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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 BRAF^{V600} mutations

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Abstract

PURPOSE—Loss of function of PTEN is a frequent event in melanoma, particularly in tumors with *BRAF*^{V600} mutations. The prevalence, pathological 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 were assessed by DNA sequencing for activating *BRAF* and *NRAS* mutations, and by immunohistochemistry (IHC) for the expression of PTEN protein. Associations of these molecular aberrations with demographics, tumor characteristics, and clinical outcomes were determined.

RESULTS—The prevalence of *BRAF*^{V600} mutations (40% overall), *NRAS* mutations (10%), and PTEN loss (25%) did not vary by pathological substage. *BRAF*/*NRAS* mutation status did not

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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. Analysis of PTEN in mutationally-defined subsets showed that PTEN loss was significantly associated with OS and time to MBM in patients with *BRAF*^{V600} mutations.

CONCLUSIONS—Loss of PTEN protein expression correlates significantly with decreased OS and time to MBM in stage IIIB/C melanoma patients with *BRAF*^{V600} mutations. The findings add to evidence supporting a significant role for PTEN loss and the PI3K-AKT pathway in melanoma.

Keywords

melanoma; BRAF; NRAS; PTEN; brain metastasis

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 BRAF serine-threonine kinase are the most common activating mutation detected in cutaneous melanoma patients (40–45%).(1, 2) *BRAF*^{V600} mutations increase the kinase activity of BRAF 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%).(2) Previous studies have demonstrated that *NRAS* mutations are essentially mutually exclusive with *BRAF*^{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 BRAF inhibitors (vemurafenib, dabrafenib) and MEK inhibitors (trametinib) have been approved by the FDA for the treatment of metastatic melanoma patients with *BRAF*^{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 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 *BRAF*^{V600} 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 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 *BRAF*^{V600} 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 stage III melanoma patients. 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 pathological features.(22) The identification of markers that could improve risk stratification in stage III melanoma patients is important, as the only currently approved adjuvant therapies for these patients (interferon- α -2B and pegylated-interferon- α -2b) are given for prolonged periods of time and have significant associated toxicities. Further, evaluating the prognostic significance of such markers in stage III melanoma patients 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.

In order 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 and shorter time to melanoma brain metastasis.

Methods

Patients

Under an Institution Review Board-approved protocol, patients were identified who underwent standard-of-care lymphadenectomy for stage IIIB/C melanoma, for whom archival material was available, and that had clinical follow-up of at least 5 years for surviving patients. The patients underwent lymphadenectomy surgery between 1988 to 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 pattern(s) of distant metastasis(es), and overall survival.

DNA Mutation Detection

DNA was extracted from unstained formalin-fixed paraffin embedded (FFPE) slides that had undergone H&E-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 immunohistochemistry (IHC) (6H2.1 antibody, Cascade Bioscience) as previously described.(23) Tumor samples were assessed as part of a tissue microarray (TMA), which included 3 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 to molecular features using Fisher's exact, Wilcoxon rank sum or Kruskal-Wallis tests. Time to any (local or distant) relapse (RFS), time to first distant metastasis (DDFS), and overall survival (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-AKT^{Ser473}, Phospho-AKT^{Thr308}) in a cohort (n=96) 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 (n=94) used in the RPPA analysis. Five patterns of PTEN protein expression were observed. Complete absence of PTEN protein expression in the tumor cells (Figure 1A) was observed in 20 samples (21%). PTEN expression was markedly reduced compared to 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 Figure S1). The staining in virtually all samples was homogenous; however, 2 tumors (2.1%) demonstrated distinct areas of PTEN absence and PTEN presence (termed 'clonal-like' due to its appearance) (Figure 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 (Figure 1B). In contrast, expression levels of P-AKT^{Ser473} and P-AKT^{Thr308} (RPPA) were significantly elevated only in the tumors with complete absence of PTEN expression (Figure 1C/D). The levels of both P-AKT proteins did not differ significantly between the other 3 groups. Based on 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*^{V600} 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 were identified for molecular analysis (Figure 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 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 to patients with stage IIIB disease, patients with stage IIIC disease had shorter OS (median 1.9 versus 5.0 years, p=0.005) and DDFS (median 0.8 versus 1.6 years, p=0.057). Thirty two patients received adjuvant interferon, which did not correlate significantly with OS.

In the full cohort, 40% of the patients had *BRAF*^{V600} mutations, 10% had *NRAS* mutations, and 50% had no hotspot mutation in either gene ("WT"). No patients had mutations in both *BRAF*^{V600} and *NRAS*. Over 90% (51/55) of the detected *BRAF* mutations resulted in *BRAF*^{V600E} 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*^{V600} mutations were detected in acral and mucosal melanomas compared to cutaneous melanomas (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 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 pathological substage (IIIB/C) (p=1.00), DDFS (p=0.34), or OS (p=0.89) (Table S1).

Clinical associations of PTEN expression

PTEN IHC was performed on a tissue microarray (TMA) comprised of lymph node metastases from the 136 patients (Table 1) with stage IIIB or IIIC metastatic melanoma. All samples had tissue cores from 3 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%) (Figure 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, $p=0.03$) (Figure 2A). However, PTEN status did not correlate with DDFS (PTEN Absent vs PTEN Not Absent, 0.9 years vs. 1.2 years, $p=0.35$) (Figure 2B), nor RFS (median 0.7 years for both, $p=0.91$).

The anatomic site(s) of distant metastasis are significantly associated with overall survival in stage IV melanoma patients 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 compared to lung and liver metastases(9). Since 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 melanoma brain metastases (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, $p=0.03$) (Figure 2C). In contrast, there was no significant difference in the median time to lung ($p=0.92$) (Figure 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 (Table S2). Both increased stage (HR 1.8, $p=0.016$) and PTEN Absent (HR1.6, $p=0.046$) were significant predictors of OS. No factors were significantly ($p<0.05$) associated with time to brain metastasis on multivariate analysis (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 *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 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, $p=0.10$) (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 versus 3.6 years, $p<0.001$) (Figure 3A). *BRAF*^{V600} melanoma patients with *PTEN* Absent also had significantly shorter time to melanoma brain metastasis (1.2 vs. 4.2 years, $p<0.001$) (Figure 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 (Figure 3C), DDFS, or time to brain metastasis (Figure 3D) (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).(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 stage III melanoma patients. The results of this analysis add to existing data from preclinical models and clinical trials implicating loss of PTEN as a clinically significant event in this disease, specifically in tumors with *BRAF*^{V600} mutations.

PTEN was discovered in the minimal region of loss of chromosome 10 that occurs frequently in glioblastoma multiforme (GBM).(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}/*NRAS* Wild-Type tumors. While 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 lab to measure *PTEN* 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* Absent IHC pattern correlated with AKT activation 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 to 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 wild-type *BRAF*^{V600} and *NRAS*. Notably, the prognostic significance of PTEN appears to be due to shorter survival after the diagnosis of stage IV disease, and 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*^{V600} mutation in melanocytes resulted in hyperplasia only. However, concurrent loss of PTEN in the melanocytes with *BRAF*^{V600} mutations resulted in 100% penetrance of invasive, metastatic melanomas.(29) Loss of PTEN in murine melanocytes without *BRAF*^{V600} 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}/*NRAS* Wild-Type 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 to 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 to patients with a normal *PTEN*.(33) Lower PTEN expression was also observed in non-responders compared to responders in the phase II clinical trial of vemurafenib.(34) Interestingly, recent data also suggests that PTEN loss can also promote resistance to the anti-tumor 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 melanoma brain metastases (n=10) compared to 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 to extracranial metastases (p=0.008 by paired t-test). Preliminary studies have also shown that activated forms of AKT can promote melanoma brain metastasis formation in preclinical models.(40) Together these findings suggest that the PI3K-AKT pathway may be a rational, actionable therapeutic target for melanoma brain metastases.

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 overall survival. The low prevalence of *NRAS* mutations limited our power to detect significant clinical associations with this molecular event. The rate of *BRAF*^{V600K} mutations (n=4) was also too low to make meaningful comparisons to *BRAF*^{V600E} mutations, which have demonstrated significant differences in larger cohorts of stage IV patients.(41, 42) Notably, retrospective analyses of melanoma patients 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, histological, and molecular data to further refine risk models for, and ultimately to improve the management of, melanoma patients with regionally advanced disease. Based on 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*^{V600} mutations, *NRAS* mutations, and PTEN loss in patients with stage IIIB/C melanoma. Our findings implicate PTEN loss, which was detected by a routine IHC assay, as a significant predictor of overall survival and time to brain metastasis in patients with *BRAF*^{V600} 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of 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 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 *BRAF*^{V600} mutations, 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 to further examine the role of the PI3K-AKT pathway in this common and devastating complication of advanced melanoma.

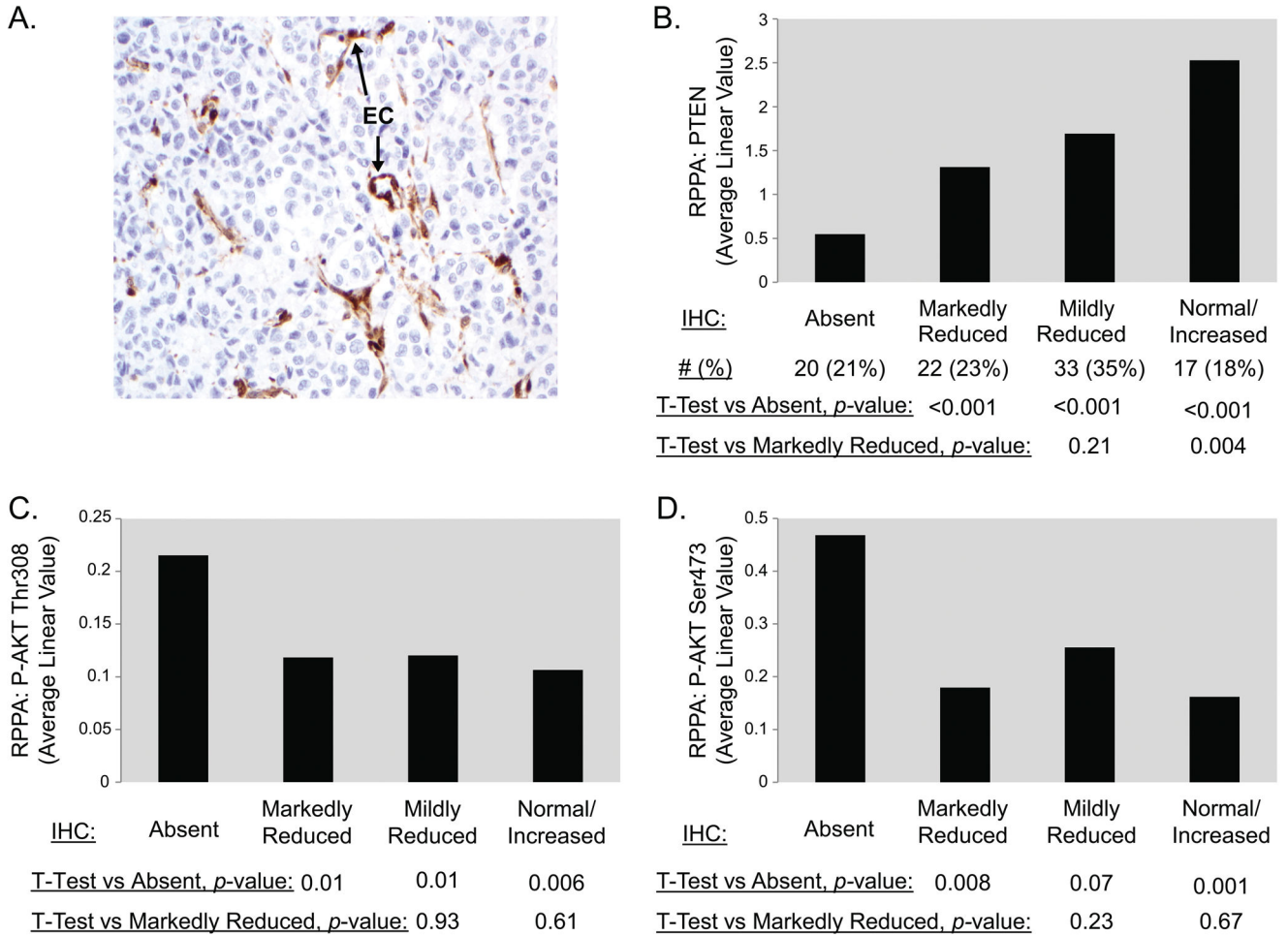


Figure 1. Evaluation of PTEN expression in melanoma by immunohistochemistry (IHC). (A) Representative image of a melanoma with complete loss of PTEN protein expression in the tumor cells. Endothelial cells (labeled “EC”) serve as an internal positive control and comparator. (B) Comparison of PTEN protein expression measurement by RPPA (on frozen tumor tissue) and by IHC (FFPE from the same surgical accessions). PTEN expression in the melanomas was categorized (x-axis) based on the observed results of IHC analysis. The average PTEN protein expression for each group of tumors as measured by RPPA is shown on the y-axis (arbitrary units). The RPPA results for each group were compared by un-paired t-tests, *p*-values are shown below the bar graph. Similar comparison of PTEN IHC results to levels of AKT^{Thr308} and P-AKT^{Ser473}, each measured by RPPA, are shown in panels (C) and (D), respectively. Melanomas with Clonal PTEN expression (by IHC) were not analyzed due to insufficient number of samples (n=2) to make meaningful conclusions.

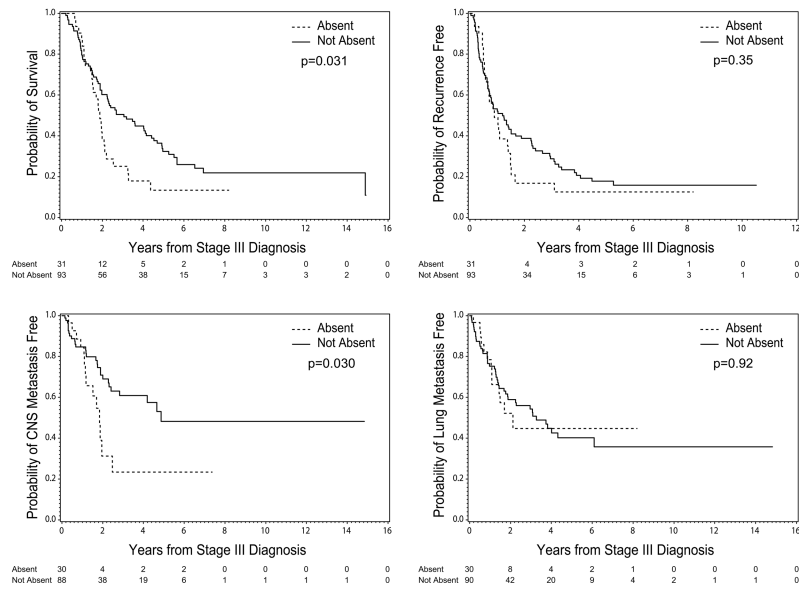


Figure 2. Clinical outcomes by PTEN status in stage IIIB/C melanoma patients (N=124). Kaplan-Meier analyses are shown for (A) OS, (B) DDFS, (C) CNS metastasis-free survival, and (D) lung metastasis-free survival.

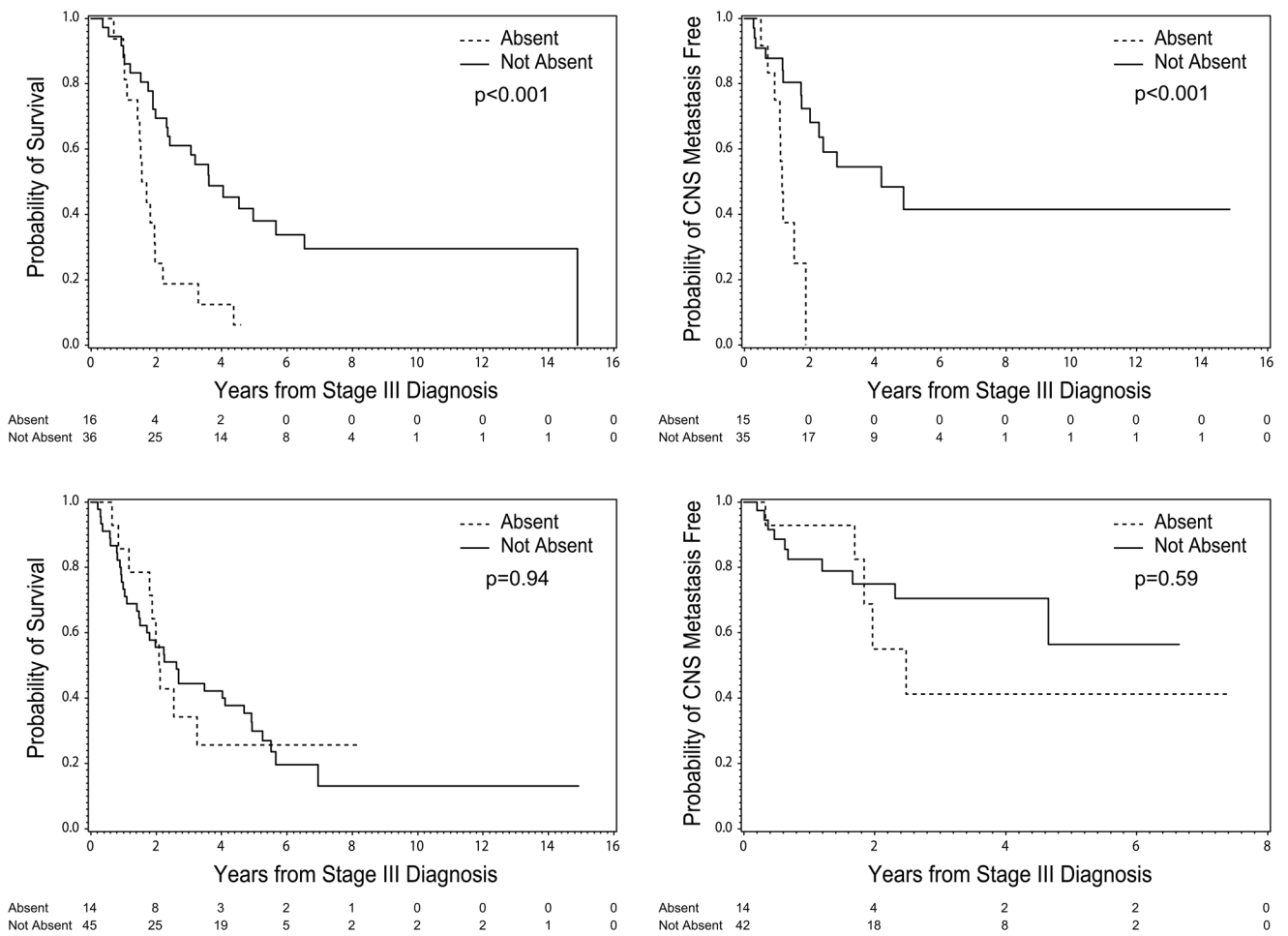


Figure 3. Clinical outcomes by PTEN status in stage IIIB/C melanoma patients with *BRAF*^{V600} mutations (A, B) and in patients without *BRAF*^{V600} or *NRAS* mutations (C, D). Kaplan-Meier analysis are shown for (A, C) OS and (B, D) CNS metastasis-free survival.

Table 1

Demographics, Tumor Characteristics, and Clinical Outcomes in Stage IIIB and IIIC Patients.

Measure	All Patients (N=136)	Stage IIIB (N=38)	Stage IIIC (N=98)	p-value
Gender, n (%)				
Male	94 (69)	25 (66)	69 (70)	0.680 ^a
Female	42 (31)	13 (34)	29 (30)	
Age at Stage III diagnosis (years), median (range)	55.4 (24.9, 85.4)	54.3 (26.1, 85.4)	55.7 (24.9, 84.0)	0.684 ^b
Mutation, n (%)				
BRAF	55 (40)	15 (39)	40 (41)	1.000 ^a
NRAS	13 (10)	4 (11)	9 (9)	
Wild-type	68 (50)	19 (50)	49 (50)	
PTEN, n (%)				
Absent	31 (25)	8 (24)	23 (25)	1.000 ^a
Not Absent	93 (75)	25 (76)	68 (75)	
Missing	12	5	7	
Time to any distant recurrence (years), median (95% CI)	1.1 (0.8, 1.4)	1.6 (1.0, 3.3)	0.8 (0.6, 1.3)	0.057 ^c
Overall survival (years), median (95% CI)	2.2 (1.9, 3.2)	5.0 (2.3, 6.5)	1.9 (1.5, 2.4)	0.005 ^c

^aFisher's exact test.^bWilcoxon rank sum test.^cLog-rank test.

Table 2

Demographics, Tumor Characteristics, and Clinical Outcomes by PTEN Status.

Measure	PTEN Absent (N=31)	PTEN Not Absent (N=93)	p-value
Gender, n (%)			
Male	22 (71)	63 (68)	0.826 ^a
Female	9 (29)	30 (32)	
Age at Stage III diagnosis (years), median (range)	48 (26.3, 82.4)	58.1 (24.9, 85.4)	0.073 ^b
Clinical/Pathological Type, n (%)			
Acral	4 (13)	12 (13)	0.878 ^a
Cutaneous	20 (65)	65 (70)	
Mucosal	1 (3)	3 (3)	
Unknown Primary	6 (19)	13 (14)	
Breslow thickness (mm), median (range)	3.2 (0.4, 25.0)	3.2 (0.3, 33.0)	0.462 ^b
Ulceration, n (%)			
No	6 (30)	10 (15)	0.188 ^a
Yes	14 (70)	56 (85)	
Missing	11	27	
Time to any recurrence (years), median (95% CI)	0.7 (0.5, 1.1)	0.7 (0.5, 0.9)	0.913 ^c
Time to any distant recurrence (years), median (95% CI)	0.9 (0.6, 1.4)	1.2 (0.7, 1.7)	0.352 ^c
Overall survival (years), median (95% CI)	1.9 (1.5, 2.1)	3.1 (2.0, 4.1)	0.031 ^c
Time to CNS metastasis (years), median (95% CI)	1.8 (1.2, 2.5)	4.9 (2.4, NE)	0.030 ^c
Time to lung metastasis (years), median (95% CI)	2.1 (1.1, NE)	3.3 (1.9, 6.1)	0.920 ^c
Time to liver metastasis (years), median (95% CI)	NE (1.4, NE)	6.3 (3.0, NE)	0.522 ^c
Time to bone metastasis (years), median (95% CI)	NE (1.4, NE)	NE (6.3, NE)	0.102 ^c

^aFisher's exact test.^bWilcoxon rank sum test.^cLog-rank test.

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

Table 3Multivariable analysis of Overall Survival and Time to CNS metastasis in Patients with BRAFV⁶⁰⁰ Mutations

Measure	Overall Survival		Time to CNS Metastasis	
	Hazard Ratios (95% CI)	p-value	Hazard Ratios (95% CI)	p-value
Stage III status (IIC v IIIB)	2 (0.9, 4.2)	0.09	1.4 (0.5, 3.7)	0.503
PTEN (Absent v Not Absent)	2.7 (1.3, 5.5)	0.008	4.2 (1.5, 12.0)	0.008
BRAF Inhibitor use (No v Yes)	3 (0.4, 23.3)	0.285	3 (0.4, 23.8)	0.299
Ipilimumab use (No v Yes)	1 (0.3, 3.0)	0.976	1.2 (0.4, 4.2)	0.741
Age	1 (1.0, 1.0)	0.549	1 (1.0, 1.0)	0.98
Gender (Male v Female)	1.5 (0.7, 3.8)	0.293	2.1 (0.7, 6.2)	0.179