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ORIGINAL ARTICLE

BRAF Mutation, *NRAS* Mutation, and the Absence of an Immune-Related Expressed Gene Profile Predict Poor Outcome in Patients with Stage III Melanoma

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Prediction of outcome for melanoma patients with surgically resected macroscopic nodal metastases is very imprecise. We performed a comprehensive clinico-pathologic assessment of fresh-frozen macroscopic nodal metastases and the preceding primary melanoma, somatic mutation profiling, and gene expression profiling to identify determinants of outcome in 79 melanoma patients. In addition to disease stage <II at initial presentation, the following clinical and pathologic factors were independent predictors of improved outcome (odds ratios for survival >4 years, 90% confidence interval): the presence of a nodular component in the primary melanoma (6.8, 0.6–76.0), and small cell size (11.1, 0.8–100.0) or low pigmentation (3.0, 0.8–100.0) in the nodal metastases. Absence of *BRAF* mutation (20.0, 1.0–1000.0) or *NRAS* mutation (16.7, 0.6–1000.0) were both favorable prognostic factors. A 46-gene expression signature with strong overrepresentation of immune response genes was predictive of better survival (10.9, 0.4–325.6); in the full cohort, median survival was >100 months in those with the signature, but 10 months in those without. This relationship was validated in two previously published independent stage III melanoma data sets. We conclude that the presence of *BRAF* mutation, *NRAS* mutation, and the absence of an immune-related expressed gene profile predict poor outcome in melanoma patients with macroscopic stage III disease.

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INTRODUCTION

Most melanoma patients who ultimately die of their disease will, at some point, have metastases resected from a draining lymph node field, and outcome in surgically resected American Joint Committee on Cancer (AJCC) (Balch *et al.*, 2009) Stage III disease is highly uncertain. Only 30–40% of AJCC stage III patients will survive beyond 5 years, and a similar proportion will die within 1 year; however, there are at present no reliable and validated biomarkers of outcome in this setting (Thompson *et al.*, 2009). The patients most likely to benefit from potentially toxic adjuvant systemic therapy are therefore difficult to define, and stratification of these patients for adjuvant therapy trials is correspondingly confounded.

Protein biomarkers have prognostic value in primary melanoma (Gould Rothberg *et al.*, 2009; Gould Rothberg and Rimm, 2010) and claims have been made for the utility of more complex molecular profiles (Winnepenninckx *et al.*, 2006). However, it is still unclear whether the latter can contribute validated information independent of well-defined and more simply determined clinical and pathologic variables, such as Breslow thickness, ulceration, and mitotic rate (Schramm *et al.*, 2012). Several studies have reported molecular signatures associated with prognosis in stage III melanoma, but these studies have not taken into account the prognostic effects of important clinical and pathologic variables, and thus the truly independent effect of gene expression profiles is uncertain (John *et al.*, 2008; Bogunovic *et al.*, 2009a).

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Abbreviations: AJCC, American Joint Committee on Cancer; MAP, mitogen-activated protein; MIA, Melanoma Institute Australia

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Furthermore, these prior studies have not assessed the effect, if any, of oncogenic mutations in BRAF or NRAS that commonly occur in melanoma. This is of particular important clinical relevance following the recent demonstration in clinical trials of the efficacy of potent inhibitors of V600 mutant BRAF in the majority of patients with BRAF mutant metastatic melanoma (Flaherty *et al.*, 2010; Chapman *et al.*, 2011). Despite the lack of effect of BRAF mutation on prognosis (disease-free survival) in patients with primary melanoma (Maldonado *et al.*, 2003; Shinozaki *et al.*, 2004; Akslen *et al.*, 2005; Edlundh-Rose *et al.*, 2006), we hypothesized that, in those with tumors that have metastasized, these mutations might influence the probability or rate of further disease progression to relapse and death. A recent analysis of a prospectively collected, consecutive series of patients from our Institute (with no overlap of the patient cohort with that of the current series) provided preliminary evidence supporting this hypothesis (Long *et al.*, 2011).

We therefore studied a series of prospectively accrued stage III melanoma patients. First, we established which clinical and pathologic variables were associated with long-term melanoma-specific survival, and showed that the absence of BRAF mutation or NRAS mutation was independently associated with better survival. Then we derived a gene expression set associated with long-term survival and established that it was equally effective as a predictor to the combined clinical, pathologic, and mutation status variables. A combined model incorporating clinical, pathologic, mutation, and gene expression status was the most effective of all. The gene set was validated as a predictor in the two independent, published stage III data sets referred to above and is strongly characterized by genes associated with immune activation.

RESULTS

Clinical, pathologic, and mutation characteristics

The prevalence of selected clinical, pathologic, and somatic genetic variables in the full cohort, and in the subsets selected for more favorable (''better'') or less favorable (''worse'') prognosis, is summarized in Table 1. Tumors in the worse prognosis group were more likely, at the time of resection of nodal metastases, to be large, to show extranodal spread, to be more strongly pigmented, and to be positive for BRAF or NRAS mutation; however, none of these differences was significant at the 5% level. The antecedent primary tumors also differed between the better and worse prognosis groups, although again no individual parameter reached significance at the 5% level: primary tumors preceding worse prognosis tumors tended to be thicker, to have more mitoses, to be ulcerated, to show more regression, and were less likely to have a nodular component or to be situated in a chronically sun-exposed site.

BRAF mutations were observed in 40.5% of tumors overall, 52% of worse prognosis tumors, and 39% of better prognosis tumors. NRAS mutations were observed in 37% overall, 40% of worse prognosis, and 22% of better prognosis tumors. No tumors carried both a BRAF and NRAS mutation. Only two cKIT mutations were observed, with 30% of the preceding primary tumors occurring on chronically sun-exposed sites. PIK3CA (PI3 kinase) mutations were infrequent in the group as a whole (3/79), and all were identified in worse prognosis tumors. Occasional tumors were identified with mutations in FLT3, PDGFR, and MET. ERBB4 mutations were observed in 3.8% of tumors.

Association of clinical, pathologic, and mutation variables with outcome

As shown in Figure 1a, multivariable logistic regression using a random forest-based multiple resampling procedure identified the following clinical, pathologic, and mutation variables as associated with more favorable prognosis: AJCC disease stage $<$II at presentation, the presence of a nodular component in the primary melanoma (odds ratio 8.0, 90% confidence interval 0.51–126), and smaller cell size (6.1, 0.58–63.4) or lower pigmentation (3.1, 0.97–9.7) in the nodal metastases. Absence of BRAF mutation (15.5, 0.49–488) or of NRAS mutation (24.3, 0.45–313) were each strong positive prognostic factors.

This model produced a 6-fold cross-validation error rate of 27%.

Gene expression profiles associated with outcome in combined model

Gene expression array data were then tested for association with outcome, allowing for the previously established clinical, pathologic, and gene mutation prognostic factors. By using differentially expressed genes alone, a minimum cross-validation error rate of 25% was achieved, using the 60 top-ranked probes, representing 46 genes (Table 2). The prevalidated expression estimates were added as an additional variable to the clinical data and the now combined expression and clinical data were analyzed using logistic regression. Variables used in the model were the original six from the clinical model and the prevalidated gene expression variable. Figure 1b shows the final model from this part of the analysis, which produces a 6-fold cross-validation error rate of 23%. The combined model thus performed somewhat better than either the clinical/pathologic/mutation profile model or the gene expression data alone.

Notably, the effect of none of the clinical, pathologic, and mutation variables was weakened significantly by incorporation of the gene expression variable. This indicates that the gene expression-profiling signature (Table 2) does not specifically reflect any of those parameters, but is an independent prognostic variable. For example, it cannot simply be a molecular footprint of the mitogen-activated protein (MAP) kinase (BRAF or NRAS) pathway mutational activation.

Figure 2 shows dendrograms based on supervised clustering using the gene expression signature, in order to visualize its association with prognosis. Figure 3 shows the performance of the gene expression classifier alone in predicting survival of the full cohort of stage III patients. The patient group in which this classifier is expressed includes all the long-term survivors.

Table 1. Selected characteristics of 79 AJCC stage III melanoma patients and their tumors

Variable	Whole cohort (*n*=79)	Poor survival group (*n*=25)	Good survival group (*n*=23)	Good versus poor (*P*-value)[1]
Age (mean)	63.4	60.0	61.3	0.76
Sex (% F)	36.7%	40.0%	43.5%	1
Survival (days) (median, range)	812 (27, 3,439)	201 (27, 362)	2,176 (1,499, 3,205)	NA
Previous primary melanoma				
Body site (% chronic sun exposure)	30.0%	19.0%	35.0%	0.31
Stage at diagnosis (% stage <II)	37.2%	16.0%	45.5%	0.05
Breslow thickness (median) (mm)	1.8	2	1.5	0.12
Mitotic rate (median, /mm^2)	3	3	2	0.44
Histologic type (% with a nodular component)	43.8%	36.8%	50.0%	0.52
Presence of regression	47.8%	45.0%	73.7%	0.10
Ulceration	27.9%	38.1%	21.1%	0.31
Nodal tumor analyzed				
Metastasis max size (median, mm)	35	45	35	0.12
Extranodal spread	42.3%	48.0%	21.7%	0.07
Cell size (large)	53.9%	58.3%	54.6%	1
Pigmentation (present)	35.1%	40.0%	27.3%	0.54
Mutation status				
BRAF	40.5%	52.0%	39.1%	0.40
NRAS	36.7%	40.0%	21.7%	0.22
PI3KCA	1.3%	4.0%	0.0%	1
cKIT	2.5%	0.0%	4.4%	0.48
ERBB4	3.8%	4.0%	4.4%	1
FLT3	1.3%	0.0%	4.4%	0.48
MET	2.5%	4.0%	4.4%	1
PDGFRA	1.3%	4.0%	0.0%	1
EGFR	0.0%	0.0%	0.0%	NA

Abbreviations: AJCC, American Joint Committee on Cancer; NA, not applicable.
[1]By Fisher's exact test, except for: age (*t*-test), and Breslow thickness, mitoses, and metastasis size (Mann–Whitney).

Molecular subgroups revealed by unsupervised clustering are not correlated with prognosis

In supplementary Figure S2 online, we show dendrograms of the full Stage III tumor cohort based on unsupervised hierarchical clustering of expression levels of the 1,000 probes with the highest variance in expression. As others have shown in metastatic melanoma, there are distinct subsets of tumors; however, both more favorable and less favorable prognosis tumors are present in all these subsets, as are BRAF and NRAS mutant tumors (data not shown).

Molecular pathways overrepresented in genes differentially expressed by prognosis

The genes from the 60-probe expression signature associated with a more favorable prognosis have a distinctive biological profile that can be characterized as immune activation. The top ten canonical biological pathway maps most significantly enriched by that gene list, ranked from lowest to highest map significance *P*-value, are shown in Supplementary Table S1 online.

External validation of the gene expression signature

The 60-probe signature identified in this study was applied to two independent data sets providing gene expression and prognostic data for patients with stage III melanomas (John *et al.*, 2008; Bogunovic *et al.*, 2009a). As shown in Table 3, the signature identified in this study was significantly associated with more favorable outcome in both the external data sets. Owing to the limited clinical, pathologic, and mutation data provided in those studies, the combined model could not be tested.

DISCUSSION

The patient with AJCC stage IIIb or IIIc melanoma has a highly uncertain prognosis (Thompson *et al.*, 2009). Recent

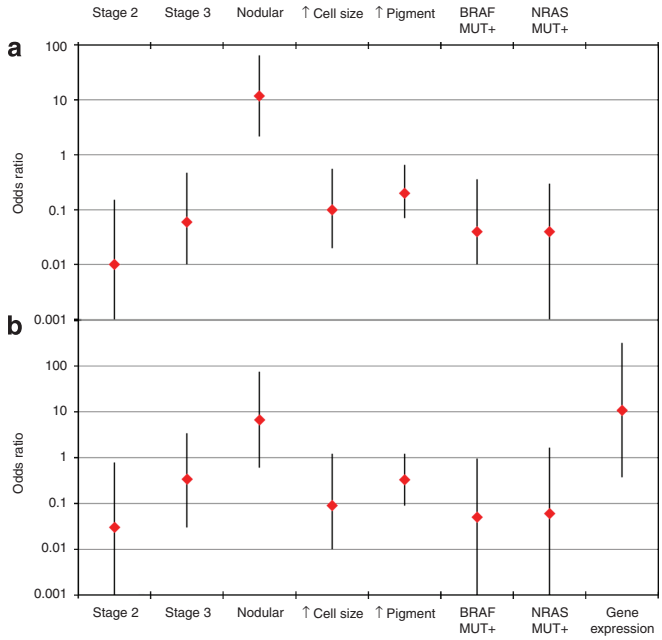

Figure 1. Factors independently associated with melanoma-specific survival >4 years on multivariable logistic regression analysis. (**a**) Model incorporating clinico-pathologic variables* alone; (**b**) model incorporating clinico-pathologic variables and signature of differentially expressed genes (according to Tibshirani and Efron (2002)). *Stage II or stage III at presentation; the presence of nodular histological component; large cell size; increased pigmentation; the presence of activating BRAF or NRAS mutation.

studies have indicated that the molecular phenotype of metastatic melanomas, as indicated by their gene expression profile, might help identify those with a more or less favorable prognosis (John *et al.*, 2008; Bogunovic *et al.*, 2009a; Jonsson *et al.*, 2010). However, these studies have not fully taken into account factors already known to influence clinical outcome, nor have they tested for any effect of BRAF mutations, which we have recently shown to be prognostically significant in patients with metastatic melanoma (Long *et al.*, 2011). The latter is particularly important now that potent inhibitors of V600 mutant-BRAF are not only available but have revolutionized the treatment of metastatic melanoma (Flaherty *et al.*, 2010; Chapman *et al.*, 2011).

We therefore conducted a multivariate analysis that initially constructed a model of clinical, pathologic, and mutational determinants of outcome (time from nodal resection to death), and then tested for any additional value contributed by gene expression profiling. Patients were selected from a single institution in which tumor is routinely banked, analyzed, and correlated to systematically reviewed characteristics of the preceding primary melanoma and to long-term follow-up. We hypothesized that the strongest determinants of outcome in this setting, where clinically detectable nodal metastasis has already occurred in all patients, would differ from those already well identified to affect long-term survival in primary melanoma.

The analysis showed that, although better and worse prognosis tumors tended to differ by several features of their antecedent primary melanoma, only early stage at presentation (I or II) and the presence of a nodular component were predictive of better survival once resectable stage III disease was present. Other features, such as Breslow thickness, ulceration, and mitotic count, important to prognostication in primary melanoma, were not independently predictive when stage, nodularity, and features of the resected stage III disease were taken into account. This suggests that they are more strongly associated with the probability of metastasis than with the rate of progression of metastatic disease. The association of a nodular component with better prognosis might reflect a propensity for an expansile but localized, rather than spreading, growth pattern.

MAP kinase pathway activating mutations in BRAF and NRAS each conferred an adverse prognosis in patients with stage III melanoma. BRAF mutation is not associated with outcome in primary melanoma (Maldonado *et al.*, 2003; Shinozaki *et al.*, 2004; Akslen *et al.*, 2005; Edlundh-Rose *et al.*, 2006); however, our finding in stage III melanoma is consistent with our recent report that BRAF mutation may be associated with worse outcome in stage IV melanoma (Long *et al.*, 2011). The finding of an association of NRAS mutation with worse outcome in the present study of patients with stage III melanoma is consistent with preliminary results recently reported in abstract form (Jakob *et al.*, 2011) and helps clarify the discrepancy between studies of BRAF in primary and metastatic melanoma. This suggests that the presence of MAP kinase–activating mutations provides an essential genetic background for more rapid evolution of the metastatic phenotype once early metastatic events have occurred. The probability of metastasis *per se* is possibly less strongly influenced by these mutations than by others present in poor prognosis primary melanomas, and currently may be most sensitively indicated by clinico-pathologic features such as Breslow thickness, ulceration, and mitotic rate.

The study lacked power to assess the prognostic effect of lower-frequency mutations, which most commonly affected cKIT, PIK3CA, and ERBB4.

Our analysis is more definitive than previous reports of a prognostically significant gene expression profile in metastatic melanoma, because it was independently prognostically significant after allowing for the effect of other factors: not only a comprehensive profile of clinical and pathologic variables but also somatic mutations. The gene expression signature alone had equivalent predictive power, based on cross-validation error rates, to the combined model of clinical, pathologic, and mutation status (stage at presentation, cell size, pigmentation, BRAF mutation, NRAS mutation). A model combining these clinical, pathologic, and molecular data performed best to predict outcome; however, the gene expression signature alone was effective in identifying all long-term survivors in the cohort as a whole.

The gene expression signature that performed best in the combined model was readily characterized by a simple gene ontology analysis as immune response gene activation. However, it did not identify a specific molecular subset of tumors in hierarchical clustering analysis, nor did the subgroups evident in unsupervised clustering show any

Table 2. Top-ranked differentially expressed genes (in alphabetical order)

Gene symbol	Entrez ID	Gene description	Probe ID
ADAMDEC1	27,299	ADAM-like, decysin 1	ILMN_2103107
ALDH1A3[1]	220	Aldehyde dehydrogenase 1 family, member A3	ILMN_1807439; ILMN_2139970
ALOX5	240	Arachidonate 5-lipoxygenase	ILMN_1680996
APOL3	80,833	Apolipoprotein L, 3	ILMN_1756862
BIRC3	330	Baculoviral IAP repeat-containing 3	ILMN_1776181
CCL5[1]	6,352	Chemokine (C–C motif) ligand 5	ILMN_1773352; ILMN_2098126
CCL8	6,355	Chemokine (C–C motif) ligand 8	ILMN_1772964
CD2	914	CD2 molecule	ILMN_1695025
CD247[1]	919	CD247 molecule	ILMN_2377669; ILMN_1676924
CD3D[1]	915	CD3d molecule, delta (CD3–TCR complex)	ILMN_2325837; ILMN_2261416
CD52	1,043	CD52 molecule	ILMN_2208903
CD79A	973	CD79a molecule, immunoglobulin-associated alpha	ILMN_1734878
CD8A[1]	925	CD8a molecule	ILMN_2353732; ILMN_1768482
CXCL10	3,627	Chemokine (C-X-C motif) ligand 10	ILMN_1791759
CXCL9	4,283	Chemokine (C-X-C motif) ligand 9	ILMN_1745356
EPSTI1	94,240	Epithelial stromal interaction 1 (breast)	ILMN_2388547
GBP1[1]	2,633	Guanylate binding protein 1, interferon-inducible, 67 kDa	ILMN_1701114; ILMN_2148785
GBP2	2,634	Guanylate binding protein 2, interferon-inducible	ILMN_1774077
GBP4	115,361	Guanylate binding protein 4	ILMN_1771385
GBP5	115,362	Guanylate binding protein 5	ILMN_2114568
GIMAP4	55,303	GTPase, IMAP family member 4	ILMN_1748473
GZMA	3,001	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	ILMN_1779324
GZMB	3,002	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	ILMN_2109489
HLA-DPA1	3,113	Major histocompatibility complex, class II, DP alpha 1	ILMN_1772218
HLA-DQA1	3,117	Major histocompatibility complex, class II, DQ alpha 1	ILMN_1808405
HLA-DRB3	3,125	Major histocompatibility complex, class II, DR beta 3	ILMN_1717261
HLA-DRB4	3,126	Major histocompatibility complex, class II, DR beta 4	ILMN_1752592
IGJ	3,512	Immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	ILMN_2105441
IRF8	3,394	Interferon regulatory factor 8	ILMN_1666594
IRX3	79,191	Iroquois homeobox 3	ILMN_1811468
LCP1	3,936	Lymphocyte cytosolic protein 1 (L-plastin)	ILMN_1662932
LOC652493	652,493	Ig kappa chain V-I region HK102-like	ILMN_1739508
LOC652694	652,694	Similar to Ig kappa chain V-I region HK102 precursor	ILMN_1680274
LYZ	4,069	Lysozyme	ILMN_1815205
MAGEC2	51,438	Melanoma antigen family C, 2	ILMN_2088876
MS4A6A	64,231	Membrane-spanning 4-domains, subfamily A, member 6A	ILMN_1797731
PLEK	5,341	Pleckstrin	ILMN_1795762
PLEKHB1	58,473	Pleckstrin homology domain containing, family B (evectins) member 1	ILMN_1783231
PMEL	6,490	Premelanosome protein	ILMN_1665994
QPCT	25,797	Glutaminyl peptide cyclotransferase	ILMN_1741727

Table 2 Continued on following page

Table 2. Continued

Gene symbol	Entrez ID	Gene description	Probe ID
RAC2	5,880	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	ILMN_1709795
RGS1	5,996	Regulator of G-protein signaling 1	ILMN_1656011
STAT1[1]	6,772	Signal transducer and activator of transcription 1, 91 kDa	ILMN_1691364; ILMN_1690105
TSPAN10	83,882	Tetraspanin 10	ILMN_1656194
UBD	10,537	Ubiquitin D	ILMN_1678841
VCAM1	7,412	Vascular cell adhesion molecule 1	ILMN_2307903

[1]Gene identified among the top-ranked differentially expressed genes from two separate probes.

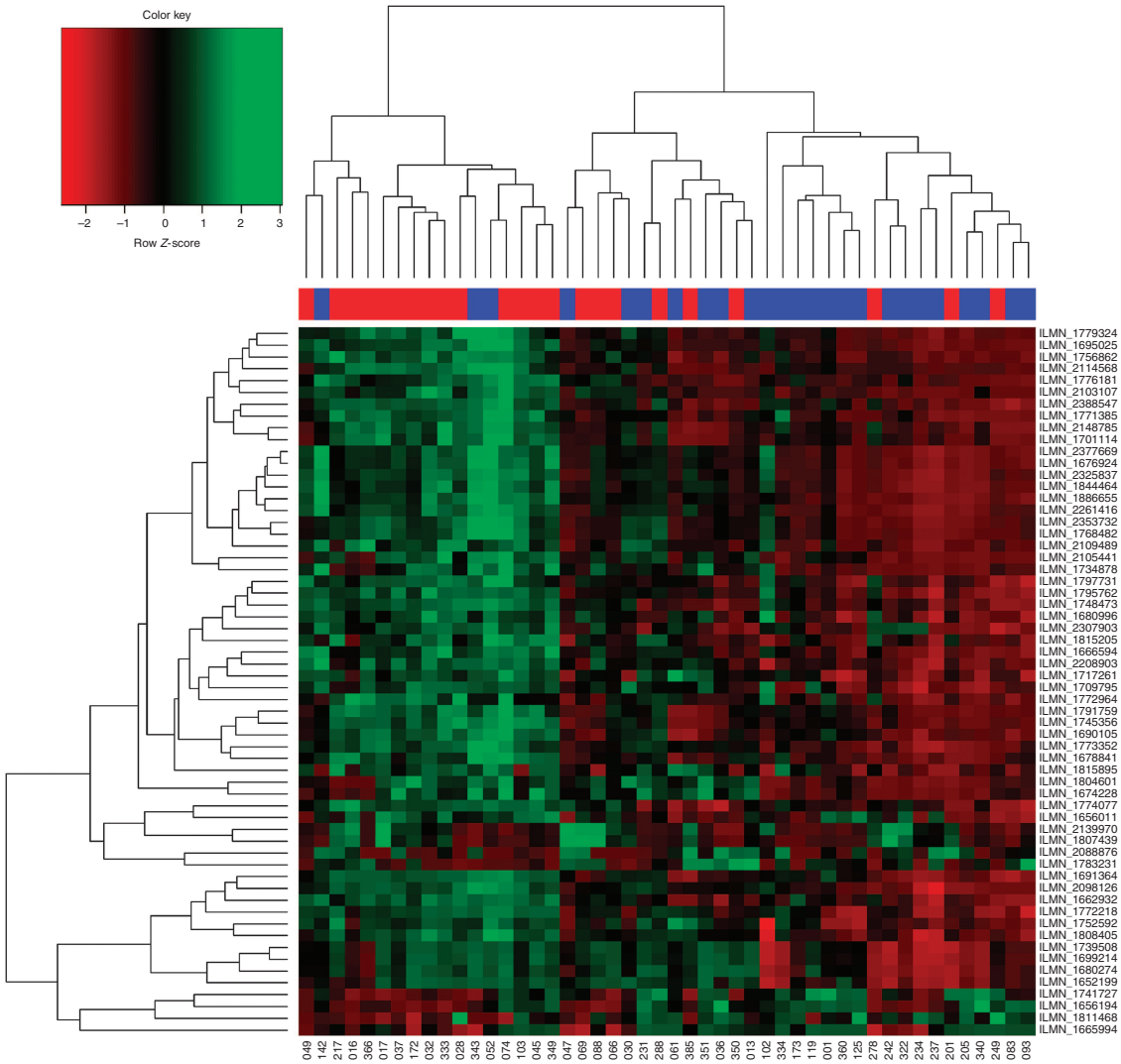

Figure 2. Supervised hierarchical clustering gene expression based on the 60-probe (46 gene) signature with strongest association with prognosis in 48 American Joint Committee on Cancer (AJCC) stage III melanoma patients.

relationship with prognosis. This contrasts somewhat with the data of Jonsson *et al.* (2010), who have made a related observation in stage IV melanoma. However, their signature was derived and tested differently: they first observed that a distinct subset of tumors was characterized by immune activation, and then showed that the expression of a selected panel of immune activation genes was associated with survival.

We have recently shown, by a systematic review and detailed cross-validation of all reported prognostically significant gene expression signatures in metastatic melanoma, that immune response activation and MAP kinase

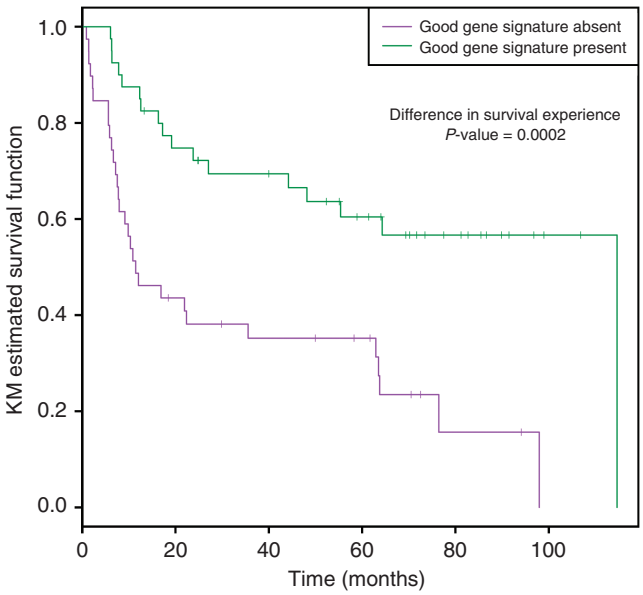

Figure 3. Melanoma-specific survival in 79 American Joint Committee on Cancer (AJCC) stage III patients according to the presence or absence of a favorable 60-probe (46 gene) signature. KM, Kaplan–Meier.

Table 3. Results of external validation of gene expression signature on previously published data sets

		Independent validation, logFC used to rank genes from the two public data sets	
Feature selection method	**Number of selected genes**	**John *et al.* (2008)**	**Bogunovic *et al.* (2009b)**
2-Fold change[1]	111	>0.001	0.004
2-Fold change using robust modeling[2]	141	>0.001	0.003
Bss/Wss	60	0.290	0.980
Median robust method	60	>0.001	0.038

Abbreviations: Bss, between sum of squares; Wss, within sum of squares.
[1]This was performed with the package limma where log(M) >1 was selected; where *M* is represents the logFC of the coefficient estimates to the linear model $y=X\beta+\varepsilon$ (see Materials and Methods).
[2]Robust fold change was calculated as per the fold change case, but using robust linear modeling.

activation via NRAS are common features of all these signatures (Schramm *et al.*,2012). Importantly, the gene expression signature identified in the present study was shown to be associated with better outcome in two independent stage III melanoma data sets (John *et al.*, 2008; Bogunovic *et al.*, 2009a), although our full model could not be tested because comprehensive clinico-pathologic data were not analyzed or reported in those studies.

In summary, we have derived a model that is effective in identifying patients with stage III melanoma who will survive long term after nodal resection, and those who will not. Unlike prior studies, the model takes account of clinical, pathologic, gene mutation, and gene expression data and, to the best of our knowledge, has identified the previously unreported key negative prognostic effect of MAP kinase activating mutations, as well as a positive effect of a gene expression signature indicating immune response activation.

MATERIALS AND METHODS

Specimens, clinical, and pathologic data

The study was conducted according to Declaration of Helsinki Principles. Tumor samples were obtained from the Melanoma Institute Australia (MIA) Biospecimen Bank, a prospective collection of fresh-frozen tumors accrued with written informed patient consent and Institutional Review Board approval (Sydney South West Area Health Service institutional ethics review committee (Royal Prince Alfred Hospital (RPAH) Zone) Protocol No. X08-0155/HREC 08/RPAH/262, No. X11-0023/HREC 11/RPAH/32, and No. X07-0202/HREC/07/RPAH/30) since 1996 through MIA, formerly the Sydney Melanoma Unit (Carter *et al.*, 2010). Samples eligible for this study ($n=79$) were obtained from lymph node specimens in which macroscopic tumor was observed, obtained from patients believed to be without distant metastases at the time of tumor banking based on clinical examination and computerized axial tomographic scanning of the brain, chest, abdomen, and pelvis. Specimens were macrodissected at the time of banking and subsequently reviewed to meet minimum criteria for tumor cell content (>80%) and amount of necrosis (<30%).

Banked fresh-frozen nodal melanoma metastasis samples selected for analysis were reviewed by a pathologist (Richard A. Scolyer) and scored for the following parameters: % of nontumor cells, % of necrosis, degree of pigmentation, predominant cell shape, and cell size of the most cellular portion of the tumor, as previously described (Viros *et al.*, 2008). Linked pathologic data were obtained for number of nodes involved, largest nodal metastasis size, and the presence of extranodal spread. Linked clinical and pathologic data were obtained from the MIA melanoma research database for any previous primary melanoma, including the following: age, sex, stage at diagnosis, body site (classified by pattern of sun exposure: chronic/continuous, intermittent, rarely exposed), the presence of an associated nevus, degree of solar elastosis in the peritumoral skin, Breslow thickness (mm), Clark level, histologic melanoma subtype, and the presence of regression, ulceration, vascular, or lymphatic invasion. In cases in which more than one previous primary melanoma had been diagnosed ($n=16$), the one with greatest Breslow thickness was designated the index melanoma determining prognosis; in all but four cases this was also the most probable melanoma to have given rise to the banked metastatic tumor on clinical and anatomic grounds.

Genotyping and transcriptome analysis

DNA was extracted from 20–30 mg tumor tissue sample using the QIAamp DNA mini kit (Qiagen, Clifton Hill, Victoria, Australia) with RNAse digestion on the column. Briefly, tissue was pulverized using liquid nitrogen, and then incubated with Buffer ATL (Qiagen) and proteinase K for 96 hours at 56 °C for complete digestion. Somatic mutation profiling was conducted using the Sequenom OncoCarta v1.0, MelaCarta v1.0, and MassARRAY mass spectroscopy (Thomas *et al.*, 2007).

Transcriptome analysis

Total RNA was extracted from 20–30 mg of fresh frozen tissue. Tissue samples were homogenized using a high-speed agitation polytron blender (Kinematica, Luzern, Switzerland) in the presence of Trizol. The RNA was isolated and purified with an RNeasy purification kit (Qiagen RNeasy purification kit; Qiagen) with DNAse I digestion on the column. The quality of the RNA preparations was assessed using the Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA). RNA integrity scores were >8 for all the samples analyzed.

cRNA amplification and labeling with biotin were performed using the Illumina TotalPrep RNA amplification kit according to the manufacturer's directions (Ambion, Austin, TX) with 250 ng total RNA as input material. Gene expression analysis was performed using the Sentrix Human-6 v3 Expression BeadChips (Illumina, San Diego, CA) and BeadStation system from Illumina according to the manufacturer's instructions.

Expression BeadChip using array annotation based on R-2.11.0 and illuminaHumanv3.db. Quality control was performed on all chips using R/Bioconductor and the lumi package (www.bioconductor.org). Data normalization was performed using a variance-stabilizing transform and quantile normalization as implemented in the lumi package for R/Bioconductor. To reduce false positives, unexpressed genes (detection P-value >0.01) were removed, reducing the number of probes analyzed from 48,802 to 26,085.

Statistical analysis

The clinical, pathologic, mutation, and gene expression data sets were interrogated systematically to identify a combined model predictive of melanoma-specific survival after resection of macroscopic AJCC stage III disease. First, the distribution of survival times was analyzed and more favorable (''better'') and less favorable (''worse'') prognosis groups were defined as having time from surgery to death from melanoma greater than 4 years with no sign of relapse ($n=23$) or less than 1 year ($n=25$), respectively, as shown in Supplementary Figure S1 online.

A model predicting better survival in the absence of gene expression information was then derived. Clinical and pathologic data comprised 19 continuous, discrete, and categorical variables with an overall 10.4% proportion of missing datum. Multiple imputation was applied using Amelia II (King *et al.*, 2001) with five imputed data sets constructed. For each of the five complete data sets, logistic regression was applied with variables reduced using the Bayesian information criterion. The five models were aggregated using their mean coefficient value with an inclusion frequency of 50%. The final model contained six variables: stage at primary diagnosis, nodular histology, BRAF mutation status, NRAS mutation status, tumor cell size, and tumor cell pigmentation (Model 1).

To identify differentially expressed gene probes according to prognostic groups, gene probes were selected by ranking them using the ''median robust method'', i.e., by absolute difference in the groups' medians for each probe. The optimal number of expressed probes classifying the tumors by prognostic group was derived using leave-one-out cross-validation error rate. The lowest leave-one-out cross-validation error rate was obtained using the 60 top-ranked probes.

The clinical, pathologic, and mutation variables comprising Model 1 were combined with the expression data as previously described (Tibshirani and Efron, 2002). The expression data were added to the regression in the form of a single prevalidated variable, which had been obtained via 6-fold cross-validation using the top 60 probes ranked by the median robust method. To obtain the final regression model, all eight coefficients (seven variables plus the intercept) were remodeled without any variable selection (Model 2). Confidence intervals for these coefficients were obtained, with an inflated SE associated with multiple imputations at the 90% level.

Independent data set validations, correlating gene expression with survival in resected stage III melanoma, were obtained from the study by John *et al.* (2008) and Bogunovic *et al.* (2009a). Such a validation applied a gene set test using the obtained *DE* gene list from our study and evaluated whether this list was more significantly associated with survival than random in the two published data sets in which their genes were ranked via logFC. Other gene lists, selected in alternate ways including fold change, fold change modeled through robust regression, and ''between sum of squares over within sum of squares'' were also obtained for our data set and compared with the independent data for validation purposes. These gene set tests were applied using the limma package in R (Ihaka and Gentleman, 1996; Smyth, 2004).

Biological pathway analysis of genes from the 60-probe expression signature was undertaken in MetaCore (from GeneGo, CA), identifying the most strongly represented canonical pathway maps using the ''enrichment analysis'' workflow, default parameters.

Supervised hierarchical clustering was performed (Figure 2) using the 60-probe gene expression signature to illustrate its association with prognosis. Unsupervised hierarchical clustering was also performed, based on the 1,000 probes with the greatest variance in the cohort (Supplementary Figure S2 online). The dendrograms of samples were created using the complete agglomeration method, using both correlation and Euclidean distance measures.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Akslen LA, Angelini S, Straume O *et al.* (2005) BRAF and NRAS mutations are frequent in nodular melanoma but are not associated with tumor cell proliferation or patient survival. *J Invest Dermatol* 125:312–7

Balch CM, Gershenwald JE, Soong SJ *et al.* (2009) Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol* 27:6199–206

Bogunovic D, O'Neill DW, Belitskaya-Levy I *et al.* (2009a) Immune profile and mitotic index of metastatic melanoma lesions enhance clinical staging in predicting patient survival. *Proc Natl Acad Sci U S A* 106: 20429–34

Bogunovic D, O'Neilla DW, Belitskaya-Levya I *et al.* (2009b) Immune profile and mitotic index of metastatic melanoma lesions enhance clinical staging in predicting patient survival. *Proc Natl Acad Sci* 106:20429–34

Carter C, de Silva C, Synnott M *et al.* (2010) Bio-specimen banking: Melanoma Institute Australia. *Pigment Cell Melanoma Res* 23:946–7

Chapman PB, Hauschild A, Robert C *et al.* (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 364:2507–16

Edlundh-Rose E, Egyhazi S, Omholt K *et al.* (2006) NRAS and BRAF mutations in melanoma tumours in relation to clinical characteristics: a study based on mutation screening by pyrosequencing. *Melanoma Res* 16:471–8

Flaherty KT, Puzanov I, Kim KB *et al.* (2010) Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med* 363:809–19

Gould Rothberg BE, Bracken MB, Rimm DL (2009) Tissue biomarkers for prognosis in cutaneous melanoma: a systematic review and meta-analysis. *J Natl Cancer Inst* 101:452–74

Gould Rothberg BE, Rimm DL (2010) Biomarkers: the useful and the not so useful—an assessment of molecular prognostic markers for cutaneous melanoma. *J Invest Dermatol* 130:1971–87

Ihaka R, Gentleman R (1996) R: a language for data analysis and graphics. *J Computat Graph Stat* 5:299–314

Jakob JA, Bassett RL, Ng CS *et al.* (2011) Clinical characteristics and outcomes associated with BRAF and NRAS mutations in metastatic melanoma. *J Clin Oncol* 29: Abstr: 8500

John T, Black MA, Toro TT *et al.* (2008) Predicting clinical outcome through molecular profiling in stage III melanoma. *Clin Cancer Res* 14:5173–80

Jonsson G, Busch C, Knappskog S *et al.* (2010) Gene expression profiling-based identification of molecular subtypes in stage IV melanomas with different clinical outcome. *Clin Cancer Res* 16:3356–67

King G, Honaker J, Joseph A *et al.* (2001) Analysing incomplete political science data: an alternative algorithm for multiple imputation. *Am Polit Sci Rev* 95:49–69

Long GV, Menzies AM, Nagrial AM *et al.* (2011) Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. *J Clin Oncol* 29:1239–46

Maldonado JL, Fridlyand J, Patel H *et al.* (2003) Determinants of BRAF mutations in primary melanomas. *J Natl Cancer Inst* 95:1878–80

Schramm S-J, Campain AE, Scolyer RA *et al.* (2012) Review and cross-validation of gene expression signatures and melanoma prognosis. *J Invest Dermatol* 132:274–83

Shinozaki M, Fujimoto A, Morton DL *et al.* (2004) Incidence of BRAF oncogene mutation and clinical relevance for primary cutaneous melanomas. *Clin Cancer Res* 10:1753–7

Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 3: Article3

Thomas RK, Baker AC, DeBiasi RM *et al.* (2007) High-throughput oncogene mutation profiling in human cancer. *Nat Genet* 39:347–51

Thompson JF, Scolyer RA, Kefford RF (2009) Cutaneous melanoma in the era of molecular profiling. *Lancet* 374:362–5

Tibshirani RJ, Efron B (2002) Pre-validation and inference in microarrays. *Stat Appl Genet Mol Biol* 1: Article1

Viros A, Fridlyand J, Bauer J *et al.* (2008) Improving melanoma classification by integrating genetic and morphologic features. *PLoS Med* 5:e120

Winnepenninckx V, Lazar V, Michiels S *et al.* (2006) Gene expression profiling of primary cutaneous melanoma and clinical outcome. *J Natl Cancer Inst* 98:472–82