

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

Graham J. Mann<sup>1,2</sup>, Gulletta M. Pupo<sup>1,2</sup>, Anna E. Campain<sup>3</sup>, Candace D. Carter<sup>2</sup>, Sarah-Jane Schramm<sup>1,2</sup>, Svetlana Pianova<sup>1,2</sup>, Sebastien K. Gerega<sup>4</sup>, Chitra De Silva<sup>2,5</sup>, Kenneth Lai<sup>2,5</sup>, James S. Wilmott<sup>2,5</sup>, Maria Synnott<sup>2,5</sup>, Peter Hersey<sup>2</sup>, Richard F. Kefford<sup>1,2</sup>, John F. Thompson<sup>2,6</sup>, Yee Hwa Yang<sup>3</sup> and Richard A. Scolyer<sup>2,5,7</sup>

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 (Winnepeninckx *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).

<sup>1</sup>The University of Sydney at Westmead Millennium Institute, Westmead, New South Wales, Australia; <sup>2</sup>Melanoma Institute Australia (formerly the Sydney Melanoma Unit), Sydney, New South Wales, Australia; <sup>3</sup>School of Mathematics and Statistics, The University of Sydney, Sydney, New South Wales, Australia; <sup>4</sup>Sydney Bioinformatics, Sydney, New South Wales, Australia; <sup>5</sup>Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia; <sup>6</sup>Discipline of Surgery, The University of Sydney, Sydney, New South Wales, Australia and <sup>7</sup>Discipline of Pathology, The University of Sydney, Sydney, New South Wales, Australia

Correspondence: John F. Thompson, Melanoma Institute Australia, 40 Rocklands Road, (formerly the Sydney Melanoma Unit), Sydney, New South Wales 2060, Australia. E-mail: john.thompson@melanoma.org.au

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<br>(n=79) | Poor survival<br>group (n=25) | Good survival group<br>(n=23) | Good versus<br>poor (P-value) <sup>1</sup> |
|--|------------------------|-------------------------------|-------------------------------|--|
| 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   |
| <i>Previous primary melanoma</i>             |                        |                               |                               |  |
| 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 <sup>2</sup> )     | 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                                       |
| <i>Nodal tumor analyzed</i>                  |                        |                               |                               |  |
| 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                                       |
| <i>Mutation status</i>                       |                        |                               |                               |  |
| 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.

<sup>1</sup>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

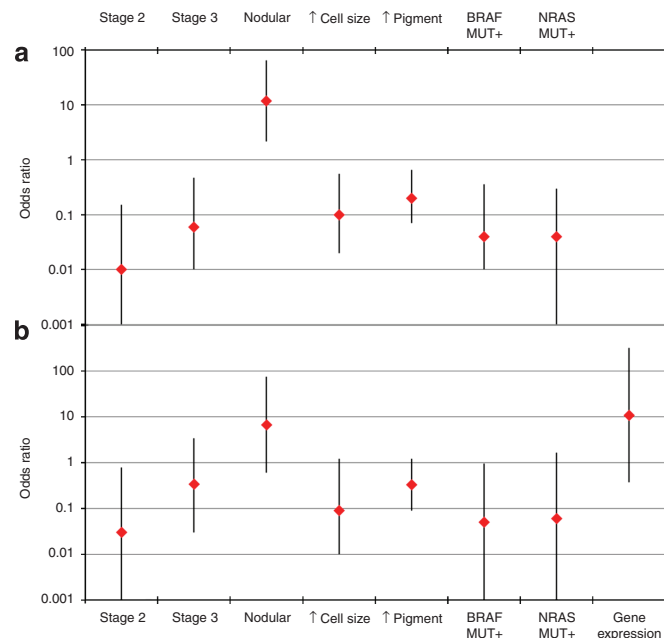
lowest to highest map significance *P*-value, are shown in Supplementary Table S1 online.

### 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; Shinzaki *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                      |
|-----------------------------|-----------|---|-------------------------------|
| <i>ADAMDEC1</i>             | 27,299    | ADAM-like, decysin 1  | ILMN_2103107                  |
| <i>ALDH1A3</i> <sup>1</sup> | 220       | Aldehyde dehydrogenase 1 family, member A3  | ILMN_1807439;<br>ILMN_2139970 |
| <i>ALOX5</i>                | 240       | Arachidonate 5-lipoxygenase   | ILMN_1680996                  |
| <i>APOL3</i>                | 80,833    | Apolipoprotein L, 3   | ILMN_1756862                  |
| <i>BIRC3</i>                | 330       | Baculoviral IAP repeat-containing 3   | ILMN_1776181                  |
| <i>CCL5</i> <sup>1</sup>    | 6,352     | Chemokine (C-C motif) ligand 5  | ILMN_1773352;<br>ILMN_2098126 |
| <i>CCL8</i>                 | 6,355     | Chemokine (C-C motif) ligand 8  | ILMN_1772964                  |
| <i>CD2</i>                  | 914       | CD2 molecule  | ILMN_1695025                  |
| <i>CD247</i> <sup>1</sup>   | 919       | CD247 molecule  | ILMN_2377669;<br>ILMN_1676924 |
| <i>CD3D</i> <sup>1</sup>    | 915       | CD3d molecule, delta (CD3–TCR complex)  | ILMN_2325837;<br>ILMN_2261416 |
| <i>CD52</i>                 | 1,043     | CD52 molecule   | ILMN_2208903                  |
| <i>CD79A</i>                | 973       | CD79a molecule, immunoglobulin-associated alpha   | ILMN_1734878                  |
| <i>CD8A</i> <sup>1</sup>    | 925       | CD8a molecule   | ILMN_2353732;<br>ILMN_1768482 |
| <i>CXCL10</i>               | 3,627     | Chemokine (C-X-C motif) ligand 10   | ILMN_1791759                  |
| <i>CXCL9</i>                | 4,283     | Chemokine (C-X-C motif) ligand 9  | ILMN_1745356                  |
| <i>EPSTI1</i>               | 94,240    | Epithelial stromal interaction 1 (breast)   | ILMN_2388547                  |
| <i>GBP1</i> <sup>1</sup>    | 2,633     | Guanylate binding protein 1, interferon-inducible, 67 kDa                                 | ILMN_1701114;<br>ILMN_2148785 |
| <i>GBP2</i>                 | 2,634     | Guanylate binding protein 2, interferon-inducible   | ILMN_1774077                  |
| <i>GBP4</i>                 | 115,361   | Guanylate binding protein 4   | ILMN_1771385                  |
| <i>GBP5</i>                 | 115,362   | Guanylate binding protein 5   | ILMN_2114568                  |
| <i>GIMAP4</i>               | 55,303    | GTPase, IMAF family member 4  | ILMN_1748473                  |
| <i>GZMA</i>                 | 3,001     | Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)              | ILMN_1779324                  |
| <i>GZMB</i>                 | 3,002     | Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)              | ILMN_2109489                  |
| <i>HLA-DPA1</i>             | 3,113     | Major histocompatibility complex, class II, DP alpha 1                                    | ILMN_1772218                  |
| <i>HLA-DQA1</i>             | 3,117     | Major histocompatibility complex, class II, DQ alpha 1                                    | ILMN_1808405                  |
| <i>HLA-DRB3</i>             | 3,125     | Major histocompatibility complex, class II, DR beta 3                                     | ILMN_1717261                  |
| <i>HLA-DRB4</i>             | 3,126     | Major histocompatibility complex, class II, DR beta 4                                     | ILMN_1752592                  |
| <i>IGJ</i>                  | 3,512     | Immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides | ILMN_2105441                  |
| <i>IRF8</i>                 | 3,394     | Interferon regulatory factor 8  | ILMN_1666594                  |
| <i>IRX3</i>                 | 79,191    | Iroquois homeobox 3   | ILMN_1811468                  |
| <i>LCP1</i>                 | 3,936     | Lymphocyte cytosolic protein 1 (L-plastin)  | ILMN_1662932                  |
| <i>LOC652493</i>            | 652,493   | Ig kappa chain V-I region HK102-like  | ILMN_1739508                  |
| <i>LOC652694</i>            | 652,694   | Similar to Ig kappa chain V-I region HK102 precursor                                      | ILMN_1680274                  |
| <i>LYZ</i>                  | 4,069     | Lysozyme  | ILMN_1815205                  |
| <i>MAGEC2</i>               | 51,438    | Melanoma antigen family C, 2  | ILMN_2088876                  |
| <i>MS4A6A</i>               | 64,231    | Membrane-spanning 4-domains, subfamily A, member 6A                                       | ILMN_1797731                  |
| <i>PLEK</i>                 | 5,341     | Pleckstrin  | ILMN_1795762                  |
| <i>PLEKHB1</i>              | 58,473    | Pleckstrin homology domain containing, family B (evectins) member 1                       | ILMN_1783231                  |
| <i>PMEL</i>                 | 6,490     | Premelanosome protein   | ILMN_1665994                  |
| <i>QPCT</i>                 | 25,797    | Glutamyl 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 <sup>1</sup> | 6,772     | Signal transducer and activator of transcription 1, 91 kDa                              | ILMN_1691364;<br>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                  |

<sup>1</sup>Gene identified among the top-ranked differentially expressed genes from two separate probes.

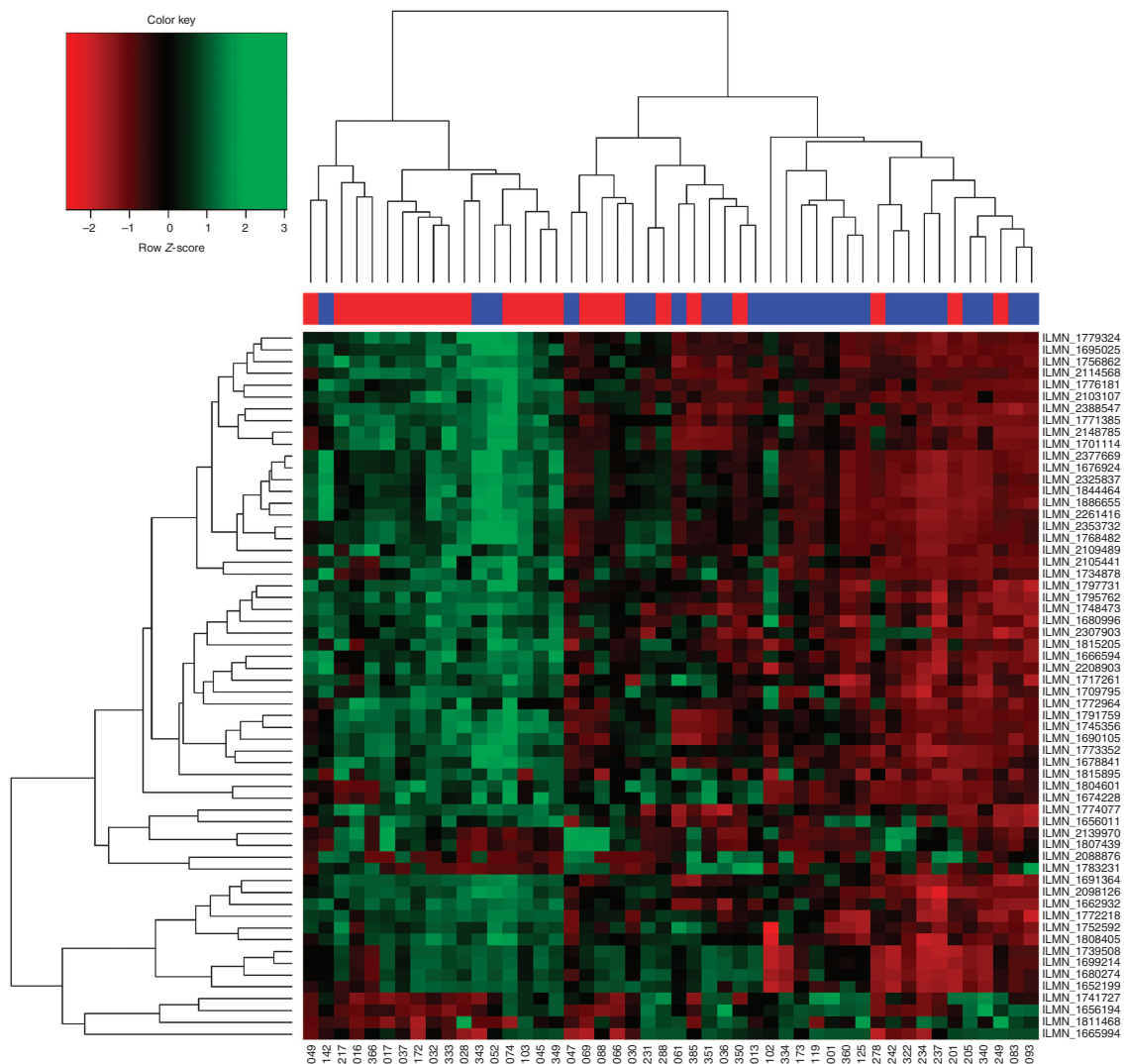
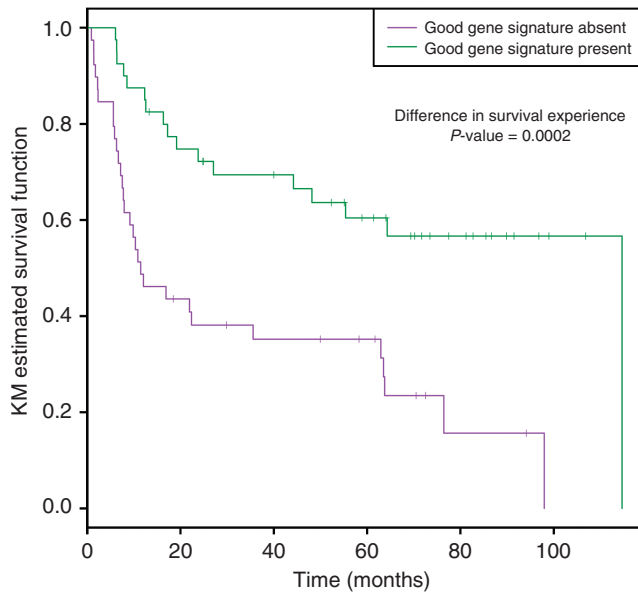


Figure 2. Supervised hierarchical clustering gene expression based on the 60-probe (46 gene) signature with strongest association with prognosis in 48 American Joint Committee on Cancer (AJCC) stage III melanoma patients.

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**

| Feature selection method                         | Number of selected genes | Independent validation, logFC used to rank genes from the two public data sets |                                 |
|--|--------------------------|--|---------------------------------|
|  |                          | John <i>et al.</i> (2008)  | Bogunovic <i>et al.</i> (2009b) |
| 2-Fold change <sup>1</sup>                       | 111                      | >0.001   | 0.004                           |
| 2-Fold change using robust modeling <sup>2</sup> | 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.

<sup>1</sup>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).

<sup>2</sup>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](http://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>

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