent emergence of several new therapies in melanoma that have, for the first time in over 20 years, been shown to improve survival for stage IV patients. In addition, a personalized, molecular-based prognostic test could decrease surveillance plan intensity and some of the emotional burden and anxiety associated with cancer diagnosis in patients with a low-risk genetic signature.

The current study used published genomic analyses of cutaneous melanoma tumors to develop a unique prognostic genetic signature for metastatic risk (12–19). Genes were selected on the basis of significant genetic expression variation within and across each of the studies compared. A putative signature comprising 28 prognostic genetic targets and 3 control genes was developed from the expression data available, and RT-PCR analysis of more than 260 primary cutaneous melanoma cases was performed. The current study reports that the novel signature can accurately determine metastatic risk profile for cutaneous melanoma tumors and adds value to current AJCC staging methods that can overlook potential for metastasis in low-stage, sentinel node–negative cutaneous melanoma tumors.

Materials and Methods

Sample and clinical data collection

Archived formalin-fixed, paraffin-embedded (FFPE) primary cutaneous melanoma tissue and associated de-identified clinical data were obtained from 6 independent institutions following Institutional Review Board (IRB) approval. Initial inclusion in the study required biopsy confirmed stage I–IV cutaneous melanoma with at least 5 years of follow-up, with the exception that fewer than 5 years was acceptable if there was a well-documented metastatic event. However, for preliminary analysis of the GEP, samples without evidence of metastasis that had less than 5 years of follow-up in clinical data were accepted ($n = 34$; $n = 17$ with <3 years follow-up). In the course of sample recruitment for the study, 15 stage 0 *in situ* melanomas were analyzed and included in the training set, based upon the hypothesis that these cases would have low-risk GEP signatures, and thus provide increased stratification of classes 1 and 2 cases. Primary tumors from cases determined to be stage III and IV at the time of diagnosis were also included in the analysis, based upon the assumption that the GEP predictor would be applied before diagnosis of metastasis in the clinical setting. All cases were originally diagnosed between 1998 and 2009. Patients younger than 18 years or previously diagnosed with another malignant tumor type were excluded.

The starting point for all cases was the initial time of diagnosis. The time for 4 possible endpoints was calculated: (i) time to any type of metastasis or local regional recurrence including involvement of sentinel nodes, in transit metastasis or distant metastasis for disease-free survival (DFS), (ii) time to distant metastasis or distant metastasis-free survival (DMFS), (iii) time to melanoma-specific death (MSS), and (iv) death from any cause [overall survival (OS)]. All cases without evidence of metastatic disease (for DFS or DMFS) or death (for MSD or OS) were censored at the time of last contact.

A hematoxylin and eosin–stained tissue section was requested for each case to perform independent confirmation of (i) the original diagnosis of melanoma and (ii) a dissectible area of tissue with tumor density greater than 60%. Specimens were rejected for study if these 2 parameters were not met. Tissue obtained from either primary biopsy or wide local excision procedures was accepted. Source documentation including clinical record and pathology report review were completed for all cases.

Cutaneous melanoma tumor sample preparation and RNA isolation

FFPE primary cutaneous melanoma tumor specimens arranged in 5-μm sections on microscope slides were used to carry out the study. Tumor tissue was macrodissected from the slide using a sterile disposable scalpel, collected into a microcentrifuge tube, and deparaffinized using xylene. Total RNA was isolated from FFPE melanoma specimens using the Ambion RecoverAll Total Nucleic Acid Isolation Kit (Life Technologies). RNA quantity and quality were assessed using the NanoDrop 1000 system and the Agilent Bioanalyzer 2100.

cDNA generation and RT-PCR analysis

RNA isolated from melanoma samples was converted to cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies). Before performing the RT-PCR assay, each cDNA sample underwent a 14-cycle preamplification step. Preamplified cDNA samples were diluted 20-fold in TE buffer. Fifty microliters of each diluted sample was mixed with 50 μl of 2× TaqMan Gene Expression Master Mix, and the solution was loaded to a custom high-throughput microfluidics gene card containing primers specific for 28-class discriminating gene targets and 3 endogenous control genes. Each sample was run in triplicate. The gene expression assay was performed on an Applied Biosystems HT7900 machine (Life Technologies).

Expression analysis and class assignment

Mean C_t values were calculated for triplicate sample sets. The 3 control genes were selected on the basis of analysis using geNorm.

ΔC_t values were calculated by subtracting the mean C_t of each discriminating gene from the geometric mean of the C_t values of all 3 control genes. ΔC_t values were standardized according to the mean of the expression of all discriminant genes with a scale equivalent to the SD. Radial basis machine (RBM) predictive modeling was performed using JMP Genomics SAS-based software (SAS). RBM is a nonlinear classification based upon the normalized ΔC_t values for each gene of the 28-gene prognostic signature. RBM uses the GLIMMIX procedure in SAS to fit a radial smoothing kernel according to the continuous variable of gene expression. RBM transforms the gene measurements using a kernel function to find an optimal hyperplane in multivariate dimension, thus providing a predicted classification of high- and low-risk tumor biology. Kaplan–Meier curves reflecting DFS, DMFS, metastasis-specific survival, and OS were also generated in JMP Genomics, and statistical significance was calculated according to the log-rank method. Cox univariate and multivariate regression analyses were performed using WinSTAT for Microsoft Excel version 2012.1.

Results

Selection of prognostic gene signature

Several studies reported microarray analysis comparing primary cutaneous melanoma or uveal melanoma to metastatic tissue, along with the resulting genetic expression data, before the initiation of the current study (4, 12–17). We undertook an analysis of the expression data from public databases to identify genes that were similarly up- or downregulated in metastatic tissue across the studies. Our analysis of cutaneous melanoma and uveal melanoma tumors led to the selection of 54-gene targets with notable expression profile differences in primary tumors compared with metastatic tumors. Of the 54 targets of interest, 20 were selected for further RT-PCR analysis based upon genetic loci, with the majority located on chromosomes 1 (*CRABP2*, *TACSTD2*, *CLCA2*, *S100A9*, *SPRR1B*, and *S100A8*), 6 (*GJA1* and *ARG1*), 9 (*TYRP1* and *AQP3*), and 12 (*MGP*, *KRT6B*, and *BTG1*). In addition, analysis of metastatic and nonmetastatic primary cutaneous melanoma tumors using a previously developed and clinically available 15-gene prognostic expression profile assay for uveal melanoma led to the selection of 5 additional gene targets for inclusion in the RT-PCR analysis (unpublished data). The same prognostic gene set study allowed for the selection of 4 genes that exhibited minimal expression level changes in both metastatic and nonmetastatic melanoma tumors that we hypothesized would serve as control genes within a prognostic signature for metastatic risk in cutaneous melanoma. The final 2 targets included in the preliminary prognostic gene set were selected on the basis of previous mutational studies that found a significant correlation between BRCA1-associated protein 1, *BAP1*, and the metastatic potential of uveal melanoma tumors. Given the putative importance of predisposing *BAP1* mutations in cutaneous melanoma tumors and the description of *BAP1* transcript truncation due to single-nucleotide mutations prevalent in uveal melanoma tumors, probes for both the 3′ and 5′ prime ends of the *BAP1* transcript were included in the final gene set (Table 1; ref. 20).

Gene ontology analysis using the DAVID and WebGestalt programs suggested a representation of genes involved in the biologic processes of epithelial differentiation and development, whereas the cellular components represented included cell–cell junction and non–membrane-bound organelle classes (data not

Table 1. Discriminant genes included in the prognostic genetic signature for cutaneous melanoma metastatic risk

Gene symbol	Gene title	Direction of regulation in class 2	*P*[a]
BAP1[b]	BRCA1-associated protein-1	Down	0.007
MGP	Matrix Gla protein	Down	0.486
SPP1	Secreted phosphoprotein 1	Up	6.08 e-16
CXCL14	Chemokine (C-X-C motif) ligand 14	Down	3.31 e-12
CLCA2	Chloride channel accessory 2	Down	1.02 e-08
S100A8	S100 calcium-binding protein A8	Down	0.031
BTG1	B-cell translocation gene 1, antiproliferative	Down	0.024
SAP130	Sin3A-associated protein, 130 kDa	Down	0.024
ARG1	Arginase 1	Down	1.05E-08
KRT6B	Keratin 6B	Up	0.160
GJA1	Gap junction protein, alpha 1, 43 kDa	Down	0.034
ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	Down	3.91 e-06
EIF1B	Eukaryotic translation initiation factor 1B	Up	0.024
S100A9	S100 calcium-binding protein A9	Down	0.012
CRABP2	Cellular retinoic acid binding protein 2	Down	0.0006
KRT14	Keratin 14	Down	1.75 e-05
ROBO1	Roundabout, axon guidance receptor, homolog 1 (Drosophila)	Down	0.0004
RBM23	RNA-binding motif protein 23	Down	0.018
TACSTD2	Tumor-associated calcium signal transducer 2	Down	0.037
DSC1	Desmocollin 1	Down	7.00 e-09
SPRR1B	Small proline-rich protein 1B	Down	0.001
TRIM29	Tripartite motif containing 29	Down	2.34 e-09
AQP3	Aquaporin 3 (Gill blood group)	Down	5.08 e-06
TYRP1	Tyrosinase-related protein 1	Down	2.41 e-06
PPL	Periplakin	Down	5.59 e-11
LTA4H	Leukotriene A4 hydrolase	Down	0.0001
CST6	Cystatin E/M	Down	1.02 e-08

[a]*P* value reflects *t*-test analysis of ΔC_t values from nonmetastatic cases compared with metastatic cases within the 268 sample cohort.
[b]Two assays for BAP1 were included to target both the 5′ and 3′ regions of the gene.

shown). As both processes have been implicated in the transition of localized tumor cells to active, metastatic cells, the expression of the signature was evaluated in a cohort of primary cutaneous melanoma tumors with and without documented metastasis.

Development cohort characteristics and cutaneous melanoma GEP class assignment

The cutaneous melanoma sample set used for initial development of the genetic signature was composed of 107 stage I and II primary melanoma tumor cases from 3 separate institutions within the United States. Twenty cases included in the cohort had documented evidence of metastatic disease and 5 cases had regional recurrence. Statistically significant differences between the nonmetastatic and metastatic groups of samples were observed for median age and Breslow thickness ($P < 0.01$ for each factor but primary tumor location).

Prediction of metastatic risk using RBM modeling results in the classification of melanoma tumors as either class 1 or class 2, with a low or high risk of metastasis, respectively. In the development cohort, 43 of 107 cases were predicted to be class 2. All cases with documented metastatic progression were called class 2 (100% sensitivity), whereas 64 of 82 nonmetastatic cases were called class 1 (78% specificity). The accuracy of the predictive model, as determined by the area under the receiver operating characteristic (ROC) curve, was 0.93, consistent with a clinically relevant predictive model. Further assessment of the model by Kaplan–Meier survival analysis revealed that DFS for the predicted classes was significantly different ($P < 0.0001$), and that median time to metastasis for class 2 cases was 2.5 years, whereas the median time for class 1 cases was not reached. Five-year DFS was 100% for class 1 cases compared with 38% for class 2 cases.

Training set development

Following completion of the development study, sample recruitment was expanded to increase the number of melanoma samples included in the study. When recruitment was stopped in May 2013, 268 stage 0 through IV cases were collected from 7 independent centers (which includes the 107 cases in the development set). Clinical characteristics for the 268 patient population and tumor characteristics are shown in Table 2.

Among the 268 total cases, 30 patients had a positive sentinel lymph node (SLN) biopsy result at the time of the initial diagnosis (18 had subsequent distant metastasis). The presence of a positive SLN was considered a metastasis in the initial data analysis. A total of 102 patients developed metastatic disease whereas 166 had no evidence of metastasis. Characteristics of the patient group with metastasis and the patient group without metastasis are listed in Table 2. Of the 166 patients without evidence of metastasis, source documentation review identified 34 of these patients who had follow-up of less than 5 years. These patients were maintained in the overall analysis, comparable with an intent-to-treat analytical approach, to develop a clinically useful cutaneous melanoma training set. The median time of clinical follow-up for the 166 patients without evidence of metastasis was 7 years (range, 0–14 years; $n = 133$ with >5 years follow-up; $n = 150$ with >3 years follow-up), whereas the median time to metastasis was 1.5 years (range, 0–9 years).

On the basis of the prognostic accuracy of RBM modeling in the analysis of the described 107 cutaneous melanoma sample set, we hypothesized that a training set of cases could be identified and used to predict the metastatic risk for all other independent cases (21). Expression levels of the genes in the prognostic signature were determined for the 268 samples collected to the censor date, and Learning Curve Model Comparison (LCMC) computational modeling was used to predict the optimal size for a clinically applicable training set. According to LCMC, the optimal training set would be composed of 150 to 180 cutaneous melanoma cases (data not shown). A final training set of cases was selected for further development and clinical application of the assay. Aside from 15 *in situ* melanoma cases that were included in the training set, all other samples stratified to the training set were randomly selected. Clinical features of training set cases are shown in Table 2. Of the 164 cases, 67 patients developed metastatic disease.

The final gene signature identified 88 training set cases as class 1 (low metastatic potential) and 76 cases as class 2 (high metastatic potential). RBM analysis revealed an ROC of 0.91 with an overall risk prediction accuracy of 83%. Kaplan–Meier analysis curves demonstrated a significant difference in DFS, with the class 1 group 5-year DFS of 91% and the class 2 group 5-year DFS of 25% ($P < 0.0001$, Fig. 1A). The negative predictive value (NPV) was 89% with a positive predictive value (PPV) of 75%.

Analysis of an independent validation set

Of the 104 cases stratified to the independent validation set, 35 patients developed metastatic disease and 69 did not (Table 2). Median time of follow-up for cases in the validation set that did not have a metastatic event (all stage I and II) was 7.3 years (range, 0.5–11.9). The 28-gene prognostic expression profile for the validation set cases was compared with the training set by RBM and revealed a highly accurate model with ROC of 0.91. According to the model, 61 cases were identified as low-risk class 1, whereas 43 were predicted to be high-risk class 2 (Table 2). Five-year DFS rate was 97% for class 1 and 31% for class 2 cases ($P < 0.0001$; Fig. 1B). NPV and PPV were 93% and 72%, respectively.

When the analysis was focused only on the stage I and II cases in the validation cohort that had either a metastatic event or more than 5 years of follow-up without metastasis ($n = 78$), class 1 5-year DFS rate was 98% compared with class 2 DFS rate of 37% ($P < 0.0001$, Fig. 1C). Median follow-up for cases in this cohort that did not have evidence of metastasis was 7.6 years (range, 5–11.9). The NPV was 94% and the PPV was 67%.

Analysis of DMFS, MSS, and OS for stage I and II cases in the validation set also revealed a significant stratification of high-risk and low-risk cutaneous melanoma cases (Fig. 2). For each survival endpoint, 5-year rates for predicted class 1 cases were 100%. Conversely, 5-year class 2 DMFS rate was 58% (Fig. 2A), MSS rate was 77% (Fig. 2B), and OS rate was 68% (Fig. 2C).

Prognostic accuracy of GEP compared with AJCC T-factors in stage I and II cases

Following validation of the cutaneous melanoma training set, we next began to compare the prognostic accuracy of the GEP signature to the AJCC T-factors of Breslow thickness, ulceration, mitotic rate, and age using Cox regression analysis. Both univariate and multivariate analyses characterized the GEP signature as an independent predictor of metastatic risk compared with AJCC stage and individual T-factors (Table 3). The 28-gene signature

Table 2. Clinical characteristics by metastasis status and class prediction for the development, training, and validation cohorts of patients with cutaneous melanoma

	Training set ($n = 164$)					Validation set ($n = 104$)				
	Nonmetastasis ($n = 97$)	Metastasis ($n = 67$)	Class 1 ($n = 88$)	Class 2 ($n = 76$)	P^a	Nonmetastasis ($n = 69$)	Metastasis ($n = 35$)	Class 1 ($n = 61$)	Class 2 ($n = 43$)	*P*
Follow-up time (y), median (range)	6.8 (0.06–13.7)		7.3 (2.3–13.7)	6.6 (0.1–11.5)		7.3 (0.5–11.9)		7.3 (0.8–11.9)	7.4 (0.5–10.5)	
Time to metastasis (y), median (range)		1.5 (0–7.8)	2.7 (0.6–7.8)	1.3 (0–7.6)			1.4 (0–9.17)	4.1 (0–9.2)	1.4 (0–5.8)	
Age (y), median (range)	57 (23–87)	68 (23–89)	58 (31–86)	64 (23–89)	0.04	55 (18–86)	63 (28–94)	54 (18–82)	66 (28–92)	0.002
AJCC stage					<0.001					<0.001
0	15	0	15	0		0	0	0	0	
I	54	9	57	6		56	0	50	6	
IA	32	5	35	2		38	0	37	1	
IB	21	3	20	4		15	0	10	5	
II	28	39	16	51		13	21	10	24	
IIA	16	15	10	21		7	6	5	8	
IIB	8	17	5	20		6	11	5	12	
IIC	3	7	0	10		0	4	0	4	
III	0	18	0	18		0	12	1	11	
IV	0	1	0	1		0	2	0	2	
Breslow thickness (mm)					<0.001					<0.001
Median (range)	1.0 (0.3–10.4)	2.8 (0.15–16)	0.8 (0–5.2)	2.9 (0.15–16)		0.8 (0.13–7)	3.99 (0.9–10)	0.7 (0.1–7)	3.4 (0.9–10)	
<1 mm	38	6	57	2		43	1	43	1	
1–1.99 mm	19	11	18	12		11	4	7	8	
2–3.99 mm	18	28	10	36		11	12	7	16	
>4 mm	7	20	1	26		4	17	4	17	
Ulceration					<0.001					<0.001
Absent	78	26	73	31		56	9	48	17	
Present	11	35	6	40		5	23	4	24	
Mitotic rate					0.32					0.06
<1/mm^2	18	16	18	15		13	3	13	4	
>1/mm^2	52	45	42	54		38	26	32	33	
Location					0.18					0.25
Head and neck	24	26	21	28		15	10	13	12	
Trunk	22	11	19	15		23	8	22	9	
Extremity	51	30	48	33		31	17	26	22	

NOTE: *P* values reflect differences in class prediction and were determined by χ^2 or Fisher exact tests.

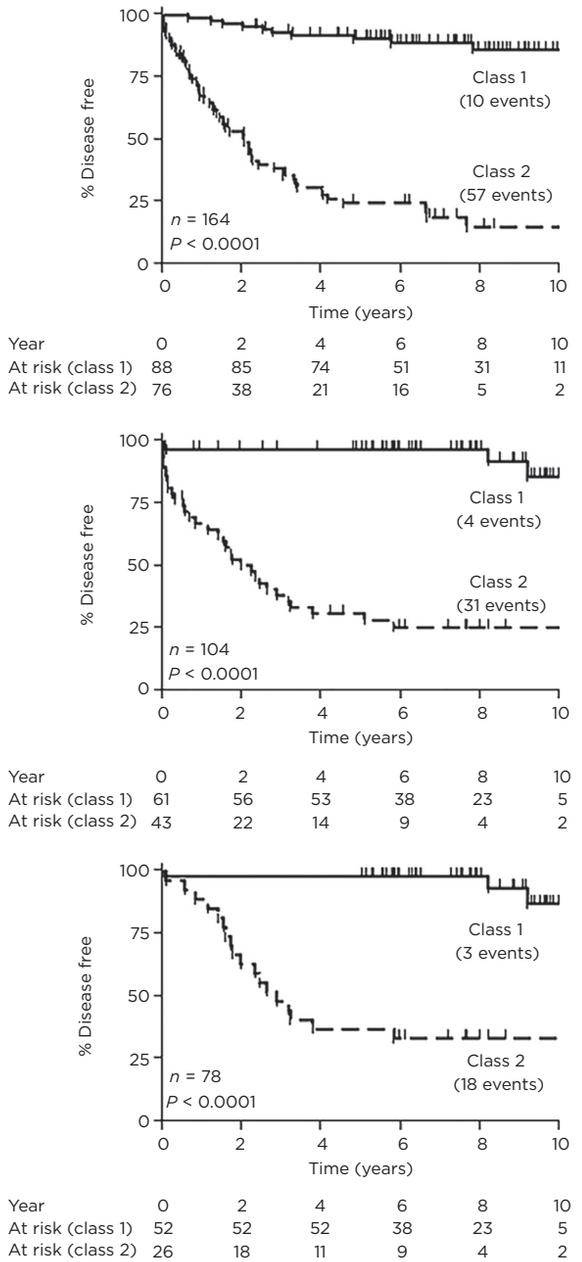

Figure 1.
Kaplan–Meier analyses of a clinically useful cutaneous melanoma training set to predict low risk (class 1) or high risk (class 2) of regional or distant metastasis. Five-year DFS rate for the 164 sample training set (A) was 91% for class 1 and 25% for class 2 ($P < 0.0001$). Five-year DFS rate for the 104 sample validation set (B) that includes stage I–IV cases was 97% for class 1 and 31% for class 2 ($P < 0.0001$). Analysis of only stage I and II cases ($n = 78$) from the validation cohort (C) resulted in 5-year DFS rates of 98% for class 1 and 37% for class 2 ($P < 0.0001$).

was a strong prognostic indicator of metastasis (HR, 20.3, $P =$ 82.88E-06) according to univariate analysis. AJCC higher risk stage II cases (IIB/IIC), Breslow thickness > 0.75 mm, presence of ulceration, and age were also highly predictive (HR. 15.2, $P =$ 5.97E-07; HR, 165.6, $P = 0.0003$; HR, 13.1, $P = 8.10$E-07; and HR, 5.6, $P = 0.021$, respectively). It should be noted that a cutoff of 0.75 mm for analysis of Breslow thickness was chosen based upon NCCN guidelines that suggest consideration of SLN biopsy for all tumors > 0.75 mm thick. Direct comparison of the GEP test to AJCC stage by multivariate Cox regression analysis again showed that the class 2 signature is an independent predictor of metastatic risk. GEP prediction was associated with an HR of

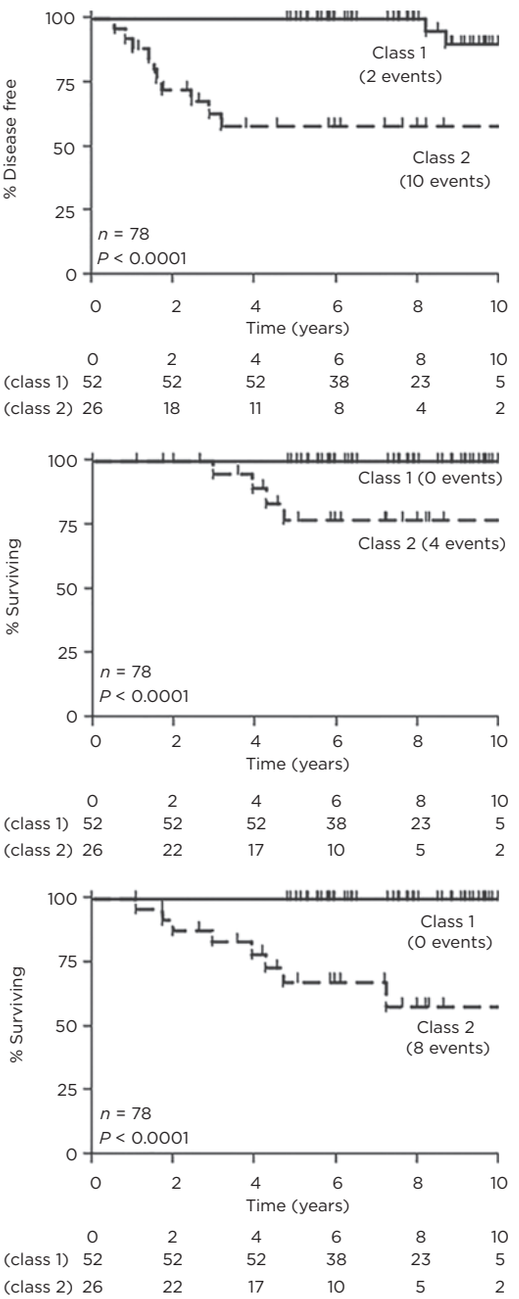

Figure 2.
Kaplan–Meier analysis of DMFS (A), MSS (B), and OS (C). Stage I and II cases ($n = 78$) with evidence of metastasis or greater than 5 years of follow-up without a metastatic event were analyzed and reflect 100% survival of predicted class 1 cases for all endpoints. Class 2 DMFS (A), MSS (B), and OS (C) 5-year rates were 58%, 77%, and 68%, respectively.

Table 3. Univariate and multivariate Cox regression analyses of DFS for stage I and II ($n = 78$) validation cases comparing the prognostic GEP with AJCC stage and individual staging factors

	Univariate		Multivariate	
Factor (high-risk variable)	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
GEP (class 2)	20.3 (5.8–70.8)	2.88E−06	9.55 (2.3–39.5)	0.002
AJCC (IIB/IIC)	15.2 (5.8–39.7)	5.97E−07	5.40 (1.8–15.7)	0.002
Breslow thickness (>0.75)	165.6 (10.7–25.59)	0.0003		
Ulceration (present)	13.1 (4.8–35.6)	8.10E−07		
Mitotic rate (>1/mm^2)	1.69 (0.5–5.8)	0.407		
Age (>50 y)	5.6 (1.3–23.9)	0.021		

9.55 ($P = 0.002$) compared with the AJCC staging HR of only 5.4 ($P = 0.002$) in the 78 stage I and II cases from the validation cohort.

Accuracy of GEP risk predictor in AJCC substages

A total of 220 stage I and II cutaneous melanoma cases were included in the training and validation cohorts. Table 4 reflects the GEP accuracy of DFS risk prediction for the AJCC stage I and II subgroups. Importantly, of the 9 stage I cases with documented metastasis, 5 (56%) were accurately called class 2. Conversely, 104 of 110 (95%) stage I cases without documented metastasis were accurately called class 1 according to the GEP signature. Overall, the GEP predictor accurately identified 120 of 134 (90%) "low-risk" stage I and IIA cases without documented evidence of metastasis as class 1 and 24 of 30 (80%) stage I and IIA cases with documented metastasis as class 2.

Discussion

Molecular-based prognostic tests have greatly impacted the classification and management of a number of neoplastic diseases, including breast cancer, uveal myeloma, and thymoma (6, 22, 23). The clinical behavior of cutaneous melanoma is highly variable and like many other tumors cannot be fully accounted for by traditional staging methods. Some patients with thin melanomas (<1 mm Breslow) will develop distant metastasis and die from their tumor, and conversely, some patients with thicker melanomas may be cured by surgical management alone (2). In this study, we have identified a distinct gene expression profile signature that characterizes high- and low-risk subtypes of cutaneous melanoma. Importantly, the signature was shown to be an independent prognostic marker in multivariate analysis when analyzed alongside traditional AJCC staging.

A number of studies over the past decade have identified differential genetic expression patterns in primary cutaneous melanoma tumors compared with metastatic tumors (12, 15–17, 19). The studies primarily analyzed fresh-frozen tissue and had restricted numbers of primary and metastatic cases available, limiting the utility and clinical applicability of the genes identified as prognostic biomarkers in each individual study. Using a meta-analysis of those reports, we compared gene expression data to determine which genes were similarly dysregulated across the studies. We sought to identify genes that would most likely be involved with changes in the primary tumor leading to cellular differentiation and metastatic progression. In addition, we considered data from an independent analysis of cutaneous melanoma tumors that used a clinically available prognostic gene signature for uveal melanoma to select genes that could have prognostic value for both diseases (data not shown). Through these methods, a genetic signature (Table 1) that includes 22 cutaneous melanoma–related genes and 9 genes related to uveal melanoma metastatic potential was selected for further expression analysis.

Table 4. Accuracy of class prediction for stage I and II cutaneous melanoma subgroups

Stage	Total cases	Cases without documented metastasis	Cases called class 1	Cases with documented metastasis	Cases called class 2
I/IA/IB	119	110	104 (95%)	9	5 (56%)
IIA	45	24	16 (67%)	21	19 (90%)
IIB	42	14	6 (43%)	28	27 (96%)
IIC	14	3	1 (33%)	11	11 (100%)

We hypothesized, on the basis of the results of studies used to develop the prognostic signature, that gene ontology analysis would uncover biologic and cellular pathways related to cell differentiation and cancer progression. Web-based gene ontology analysis tools revealed that genes related to the biologic processes of tissue development and epithelial differentiation, as well as cell junction–related genes, are highly represented. In comparison, a subsequently reported signature discovered by Harbst and colleagues reflects genes related to wound/immune responses, DNA repair, and cell-cycle, whereas a subsequently reported 9-gene signature from Brunner and colleagues contains genes encoding small secretory peptides and cell invasion–related extracellular and cytoskeletal genes (24, 25). Of note when directly comparing the gene panels is that *CXCL14* was the only gene identified in the current analysis that was included in those genetic signatures. Further similarities include (i) related genes from the keratin family (*KRT6B* and *KRT14*) that are similar to *KRT9* included in the signature from Brunner and colleagues and (ii) a correlation between *BAP1* and the *BRCA1* DNA damage response pathway identified in Harbst and colleagues In fact, it is biologically interesting that this gene set has the greatest similarity to a signature reported by Koh and colleagues that has been shown to be important for predicting nodal metastasis (26). Eight genes that are downregulated in that study in SLN melanoma metastases compared with primary melanomas are included in the current signature (*KRT6B*, *SPRR1B*, *S100A8*, *KRT14*, *TACSTD2*, *AQP3*, *DSC1*, and *CLCA2*).

Several other genes in our signature have previously been shown to be involved in cancer progression and metastasis. The protein encoded by *SPP1* is widely recognized as a modulator of tumor progression and was previously reported as an integral marker for determining disease-specific survival in cutaneous melanoma–focused protein- and genetic-based microarray studies (27, 28). *CLCA2* has been implicated in the mediation of lung metastasis (29). *S100A8* and *S100A9* encode proinflammatory proteins that have recently been implicated in the epithelial–

mesenchymal transition of breast cancer cells and have been shown to be overexpressed in prostate cancer cells (30, 31). Genes encoding gap junction proteins, including *GJA*, *DSC1*, and *PPL*, are also included in the signature and have been implicated in metastatic progression. Downregulation of *CRABP2* and *TACSTD2* in head and neck cancers has been documented, and *CST6* downregulation is associated with increased metastasis in breast cancer (32–34). In addition, *BAP1*, *ROBO1*, *LTA4H*, *ID2*, and *EIF1B*, genes previously reported to be important for uveal melanoma metastatic prognosis, are regulators of metastasis, angiogenesis, migration, or immunomodulation or are downregulated in other types of cancer (9). Removal of these genes from the analysis does not dramatically impact the accuracy of the genetic signature but does slightly reduce the accuracy of the training set (82% compared with 83% reported in Fig. 1). This is a valuable point with regard to future analysis of genetic data obtained from studies of biologically similar, but pathologically different, diseases.

To our knowledge, this is the first study to combine quantitative genetic expression analysis of a large, multicenter cohort of primary FFPE melanoma cases with the development of a clinically validated prognostic training set. The prognostic power of this assay is considerably greater than other reported prognostic assays for melanoma. For example, the Kaplan–Meier analysis of a protein-based prognostic signature from Meyer and colleagues resulted in 5-year MFS rates of about 70% (median, 7.3 years) versus 35% (median, 2.75 years) for low-risk compared with high-risk patients, respectively, whereas Harbst and colleagues reported 5-year MFS rates of approximately 85% (median not reached) and 40% (median, 3.75 years) for low- and high-grade forms of melanoma, respectively (25, 35).

The development of a highly accurate and robust molecular prognostic test for cutaneous melanoma could significantly impact melanoma management from multiple perspectives. The identification of stage I and II SLN-negative patients at high risk for recurrence allows these patients to take part in more aggressive imaging protocols for early detection of metastatic disease and to be considered for adjuvant therapy. In addition, the psychologic and emotional burden of a melanoma diagnosis may be somewhat dampened for patients identified as having a class 1 signature. In this study, the assay was clearly shown to be an independent and powerful prognosticator of metastasis in stage I and II patients. In future studies, it will be important to evaluate the efficacy of the assay for stage III patients as a significant proportion of these patients, particularly those with only microscopic disease identified with SLN biopsy, will never have disease progression.

Disclosure of Potential Conflicts of Interest

P. Gerami is a consultant for Castle Biosciences. C.E. Johnson is an employee of Castle Biosciences. R.W. Cook, K.M. Oelschlager, and D. Maetzold are employees of and have ownership interests (including patents) in Castle Biosciences. No potential conflicts of interest were disclosed by the other authors.

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