

Osteopontin as a Molecular Prognostic Marker for Melanoma

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BACKGROUND. Osteopontin has been suggested as a marker of disease progression in patients with melanoma because of its overexpression in recent microarray analyses. However, its prognostic role in melanoma has not been fully defined.

METHODS. Osteopontin expression status was examined using immunohistochemical analysis of a tissue microarray that contained primary cutaneous melanomas from 345 patients. The correlation between osteopontin expression and several histologic markers for melanoma was assessed by using the Chi-square test and the Le directional test. The impact of osteopontin expression on recurrence-free survival (RFS) and disease-specific survival (DSS) of patients with melanoma was examined using Cox regression and Kaplan-Meier analyses. The impact of increasing osteopontin expression on sentinel lymph node (SLN) metastasis was assessed using logistic regression analysis.

RESULTS. High osteopontin expression was associated with increased tumor thickness ($P = .037$), Clark level ($P = .035$), and mitotic index ($P = .046$). Kaplan-Meier analysis demonstrated an association between osteopontin expression and reduced RFS ($P < .03$) and DSS ($P = .05$). Multivariate Cox regression analysis demonstrated that high osteopontin immunostaining had an independent impact on the DSS of this melanoma cohort ($P = .049$). In addition, osteopontin expression was significantly predictive of SLN metastasis ($P = .009$) and SLN burden, as assessed by the mean number of SLN metastases ($P = .0025$). Multivariate logistic regression analysis demonstrated an independent role for osteopontin expression in predicting SLN status ($P = .0062$).

CONCLUSIONS. The current results validated the role of osteopontin as an independent prognostic marker for melanoma and provided new evidence for its predictive role in melanoma lymph node metastasis. *Cancer* 2008;112:144-50. © 2007 American Cancer Society.

KEYWORDS: osteopontin, prognostic modeling, melanoma, molecular markers.

Osteopontin (also known as secreted phosphoprotein-1 or SPP1) is a secreted, integrin-binding protein that has been implicated in the progression of various cancers, including lung, breast, prostate, and colon cancer, as well as melanoma (for reviews, see Rittling and Chambers, 2004¹ and Rangaswami et al., 2006²). Initial studies demonstrated increased levels of osteopontin upon malignant transformation,³ and subsequent analyses demonstrated a correlation between osteopontin expression and metastatic potential.^{4,5} Studies using human tumor specimens have suggested the importance of osteopontin as a tumor progression marker, because increased expression of osteopontin has been correlated with advancing stage or worsened survival in several epithelial malignancies.⁶⁻⁸

In melanoma, several in vitro studies using melanoma cell lines have implicated osteopontin in disease progression.^{9,10}

The potentially important role of osteopontin in melanoma progression has been supported by several recent microarray analyses of melanoma.^{10–13} Two of those analyses independently reported that the osteopontin gene was overexpressed significantly in primary or metastatic melanomas compared with nevi.^{10,12} In 2 additional studies, osteopontin reportedly was expressed differentially in advanced-stage melanomas that had a more invasive phenotype.^{11,13} Taken together, these studies suggest that osteopontin may represent an important molecular marker of melanoma progression and outcome. However, to date, the prognostic role of osteopontin in melanoma has not been demonstrated convincingly. In the current study, we analyzed the role of osteopontin expression in predicting the outcome associated with melanoma in a tissue microarray that contained a large cohort of primary melanoma specimens.

MATERIALS AND METHODS

Characterization and Construction of Melanoma Tissue Microarray

We constructed a tissue microarray of 345 primary melanomas with the following patient-eligibility criteria: ≥ 2 years of follow-up, a documented relapse, or sentinel lymph node (SLN) biopsy. The demographic breakdown of the cohort appears in Table 1. The dates of initial melanoma diagnosis ranged from 1985 to 2005. The histologic prognostic factors analyzed all were established on sections from the initial tumor excision and were read prospectively at the time of the original melanoma diagnosis. Of the 345 patients, information regarding SLN status was available in 256 patients who underwent SLN biopsy. The criteria for undergoing SLN biopsy have been reported previously.¹⁴ The mean follow-up of this cohort was 49 months, and the median follow-up was 44 months. This molecular prognostic factor analysis was approved by the Committee on Human Research. The tissue microarrays were constructed as described previously¹⁵ by taking tissue cores that measured 1.0 mm in greatest dimension from the paraffin blocks.¹⁵

Immunohistochemistry

Slides were deparaffinized, rehydrated in xylene, then microwaved in 10 mmol/L citrate buffer. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and then incubated with normal goat serum blocking reagent. Then, the primary antibody,

TABLE 1
Summary of Patient Demographics and Osteopontin Score by Clinical or Histologic Variable*

Demographic variable	No. of patients (%)	Osteopontin score, %			
		0	1	2	3
Age, y					
<60	218 (63.2)	10.1	28	40.8	21.1
≥ 60	127 (36.8)	12.6	35.4	36.2	15.8
Median, y	53				
Sex					
Men	223 (64.6)	11.2	29.2	42.2	17.5
Women	122 (35.4)	10.7	33.6	33.6	22.1
Anatomic location					
Head and neck	59 (17.1)	15.3	44.1	28.8	11.9
Trunk	144 (41.7)	10.4	26.4	38.2	25
Extremity	142 (41.2)	9.8	29.6	44.4	16.2
Histologic tumor type					
Superficial spreading	165 (47.8)	13.9	28.5	38.2	19.4
Nodular	121 (35.1)	5.8	26.5	43	24.8
Acral	20 (5.8)	5	60	35	0
Desmoplastic	10 (2.9)	10	60	20	10
Lentigo maligna	9 (2.6)	11.1	44.4	44.4	0
Not otherwise classified	20 (5.8)	25	25	35	15
Tumor thickness					
T1 (≤ 1 mm)	15 (4.4)	6.7	26.7	53.3	13.3
T2 (1.01–2 mm)	106 (30.7)	12.3	28.3	43.4	16
T3 (2.01–4 mm)	101 (29.3)	14.9	25.7	41.6	17.8
T4 (> 4 mm)	121 (35)	7.4	38	30.6	24
Not available	2 (0.6)	0	0	100	0
Median, mm	2.7				
Mean, mm	3.98				

* Percentages may not total 100 because of rounding.

rabbit polyclonal antiosteopontin immunoglobulin G (IgG) (no. ab8448; Abcam, Cambridge, Mass) was added at a 1:200 dilution and incubated overnight at 4°C. Biotinylated goat antirabbit IgG antibody (Vector Laboratories, Burlingame, Calif) was used as a secondary antibody for amplification, followed by incubation with avidin-biotin complex-horseradish peroxidase (Vector Laboratories) for 30 minutes, and diaminobenzidine/hydrogen peroxide solution (Sigma; St. Louis, Mo).

Evaluation of Immunohistochemical Staining

The regions of most intense staining were scored for each tissue array core. Expression of osteopontin protein was graded from 0 to 3 according to the following scale: 0, no staining; 1, weak staining; 2, moderate staining; 3, intense staining. The arrays were scored by a pathologist (R.W.S.) who was blinded to the identity of the patients, and each score was replicated by a separate, independent scoring trial. A consensus score was determined for the few instances of discrepant scoring across replicated trials. Specificity

positive controls for osteopontin staining included breast tumor, melanoma cell lines (LOX and FEM), and melanoma tissue sections. The negative control that was used for immunohistochemistry included the use of phosphate-buffered saline instead of primary antibody.

Statistical Analyses

Statistical methods for assessing the significance of various prognostic factors on outcome in patients with melanoma were described previously,¹⁴⁻¹⁷ and the histologic attributes were analyzed as follows: high mitotic index ($>4/\text{mm}^2$) versus low mitotic index ($\leq 4/\text{mm}^2$) and absent versus present ulceration, microsatellites, vascular involvement, and regression. Relapse-free survival (RFS) was defined as the interval between the date of diagnosis and date of first relapse. The cutoffs for predictive value of osteopontin expression were selected on the basis of univariate analyses, and the same cutoffs were used for multivariate analyses. For SLN status and RFS, the cutoffs used were no osteopontin expression (score of 0) versus osteopontin expression (score of 1, 2, or 3). For disease-specific survival (DSS), the cutoffs used were low osteopontin expression (score of 0 or 1) versus high osteopontin expression (score of 2 or 3). The impact of osteopontin expression on the risk of relapse or disease-specific death was analyzed by using the Mann-Whitney test (corrected for tied observations). The association between osteopontin expression and SLN metastasis was assessed using the Fisher exact test and logistic regression. The associations between osteopontin expression and 1) mean tumor thickness, 2) mean mitotic index, and 3) mean SLN count were assessed by using the directional Le test.¹⁸ With the exception of these directional analyses, all *P* values reported are 2 sided.

RESULTS

To assess the significance of osteopontin as a prognostic marker for primary cutaneous melanoma, we analyzed osteopontin expression by using a commercially available antibody that targets human osteopontin in a tissue microarray that contained 345 primary melanoma cores. The intensity of osteopontin immunostaining was scored on a 4-point scale (from 0 to 3) (Fig. 1). A score of 0 was observed in 38 cores (11%), a score of 1 was observed in 106 cores (30.7%), a score of 2 was observed in 135 cores (39.1%), and a score of 3 was observed in 66 cores (19.2%).

Initially, we assessed the potential correlation between osteopontin expression and several histolo-

gic markers for melanoma. Increasing osteopontin expression was associated significantly with increasing tumor thickness, Clark level, and mitotic index. The mean tumor thickness in cores with an osteopontin immunostaining score of 0 was 3.05 mm; this increased to 3.93 mm in cores with scores of 1 or 2 and to 4.71 mm in cores with a score of 3 ($P = .037$; Le directional test). Increasing osteopontin scores also were associated with a higher Clark level ($P = .035$; Chi-square test). Finally, increasing osteopontin expression levels were correlated with a higher mitotic index, because the mean mitotic index increased from 4.16 mitoses/ mm^2 in cores with an osteopontin immunostaining score of 0 to 5.52 mitoses/ mm^2 in cores with an osteopontin score of 3 ($P = .046$; Le directional test). There were no other statistically significant correlations between osteopontin expression and other clinical or histologic prognostic factors (Table 1 and data not shown).

Next, we analyzed the association between osteopontin expression levels and relapse. Osteopontin expression (defined as a score of 1, 2, or 3) was correlated with an increased of risk relapse, increasing from 32% in patients with no osteopontin expression to 51% in patients with some osteopontin expression ($P < .03$; Mann-Whitney test). In addition, osteopontin expression was associated with significantly reduced RFS ($P < .03$; log-rank test) (Fig. 2A) in a Kaplan-Meier analysis.

We also evaluated the impact of osteopontin expression on DSS. High osteopontin expression (defined as a score of 2 or 3) was associated with reduced DSS ($P = .05$; log-rank test) (Fig. 2B) in our cohort of patients with melanoma in Kaplan-Meier analysis. The risk of death from melanoma for patients with low osteopontin expression was 22%, compared with 33% for patients with high osteopontin expression ($P = .03$; Mann-Whitney test). Multivariate Cox regression analysis of DSS was performed and included the osteopontin expression level and the 6 clinical and histologic prognostic factors that have been evaluated by the American Joint Committee on Cancer Staging Committee for Melanoma.^{19,20} In this analysis, high osteopontin expression emerged as an independent predictor of DSS after Clark level and ulceration (Table 2). With the inclusion of the osteopontin expression level, tumor thickness was not predictive of DSS in this cohort.

In addition, we examined the association between osteopontin immunostaining and SLN status. Osteopontin expression was associated significantly with SLN metastasis in univariate logistic regression analysis ($P = .009$). Osteopontin expression also was

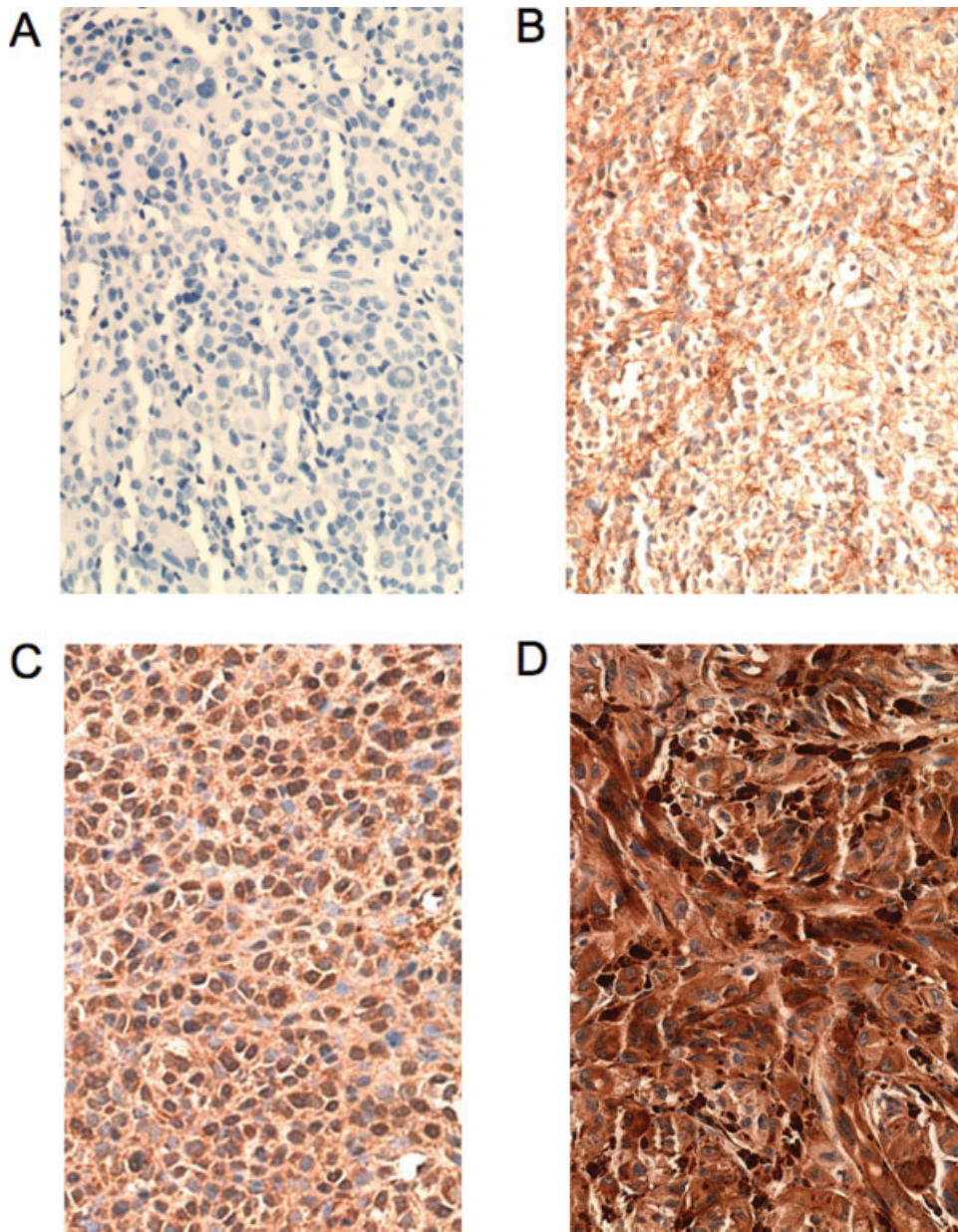


FIGURE 1. These photomicrographs of primary melanoma demonstrate osteopontin immunostaining scores of 0 (A), 1 (B), 2 (C), and 3 (D) (original magnification, $\times 200$).

correlated significantly with an increased risk of positive SLN status. Thus, among the patients who had an osteopontin score of 0, SLN metastasis was observed in 8.8%; this increased to 32.9% in patients who had a score of 1, 2, or 3 ($P = .0082$; Fisher exact test). Osteopontin expression also was correlated with increased SLN metastatic burden, as determined by the mean number of positive SLNs. In patients who had an osteopontin immunostaining score of 0, the mean number of involved lymph nodes was 0.15. This increased to a mean of 0.53 lymph nodes in

patients who had a score of 1, 2, or 3 ($P = .0025$; T test).

Finally, we assessed the predictive value of osteopontin expression level on SLN status by using multivariate logistic regression analysis. Osteopontin expression was predictive both significantly and independently of SLN metastasis with the inclusion of the 6 aforementioned prognostic factors after age and tumor thickness (Table 3). Given the powerful impact of the osteopontin expression level on SLN status, an additional multivariate analysis was

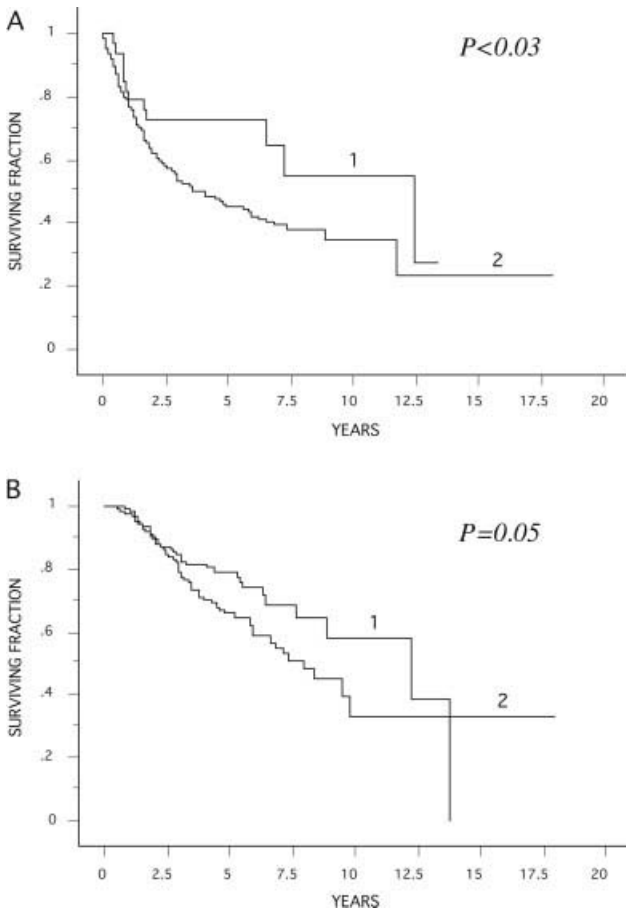


FIGURE 2. (A) Kaplan-Meier analysis of recurrence-free survival according to no osteopontin expression (score of 0, curve 1) versus the presence of osteopontin expression (score of 1, 2, or 3, curve 2). (B) Kaplan-Meier analysis of disease-specific survival in patients with low osteopontin expression levels (score of 0 or 1, curve 1) versus patients with high osteopontin expression levels (score of 2 or 3, curve 2).

TABLE 2
Cox Regression Analysis of the Impact of Various Factors on Disease-specific Survival in the Melanoma Cohort

Prognostic factor	Risk ratio	Chi-square test	P
Clark level	1.56	6.74	.009
Ulceration	1.69	5.58	.018
Osteopontin score (2 and 3 vs 0 and 1)	1.55	3.88	.049
Tumor thickness	1.26	2.73	.098
Site	1.35	1.80	.18
Sex	1.06	0.06	.81
Age	1.00	0.001	.97

performed that included 5 histologic factors that have not been analyzed by the American Joint Committee on Cancer Staging Committee for Melanoma, namely, mitotic index, degree of tumor vascularity, and the presence or absence of vascular involvement,

TABLE 3
Logistic Regression Analysis of the Impact of Various Prognostic Factors on Sentinel Lymph Node Metastasis

Prognostic factor	Chi-square test	P	Odds ratio
Decreasing age	14.55	.0001	.66
Tumor thickness	10.18	.0014	1.98
Osteopontin score (1, 2, and 3 vs 0)	7.50	.0062	8.59
Sex	2.07	.15	1.64
Clark level	1.77	.18	1.63
Site	0.93	.33	.73
Ulceration	0.53	.47	1.28

TABLE 4
Logistic Regression Analysis of the Impact of All Available Prognostic Factors on Sentinel Lymph Node Metastasis

Prognostic factor	Chi-square test	P	Odds ratio
Decreasing age	6.40	.011	.72
Vascular involvement	6.24	.0125	2.96
Tumor thickness	6.19	.013	1.96
Osteopontin score (1, 2, and 3 vs 0)	5.94	.015	7.63
Sex	2.64	.10	1.98
Microsatellites	2.55	.11	2.80
Site	1.97	.16	.57
Clark level	0.79	.37	1.46
Regression	0.65	.42	.57
Mitotic index	0.36	.55	1.27
Tumor vascularity	0.28	.60	.79
Ulceration	0.13	.72	.85

microsatellites, and regression. Osteopontin overexpression remained significantly predictive of SLN status with the inclusion of all 12 factors in the model (Table 4). Step-wise logistic regression of these 12 factors revealed that decreasing age ($P < .001$), tumor thickness ($P < .001$), and osteopontin immunostaining score ($P = .0066$) were the only factors to remain significantly predictive of SLN status.

DISCUSSION

In this study, we examined the prognostic impact of osteopontin overexpression in the outcome associated with melanoma in a large tissue microarray cohort of primary melanoma specimens with defined histology and follow-up. Increasing osteopontin immunostaining was associated with increasing tumor thickness, Clark level of invasion, and mitotic index, which represent well-known histologic prognostic markers for cutaneous melanoma. Osteopontin expression was associated with decreased RFS and DSS in this melanoma cohort. In multivariate analysis, osteopontin expression level was an independent predictor of DSS and had a stronger impact than

tumor thickness. Unexpectedly, increasing osteopontin expression was identified as a powerful and independent predictor of SLN metastasis.

The potentially important role of osteopontin in melanoma proliferation, invasion, and metastasis has been suggested previously by a growing body of *in vitro* evidence. It has been demonstrated that osteopontin plays an important role in melanoma growth and adhesion through activation of the nuclear factor κ B (NF- κ B) signaling pathway,^{9,21} which, itself, has a demonstrated role in melanoma progression and metastasis.^{13,22,23} Specifically, osteopontin induced cell motility, tumor growth, and NF- κ B-mediated activation of matrix metalloproteinases through the phosphatidylinositol 3-kinase/I- κ B kinase/Akt signaling pathways.^{9,21}

More recently, osteopontin has consistently appeared as a differentially expressed gene in several gene expression profiling studies using different array platforms and clinical samples.¹⁰⁻¹³ It was observed that osteopontin was expressed differentially in the original profiling analysis of metastatic melanomas with increased invasiveness.¹¹ In our own analysis of the different phases of melanoma progression, osteopontin was the top gene in the statistical analysis of microarrays comparing nevi with primary melanomas.¹² Three other profiling analyses demonstrated the overexpression of osteopontin in advanced melanomas compared with earlier stage lesions.^{10,13,24} Recently, osteopontin was identified as a key factor in the epithelial-mesenchymal transition from non-metastatic to metastatically competent melanoma cells.²⁵ Taken together, these studies, along with a consensus panel at the Markers and Tissue Resources for Melanoma Meeting convened by the National Cancer Institute,²⁶ have suggested an important role for osteopontin as a tumor progression marker in melanoma. However, evidence to support its role as a novel molecular prognostic marker for melanoma has been lacking.

To our knowledge, the current study shows for the first time that there is an independent role for osteopontin in the prognosis associated with melanoma. Osteopontin expression was associated significantly with RFS and DSS in this cohort. These studies represent an important confirmation of the gene expression profiling studies performed by various groups in melanoma, and they suggest the relevance of the genes identified by such analyses as plausible melanoma progression genes. The potential role of osteopontin as a molecular prognostic marker also has been supported by studies showing an association between osteopontin expression and advancing tumor stage in various epithelial malignancies.⁶⁻⁸

However, few of those studies demonstrated an independent prognostic role for osteopontin when complete clinical and histologic prognostic factors were included in multivariate models.

It is noteworthy that osteopontin appeared to have its greatest prognostic impact in predicting SLN metastasis, because our results indicated a direct link between osteopontin expression and the risk of lymph node metastasis as well as SLN tumor burden. Consequently, osteopontin expression may prove useful in selecting patients to undergo SLN biopsy, which is a powerful predictor of melanoma prognosis that may identify candidates for adjuvant therapy. Because the data set amassed for the current analysis contained a high proportion of patients undergoing SLN biopsy, it may result in selection bias. Thus, the impact of osteopontin expression may be most relevant to patients who are candidates for SLN biopsy. Further studies of the prognostic role of osteopontin in population-based series are warranted to assess the more broad-based role of osteopontin as a molecular marker of melanoma outcome.

The link between osteopontin expression and SLN metastasis reported here is consistent with a recent study that analyzed breast cancer specimens and animal models and suggested a role for osteopontin in lymphatic metastasis of breast cancer.²⁷ In that study, osteopontin protein levels were significantly higher in lymph node metastases than in the primary tumors. Moreover, human breast cancer cells that overexpressed osteopontin exhibited increased lymphovascular invasion and axillary lymph node metastasis when they were injected into the mammary fat pad. Our results have extended those studies by demonstrating that increasing osteopontin immunostaining in primary melanomas was associated with an increased risk of SLN metastasis and SLN tumor burden and was an independent predictor of SLN status. Taken together, these studies strongly suggest osteopontin as both an important molecular mediator and an important predictive marker of lymphatic metastasis both in melanoma and breast cancer.

The current results also shed light on the potential mechanisms by which osteopontin may promote tumor metastasis in melanoma. Increasing osteopontin immunostaining was correlated significantly with tumor thickness, Clark level of invasion, and mitotic index, suggesting a role for osteopontin in melanoma cell invasiveness and proliferation. These results are consistent with prior studies from both *in vitro* and *in vivo* models demonstrating a role for osteopontin in these cellular pathways.^{9,10,21} Osteopontin has been correlated with melanoma invasion

in a prior study,¹⁰ it has been implicated in mediating tumor cell adhesion,²⁰ and others have demonstrated that it is important for melanoma cell proliferation.^{9,10,21}

In summary, the current results, which were derived from a large cohort of patients with primary melanoma, define a role for osteopontin as an independent molecular prognostic marker for melanoma. Moreover, they represent an important validation of the multiple gene expression profiling efforts that identified osteopontin as a potential melanoma progression gene. Finally, these studies identify a novel role for osteopontin as an independent molecular predictor of melanoma lymph node metastasis, suggesting its potential clinical relevance in identifying candidates to undergo lymphatic mapping and SLN biopsy.

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