Revisiting the Clinical and Biologic Relevance of Partial PTEN Loss in Melanoma

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The extent of PTEN loss that confers clinical and biological impact in melanoma is unclear. We evaluated the clinical and biologic relevance of PTEN dosage in melanoma and tested the postulate that partial PTEN loss is due to epigenetic mechanisms. PTEN expression was assessed by immunohistochemistry in a stage III melanoma cohort (n = 190) with prospective follow up. Overall, 21 of 190 (11%) tumors had strong PTEN expression, 51 of 190 (27%) had intermediate PTEN, 44 of 190 (23%) had weak PTEN, and 74 of 190 (39%) had absent PTEN. Both weak and absent PTEN expression predicted shorter survival in multivariate analyses (hazard ratio = 2.13, P < 0.01). We show a continuous negative correlation between PTEN and activated Akt in melanoma cells with titrated PTEN expression and in two additional independent tumor datasets. PTEN genomic alterations (deletion, mutation), promoter methylation, and protein destabilization did not fully explain PTEN loss in melanoma, whereas PTEN levels increased with treatment of melanoma cells with the histone deacetylase inhibitor LBH589. Our data indicate that partial PTEN loss is due to modifiable epigenetic mechanisms and drives Akt activation and worse prognosis, suggesting a potential approach to improve the clinical outcome for a subset of patients with advanced melanoma.


INTRODUCTION

PTEN is a well-characterized tumor suppressor (Li et al., 1997) that antagonizes oncopgenic PI3K/Akt signaling through its lipid phosphatase activity (Li et al., 1998). Additionally, PTEN has PI3K-independent tumor suppressor functions, including regulation of cell migration (Tamura et al., 1997) and genomic stability (Puc et al., 2005). PTEN loss occurs in different cancers (Milella et al., 2015), including melanoma (Mikhail et al., 2005), leading to Akt hyperactivation and apoptosis resistance, tumor growth, and metastasis. Multiple mechanisms are proposed for PTEN loss, including mutations, deletions, transcriptional and epigenetic silencing, posttranscriptional and posttranslational regulation, and protein-protein interactions (Bermúdez Brito et al., 2015).

As a “haploinsufficient” tumor suppressor gene, PTEN protein dose is thought to dictate the extent of its inactivation and regulates tumor development and progression (Carracedo et al., 2011). Genetic mouse models and human tumors suggest that even subtle reductions in PTEN expression promote tumorigenesis (Trothman et al., 2003). Pten hyper mice expressing 80% of normal Pten develop a spectrum of tumors, sharing gene expression profiles with human breast tumor specimens that have PTEN levels slightly below normal (Alimonti et al., 2010). Immunohistochemical (IHC) analysis found absent PTEN in 5 of 34 melanomas (15%) and low PTEN expression in 17 melanomas (50%) (Zhou et al., 2000). Another study found reduced PTEN in 29 of 92 (56%) primary melanomas and absent PTEN in 1 of 92 (1%) tumors (Whiteman et al., 2002). In stage III melanoma, PTEN protein expression (by IHC) was decreased in 55 of 94 (58%) tumors and was undetectable in 20 of 94 (21%) tumors (Buchet et al., 2014); however, only complete PTEN absence in tumor cells was associated with significantly shorter overall survival (OS) and elevated Akt activity. This finding is inconsistent with PTEN’s purported role as a haploinsufficient tumor suppressor, raising critical questions regarding PTEN’s role in melanoma. What is the clinical and biological impact of partial PTEN loss in melanoma, and is there a significant threshold for reduced PTEN dosage? What are the mechanisms underlying reduced PTEN levels in melanoma, and could they be exploited to restore PTEN for therapeutic benefit? In this study, we hypothesized that there is a threshold of partial PTEN loss with significant clinical and biological impact in melanoma and that epigenetic mechanisms are critical for deregulated PTEN.
Table 1. Baseline patient characteristics\(^1\)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PTEN Absent (n = 74)</th>
<th>PTEN Weak (n = 44)</th>
<th>PTEN Intermediate (n = 51)</th>
<th>PTEN Strong (n = 21)</th>
<th>All Patients (n = 190)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Sex, n (%)</td>
<td>30 (40.5)</td>
<td>18 (40.9)</td>
<td>18 (35.3)</td>
<td>9 (42.9)</td>
<td>75 (39.5)</td>
<td>0.91</td>
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<tr>
<td>Male</td>
<td>44 (59.5)</td>
<td>25 (59.1)</td>
<td>33 (64.7)</td>
<td>12 (57.1)</td>
<td>115 (60.5)</td>
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<tr>
<td>Female</td>
<td></td>
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<tr>
<td>Age in years at stage III diagnosis, median (range)</td>
<td>62.8 (20.6–91.4)</td>
<td>67.8 (23.5–92.2)</td>
<td>63.3 (26.7–83.6)</td>
<td>55.6 (28.2–92.3)</td>
<td>61.2 (20.6–92.3)</td>
<td>0.36</td>
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<tr>
<td>Substage, n (%)</td>
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<td></td>
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<tr>
<td>IIA</td>
<td>7 (9.7)</td>
<td>7 (15.9)</td>
<td>7 (13.7)</td>
<td>3 (14.3)</td>
<td>24 (12.8)</td>
<td>0.23</td>
</tr>
<tr>
<td>IIB</td>
<td>23 (31.9)</td>
<td>15 (34.1)</td>
<td>26 (51)</td>
<td>10 (47.6)</td>
<td>74 (39.4)</td>
<td></td>
</tr>
<tr>
<td>IIIC</td>
<td>42 (58.3)</td>
<td>22 (50)</td>
<td>18 (35.3)</td>
<td>8 (38.1)</td>
<td>90 (47.9)</td>
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<tr>
<td>BRAF status, n (%)</td>
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<tr>
<td>Mutant</td>
<td>21 (41.2)</td>
<td>13 (44.8)</td>
<td>20 (50)</td>
<td>6 (37.5)</td>
<td>60 (44.1)</td>
<td>0.79</td>
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<tr>
<td>Wild type</td>
<td>30 (58.8)</td>
<td>16 (55.2)</td>
<td>20 (50)</td>
<td>10 (62.5)</td>
<td>76 (55.9)</td>
<td></td>
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<tr>
<td>NRAS status, n (%)</td>
<td></td>
<td></td>
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<tr>
<td>Mutant</td>
<td>6 (12.8)</td>
<td>8 (32)</td>
<td>20 (27.8)</td>
<td>4 (28.6)</td>
<td>28 (23.0)</td>
<td>0.20</td>
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<tr>
<td>Wild type</td>
<td>41 (87.2)</td>
<td>17 (68)</td>
<td>26 (72.2)</td>
<td>10 (71.4)</td>
<td>94 (77.0)</td>
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<td>Adjuvant therapy, n (%)</td>
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<tr>
<td>No</td>
<td>52 (70.3)</td>
<td>33 (75)</td>
<td>31 (60.8)</td>
<td>14 (66.7)</td>
<td>130 (68.4)</td>
<td>0.49</td>
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<tr>
<td>Yes</td>
<td>22 (29.7)</td>
<td>11 (25)</td>
<td>20 (39.2)</td>
<td>7 (33.3)</td>
<td>60 (31.6)</td>
<td></td>
</tr>
<tr>
<td>Patient follow-up in years, mean (range)</td>
<td>3.7 (0.5–15.6)</td>
<td>3.3 (0.1–9)</td>
<td>4.8 (0.3–15.9)</td>
<td>4.9 (0.6–16.1)</td>
<td>3.1 (0.1–18.1)</td>
<td>0.04</td>
</tr>
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</table>

\(^1\)Summary of baseline clinical features from 190 patients with stage III melanoma enrolled in the New York University Interdisciplinary Melanoma Cooperative Group database, stratified by the level of tumor PTEN as determined by immunohistochemistry (see Materials and Methods).

RESULTS

Partial loss of PTEN expression predicts worse overall survival in stage III melanoma

Complete PTEN loss is associated with worse OS and shorter progression to brain metastasis in stage III melanoma (Buchheit et al., 2014); however, multiple studies suggest that PTEN down-regulation is more common than complete PTEN loss. To test our hypothesis that partial loss of PTEN has significant clinical and biological impact in melanoma, we analyzed PTEN expression in tumors from patients with stage III melanoma (n = 190) enrolled in the New York University (NYU) Interdisciplinary Melanoma Cooperative Group database (see Materials and Methods). We focused on stage III melanoma as a heterogeneous patient population with a median recurrence-free survival of approximately 17 months (Eggermont et al., 2016). Table 1 summarizes patient characteristics and clinicopathological features. Median follow-up time was 3.1 years. Most tumors were regional nodal metastases (116/190, 61.1%); the remainder were in-transit lesions (34/190, 17.9%), satellite lesions (22/190, 11.6%), or local recurrences (18/190, 9.5%). Approximately one third (31.6%) of patients received adjuvant chemotherapy, interferon, leukine, or a vaccine. Activating BRAF and NRAS mutations were identified in 44.1% and 23.0% of tumors, respectively, consistent with their reported prevalence (Smalley, 2003).

PTEN expression was determined by IHC using a validated anti-PTEN monoclonal antibody (D4.3). PTEN staining was optimized by primary antibody titration and using positive and negative controls (see Supplementary Figure S1 online). A composite PTEN expression score for tumors was derived that incorporated staining intensity (0, 1, 2) and distribution (0, 1, 2) (see Materials and Methods) (McCarty et al., 1986): a composite score of 0 indicated absent PTEN, 2 indicated weak PTEN, 3 indicated intermediate PTEN, and 4 indicated strong PTEN (Figure 1a). Overall, 74 of 190 tumors (38.9%) had absent PTEN, 44 of 190 (23.2%) had weak PTEN, 51 of 190 (26.8%) had intermediate PTEN, and 21 of 190 (11.1%) had strong PTEN (Table 1). Thus, 169 of 190 (88.9%) of stage III tumors had some PTEN loss (intermediate, weak, or absent PTEN expression); of these tumors, PTEN was not absent in 95 of 169 (56.2%) cases. A subset of tumors (n = 11) was also stained with two additional PTEN antibodies: (i) 6H2.1 (mouse monoclonal), used by Buchheit and coworkers (2014), and (ii) EPR9941-2 (rabbit monoclonal) (see Supplementary Figure S2 online). Although the EPR9941-2 antibody (1:100 dilution) showed similar results for PTEN expression as we obtained with the D4.3 antibody (1:200 dilution), the 6H2.1 antibody (1:50 dilution) showed less sensitivity for detection of PTEN expression. Several tumors with detectable PTEN expression using D4.3 or EPR9941-2 antibodies had absent PTEN staining using the 6H2.1 antibody. This observation is consistent with a previous study that found the D4.3 antibody used in our study was the most sensitive and specific of five different PTEN antibodies that were tested, including 6H2.1 (Lotan et al., 2011).

Age, sex, primary tumor characteristics, mutation status, clinical substage, or adjuvant treatment did not differ significantly between the four PTEN expression groups (Table 1). Although BRAF mutations have been associated with loss of PTEN expression in primary melanoma (Goel et al., 2006), we did not observe a significant association between BRAF or NRAS mutation status and PTEN expression in our cohort (Table 1, and see Supplementary Figure S3 online). OS of stage III melanoma patients was significantly different when stratified by PTEN expression (strong, intermediate, weak, or absent) (P = 0.012) (Supplementary Figure S4a online). OS was not significantly different between patients whose tumors had absent or weak PTEN or between patients whose tumors had strong or intermediate PTEN (P = 0.99).
Figure 1. A threshold of PTEN loss associated with worse overall survival and Akt activation in stage III melanoma. (a) Representative photomicrographs of PTEN expression in stage III melanoma tissues assessed by immunohistochemical staining. Original magnification ×10. Tumors were stratified according to PTEN expression, which was assessed semiquantitatively as the combination of PTEN staining intensity and staining distribution (see Materials and Methods). PTEN scores for tumors were either 0 (absent), 2 (weak), 3 (intermediate), or 4 (strong). Insets show high-magnification images. Original magnification ×40. Scale bars = 0.2 mm (0.1 mm for insets). (b) Kaplan-Meier survival curves for “PTEN high” tumors (n = 72) vs. “PTEN low” tumors (n = 118). Tumors with strong or intermediate PTEN expression were designated as “PTEN high,” whereas those with absent or weak PTEN expression were designated as “PTEN low.” *P = 0.012. (c) Graph showing inverse correlation between PTEN expression (titrated by siRNA knockdown in SKMEL19 melanoma cells) and Akt1 activity. Duplicate protein samples for control, negative control siRNAs, or PTEN siRNAs were analyzed by RPPA (see Materials and Methods). Normalized, median-centered expression data were log2-transformed and plotted. Top, PTEN vs. p-Akt1 serine473: Pearson r = −0.86, P < 0.0001; bottom, PTEN vs. p-Akt1 threonine 308: Pearson r = −0.73, P = 0.003. (d) Graphs showing inverse correlation between PTEN expression and Akt activity (top, pSer473; bottom, pThr308) in TCGA melanoma cohort (left, n = 355 tumors with RPPA data) (TCGA Network, 2015) and Wistar melanoma patient-derived xenograft cohort (n = 61 tumors with RPPA data) (Krepler et al., 2017). TCGA melanoma cohort: Spearman r = −0.23 and P = 1.6 × 10−6 for PTEN vs. p-Akt1 Ser473; Spearman r = −0.26 and P = 1.0 × 10−6 for PTEN vs. p-Akt1 Thr308. Wistar melanoma patient-derived xenograft cohort: Pearson r = −0.47 and P < 0.0001 for PTEN vs. p-Akt1 Ser473; Pearson r = −0.50 and P < 0.0001 for PTEN vs. p-Akt1 Thr308. RPPA, reverse phase protein array; siRNA, small interfering RNA; TCGA, The Cancer Genome Atlas.
Akt activation is a biological consequence of reduced PTEN dosage in melanoma

Given the clinical impact of reduced PTEN protein expression, we sought to clarify the biological consequences of PTEN dosage in melanoma. To titrate PTEN knockdown, PTEN-expressing SKMEL19 melanoma cells were transfected with PTEN-specific or nontargeting small interfering RNAs (siRNAs) at final siRNA concentrations ranging from 0.3 to 30 nmol/L. Protein lysates were extracted for reverse phase protein assay (RPPA) analysis of PTEN, P-Akt Ser473, and P-Akt Thr308. A significant negative correlation was found between PTEN expression and P-Akt1 Ser473 or P-Akt1 Thr308 (r = -0.86 and r = -0.73, P < 0.0001 and P = 0.003, respectively) (Figure 1c). Similar results were obtained for P-Akt2 and P-Akt3 (data not shown). We confirmed the correlation between PTEN and P-Akt in two independent melanoma RPPA cohorts: (i) The Cancer Genome Atlas (TCGA) melanoma dataset (n = 355 tumors with RPPA data) (TCGA Network, 2015), (ii) a recently published patient-derived xenograft (PDX) melanoma dataset (n = 61 tumors with RPPA data) (Krepler et al., 2017). Significant negative correlations between PTEN expression and Akt activity (Ser473 or Thr308) were also found (TCGA melanoma: PTEN vs. P-Akt1 Ser473, r = -0.20, P = 0.0012; PTEN vs. P-Akt1 Thr308, r = -0.23, P = 0.0003; Wistar PDX: PTEN vs. P-Akt1 Ser473, r = -0.47, P < 0.0001; PTEN vs. P-Akt1 Thr308, r = -0.50, P < 0.0001) (Figure 1d; data not shown for Akt2 and Akt3). Thus, even modest reductions in PTEN expression can activate Akt, suggesting that PTEN dosage has a biological as well as a clinical impact in melanoma.

PTEN deletion and mutation are uncommon in melanoma

PTEN loss in melanoma has been attributed to gene deletions and mutations (Pollock et al., 2002; Tsao et al., 1998). To reconcile this issue, we used PTEN-specific TaqMan PCR assays (Thermo Fisher Scientific, Waltham, MA) to determine PTEN copy number status in 48 stage III/IV melanomas from the NYU Interdisciplinary Melanoma Cooperative Group database and in 17 short-term cultures (STCs) (de Miera et al., 2012) derived from NYU melanoma patients. Primer-probe sets were directed against three different PTEN introns (1, 3, and 8) to exclude detection of PTENPII, an intronless PTEN pseudogene sharing more than 98% homology with the PTEN coding region (Dahia et al., 1998; Poliseno et al., 2010). PTEN was intact in 30 of 48 (62.5%) tumors and 10 of 17 (58.8%) of STCs (Figure 2a; data not shown for STCs). Complete PTEN deletion was identified in 5 of 48 (10.4%) tumors and 1 of 17 (5.9%) STCs, and hemizygous PTEN deletions were identified in 13 of 48 (27.1%) tumors and 6 of 17 (35.3%) STCs (Figure 2a; data not shown for melanoma STCs). We also assessed the frequency of PTEN genomic alterations in TCGA melanoma samples (n = 287 tumors). Of these, 18 of 287 tumors (6.3%) had deep PTEN deletions, and 24 of 87 tumors (8.4%) carried PTEN mutations (Figure 2b). In contrast, deep PTEN deletions were observed in 150 of 242 (62.0%) uterine corpus endometrial carcinomas, whereas 84 of 273 (30.8%) of glioblastomas (glioblastoma multiforme) harbored PTEN mutations. Thus, genomic PTEN alterations are common in specific tumor types but are relatively uncommon in melanoma and cannot explain the high frequency of PTEN loss.

PTEN promoter hypermethylation does not fully explain reduced PTEN expression in melanoma

PTEN transcriptional silencing by promoter hypermethylation has been reported in several cancer types, including melanoma (Kang et al., 2002; Mirmohammadsadegh et al., 2006; Soria et al., 2002). TCGA melanoma DNA methylation data (n = 473 samples) were analyzed to assess the contribution of methylation to reduced PTEN expression. When PTEN mRNA
expression was plotted against PTEN methylation, most (463/473, 97.9%) tumors showed less than 10% PTEN methylation (Figure 2c), and only 3 of 473 (0.6%) of tumors showed more than 30% PTEN promoter methylation. However, a large range (＞32-fold) of tumor PTEN mRNA expression was observed, suggesting that DNA hypermethylation is not a major determinant of PTEN expression. To corroborate this finding, we quantified PTEN methylation by pyrosequencing at five CpG regions (CpG1, CpG2, CpG3, CpG4, and CpG5) upstream of PTEN in DNA from seven stage III/IV tumors that had reduced PTEN expression (by IHC) but lacked PTEN deletion. No relationship was found between methylation at CpG1-5 and PTEN expression (Figure 2d). With the exception of CpG1, PTEN promoter methylation did not exceed 3.7% in 6 of 7 (85.7%) tumors, suggesting that PTEN promoter hypermethylation is uncommon and cannot fully explain the reduced PTEN expression in a majority of melanomas.

Histone deacetylase inhibition restores PTEN expression and inhibits Akt activity in melanoma cell lines

The histone deacetylase (HDAC) superfamily mediates histone deacetylation, causing deregulated tumor suppressor gene expression in cancers (e.g., CDKN1A, BRCA1, ATR) (Ocker and Schneider-Stock, 2007; West and Johnstone, 2014). HDAC inhibitors (HDACi) are approved for treatment of T-cell lymphomas (Rangwala et al., 2012). Although HDACi monotherapy shows limited efficacy in solid tumors, additional clinical trials with combination therapies have been proposed (Thurn et al., 2011).

We postulated that HDACs mediate partial PTEN silencing in some melanomas. To test this we treated seven melanoma cell lines (SKMEL239, WM4235, WM983, SKMEL173, SKMEL103, SKMEL192, and SKMEL147) with the pan-HDACi LBH589 (panobinostat, 15 nmol/L) for 24 hours. After protein extraction, we assessed the impact of HDACi on PTEN
expression by Western blotting. As a control, we analyzed protein levels of p21, whose expression is suppressed in tumor cell lines by histone deacetylation (Sowa et al., 1997). LBH589 treatment increased PTEN and p21 levels in 4 of 7 (57%) melanoma cell lines tested (Figure 3a), an effect that was accompanied by decreased P-Akt (Ser473) (Figure 3a), showing a functional impact of restoring PTEN expression upon PI3K/Akt signaling in melanoma.

**PTEN protein is stable in melanoma cell lines**

Several reports suggest that PTEN function in cancers can be disrupted by aberrant activity of ubiquitin ligases and proteases that control PTEN protein stability (Lee et al., 2015; Wang et al., 2007). We postulated that PTEN destabilization explains PTEN loss in melanoma and tested this by treating SKMEL239 and SKMEL28 melanoma cells with the proteasome inhibitor MG132 or cycloheximide, a translation initiation inhibitor. MG132 treatment (10 μmol/L) rapidly induced expression of p27, an unstable protein that is rapidly degraded in cancer cell lines (Wu et al., 2012) but did not induce PTEN expression (Figure 3b, data not shown for SKMEL28 cells). Cycloheximide treatment (50 μg/ml) did not decrease PTEN levels over a 12-hour period (Figure 3c, data not shown for SKMEL28 cells), suggesting that the PTEN half-life exceeds 12 hours in these cells.

**DISCUSSION**

Genetic mouse models suggest that PTEN dosage confers the degree of PTEN inactivation to regulate tumorigenesis (Alimonti et al., 2010). Despite multiple studies showing reduced, not absent, PTEN in approximately 50% of cases (Mikhail et al., 2005; Zhou et al., 2000), evidence supporting the clinical and biological impact of reduced PTEN in melanoma is lacking. One study found that 58% of stage III melanomas (n = 94 total) had reduced PTEN levels (Bucheit et al., 2014); however, only complete absence of detectable PTEN (21% of melanomas) was associated with significantly worse OS and increased Akt activity (by RPPA). These data are inconsistent with our observation that partial PTEN loss is associated with worse OS and Akt hyperactivation, and they cannot be reconciled with PTEN functioning as a haploinsufficient tumor suppressor. There are several differences between our study and that of Bucheit and coworkers (2014). First, Bucheit et al. used a tissue microarray with three cores per tumor sample and found homogeneous PTEN staining in all but 2.1% of tumors. In contrast, we analyzed full tumor sections from twice as many samples and observed focal PTEN expression in a substantial proportion of samples, possibly reflecting intra-tumor heterogeneity given PTEN’s critical tumor suppressor function. Second, the Bucheit study used a PTEN antibody (6H2.1) that is reported to be less sensitive and specific for PTEN than the antibody used in this study (D4.3) (Lotan et al., 2011). We used the same RPPA platform to assess the relationship between PTEN dosage and Akt activity in melanoma cells with titrated PTEN knockdown and in two large, independent melanoma cohorts (TCGA and Wistar PDX studies). Our results showed an unequivocal negative correlation between PTEN dosage and Akt activation in melanoma.

Data from our NYU cohort and TCGA suggest that PTEN mutations and deletions are relatively uncommon in melanoma, and hence PTEN loss cannot be fully explained by genomic events. Despite this, early reports suggested that PTEN disruption in melanoma cell lines was mediated by deletion or mutation (Pollock et al., 2002), although parallel analysis of 49 matched melanoma/normal tissues found hemizygous PTEN deletions in 10 tumors (20%) and a single PTEN mutation, suggesting that genomic PTEN alterations are more common in cultured cells than noncultured melanoma tissues. We found absent PTEN expression in 74 of 190 (39%) stage III tumors and reduced expression in 95 of 190 (50%) tumors, which is consistent with the Bucheit study (2014); however, we used our samples and those from TCGA to confirm that PTEN loss cannot be fully explained by deletion...
or mutation. PTEN promoter hypermethylation has also been associated with loss of PTEN in melanoma. However, our samples and TCGA data did not show evidence of PTEN promoter hypermethylation in a substantial proportion of tumors, nor was there a strong correlation between PTEN methylation and PTEN expression. Roh and coworkers (2016) found that PTEN promoter hypermethylation was independently associated with worse OS in Korean and TCGA melanoma cohorts, yet paradoxically, PTEN mRNA expression was not correlated with PTEN methylation (Roh et al., 2016). TCGA data indicate that PTEN promoter methylation is inversely correlated with expression of KLLN, a tumor suppressor gene sharing a transcriptional start site with PTEN in the opposite strand (Wang et al., 2013). Furthermore, Hesson and coworkers (2012) concluded that PTEN hypermethylation is rare in tumor cell lines and can be attributed to hypermethylation of PTENP1, a PTEN pseudogene (Dahia et al., 1998) sharing 91% identity with the PTEN promoter. Our experiments and TCGA data reported methylation at PTEN-specific CpG sites and excluded PTENP1. Other studies confirm PTENP1, and not PTEN, hypermethylation in cancer cell lines and tissues (Zysman et al., 2002), possibly explaining the discordance between PTEN methylation and expression in melanoma.

Treatment of melanoma cell lines with HDACi LBH589 led to rapid up-regulation of PTEN expression and reduced Akt activity. Previously, treatment of low PTEN-expressing lung cancer cells with the pan-HDACi trichostatin A induced PTEN expression and cell growth inhibition (Noro et al., 2007). Su and coworkers (2010) reported HDACi-mediated induction of PTEN in synovial sarcoma via stimulation of EGR1, a known transcriptional regulator of PTEN and a putative melanoma tumor suppressor (Nair et al., 1997; Virolle et al., 2001). Thus, HDACi could modulate PTEN transcription directly but also indirectly through induction of factors such as EGR1 that regulate PTEN expression. We did not observe a role for increased protein degradation in mediating PTEN loss in melanoma. Nuclear precursor cell-expressed NEDD-4 inhibits PTEN stability, and levels of NEDD-4 are inversely correlated with PTEN in human bladder cancer (Wang et al., 2007). However, studies in different experimental systems do not support regulation of PTEN ubiquitination by NEDD-4, and the impact of PTEN ubiquitin ligases on PTEN expression may be context specific (Chen et al., 2016; Foulaikou et al., 2008). Additionally, PTEN protein half-life exceeds 25 hours in 786-0 kidney cancer cells (Vazquez et al., 2000), consistent with our observation that PTEN is stable in melanoma cells.

Our findings have implications for management of melanoma. There is a lack of robust biomarkers to predict OS for patients with stage III melanoma, which ranges from 40% to 78% depending on tumor stage (Balch et al., 2010). PTEN assessment might identify patients at higher risk for recurrence and death or candidates for adjuvant therapy; this requires testing in larger, independent cohorts. PTEN loss in melanoma is associated with increased expression of PD-L1 and immunosuppressive cytokines, decreased T-cell infiltration, and resistance to T cell-mediated tumor cell lysis (Parsa et al., 2007; Peng et al., 2016). Adjuvant anti-PD-1 therapy delays recurrence in resected stage III/IV melanoma (Weber et al., 2017); thus, monitoring tumor PTEN expression may guide patient selection for adjuvant immunotherapy. The importance of histone deacetylation to PTEN deregulation in melanoma supports the testing of combinations of immune checkpoint inhibitors and HDACi (Dunn and Rao, 2017). In selected melanoma patients, induction of tumor PTEN expression above a threshold level, or “PTEN-restoring therapy” (Hopkins and Parsons, 2014), might inhibit Akt activity and tumor growth and promote anti-tumor immunity to improve patient survival.

**MATERIALS AND METHODS**

**Patients and tissue specimens**

Patients with stage III melanoma (n = 190) were enrolled in the NYU Langone Medical Center Interdisciplinary Melanoma Cooperative Group biospecimen database (August 2002–December 2015), with prospectively recorded demographic, clinical, and pathological data (Wich et al., 2009). All patients were diagnosed with stage III melanoma according to American Joint Committee on Cancer staging criteria and gave written, informed consent before the study. The NYU School of Medicine Institutional Review Board approved the study protocol. OS was defined as the time from stage III melanoma diagnosis until death from melanoma.

**PTEN IHC**

IHC was performed on formalin-fixed paraffin-embedded tumors, as described (Scanlon et al., 2014), using PTEN rabbit monoclonal antibody D4.3 XP (#9188, 1:200 dilution; Cell Signaling Technology, Danvers, MA). For validation, a subset of tumors was also stained for PTEN using mouse monoclonal antibody 6H2.1 (ABM-2052, 1:50 dilution; Cascade Bioscience, Winchester, MA) or rabbit monoclonal antibody EPR9941-2 (ab70941, 1:100 dilution; Abcam, Cambridge, MA). PTEN expression was scored based on staining intensity and distribution in tumors (McCarty et al., 1986) by a melanoma pathologist (FD) who was blinded to the clinical data (see Supplementary Materials and Methods online).

**Reverse phase protein array**

RPPA was performed at the MD Anderson Cancer Center (Houston, TX) (see Supplementary Materials and Methods). Additional RPPA data was obtained from the TCGA Skin Cutaneous Melanoma study (n = 355 tumors) (TCGA Network, 2015) (see “Bioinformatics analysis of TCGA data” section) and from a published melanoma PDX study (n = 61 tumors) (Krepler et al., 2017).

**PTEN copy number assays**

DNA was extracted from stage III/IV melanoma samples (n = 48) and melanoma patient-derived short-term cultures (n = 17) (de Miera et al., 2012), using the QIamp DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. PTEN DNA copy number was determined using TaqMan Copy Number Assays (Thermo Fisher Scientific) (see Supplementary Materials and Methods).

**Bioinformatics analysis of TCGA data**

Pre-processed genomic, transcriptomic, methylation, and proteomic data from the TCGA Skin Cutaneous Melanoma, Uterine Corpus Endometrial Carcinoma, and Glioblastoma Multiforme studies were visualized and analyzed using the cBioPortal (http://www.cbioportal.org/) browser and analyzed using the cBioPortal (http://www.cbioportal.org/) browser. Spearman correlation was used to assess log-transformed PTEN and P-Akt RPPA data and PTEN RNA sequencing and PTEN methylation data. TCGA
methylated DNA data represents the PTEN-associated CpG island whose methylation is most anti-correlated with PTEN expression.

Cell culture, chemicals, and transfection
Melanoma cell lines SKMEL19, SKMEL28, SKMEL103, SKMEL147, SKMEL173, SKMEL192, and SKMEL239 were from Memorial Sloan-Kettering Cancer Center (New York, NY). WM4235 and WM983 cells were from Meenhard Herlyn (Wistar Institute, Philadelphia, PA). Cells were cultured with DMEM media (Fisher Scientific, Pittsburgh, PA) and 10% fetal bovine serum and 100 mg/ml Normocin (InvivoGen, San Diego, CA), used within 20 passages of original stock, and PCR-tested to confirm absence of mycoplasma contamination. For details of drugs, siRNA, and transfection, see Supplementary Materials and Methods.

PTEN methylation assessment
Genomic DNA was extracted from melanoma tissues using the QIAamp DNA Mini kit (Qiagen, Germantown, MD), according to the manufacturer's instructions. Bisulfite conversion and pyrosequencing were performed by the Genetic Resources Core Facility at Johns Hopkins University School of Medicine (Baltimore, MD) (see Supplementary Materials and Methods).

Statistical analysis
Descriptive analyses were performed between expression groups. Analysis of variance was used to compare mean values for continuous variables between the expression groups, and the chi-square test was used to compare the frequency distribution of categorical variables. Log-rank testing was used to assess differences between PTEN expression groups. The association between PTEN expression and OS was assessed using univariate and multivariable Cox proportional hazards regression models that included primary tumor thickness and was assessed using univariate and multivariable Cox proportional hazards regression models that included primary tumor thickness and ulceration, clinical substage, and adjuvant therapy. Error bars represent standard deviations. All reported P-values were two sided, and P less than 0.05 was considered statistically significant.

Western blotting
Western blotting was performed as described (Segura et al., 2013). The following antibodies (Cell Signaling Technology) were used: PTEN (D4.3 XP Rabbit mAb #9188), Total Akt (pan) (C67E7 Rabbit mAb #4691), Phospho-Akt (Ser473) (D9E XP Rabbit mAb #4060), p21 (12D1 Rabbit mAb #2947), p27 (D69C12 XP Rabbit mAb #3686), HSP90 (C45S5 Rabbit mAb #4877), and goat anti-rabbit IgG-HRP (#7074).

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.07.031.

REFERENCES

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