Melanoma is estimated to be the fifth most common cancer in the United States in 2009 (1). The unpredictable behavior of melanoma has prompted the search for prognostic factors to better predict its outcome. The vertical thickness of the primary tumor consistently emerges as a dominant prognostic factor for melanoma but does not account adequately for its heterogeneity. Numerous clinical and histologic prognostic factors have been examined for their ability to predict melanoma progression (2). Ulceration was included in the American Joint Cancer Committee (AJCC) staging classification for cutaneous melanoma because of its independent effect on melanoma survival (3, 4). Despite this development, further advances in the prognostic assessment of melanoma are still essential to improve prognostic predictions for all patients diagnosed with melanoma.

One such approach to improving the prognostic assessment of cancer is the use of molecular markers. In the genomic era, the sequencing of the human genome and the availability of genome-wide approaches to interrogate the malignant phenotype have raised the promise that molecular markers will be routinely incorporated into the clinical assessment of cancer patients. Recent studies have shown the efficacy of multimarker prognostic assays for several malignancies, including breast cancer, lung cancer, and B-cell lymphomas (5–7). To date, no molecular markers are routinely used in the prognostic assessment of melanoma patients. Gene expression profiling analyses of melanoma have identified a plethora of putative biomarkers (8–10). However, the prognostic significance of these gene signatures has not been validated. Three markers (NCOA3, SPP1, and RGS1) derived from a cDNA microarray study conducted by our group have been shown to play an
Translational Relevance

This article describes the prognostic role of an immunohistochemical assay combining the expression levels of three molecular markers for cutaneous melanoma derived from gene expression profiling studies. The multimarker assay described here was an independent predictor of disease-specific survival in two distinct cohorts and could be used to identify patients at higher risk of relapse or death that may be candidates for sentinel lymph node biopsy or adjuvant therapy.

Materials and Methods

Study population. We previously assessed expression levels of NCOA3, SPP1, and RGS1, separately, on a primary melanoma tissue microarray obtained from a retrospective cohort of University of California-San Francisco patients with at least 2 years of follow-up or documented relapse or following sentinel lymph node (SLN) biopsy. All patients underwent wide excision of their primary melanoma and, where indicated, SLN biopsy. This study focuses on the 395 of these patients on whom marker expression data were available. According to the Reporting Recommendations for Tumour Marker Prognostic Studies guidelines (14), the breakdown of tumor thickness within this tissue microarray cohort was as follows: T1 (<1.0 mm), 5%; T2 (1.01-2.0 mm), 33%; T3 (2.01-4.0 mm), 28%; and T4 (>4.0 mm), 34%. The median age of this cohort was 53, with males comprising 65% of the patients. Median follow-up times were 68 and 57 months, respectively.

The prognostic effect of the multimarker assay was separately evaluated in a cohort of melanomas with defined histology and follow-up (11-13). NCOA3 (also known as AIB1 or SRC-3) is a member of the steroid receptor coactivator 1 family. SPP1 (also known as osteopontin) is a secreted integrin-binding protein implicated in the progression of several cancers. Regulator of G protein signaling 1 (RGS1) is a GTPase-activating protein and a member of the regulator of G-protein family.

In this study, we both assess the predictive efficacy of a multimarker prognostic assay combining the effects of these three biomarkers, drawn from a tissue microarray cohort of 395 patients with primary cutaneous melanoma, and evaluate its efficacy in an independent cohort of 141 patients.

Immunohistochemistry. Tissue microarray construction and immunohistochemical staining of NCOA3, SPP1, and RGS1 were carried out in the 395-patient cohort as described previously (11-13). In the case of the 141-patient Heidelberg/Kiel tissue set, immunohistochemical analysis was done on 5 μm tissue sections, and the following primary antibodies used: mouse monoclonal anti-NCOA3 IgG (Abcam; 1:10 dilution), rabbit polyclonal anti-SPP1 IgG (Abcam; 1:200 dilution), and chicken anti-RGS1 IgG (GeneTec; 1:50 dilution). All of the testing was done on paraffin-embedded tissues, requiring no special storage or handling.

Evaluation of immunohistochemical staining. For both tissue microarrays and tissue sections, in cases in which marker expression was homogeneous, the region(s) of most intense staining was scored for each tissue specimen. In the event of heterogeneity of marker expression within a tumor, the largest region of homogeneous marker expression was scored. Marker expression was graded using the following scale: no staining (0), weak staining (1), moderate staining (2), and intense staining (3). All tissue specimens in both tissue sets were scored by the same pathologist (R.W.S.) blinded to the identity of the cases as described previously (11-13).

Imaging analysis of immunohistochemical staining. In the 395-patient cohort, mean densitometric intensity for each marker was calculated for the specimen cores on tissue arrays stained using the Carl Zeiss Mirax Scan and Axiovision 4.5 image processing system as described previously (15).

Statistical analysis. Statistical methods used to assess the individual effect of various prognostic factors on melanoma outcomes were described previously in detail (11-13, 16, 17), and the coding both for clinical and for pathologic attributes was done exactly as described by the AJCC staging committee for melanoma (3). Mitotic rate was coded as c4 versus >4, which represented the optimal cut-point for this factor in the University of California-San Francisco data set. To assess the joint effect of the combination of the three biomarkers on melanoma progression, a prognostic index was developed using cut-points for overexpression or underexpression for each outcome measure [disease-specific survival (DSS) and SLN status] that provided the best prediction for that outcome measure. We used univariate Cox regression to determine the optimal cut-points for DSS and univariate logistic regression to determine the optimal cut-points for SLN status. Each marker was given a score of +1 or -1 for each lesion based on whether the marker was overexpressed or underexpressed (above or below the cut-point) for the given outcome measure. A score of 0 was given whenever no specific degree of expression could be determined by the pathologist (e.g., insufficient tissue). Then, for each lesion, a prognostic index was calculated that reflected the net score (the sum of the scores of the three
higher prevalence of SLN positivity (31.6%) than patients in a low-risk group as all patients with negative net multimarker index scores were significantly predictive of reduced DSS (P < 0.001). A high-risk group was identified as all patients in the Heidelberg/Kiel cohort with positive net multimarker index scores and a low-risk group as all patients with negative net multimarker index scores. The significance of the difference in 5-year DSS rates between high-risk and low-risk patients was assessed via the Fisher's exact test. In the digital imaging analysis, optimal cut-points for marker overexpression or underexpression using mean densitometric intensity were identified by maximizing the average of specificity and sensitivity of each separate marker in the prediction of 5-year DSS. Then, the multimarker index was created exactly as described above using data from 353 patients possessing sufficient data to construct this index. None of our conclusions depends specifically on the linearity of our multimarker index.

In the analysis of the 141-patient Heidelberg/Kiel cohort, indices of combined marker overexpression or underexpression were constructed in exactly the same way as described above for the 395-patient cohort. Data recording mitotic rate and SLN status were not available in the Heidelberg/Kiel cohort, precluding the inclusion of these factors in both univariate and multivariate models. All P values reported are two-sided.

### Results

Given the significant prognostic effect of three molecular markers (NCOA3, SPP1, and RGS1), when analyzed individually, a multimarker index was constructed to combine the separate information provided by each marker. The cumulative effect of multimarker overexpression was evaluated relative to DSS (the primary endpoint) and, where possible, SLN status. Initially, the effect of the multimarker index (using the entire 7-point scale) on melanoma outcome was examined using univariate analysis. By Cox regression analysis, the multimarker index was significantly predictive of reduced DSS (P < 0.001). Increasing multimarker index scores were significantly predictive of SLN metastasis by logistic regression analysis (P < 0.001). A high-risk group was identified as all patients in the 395-patient cohort with positive net multimarker index scores and a low-risk group as all patients with negative net multimarker scores. Patients in the high-risk group had a significantly higher prevalence of SLN positivity (31.6%) than patients in the low-risk group (5.0%; P < 0.001, Fisher’s exact test) as well as significantly reduced DSS (P < 0.001, log-rank test; Fig. 1) by Kaplan-Meier analysis. The 5-year DSS of patients in the low-risk group was 96% compared with 60% in the high-risk group (P < 0.001, Fisher’s exact test).

Next, we analyzed the effect of the multimarker index on melanoma outcome by multivariate analyses. In these multivariate models, the six factors analyzed by the AJCC staging committee for melanoma were included (tumor thickness, ulceration, Clark level of invasion, primary tumor site, sex, and age). All of the results presented follow the conventions set by the AJCC staging committee (3), in which the relative importance of prognostic factors was measured by \( \chi^2 \) values because their interpretation is unrelated to the coding of any covariate, whereas the comparison of risk ratios depends critically on the particular manner in which each covariate is separately coded. Multivariate logistic regression analysis revealed the multimarker index to be independently and significantly predictive of SLN status (P = 0.001; Table 1), following decreasing age, but with a significance greater than tumor thickness.

Then, several multivariate Cox regression analyses of DSS were done. The first analysis again included the multimarker index and the six AJCC factors. The multimarker index was independently and significantly predictive of reduced DSS (P < 0.001), emerging as the most significant predictor of DSS (Table 2). In addition, a multivariate analysis was done to include mitotic rate, an important histologic prognostic factor that will be included in the next version of the AJCC staging classification for melanoma. The multimarker expression index remained the top factor predicting DSS in this analysis, surpassing tumor thickness, ulceration, and mitotic rate (data not shown).

Subsequently, a multivariate analysis was done incorporating SLN status in addition to the six AJCC prognostic factors. In this analysis, the multimarker index remained the most significant factor predicting DSS (P = 0.002; Table 3). Whereas ulceration and SLN status were still significantly predictive of DSS, tumor thickness was no longer significant. To achieve a legitimate comparison of risk ratios between the multimarker assay and SLN status, this particular multivariate analysis was repeated with the 7-point multimarker index rescaled and reentered into the model as a dichotomous variable, representing the high-risk versus low-risk groups. The dichotomized multimarker index still emerged as the most significant factor in the revised analysis, with a higher risk ratio (3.27) than that observed for SLN status (1.75).

Given that the multimarker expression index combines the effects of three molecular markers, we compared its prognostic

### Table 1. Multivariate logistic regression analysis of the effect of various factors on SLN metastasis (n = 274)

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>( \chi^2 )</th>
<th>Odds ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>16.42</td>
<td>0.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Multimarker index</td>
<td>10.46</td>
<td>1.47</td>
<td>0.001</td>
</tr>
<tr>
<td>Tumor thickness</td>
<td>6.90</td>
<td>1.76</td>
<td>0.009</td>
</tr>
<tr>
<td>Sex</td>
<td>2.72</td>
<td>1.73</td>
<td>0.10</td>
</tr>
<tr>
<td>Clark level</td>
<td>1.55</td>
<td>1.37</td>
<td>0.21</td>
</tr>
<tr>
<td>Ulceration</td>
<td>0.94</td>
<td>1.39</td>
<td>0.33</td>
</tr>
<tr>
<td>Site</td>
<td>0.20</td>
<td>0.87</td>
<td>0.66</td>
</tr>
</tbody>
</table>

### Table 2. Multivariate Cox regression analysis of the effect of various factors on DSS—effect of the multimarker index (n = 340)

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>( \chi^2 )</th>
<th>Risk ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multimarker index</td>
<td>13.33</td>
<td>1.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Clark level</td>
<td>9.13</td>
<td>1.59</td>
<td>0.003</td>
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<tr>
<td>Ulceration</td>
<td>8.63</td>
<td>1.82</td>
<td>0.003</td>
</tr>
<tr>
<td>Site</td>
<td>2.29</td>
<td>1.35</td>
<td>0.13</td>
</tr>
<tr>
<td>Tumor thickness</td>
<td>1.12</td>
<td>1.16</td>
<td>0.29</td>
</tr>
<tr>
<td>Age</td>
<td>0.06</td>
<td>1.01</td>
<td>0.80</td>
</tr>
<tr>
<td>Sex</td>
<td>0.03</td>
<td>1.04</td>
<td>0.96</td>
</tr>
</tbody>
</table>
To our knowledge, this is the first description of an independently predictive molecular prognostic assay for primary melanoma. Moreover, it is the first study to replicate the independent prognostic effect of any molecular markers: (a) in a data set drawn from a completely different patient population (the Heidelberg/Kiel cohort), (b) across different tissue platforms (tissue microarray in the initial study cohort versus tissue sections in the replication cohort), and (c) using different measurement techniques (pathologist scoring versus digital imaging analysis in the initial study cohort). We examined the prognostic utility of this three-marker index by incorporating into the multivariate models several powerful and commonly used prognostic factors. In these analyses, we tested the multimarker expression index against the six factors included in the AJCC staging committee analyses of prognostic factors for melanoma (3, 4). In our 395-patient cohort, the multimarker index was independently predictive of DSS and SLN status with the inclusion of these six factors.

The multimarker index described here remained significantly predictive of DSS even when SLN status was included in the multivariate model. Lymph node status in general, including SLN status, is well recognized to represent a powerful predictor of melanoma survival and has been shown to be more powerful as a predictor than most routinely coded clinical and histologic factors (3, 18, 19). Nevertheless, the multimarker index was independent of SLN status, tumor thickness, and ulceration, which comprise the three most important factors in the AJCC analysis of patients with localized cutaneous melanoma. We are unaware of any other histologic or molecular factor that has been shown to have a more significant effect on DSS and to have a higher risk ratio than SLN status. Lymph node metastasis is a complex phenomenon, likely reflecting the sum total of numerous molecular events. Thus, it is of particular interest that the combined expression levels of these three markers could provide independent outcome information relative to SLN status, because their individual expression levels can be determined from assessment of the primary tumor, without the need for additional surgery or general anesthesia. We are unaware of studies in other solid tumors showing that expression of (as few as) three molecular markers analyzed in the primary tumor provides such powerful prognostic information.

The independent and powerful prognostic efficacy of the multimarker expression index in predicting DSS was confirmed using a digital imaging analysis. Importantly, the prognostic significance of the multimarker index relative to tumor thickness, ulceration, and SLN status was confirmed, substituting this densitometric analysis of individual marker expression. Taken together, these results suggest that the multimarker assay can provide useful prognostic information beyond that provided by routine clinical and histologic factors, including SLN status or AJCC stage.

The effect of the multimarker assay on DSS was separately evaluated for 141 patients drawn from a completely different population (the Heidelberg/Kiel cohort). Univariate and
T1 patients. Despite these differences, the multimarker assay undergoing SLN biopsy. However, this was not the case with the melanoma in part due to the cohort being enriched for patients un-
studywere not identical in their composition. The initial study to have independent prognostic significance with the inclu-
second of this multimarker assay in prospectively collected, population-based series are warranted to assess its more broad-based role in the prognostic assessment of distinct subsets of melanoma patients.

Many individual biomarkers (reviewed in ref. 20) have been suggested as molecular prognostic factors for melanoma, including Ki-67 and p16, which have been shown to have independent prognostic significance in distinct cohorts (21). However, to date, none of these factors has been shown to have independent prognostic significance with the inclusion of a powerful factor such as SLN status, and none has been validated in an independent cohort. Moreover, these markers were not differentially expressed in our microarray analyses, thereby precluding their inclusion in the current analysis. However, whether addition of Ki-67, p16, or other markers could improve the prognostic efficacy of the current multimarker assay could be the subject of future studies. The multimarker assay described here incorporated three novel markers identified by gene expression profiling of melanoma (8). Although transcriptome analysis has shown promise to radically alter current approaches to cancer classification and prognosis, significant challenges have prevented the routine translation of gene expression signatures into clinically useful assays. The multimarker assay presented in our study thus provides a model for the development and validation of immunohistochemical prognostic assays for human cancer derived from gene expression profiles.

In addition to their role as biomarkers, the three markers analyzed here have plausible roles in promoting melanoma progression. The genes encoding NCOA3 and RGS1 reside on chromosomal loci with gains or amplifications in human melanoma (22). SPP1 and NCOA3 may promote melanoma cell growth and metastasis by virtue of activation of the NF-κB signaling pathway (23, 24). The RGS family has been shown to be involved in regulating signaling pathways relevant to melanoma progression, including Wnt and Rho (25). We have shown previously that SPP1 expression correlated with tumor thickness, Clark level, and mitotic index (12), whereas RGS1 expression correlated with tumor thickness, mitotic index, and vascular involvement (13), suggesting mechanisms by which these markers may contribute to their prognostic effect.

The practical significance of biomarkers that show independent prognostic information lies in their potential to affect treatment decisions. Knowledge of the expression levels of RGS1, NCOA3, and SPP1 may be useful in various clinical scenarios for patients diagnosed with primary cutaneous melanoma in which routine clinical and histologic factors fail to inform such decisions. One scenario in which this multimarker assay could be used is in the selection of patients to undergo SLN biopsy (19). Our results suggest that the multimarker assay described here could be used as an adjunct to tumor thickness in the selection of patients to undergo SLN biopsy given its significant prediction of SLN status by multivariate logistic regression analysis. It will be important to replicate the independent prognostic role of this multimarker assay on SLN status in additional cohorts given that we were unable to examine this in the Heidelberg/Kiel cohort.

Secondly, these markers can identify patients at higher risk for death due to metastatic melanoma, who would then be candidates for adjuvant therapy. Significant controversy exists regarding the patient population benefiting and the magnitude of benefit derived from IFN-α2b, the only Food and Drug Administration–approved therapy for high-risk melanoma (26–30). Our results suggest the potential utility of this multimarker assay to identify high-risk patient populations to undergo IFN therapy, given its independent ability to predict DSS, even with the inclusion of SLN status or AJCC stage. Whether the multimarker assay is predictive of response to or benefit from IFN therapy awaits evaluation in cohorts of patients undergoing this treatment.

Table 5. Multivariate Cox regression analysis of the effect of various factors on DSS— the Heidelberg/Kiel cohort (n = 102)

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>$\chi^2$</th>
<th>Risk ratio</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multimarker index</td>
<td>5.93</td>
<td>1.34</td>
<td>0.01</td>
</tr>
<tr>
<td>Tumor thickness</td>
<td>4.77</td>
<td>2.17</td>
<td>0.03</td>
</tr>
<tr>
<td>Age</td>
<td>3.00</td>
<td>0.69</td>
<td>0.08</td>
</tr>
<tr>
<td>Site</td>
<td>2.69</td>
<td>0.41</td>
<td>0.10</td>
</tr>
<tr>
<td>Ulceration</td>
<td>2.46</td>
<td>2.19</td>
<td>0.12</td>
</tr>
<tr>
<td>Sex</td>
<td>1.20</td>
<td>1.85</td>
<td>0.27</td>
</tr>
<tr>
<td>Clark level</td>
<td>0.10</td>
<td>0.86</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Disclosure of Potential Conflicts of Interest

M. Kashani-Sabet has stock in Melanoma Diagnostics, Inc. The other authors disclosed no potential conflicts of interest.

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