Complete Loss of PTEN Protein Expression Correlates with Shorter Time to Brain Metastasis and Survival in Stage IIIB/C Melanoma Patients with B\textit{RAF}\textsuperscript{V600} Mutations

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Abstract

**Purpose:** Loss of function of PTEN is a frequent event in melanoma, particularly in tumors with B\textit{RAF}\textsuperscript{V600} mutations. The prevalence, pathologic features, and clinical outcomes associated with PTEN loss in patients with stage IIIB/C melanoma were interrogated to improve our understanding of the clinical significance of this molecular event.

**Experimental Design:** Archival tissue from lymphadenectomy specimens among patients (n = 136) with stage IIIB or IIIC melanoma was assessed by DNA sequencing for activating B\textit{RAF} and NRAS mutations, and by immunohistochemistry for the expression of PTEN protein. Associations of these molecular aberrations with demographics, tumor characteristics, and clinical outcomes were determined.

**Results:** The prevalence of B\textit{RAF}\textsuperscript{V600} mutations (40% overall), NRAS mutations (10%), and PTEN loss (25%) did not vary by pathologic substage. B\textit{RAF}/NRAS mutation status did not correlate with distant disease-free survival (DDFS) or overall survival (OS). Complete loss of PTEN expression correlated with shorter OS but not DDFS. When stratified by specific sites of distant metastasis, PTEN loss was associated with significantly shorter time to melanoma brain metastasis (MBM), but not to liver, lung, or bone metastasis. Analysis of PTEN in mutationally defined subsets showed that PTEN loss was significantly associated with OS and time to MBM in patients with B\textit{RAF}\textsuperscript{V600} mutations.

**Conclusions:** Loss of PTEN protein expression correlates significantly with decreased OS and time to MBM in stage IIIB/C melanoma patients with B\textit{RAF}\textsuperscript{V600} mutations. The findings add to evidence supporting a significant role for PTEN loss and the PI3K–AKT pathway in melanoma.

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Introduction

The clinical management of melanoma is evolving rapidly due to improved understanding of the molecular drivers of this disease. Substitutions affecting the V600 residue of the B\textit{RAF} serine-threonine kinase are the most common activating mutation detected in patients with cutaneous melanoma (40%–45%; refs. 1, 2). B\textit{RAF}\textsuperscript{V600} mutations increase the kinase activity of B\textit{RAF} and result in constitutive activation of the RAS–RAF–MAPK pathway. Recurrent point mutations affecting residues G12, G13, and Q61 of NRAS are the second most common activating event in melanoma (∼20%; ref. 2). Previous studies have demonstrated that NRAS mutations are essentially mutually exclusive with B\textit{RAF}\textsuperscript{V600} mutations, likely due to the fact that they also activate signaling through RAS-RAF-MAPK (3, 4). In addition to the high prevalence of oncogenic mutations, the importance of this pathway in melanoma is supported by the clinical results observed with inhibitors targeting it. Mutation-selective B\textit{RAF} inhibitors (vemurafenib and dabrafenib) and MEK inhibitors (trametinib) have been approved by the FDA for the treatment of patients with metastatic melanoma with B\textit{RAF}\textsuperscript{V600} mutations (5–7). MEK inhibitors have also demonstrated clinical activity in patients with activating NRAS mutations, and clinical trials of MEK inhibitors alone and in combination with other agents are planned and/or ongoing (8). Mutant RAS proteins utilize multiple signaling pathways to mediate their oncogenic effects. One such pathway is the...
Translational Relevance

Multiple clinical trials with new agents are now underway or planned for patients with regional metastases from melanoma. The appropriate design and interpretation of these and future investigations will be strengthened by developing an improved understanding of the clinical characteristics and outcomes associated with molecular aberrations in this disease. This integrated analysis of BRAF and NRAS mutations, PTEN loss, and clinical outcomes in patients with stage IIIB/C disease adds to growing evidence that melanomas with loss of PTEN represent a clinically significant subpopulation. PTEN loss was associated with shorter time to brain metastasis and overall survival specifically in melanomas with concurrent BRAFV600E mutations, thus supporting functional interactions observed preclinically between these oncogenic events. The novel association of PTEN loss with shorter time to brain metastasis also supports the rationale behind further examination of the role of the PI3K–AKT pathway in this common and devastating complication of advanced melanoma.

PI3K–AKT pathway: In addition to NRAS mutations, this pathway can be activated in melanoma by loss of function of the phosphatase PTEN (9). Genetic analyses of melanoma cell lines and clinical specimens have demonstrated that inactivating mutations and deletions of the PTEN gene are relatively rare (~10%), and are largely mutually exclusive with NRAS mutations in melanoma, but frequently overlap with BRAFV600E mutations (3, 4). Loss of PTEN mRNA and protein expression has been detected more frequently (20%-30%), potentially due to epigenetic mechanisms (10-12). Our previous quantitative proteomic analyses of cell lines and frozen tumor samples in multiple tumor types have shown that loss of PTEN protein expression correlates with greater activation of the AKT and other PI3K pathway effectors than a number of other oncogenic events, including PIK3CA and NRAS mutations (9, 13). Multiple inhibitors against targets in the PI3K–AKT pathway are in various phases of clinical testing in melanoma and other malignancies (14).

In addition to serving as therapeutic targets, molecular aberrations can impact patient management in cancer by improving risk models of disease progression and mortality. Several previous studies have examined the clinical associations and prognostic significance of BRAFV600E and NRAS mutations in primary melanomas (15-19). We and others have also reported their prevalence and prognostic significance in patients with stage IV melanoma (20, 21). However, currently there is very little information about the clinical significance of BRAF, NRAS, or PTEN in patients with stage III melanoma. Patients with stage III melanoma have highly variable outcomes, and currently there are no validated molecular markers that add to existing prognostic models utilizing clinical and pathologic features (22).

identification of markers that could improve risk stratification in patients with stage III melanoma is important, as the only currently approved adjuvant therapies for these patients (IFNα-2B and pegylated-IFNα-2b) are given for prolonged periods of time and have significant associated toxicities. Furthermore, evaluating the prognostic significance of such markers in patients with stage III melanoma will also strengthen the design and interpretation of new clinical trials that have been designed for this population based on observed activity in patients with stage IV disease.

To test the hypothesis that molecular markers can improve existing prognostic models, and to improve our understanding of their potential role in the pathogenesis of this disease, we determined the mutational status of BRAF and NRAS, and the protein expression of PTEN, in lymphadenectomy specimens from patients who underwent standard-of-care surgical treatment for stage IIIB/C melanoma. This study includes analysis of PTEN expression (by IHC) in a cohort of tumors that previously underwent quantitative proteomic analysis. This analysis demonstrates that only complete loss of PTEN protein expression correlates with increased expression of activation-specific markers in the PI3K–AKT pathway, thus providing a molecular rationale to guide the evaluation of PTEN expression in melanoma clinical samples. Our subsequent analysis of BRAF, NRAS, and PTEN status in a cohort of clinically annotated lymphadenectomy specimens from patients with stage IIIB/C melanoma identifies a significant association for loss of expression of PTEN with shorter overall survival (OS) and shorter time to melanoma brain metastasis (MBM).

Materials and Methods

Patients

Under an Institution Review Board-approved protocol, patients who underwent standard-of-care lymphadenectomy for stage IIIB/C melanoma, who, in the case of survival, had clinical follow-up of at least 5 years, and for whom archival material was available were identified. The patients underwent lymphadenectomy surgery between 1988 and 2009. Patient data and primary tumor characteristics were collected for all patients. Patient records were reviewed for clinical events, including development of local recurrence, development and patterns of distant metastases, and OS.

DNA mutation detection

DNA was extracted from unstained formalin-fixed paraffin-embedded (FFPE) slides that had undergone hematoxylin and eosin-guided macrodissection to isolate portions with at least 70% viable tumor cells. DNA was extracted by the MD Anderson Biospecimen Extraction Core Facility. Samples were screened for hotspot mutations in BRAF and NRAS using the Sequenom MassArray Platform by the MD Anderson Characterized Cell Line Core facility, as previously described (9).

PTEN immunohistochemistry

PTEN protein expression in FFPE samples was assessed by IHC (6H2.1 antibody, Cascade Bioscience) as previously...
described (23). Tumor samples were assessed as part of a tissue microarray (TMA), which included three cores for each tumor. Complete absence of PTEN was defined as <10% of tumor cells with any immunoreactivity in tumors with staining observed in internal positive controls (i.e., endothelial cells).

Statistical analysis

Patient and tumor characteristics were compared with molecular features using Fisher exact, Wilcoxon rank-sum, or Kruskal–Wallis tests. Time to any (local or distant) relapse (RFS), time to first distant metastasis (DDFS), and OS from diagnosis of stage III disease were estimated using Kaplan–Meier method. For organ-site specific analyses, patients without metastases were censored at the date of their last imaging by CT and/or MRI scan. Log-rank testing was used to assess differences between groups. The association between PTEN status and OS and time to brain metastasis was further assessed using multivariable Cox proportional hazards regression models that included age, clinical stage, and gender.

Results

Evaluation of PTEN expression by immunohistochemistry

Previously we used reverse phase protein arrays (RPPA) to quantitatively assess protein expression levels of PTEN and PI3K–AKT pathway activation-specific markers (i.e., Phospho-AKTThr308, Phospho-AKTSer473) in a cohort of frozen melanoma regional and distant metastases (9). To determine the expression and molecular correlations of PTEN expression as assessed by IHC, we performed staining for PTEN protein expression on available FFPE slides from the same surgical accessions. The RPPA results for each group were compared by unpaired t tests, P values are shown below the bar graph. Similar comparisons of PTEN IHC results to levels of AKTThr308 and P-AKTSer473, each measured by RPPA, are shown in C and D, respectively. Melanomas with clonal PTEN expression (by IHC) were not analyzed because of an insufficient number of samples (n = 2) from which to draw meaningful conclusions.
expression in the tumor cells (Fig. 1A) was observed in 20 samples (21%). PTEN expression was markedly reduced compared with internal positive controls (i.e., endothelial cells) in 22 tumors (23%), mildly reduced in 33 tumors (35%), and normal or increased in 17 tumors (18%; representative images are shown in Supplementary Fig. S1). The staining in virtually all samples was homogenous; however, two tumors (2.1%) demonstrated distinct areas of PTEN absence and PTEN presence (termed "clonal-like" due to its appearance; Supplementary Fig. S1). Levels of PTEN measured by RPPA showed a good correlation with IHC-based PTEN measurement, and quantitative levels of PTEN (RPPA) demonstrated statistically significant differences among the four prevalent IHC staining patterns (Fig. 1B). In contrast, expression levels of P-AKTSer473 and P-AKTThr308 (RPPA) were significantly elevated only in the tumors with complete absence of PTEN expression (Fig. 1C and D). The levels of both P-AKT proteins did not differ significantly between the other three groups. On the basis of these findings, PTEN expression in subsequent studies was categorized as "absent" or "not absent"; tumors with "clonal-like" expression were also identified but were excluded from analyses due to their rarity and uncertain molecular category.

**Clinical associations of BRAF<sup>V600</sup> and NRAS mutations in stage IIIB/C melanoma**

An independent cohort of patients (n = 136) with archival specimens available from standard-of-care lymphadenectomy surgery for clinically apparent regional lymphadenopathy was identified for molecular analysis (Supplementary Fig. S2). Approximately two-thirds of the patients were male, and the median age at the time of surgery was 55.4 years (Table 1). Thirty-five patients previously presented with stage I or II melanoma, and three patients were previously treated for stage IIIA disease, before presenting with clinically apparent regional lymphadenopathy. For the purpose of this study, the patients were categorized as stage IIIB or IIIC based on American Joint Committee on Cancer (AJCC) criteria at the time of surgery for their clinically apparent lymphadenopathy, along with the 98 patients who were stage IIIB/C at presentation. Thirty-eight patients were classified as stage IIIB disease, and ninety-eight as stage IIIC. Consistent with validated prognostic models, compared with patients with stage IIIB disease, patients with stage IIIC disease had shorter OS (median 1.9 vs. 5.0 years, P = 0.005) and distant disease-free survival (DDFS; median 0.8 vs. 1.6 years, P = 0.057). Thirty-two patients received adjuvant IFN, which did not correlate significantly with OS. In the full cohort, 40% of the patients had BRAF<sup>V600</sup> mutations, 10% had NRAS mutations, and 50% had no hotspot mutation in either gene ("WT"). No patients had mutations in both BRAF<sup>V600</sup> and NRAS. More than 90% (51/55) of the detected BRAF mutations resulted in BRAF<sup>V600E</sup> substitution, which is the most common change observed in melanoma. Mutation status was significantly associated with primary melanoma subtype (P = 0.026). Relatively low rates of BRAF<sup>V600E</sup> mutations were detected in acral and mucosal melanomas compared with cutaneous melanomas (Supplementary Table S1). The rate of BRAF mutations was very similar in the melanomas from known cutaneous primaries (n = 94, 47.9%) and those with no

<table>
<thead>
<tr>
<th>Measure</th>
<th>All patients (N = 136)</th>
<th>Stage IIIB (N = 38)</th>
<th>Stage IIIC (N = 98)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>94 (69)</td>
<td>25 (66)</td>
<td>69 (70)</td>
<td>0.680&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Female</td>
<td>42 (31)</td>
<td>13 (34)</td>
<td>29 (30)</td>
<td></td>
</tr>
<tr>
<td>Age at Stage III diagnosis (y), median (range)</td>
<td>55.4 (24.9–85.4)</td>
<td>54.3 (26.1–85.4)</td>
<td>55.7 (24.9–84.0)</td>
<td>0.684&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Mutation, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAF</td>
<td>55 (40)</td>
<td>15 (39)</td>
<td>40 (41)</td>
<td>1.000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NRAS</td>
<td>13 (10)</td>
<td>4 (11)</td>
<td>9 (9)</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>68 (50)</td>
<td>19 (50)</td>
<td>49 (50)</td>
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<tr>
<td>PTEN, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>31 (25)</td>
<td>8 (24)</td>
<td>23 (25)</td>
<td>1.000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Not absent</td>
<td>93 (75)</td>
<td>25 (76)</td>
<td>68 (75)</td>
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<tr>
<td>Missing</td>
<td>12</td>
<td>5</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Time to any distant recurrence (y), median (95% CI)</td>
<td>1.1 (0.8–1.4)</td>
<td>1.6 (1.0–3.3)</td>
<td>0.8 (0.6–1.3)</td>
<td>0.057&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>OS (y), median (95% CI)</td>
<td>2.2 (1.9–3.2)</td>
<td>5.0 (2.3–6.5)</td>
<td>1.9 (1.5–2.4)</td>
<td>0.005&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviation: CI, confidence interval.
<sup>a</sup>Fisher exact test.
<sup>b</sup>Wilcoxon rank-sum test.
<sup>c</sup>Log-rank test.
known primary tumor (n = 20, 40%). Mutation status was not significantly associated with the primary tumor Breslow thickness (P = 0.42) or ulceration (P = 0.26). Mutation status was also not significantly associated with pathologic substage (IIIB/C; P = 1.00), DDFS (P = 0.34), or OS (P = 0.89; Supplementary Table S1).

Clinical associations of PTEN expression

PTEN IHC was performed on a TMA composed of lymph node metastases from the 136 patients (Table 1) with stage IIIB or IIIC metastatic melanoma. All samples had tissue cores from three different regions of the tumor. Nine samples were not evaluable for PTEN expression due to a lack of viable tumor tissue in the TMA samples (7%; Supplementary Fig. S2). Three samples (2%) demonstrated "clonal-like" PTEN expression and were excluded from subsequent analyses due to their rarity and uncertain molecular category. Absent PTEN expression was observed in 31 (25%) of the 124 evaluable tumors. All evaluable cores for these 31 tumors demonstrated concordant results. The prevalence of PTEN absent was similar in the stage IIIB (24.2%) and IIIC (25.0%) patients (Table 1).

PTEN status did not correlate significantly with age, gender, primary tumor type, or primary tumor characteristics (Table 2). Patients with absent PTEN survived a median of 1.9 years from stage IIIB/C diagnosis, which was significantly shorter than patients with PTEN not absent (median 3.1 years, P = 0.03; Fig. 2A). However, PTEN status did not correlate with DDFS (PTEN absent vs. PTEN not absent, 0.9 years vs. 1.2 years, P = 0.35; Fig. 2B), or RFS (median 0.7 years for both, P = 0.91).

The anatomic site(s) of distant metastasis are significantly associated with OS in patients with stage IV melanoma and have been incorporated into AJCC melanoma staging system and prognostic models (22). Our previous RPPA analysis identified decreased PTEN expression in melanoma brain metastases (MBM) compared with lung and liver metastases (9). Because brain metastases are associated with poor outcomes, we determined the time to brain, lung, liver, and bone metastasis for all patients. Patients with PTEN absent developed MBM at a median of 1.8 years after stage IIIB/C diagnosis, which was significantly shorter than for patients with PTEN not absent (median 4.9 years, P = 0.03; Fig. 2C). In contrast, there was no significant difference in the median time to lung (P = 0.92; Fig. 2D), liver (P = 0.52), or bone (P = 0.10) metastasis (Table 2).

Multivariate analysis of OS incorporating AJCC substage (IIIB/C), PTEN (absent/not absent), age, gender, and ipilimumab (received/not received) was performed on all patients (Supplementary Table S2). Both increased stage (HR 1.8, P = 0.016) and PTEN absent (HR 1.6, P =

### Table 2. Demographics, tumor characteristics, and clinical outcomes by PTEN status

<table>
<thead>
<tr>
<th>Measure</th>
<th>PTEN absent (N = 31)</th>
<th>PTEN not absent (N = 93)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, n (%)</td>
<td>Male</td>
<td>22 (71)</td>
<td>63 (68)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>9 (29)</td>
<td>30 (32)</td>
</tr>
<tr>
<td>Age at stage III diagnosis (y), median (range)</td>
<td>48 (26.3–82.4)</td>
<td>58.1 (24.9–85.4)</td>
<td>0.073b</td>
</tr>
<tr>
<td>Clinical/pathologic type, n (%)</td>
<td>Acral</td>
<td>4 (13)</td>
<td>12 (13)</td>
</tr>
<tr>
<td></td>
<td>Cutaneous</td>
<td>20 (65)</td>
<td>65 (70)</td>
</tr>
<tr>
<td></td>
<td>Mucosal</td>
<td>1 (3)</td>
<td>3 (3)</td>
</tr>
<tr>
<td></td>
<td>Unknown primary</td>
<td>6 (19)</td>
<td>13 (14)</td>
</tr>
<tr>
<td>Breslow thickness (mm), median (range)</td>
<td>3.2 (0.4–25.0)</td>
<td>3.2 (0.3–33.0)</td>
<td>0.462b</td>
</tr>
<tr>
<td>Ulceration, n (%)</td>
<td>No</td>
<td>6 (30)</td>
<td>10 (15)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>14 (70)</td>
<td>56 (85)</td>
</tr>
<tr>
<td></td>
<td>Missing</td>
<td>11</td>
<td>27</td>
</tr>
<tr>
<td>Time to any recurrence (y), median (95% CI)</td>
<td>0.7 (0.5–1.1)</td>
<td>0.7 (0.5–0.9)</td>
<td>0.913c</td>
</tr>
<tr>
<td>Time to any distant recurrence (y), median (95% CI)</td>
<td>0.9 (0.6–1.4)</td>
<td>1.2 (0.7–1.7)</td>
<td>0.352c</td>
</tr>
<tr>
<td>OS (y), median (95% CI)</td>
<td>1.9 (1.5–2.1)</td>
<td>3.1 (2.0–4.1)</td>
<td>0.031c</td>
</tr>
<tr>
<td>Time to CNS metastasis (y), median (95% CI)</td>
<td>1.8 (1.2–2.5)</td>
<td>4.9 (2.4, NE)</td>
<td>0.030c</td>
</tr>
<tr>
<td>Time to lung metastasis (y), median (95% CI)</td>
<td>2.1 (1.1, NE)</td>
<td>3.3 (1.9–6.1)</td>
<td>0.920c</td>
</tr>
<tr>
<td>Time to liver metastasis (y), median (95% CI)</td>
<td>NE (1.4, NE)</td>
<td>6.3 (3.0, NE)</td>
<td>0.522c</td>
</tr>
<tr>
<td>Time to bone metastasis (y), median (95% CI)</td>
<td>NE (1.4, NE)</td>
<td>NE (6.3, NE)</td>
<td>0.102c</td>
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</tbody>
</table>

Abbreviations: CI, confidence interval; NE, not estimated.

aFisher exact test.
bWilcoxon rank-sum test.
cLog-rank test.
0.046) were significant predictors of OS. No factors were significantly (P < 0.05) associated with time to brain metastasis on multivariate analysis (Supplementary Table S2).

**Clinical associations and significance of PTEN in BRAF^{V600}-mutant and BRAF/NRAS-wild-type melanomas**

PTEN mutations are generally mutually exclusive with the presence of hotspot NRAS mutations; however, PTEN loss is observed in BRAF^{V600}-mutant and BRAF/NRAS-WT melanomas (3). In this study, complete loss of PTEN expression was observed in 8% (1 of 13) of NRAS, 31% (16 of 52) of BRAF^{V600}, and 24% (14 of 59) of wild-type (WT) tumors.

Among the patients with BRAF^{V600} mutations, PTEN status was not significantly associated with stage (P = 0.90), or with treatment (at any time) with either ipilimumab (P = 0.98) or a selective BRAF inhibitor (P = 0.29). There was also no significant difference between PTEN absent and PTEN not absent patients in RFS (median 0.7 years in both groups, P = 0.91) or DDFS (median 0.7 vs. 0.9 years, P = 0.10; Supplementary Table S3). However, patients with BRAF^{V600} mutations with absent PTEN had significantly shorter OS than the patients with PTEN not absent (median 1.6 vs. 3.6 years, P < 0.001; Fig. 3A). BRAF^{V600} melanoma patients with PTEN absent also had significantly shorter time to MBM (1.2 vs. 4.2 years, P < 0.001; Fig. 3B) and liver metastasis (1.4 years vs. not estimable, P = 0.046), and trends for shorter time to lung (1.4 vs. 4.0 years, P = 0.08) and bone (3.0 years vs. not estimable, P = 0.14) metastasis. In contrast, in patients without activating mutations in BRAF^{V600} or NRAS, PTEN was not significantly associated with OS (Fig. 3C), DDFS, or time to brain metastasis (Fig. 3D; Supplementary Table S4).

The differences in OS and time to MBM by PTEN status in the patients with BRAF^{V600} mutations remained significant after adjusting for multiple comparisons (adjusted P = 0.02 for both; ref. 24).

Multivariate analysis of patients with BRAF^{V600} mutations incorporating stage (IIIB/C), PTEN (absent/not absent), age, gender, subsequent ipilimumab (received/not received), and subsequent BRAF inhibitor treatment identified PTEN absent (HR 2.7, P = 0.008) as the only factor significantly associated with OS (Table 3). Multivariate
analysis using the same factors also identified PTEN absent (HR 4.2, $P = 0.008$) as the only significant predictor of time to brain metastasis (Table 3). After adjusting for multiple comparisons, the $P$ value for both OS and time to MBM by PTEN status was 0.07.

Discussion

Activating $BRAF^{V600}$ and $NRAS$ mutations, and loss of expression of the tumor suppressor PTEN, are three of the most common oncogenic events in melanoma. Multiple clinical trials are planned or ongoing to evaluate the benefit of targeting these molecules and their associated pathways. In addition to providing benefit as therapeutic targets, molecular markers can enhance patient management by improving prognostic models. This study represents the first integrated analysis of $BRAF$, $NRAS$, and PTEN with clinical outcomes in patients with stage III melanoma. The results of this analysis add to existing data from preclinical models

Table 3. Multivariable analysis of OS and time to CNS metastasis in patients with $BRAF^{V600}$ mutations

<table>
<thead>
<tr>
<th>Measure</th>
<th>OS HR (95% CI)</th>
<th>$P$</th>
<th>Time to CNS Metastasis HR (95% CI)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage III status (IIIC vs. IIIB)</td>
<td>2 (0.9–4.2)</td>
<td>0.09</td>
<td>1.4 (0.5–3.7)</td>
<td>0.503</td>
</tr>
<tr>
<td>PTEN (absent vs. not absent)</td>
<td>2.7 (1.3–5.5)</td>
<td>0.008</td>
<td>4.2 (1.5–12.0)</td>
<td>0.008</td>
</tr>
<tr>
<td>BRAF inhibitor use (no vs. yes)</td>
<td>3 (0.4–23.3)</td>
<td>0.285</td>
<td>3 (0.4–23.8)</td>
<td>0.299</td>
</tr>
<tr>
<td>Ipilimumab use (no vs. yes)</td>
<td>1 (0.3–3.0)</td>
<td>0.976</td>
<td>1.2 (0.4–4.2)</td>
<td>0.741</td>
</tr>
<tr>
<td>Age</td>
<td>1 (1.0–1.0)</td>
<td>0.549</td>
<td>1 (1.0–1.0)</td>
<td>0.98</td>
</tr>
<tr>
<td>Gender (male vs. female)</td>
<td>1.5 (0.7–3.8)</td>
<td>0.293</td>
<td>2.1 (0.7–6.2)</td>
<td>0.179</td>
</tr>
</tbody>
</table>
PTEN was discovered in the minimal region of loss of chromosome 10 that occurs frequently in glioblastoma multiforme (25, 26). Subsequent studies identified loss-of-function deletions and mutations of the PTEN gene in multiple tumor types, including melanoma (27). PTEN is a lipid phosphatase that dephosphorylates the 3'-position of the inositol ring of phosphatidylinositols. Thus, PTEN is a negative regulator of PI3K, and loss of PTEN increases signaling through the PI3K–AKT signaling pathway, which is commonly assessed by measuring levels of phosphorylated (activated) AKT.

Our previous RPPA-based proteomic analysis of frozen metastatic melanoma tissue samples demonstrated that PTEN protein levels were significantly associated and inversely correlated with levels of multiple activation-specific markers in the PI3K–AKT pathway (9). PTEN loss was detected in BRAF V600-mutant and in BRAF V600/WT melanoma. Although RPPA is a powerful technology, its use is predominantly limited to frozen tumor samples. In contrast, in the current study, we used an IHC assay performed in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory to measure PTEN protein expression in FFPE tissues, which are widely available. One challenge with IHC assays is the determination of rational and reproducible scoring criteria. Our comparison of IHC and RPPA results demonstrates a strong correlation between PTEN measurements by these two methods, supporting their technical validity. In addition, the finding that only the PTEN absence is associated with enhanced AKT activity provides a molecular rationale for a dichotomous scoring system. Assessment of the interobserver agreement for PTEN IHC scoring of the pilot set of FFPE samples (n = 94) by two dermatopathologists using the kappa statistic identified a kappa coefficient of 0.73. Thus, the dichotomous scoring system also has the benefit of good reproducibility compared with more complex criteria. Previous studies in other tumor types also support the rationale to perform protein-based measurement of PTEN, as PTEN expression can be regulated epigenetically (11, 12, 28). It will be important in future studies to integrate and compare PTEN protein, mRNA, and genetic status with each other and with clinical outcomes.

In this study, complete loss of PTEN expression correlated with OS in stage IIIB/C patients with BRAF V600 mutations, but not in patients with WT BRAF V600 and NRAS. Notably, the prognostic significance of PTEN appears due to shorter survival after the diagnosis of stage IV disease, as PTEN status did not correlate significantly with time to first distant metastasis. The interaction between PTEN and BRAF in melanoma is also supported by functional preclinical studies. In genetically engineered mice, the expression of the BRAF V600E mutation in melanocytes resulted in hyperplasia only. However, concurrent loss of PTEN in the melanocytes with BRAF V600E mutations resulted in 100% penetrance of invasive, metastatic melanomas (29). Loss of PTEN in murine melanocytes without BRAF V600E mutations caused no melanocytic phenotype in that study. Similarly, loss of PTEN was not significantly associated with OS in the stage IIIB/C patients with BRAF V600/WT melanomas in our cohort. We and other groups have also observed that human melanoma cell lines with BRAF V600 mutations and loss of PTEN expression are resistant to apoptosis following BRAF inhibition compared with cell lines with normal PTEN (30–32). Analysis of patients enrolled in early-phase clinical trials with the FDA-approved BRAF inhibitor dabrafenib found that patients with genetic alterations in the PTEN gene had a strong trend for shorter PFS compared with patients with a normal PTEN (33). Lower PTEN expression was also observed in nonresponders compared with responders in the phase II clinical trial of vemurafenib (34). Interestingly, recent data also suggest that PTEN loss can also promote resistance to the antitumor immune response (35). Together, these findings support the rationale to evaluate PTEN status in ongoing and planned clinical trials of targeted and immune therapies in patients with stage III melanoma.

Brain metastases are one of the most common and devastating complications of melanoma. In this study, we observed that complete loss of PTEN expression correlated with markedly shorter time to brain metastasis among patients with BRAF V600 mutations and stage IIIB/C melanoma. As melanoma patients with CNS involvement have historically had very poor outcomes (36, 37), this association is likely primarily responsible for the observed shorter survival in patients with PTEN loss in this study. This finding also adds to a growing body of literature implicating the PI3K–AKT pathway in brain metastasis in melanoma. Previously, in a pilot RPPA analysis of a small number of frozen distant metastases we found significantly lower levels of PTEN, and higher levels of P-AKT, in MBM (n = 10) compared with lung (n = 5) or liver (n = 5) metastases (9). An independent IHC analysis of the expression of P-AKT and P-MAPK protein levels in tumors from patients who underwent synchronous resection of brain and extracranial metastases reported higher P-AKT in the brain metastases of 8 of 9 patients (38). We have recently also reported the RPPA analysis of an independent set of cohort of patients with resected brain and extracranial metastases (39). In this study, P-AKT had the highest ratio of expression in brain metastases compared with extracranial metastases (P = 0.008 by paired t test). Preliminary studies have also shown that activated forms of AKT can promote MBM formation in preclinical models (40). Together these findings suggest that the PI3K–AKT pathway may be a rational, actionable therapeutic target for MBM.

In this cohort of patients with stage IIIB/C melanoma, we did not detect a significant association between BRAF/NRAS mutation status and time to first distant metastasis or OS. The low prevalence of NRAS mutations limited our power to detect significant clinical associations with this molecular event. The rate of BRAF V600E mutations (n = 4) was also too low to make meaningful comparisons with BRAF V600D mutations, which have demonstrated significant differences...
in larger cohorts of stage IV patients (41, 42). Notably, retrospective analyses of patients with melanoma with regional disease have reported varying conclusions about the prognostic significance of BRAF and NRAS mutations (43–45). The varying results support the need for multicenter prospective collection and integrated analysis of clinical, histologic, and molecular data to further refine risk models for, and ultimately improve the management of, patients with melanoma with regionally advanced disease. On the basis of the results of our study, we believe that such studies should not be restricted to mutational analysis alone.

In summary, this study represents the first integrated analysis of BRAF<sup>V600</sup> mutations, NRAS mutations, and PTEN loss in patients with stage IIIIB/C melanoma. Our findings implicate PTEN loss, which was detected by a routine IHC assay, as a significant predictor of OS and time to brain metastasis in patients with BRAF<sup>V600</sup> mutations. These findings support the rationale for evaluation of PTEN protein expression in additional cohorts of patients, and the significance of the PI3K–AKT pathway in this disease.

Disclosure of Potential Conflicts of Interest
M.A. Davies reports receiving commercial research grants from AstraZeneca, Genentech, GlaxoSmithKline, Myriad Genetics, and Oncothyreon, and is a consultant/advisory board member for Genentech, Glaxo SmithKline, Novartis, and Sanofi. J. Gershenwald is a consultant/advisory board member for Merck. No potential conflicts of interest were disclosed by the other authors.

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Complete Loss of PTEN Protein Expression Correlates with Shorter Time to Brain Metastasis and Survival in Stage IIIB/C Melanoma Patients with \textit{BRAF}^{V600} Mutations

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