Promoter Methylation of *PTEN* Is a Significant Prognostic Factor in Melanoma Survival

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Structural compromise of the tumor suppressor gene, phosphatase and tensin homolog *(PTEN)*, occurs in 10% of melanoma specimens, and loss of PTEN expression through DNA methylation of the *PTEN* promoter region has also been reported in a number of other malignancies. However, the role of *PTEN* promoter methylation in melanoma is not well understood. We thus sought to elucidate the prevalence of *PTEN* promoter methylation in melanoma specimens, its relationship to clinical features, and its impact on the outcome of patients with melanoma. *PTEN* promoter methylation data were acquired from an archived primary Korean melanoma cohort (KMC) of 158 patients and, for validation, 234 patients from The Cancer Genome Atlas melanoma (TCGA-MEL) cohort. Hierarchical clustering was performed to identify *PTEN* "high methylated" and "low methylated" samples. Subsequently, differences in clinical features and outcomes based on *PTEN* promoter methylation status were then analyzed using SPSS and R. In the KMC, all tumors were acquired from primary tumors and 65.7% (n = 105) were acral or mucosal by site, whereas in the TCGA-MEL cohort, 90.5% of the tumors were from regional lymph node and distant metastatic lesions. Overall, 17.7% and 45.7% of the specimens harbored *BRAF* mutations in the KMC and TCGA-MEL cohort, respectively. Neuroblastoma RAS viral oncogene homolog was mutated in 12.2% and 26.9% of the tumors in the KMC and TCGA-MEL cohort, respectively. In the KMC, 31 cases (19.6%) were included in the high methylated group versus 142 cases (60.7%) in the TCGA-MEL cohort ($P < 0.001$). Multivariate Cox-regression analysis revealed promoter methylation of *PTEN* to be an independent negative prognostic factor for survival in both the KMC (hazard ratio 3.76, 95% confidence interval = 1.24–11.12, $P = 0.017$) and TCGA-MEL cohort (HR 1.88, 95% confidence interval = 1.13–3.12, $P = 0.015$). Our results indicate that *PTEN* promoter methylation is an independent predictor for impaired survival in patients with melanoma.

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INTRODUCTION

The phosphatase and tensin homologue gene (*PTEN*) is a tumor suppressor located on the human chromosome 10q arm and is an important mediator of carcinogenesis in a variety of human malignancies (Keniry and Parsons, 2008). PTEN is a phosphatase that degrades the product of phosphatidylinositol 3-kinase by dephosphorylating phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3,4-bisphosphate at the 3′ position (Simpson and Parsons, 2001). The loss of function of PTEN from tumor cells causes accumulation of these critical messenger lipids, which in turn increases AKT phosphorylation and activity, leading to decreased apoptosis and/or increased mitogenic signaling (Curtin et al., 2005; Dudek et al., 1997). Epigenetic alterations play an important role in cancer progression through hypermethylation and silencing of tumor suppressor genes, and somatic *PTEN* hypermethylation has been recognized as a means of PTEN downregulation in a subset of malignancies such as prostate cancer, colon cancer, and endometrial carcinoma (Esteller et al., 2000; Goelz et al., 1985; Salvesen et al., 2001; Whang et al., 1998). Sporadic melanomas frequently have a loss of PTEN through loss of heterozygosity, deletion, and mutation (Guldberg et al., 1997). Initial studies demonstrated a mutation rate approximately 30–40% in

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Abbreviations: CI, confidence interval; KMC, Korean melanoma cohort; NRAS, neuroblastoma RAS viral oncogene homolog; PTEN, phosphatase and tensin homolog; TCGA-MEL, The Cancer Genome Atlas melanoma; WT, wild-type

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melanoma cell lines and roughly 10% in primary melanomas (Guldberg et al., 1997; Tsao et al., 1998). Notably, loss of mRNA and protein expression have also been detected frequently without *PTEN* mutation that have been correlated with activation of AKT and other phosphatidylinositol 3-kinase pathway effectors, highlighting the potential role for epigenetic mechanisms, such as *PTEN* promoter methylation, in melanomagensis (Davies et al., 2009; Mirmohammadsadegh et al., 2006; Vasudevan et al., 2009). Previous studies have shown frequent cooccurrence of *BRAF* mutations and *PTEN* mutations or deletions (Tsao et al., 2012). Recently, The Cancer Genome Atlas (TCGA) program described the landscape of genomic alterations in cutaneous melanoma in which they showed a higher frequency of amplifications and overexpression of AKT3 in *RAS*, *NF1*, and triple wild-type (WT) (*RAF*, *RAS*, and *NF1* WT) melanomas (Cancer Genome Atlas, 2015). Also, two recent independent studies showed that a loss or decrease in PTEN expression in melanoma is associated with aggressive tumor behavior supporting a significant role for PTEN loss and the phosphatidylinositol 3-kinase-AKT pathway in melanoma (Bucheit et al., 2014; Mikhail et al., 2005). However, the effect of *PTEN* promoter methylation on clinical outcome has not been fully elucidated.

The goal of our study was to elucidate the prevalence of *PTEN* promoter methylation in melanoma specimens and to explore the prognostic significance of *PTEN* promoter methylation in survival in patients with melanoma. As the first phase of the study, we performed quantitative DNA methylation analysis by pyrosequencing on a discovery set of 158 primary melanoma samples in a Korean melanoma cohort (KMC). Then, we subsequently validated our results with the DNA methylation status of 234 melanomas from The Cancer Genome Atlas melanoma (TCGA-MEL) project (https://tcga-data.nci.nih.gov/tcga/). We utilized hierarchal clustering to uncover latent structure within the *PTEN* methylation space and univariate and multivariate analyses to assess the difference in clinical outcomes according to *PTEN* promoter methylation status in both cohorts. Our findings support the potential relevance of epigenetic alterations as clinically useful prognostic factors, suggesting that further studies are warranted to analyze associations with specific genetic mutation and patient outcomes.

RESULTS

Baseline clinical and genetic characteristics

In total, 392 melanoma samples were analyzed (Table 1; 158 from the KMC and 234 from the TCGA-MEL cohort). The mean age was 59.4 for KMC and 56.6 for TCGA-MEL patients. The gender ratio was 1:1 in the KMC and 1:1.7 in the TCGA-MEL cohort ($P = 0.009$). There was no significant difference in stage of diagnosis, although 10.2% of data in the TCGA-MEL were missing. The most common type of melanoma in the KMC was acral (n = 79, 50%), followed by nonchronic sun damage induced type (n = 32, 20.2%), mucosal type (n = 26, 16.5%), and chronic sun damage induced type (n = 17, 10.8%). Four patients (2.5%) had tumors of unknown primary origin (Supplementary Table S1 online). There were no significant differences in Breslow thickness and ulceration between the two cohorts.

Table 1. Summary and comparison of clinical, pathologic, and genetic characteristics of 392 patients with melanoma

Characteristics	N (%) Korean melanoma cohort (n = 158)	N (%) TCGA-MEL cohort (n = 234)	*P*-value
Age (y)			
Mean (SD)	59.4 (14.2)	56.6 (16)	0.077
Gender (%)			
Female	79 (50)	86 (36.8)	0.009
Male	79 (50)	148 (63.2)	
Breslow thickness			
Mean (SD)	4.7 (4.8)	4.35 (6.0)	0.569
Ulceration			
No	56 (56)	85 (52.8)	0.614
Yes	44 (44)	76 (47.2)	
Anatomic distribution of primary tumor			
Trunk	13 (8.3)	84 (41)	<0.001
Extremities	100 (64.1)	101 (49.3)	
Head and Neck	17 (10.9)	17 (8.3)	
Other specify	26 (16.7)	3 (1.5)	
Stage at diagnosis[1]			
I/II	98 (62)	114 (48.7)	0.137
III/ IV	60 (38)	96 (41.0)	
Metastatic with unknown stage at diagnosis	0	24 (10.3)	
Follow-up period (mo)			
Mean (SD)	42.7 (35.8)	63.9 (62.3)	<0.001
Genotype			
BRAF mutant	26 (17.7)	107 (45.7)	<0.001
NRAS mutant	18 (12.2)	63 (26.9)	0.001
Double WT	103 (70.1)	65 (27.8)	<0.001
PTEN promoter methylation			
Low methylation	127 (80.4)	92 (39.3)	<0.001
High methylation	31 (19.6)	142 (60.7)	
Sample type analyzed for *PTEN* promoter methylation			
Primary	158 (100)	22 (9.4)	<0.001
Regional lymph node	0 (0)	133 (56.8)	
Metastasis	0 (0)	79 (33.7)	

Abbreviations: BRAF, v-Raf murine sarcoma viral oncogene homolog; CSD, chronic sun damage; NA, not available; NRAS, neuroblastoma RAS viral oncogene homolog; PTEN, phosphatase and tensin homolog; SD, standard deviation; TCGA-MEL, The Cancer Genome Atlas melanoma; WT, wild type.

[1]Staging according to the American Joint Committee on Cancer Melanoma Staging System 2009.

The oncogene mutation status was significantly different between the two cohorts. A total of 107 (45.7%) tumors harbored *BRAF* mutations in the TCGA-MEL cohort, whereas only 26 (17.7%) harbored *BRAF* mutations in the KMC ($P < 0.001$). Neuroblastoma RAS viral oncogene homolog (*NRAS*) mutations were found in 18 (12.2%) cases in the KMC and 63 (26.9%) in the TCGA-MEL cohort. A total of 103 tumors (70.1%) in the KMC were *BRAF* and *NRAS* double WT, whereas only 65 tumors (27.8%) were double WT ($P < 0.001$). All of the specimens analyzed in the KMC were primary tumors, whereas 90.5% of the TCGA-MEL cases were from metastatic sources ($P < 0.001$). TCGA-MEL

patients had a significantly longer follow-up period than KMC patients ($P < 0.001$).

PTEN promoter methylation

Unsupervised cluster analysis was used to visualize and characterize broad methylation patterns in the two cohorts. Unsupervised clustering (Figure 1) generated two discrete classes of tumors, designated *PTEN* promoter "low methylated" and "high methylated" (divided by red line). The median methylation values for the low methylated and high methylated groups in the KMC were 17.4 (range, 4.2−47.0) and 53.6 (range, 39.4−91.2), respectively. For the TCGA-MEL cohort, the median β-values for the low methylated and high methylated groups were 0.3 (range, 0.00−0.51) and 0.72 (range, 0.52−0.97), respectively. Overall, 31 cases (19.6%) and 142 cases (60.7%) were considered *PTEN* high methylated in the KMC and TCGA-MEL cohort, respectively (Table 1; $P < 0.001$). As alluded to above, there were more metastatic lesions analyzed in the TCGA-MEL cohort than in the KMC (Table 1).

The clinical and pathologic characteristics of tumors with regard to methylation status are detailed in Table 2. In the KMC, there were significant differences in anatomic distribution of primary tumor ($P = 0.022$) and stage at diagnosis ($P = 0.003$). The *PTEN* promoter high methylated group had significantly more stage III/IV tumors, more acral and mucosal melanomas, and nondouble WT tumors. In the TCGA-MEL cohort, there were significant differences in sample type sequenced ($P = 0.02$) and oncogene mutation status. The *PTEN* high methylated group was more strongly correlated with metastatic lesions and *BRAF* WT tumors ($P = 0.033$) and *NRAS* mutant tumors ($P = 0.019$) compared with the *PTEN* hypomethylated group.

PTEN mutation status, mRNA levels, and protein levels were available for the TCGA-MEL cohort (Figure 2). *PTEN* mRNA correlated ($r = 0.66$, $P < 0.001$) with protein levels. *PTEN* mRNA level was significantly lower in *PTEN* mutated tumors compared with WT tumors ($P < 0.001$) and in tumors that exhibited deep deletions, as expected ($P < 0.0001$). Interestingly, however, no significant difference was seen according to promoter methylation status ($P = 0.378$).

We used the hypomethylation and hypermethylation designations determined by the cluster analysis and compared expression levels for all genes between these two groups using a simple *t*-test and adjusting the *P*-values using the false discovery rate method. There were 2,872 genes with a difference in expression based on methylation status. Using gene enrichment analysis (https://david.ncifcrf.gov/), the top Kyoto Encyclopedia of Genes and Genomes-curated pathways included "cytokine-cytokine receptor interaction" ($P = 5 \times 10^{-19}$) and "hematopoietic cell lineage" ($P = 2.1 \times 10^{-14}$) (Supplementary Table S2 online).

Association of *PTEN* promoter methylation status and clinical and pathologic variables with overall survival

Survival analyses were performed for all patients in the KMC and TCGA-MEL cohort (Table 3). In the KMC, univariate predictors of survival were gender ($P = 0.019$), Breslow thickness ($P = 0.003$), ulceration ($P = 0.006$), stage at diagnosis ($P < 0.001$), *BRAF* mutation ($P = 0.001$), and *PTEN* promoter

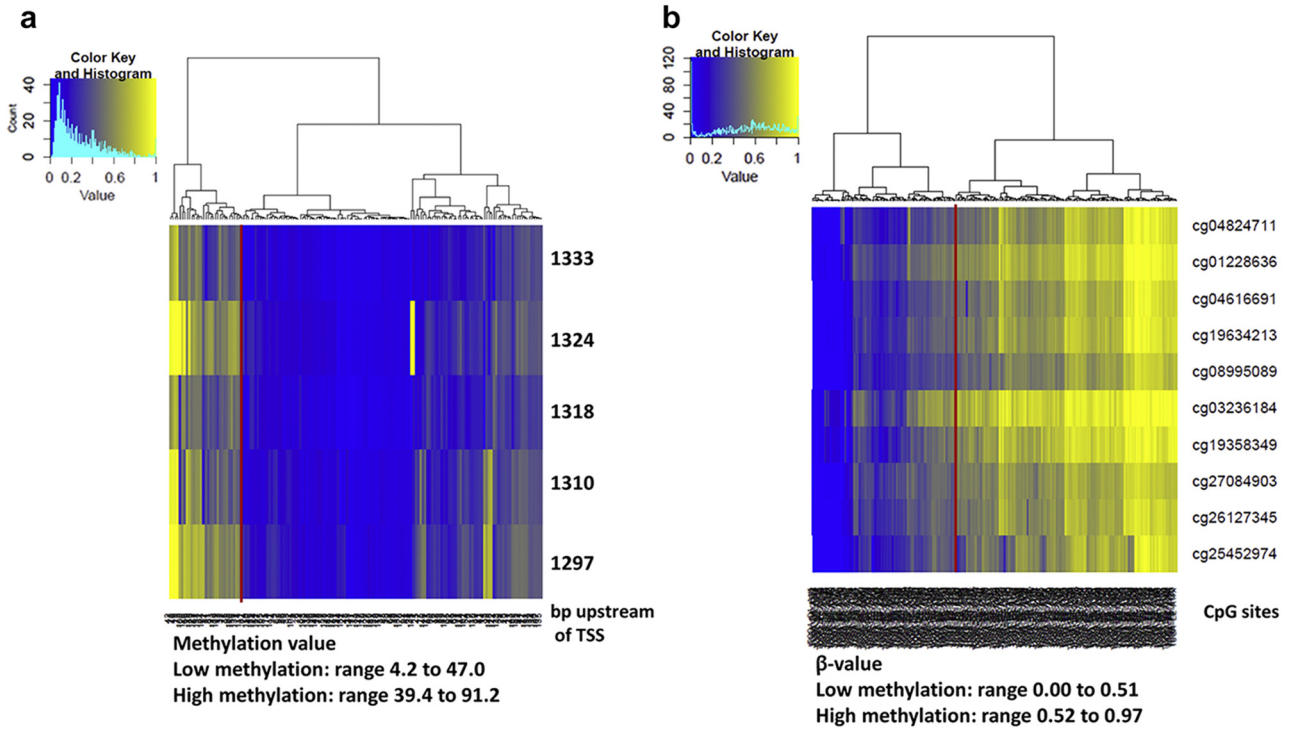

Figure 1. Identification of tumor clusters by hierarchical cluster analysis of methylation profile. Unsupervised clustering generated two discrete classes of tumors, designated *PTEN* promoter "low methylated" and "high methylated" (divided by the red line) for (**a**) the Korean melanoma cohort (n = 158) and (**b**) the TCGA-MEL cohort (n = 234). Overall, 31 cases (19.6%) and 142 cases (60.7%) were considered *PTEN* high methylated in the KMC and TCGA-MEL cohort, respectively. KMC, Korean melanoma cohort; *PTEN*, phosphatase and tensin homolog; TCGA-MEL, The Cancer Genome Atlas melanoma.

Table 2. Correlation of *PTEN* promoter methylation status with clinical, pathologic, and genetic features of patients with melanoma in the Korean melanoma cohort and TCGA melanoma cohort

	Korean melanoma cohort (n = 158)			TCGA-MEL cohort (n = 234)		
Characteristics	***PTEN* promoter low methylation**	***PTEN* promoter high methylation**	***P*-value**	***PTEN* promoter low methylation**	***PTEN* promoter high methylation**	***P*-value**
Age (y)						
Mean (SD)	59.1 (13.7)	60.4 (16.4)	0.694	56.5 (16.3)	56.7 (15.9)	0.911
Gender (%)						
Female	65 (82.3)	14 (17.7)	0.548	34 (39.5)	52 (60.5)	0.958
Male	62 (78.5)	17 (21.5)		58 (39.2)	90 (60.8)	
Breslow thickness						
Mean (SD)	4.42 (4.9)	6.01 (3.8)	0.095	4.17 (4.9)	4.46 (6.6)	0.745
Ulceration (%)						
No	51 (91.1)	5 (8.9)	0.172	33 (38.8)	52 (61.2)	0.931
Yes	36 (81.8)	8 (18.2)		29 (38.2)	47 (61.8)	
Anatomic distribution of primary tumor						
Trunk	9 (69.2)	4 (30.8)	0.022	32 (38.1)	52 (61.9)	0.753
Extremities	86 (86)	14 (14)		37 (36.6)	64 (63.4)	
Head and Neck	15 (88.2)	2 (11.8)		7 (41.2)	10 (58.8)	
Other specify	16 (61.5)	10 (38.5)		2 (66.7)	1 (33.3)	
Sample type analyzed						
Primary	127 (80.4)	31 (19.6)		9 (40.9)	13 (59.1)	0.02
Regional LN	0	0		64 (48.1)	69 (51.9)	
Metastasis	0	0		19 (24.1)	60 (75.9)	
Stage at diagnosis (%)[1]						
I/II	86 (87.8)	12 (12.2)	0.003	39 (34.2)	75 (65.8)	0.086
III/ IV	41 (68.3)	19 (31.7)		44 (45.8)	52 (54.2)	
BRAF						
WT	100 (82.6)	21 (17.4)	0.119	42 (33.1)	85 (66.9)	0.033
Mutant	18 (69.2)	8 (30.8)		50 (47.6)	57 (53.3)	
NRAS						
WT	107 (82.3)	23 (17.7)	0.117	75 (43.9)	96 (56.1)	0.019
Mutant	12 (66.7)	6 (33.3)		17 (27)	46 (73)	
BRAF & *NRAS*						
Double WT	88 (85.4)	15 (14.6)	0.023	25 (38.5)	40 (61.5)	
Nondouble WT	30 (68.2)	14 (31.8)		67 (39.6)	102 (60.4)	0.883

Abbreviations: BRAF, v-Raf murine sarcoma viral oncogene homolog; CSD, chronic sun damage; NA, not available; NRAS, neuroblastoma RAS viral oncogene homolog; PTEN, phosphatase and tensin homolog; SD, standard deviation; TCGA-MEL, The Cancer Genome Atlas melanoma; WT, wild type.
[1]Staging according to the American Joint Committee on Cancer Melanoma Staging System 2009.

methylation ($P = 0.017$). Kaplan-Meier survival analysis showed that patients whose tumors were *PTEN* promoter high methylated had a median survival of 21.6 months compared with 102.8 months for patients whose tumors were *PTEN* promoter low methylated ($P < 0.001$, Supplementary Figure S1a online). Multivariable analysis indicated that male sex (HR = 3.64, 95% confidence interval [CI]: 1.3−10.4; $P = 0.016$) and *PTEN* promoter high methylation (HR = 3.76, 95% CI: 1.2−11.1; $P = 0.017$) were independently associated with poorer survival (Figure 3a). In the TCGA-MEL cohort, univariate predictors of survival were age ($P < 0.001$), Breslow thickness ($P < 0.001$), ulceration ($P = 0.003$), stage at diagnosis ($P = 0.001$), *BRAF* mutation ($P = 0.159$), *PTEN* mRNA level ($P = 0.055$), and *PTEN* promoter methylation ($P = 0.112$). *PTEN* mutation status did not appear to correlate with outcome ($P = 0.62$). Kaplan-Meier survival analysis demonstrated that patients with *PTEN* promoter high methylation had a median survival of 64.2 months compared with 104.7 months for patients with *PTEN* promoter low methylation ($P = 0.12$, Supplementary Figure S1b). Multivariate analysis indicated that higher stage at diagnosis (HR = 2.75, 95% CI: 1.64−4.61; $P < 0.001$) and *PTEN* promoter high methylation (HR = 1.88, 95% CI: 1.13−3.12; $P = 0.015$) were independently associated with poorer survival (Figure 3b). However, *PTEN* mRNA levels did not show significant correlation with overall survival in TCGA-MEL ($P = 0.064$). In the KMC, patients with stage III/IV malignancy were more likely to be present in the *PTEN* promoter high methylated group compared with those with stage I/II malignancy ($P = 0.003$). However, there was no statistically significant difference in stage according to *PTEN* promoter methylation status in the TCGA-MEL cohort ($P = 0.086$).

DISCUSSION

In this study, we identified *PTEN* promoter methylation as an independent prognostic factor in melanoma survival. Although *PTEN* resides within the known canon of melanoma tumor suppressors, the effect of its promoter methylation on survival has not been fully elucidated though it has been previously observed (Lahtz et al., 2010). The negative

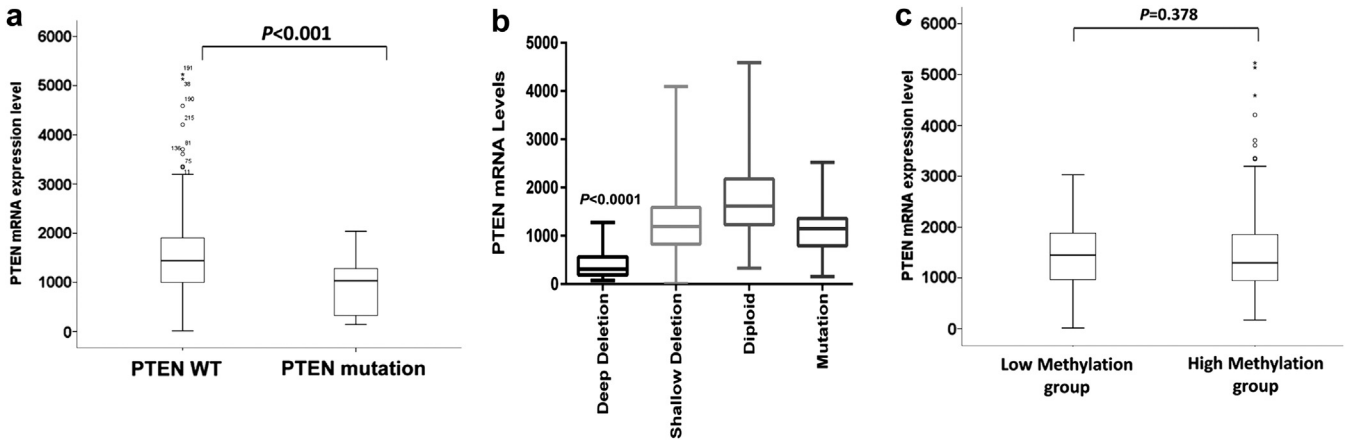

Figure 2. Correlation of *PTEN* mRNA level according to *PTEN* mutation and *PTEN* methylation status in the TCGA melanoma (TCGA-MEL) cohort. *PTEN* mRNA level was significantly lower in (**a**) *PTEN* mutated tumors compared with wild-type tumors ($P < 0.001$) and in (**b**) tumors that exhibited deep deletions ($P < 0.0001$). However, (**c**) no significant difference was seen according to methylation status ($P = 0.378$). *PTEN*, phosphatase and tensin homolog.

impact of *PTEN* promoter methylation on survival is apparently preserved between two highly disparate cohorts. The KMC comprises more primary melanomas from acral and mucosal sites compared with the TCGA-MEL cohort. The consistent effects of methylation on survival even after multivariate adjustment suggest that a core biologic mechanism may be universal.

When the two cohorts were compared, the TCGA-MEL cohort showed significantly higher proportion of high methylated tumors compared with the KMC ($P < 0.001$). This finding might be potentially explained by the fact that the majority of the KMC samples were primary melanomas, whereas most of the TCGA-MEL samples were derived from metastatic lesions. Among the TCGA-MEL tumors, 75.9% of metastatic samples and 59.1% of the primary melanomas were diagnosed as high methylated ($P = 0.02$). In addition, the prevalence of mutations in *BRAF* and *NRAS* differed between the cohorts. It is widely known that incidence of *BRAF* and *NRAS* mutation in tumors from Asian patients is much lower than compared to tumors from Caucasian patients (Kim et al., 2015; Lee et al., 2011). This could be explained by the different proportions of common histologic subtypes of melanoma between the two populations (Lee et al., 2011).

To date, approximately 50 genes have been identified to be aberrantly hypermethylated during melanoma progression and metastasis (Howell et al., 2009). Among them, epigenetic *PTEN* alteration without *PTEN* mutation was first demonstrated in melanoma by Zhou et al. (2000). They showed that weak, or absent, PTEN protein expression frequently occurred in melanomas without *PTEN* mutation, suggesting an epigenetic mechanism of biallelic functional inactivation (Zhou et al., 2000). In 2006, Mirmohammadsadegh et al. (2006) identified epigenetic *PTEN* silencing as a relevant mechanism of inactivating *PTEN* in melanoma, which may promote melanoma development by abrogating repression of the AKT pathway. More recently, Bucheit et al. (2014) showed that loss of PTEN protein expression correlates significantly with decreased overall survival and time to brain metastasis in patients with stage IIIB/C melanoma with *BRAF*(V600)-mutated tumors. Interestingly, our analysis of the TCGA-MEL data did not reveal a significant survival effect with *PTEN* mRNA levels in multivariate analysis (Table 3). In addition, *PTEN* mRNA level was not correlated with methylation status ($P = 0.378$), whereas it was significantly lower in patients with *PTEN* mutation compared with WT ($P < 0.001$). Initial reports suggested that *PTEN* promoter hypermethylation is an alternative mechanism of gene inactivation though others found that methylation state was not a strong predictor of reduced or absent PTEN protein expression in non−small-cell lung cancers (Marsit et al., 2005). Although speculative, *PTEN* promoter methylation in melanoma may have collateral effects on *KLLN*, a gene that shares a transcriptional start site with *PTEN* (in the minus strand) and is a documented tumor suppressor in genetic and functional studies (Nizialek et al., 2013; Wang et al., 2013). In our TCGA data, mean methylation of the promoter region is negatively correlated with *KLLN* mRNA levels (Pearson $r = -0.32$, $P < 0.001$; *t*-test $P < 0.001$). However, we believe that *PTEN* is the more relevant molecule for two reasons. First, the levels of *KLLN* are approximately 60-fold less than *PTEN* and are thus less likely to play a significant biologic effect compared with *PTEN* (Supplementary Figure S2a online). Second, *KLLN* levels do not appear to predict survival (HR = 0.92, 95% CI: 0.64−1.31; $P = 0.35$) (Supplementary Figure S2b). Most of the previous studies used protein-based measurement of PTEN such as reverse phase protein array-based proteomic analysis of frozen tissue or immunohistochemistry assay in formalin-fixed, paraffin-embedded tissues for PTEN expression analysis (Bucheit et al., 2014; Davies et al., 2009). Therefore, it will be important in the future studies to integrate and compare *PTEN* protein, mRNA, genetic, and epigenetic status with each other with clinical outcomes.

Currently there are few validated molecular markers that add to existing prognostic models utilizing clinical and pathologic features (Balch et al., 2009). Clinical application of aberrant DNA methylation as a molecular biomarker in the prediction of prognosis of melanoma is attractive because there is no such established biomarker to date. Recognition that methylation status plays a significant prognostic role in melanoma highlights potential therapeutic opportunities. Recently, it was shown that inhibition of signal transducer

Table 3. Association of *PTEN* promoter methylation status and clinicopathologic variables with overall survival in the Korean melanoma cohort and TCGA melanoma cohort

	Korean melanoma cohort (N = 158)						
		Univariate Cox models			Multivariable Cox model		
Variables	**N**[1]	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Age (by 1-y increment)	158	1.01	0.99–1.03	0.116	1.02	0.99–1.06	0.172
Sex							
Female	79	1.00 (referent)			1.00 (referent)		
Male	79	1.78	1.1–2.9	0.019	3.64	1.3–10.4	0.016
Breslow thickness	120	1.09	1.0–1.2	0.003	[3]	[3]	[3]
Ulceration	100	3.06	1.4–6.8	0.006	1.04	0.4–2.6	0.932
Stage at diagnosis[2]							
I/II	98	1.00 (referent)			1.00 (referent)		
III/IV	60	4.59	2.9–7.5	<0.001	2.02	0.8–5.4	0.164
Anatomic distribution of primary tumor				0.002			0.567
Trunk	13	1.00 (referent)			1.00 (referent)		
Extremities	100	0.5	0.2–1.2	0.116	0.42	0.1–2.1	0.29
Head and neck	17	0.71	0.2–2.1	0.525	0.18	0–2.3	0.188
Other specify	26	1.5	0.6–3.8	0.395	0.75	0.1–9.1	0.819
BRAF							
WT	121	1.00 (referent)			1.00 (referent)		
Mutant	26	2.62	1.5–4.6	0.001	2.87	0.9–9.4	0.082
NRAS							
WT	130	1.00 (referent)					
Mutant	18	0.77	0.4–1.7	0.511			
PTEN promoter methylation							
Low methylation	85	1.00 (referent)					
High methylation	73	3.025	1.8–5.0	<0.001	3.76	1.2–11.1	0.017
	TCGA-MEL melanoma cohort (N = 234)						
		Univariate Cox models			Multivariable Cox model		
Variables	**N**[1]	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Age *(by 1-y increment)*	234	1.02	1.01–1.04	<0.001	1.02	0.99–1.03	0.059
Sex							
Female	148	1.00 (referent)					
Male	86	1.12	0.77–1.64	0.552			
Breslow thickness	168	1.04	1.02–1.06	<0.001	[3]	[3]	
Ulceration	150	1.94	1.26–2.97	0.003	1.32	0.8–2.18	0.286
Stage at diagnosis[2]							
I/II	114	1.00 (referent)			1.00 (referent)		
III/IV	96	1.88	1.27–2.76	0.001	2.75	1.64–4.61	<0.001
Anatomic distribution of primary tumor				0.315			
Trunk	84	1.00 (referent)					
Extremities	101	0.94	0.64–1.39				
Head and neck	17	1.77	0.83–3.77				
Other specify	3	1.98	0.48–8.18				
BRAF							
WT	127	1.00 (referent)			1.00 (referent)		
Mutant	107	0.77	0.54–1.11	0.159	0.91	0.53–1.56	0.722
NRAS							
WT	171	1.00 (referent)					
Mutant	63	1.04	0.7–1.55	0.852			
PTEN							
WT	208	1.00 (referent)					
Mutant	26	1.16	0.65–2.07	0.62			
PTEN mRNA level							
PTEN $mRNA_{low}$	117	1.42	0.99–2.03	0.055	1.57	0.97–2.53	0.064
PTEN $mRNA_{high}$	117	1					

(*continued*)

Table 3. (Continued)

Variables	TCGA-MEL melanoma cohort (N = 234)						
		Univariate Cox models			Multivariable Cox model		
	N[1]	HR	95% CI	*P*-value	HR	95% CI	*P*-value
PTEN promoter methylation							
Low methylation	92	1.00 (referent)					
High methylation	142	1.36	0.99–2.00	0.122	1.88	1.13–3.12	0.015

Abbreviations: BRAF, v-Raf murine sarcoma viral oncogee homolog; CI, confidence interval; CSD, chronic sun damage; HR, hazard ratio; NRAS, neuroblastoma RAS viral oncogene homolog; PTEN, phosphatase and tensin homolog; TCGA-MEL, The Cancer Genome Atlas melanoma; WT, wild type.
All predictors with univariate $P \leq 0.20$ are included for Cox multivariate analysis.
[1]The number of available data for a particular variable in the univariate analysis.
[2]Staging according to the American Joint Committee on Cancer (AJCC) Melanoma Staging System 2009.
[3]There was strong multicollinearity between "Stage at diagnosis" and "Breslow thickness," so we decided to only include "Stage at diagnosis," given that this resulted in the largest number of complete data sets (n = 158) with no impact on the conclusions.

and activator of transcription 3 acetylation in melanoma results in demethylation and reactivation of multiple tumor suppressor genes, which indicates the feasibility of targeted therapeutic reversal of melanoma suppressor gene hypermethylation through interference with a common epigenetic silencing mechanism (Lee et al., 2012).

There are several limitations of this study. First, there are ascertainment differences given the geography and nature of lesions (i.e., primary vs. metastatic). However, as alluded to above, the overall consistency may undergird an important physiologic effect that was fortuitously uncovered. Second, the methodology used to assess methylation was different between the two cohorts. Given the extremely limited amount of primary archival tissue available for evaluation in the KMC, we utilized pyrosequencing, which restricts us to a highly focused analysis. In contrast, the TCGA-MEL samples were scored using a genome-wide bead-based microarray (Bibikova et al., 2011). In an ideal setting, identical approaches would be employed, but such is not the case given practical limitations. To make the comparison meaningful between the two cohorts, we restricted the TCGA-MEL methylation data to 10 CpG sites corresponding to the region analyzed in the KMC. Lastly, there are also a variety of methods to define methylation status itself (Cancer Genome Atlas Research, 2008, 2011, 2013, 2014). Here, we used Ward's agglomerative hierarchical clustering method to define methylation status rather than relying on prespecified cutoffs because methylation data is a continuous variable. Nevertheless, an abbreviated analysis indicates that the *PTEN* methylation effect is even conserved using prespecified cutoffs (Supplementary Figure S3 online).

In conclusion, we show that *PTEN* promoter methylation is a significant negative prognostic marker in survival in patients with melanoma. Additional studies are warranted to investigate whether *PTEN* promoter methylation status is of therapeutic relevance, both in shaping the efficacy of established therapies (i.e., BRAF inhibitors or immunotherapies) and as a potential target for novel therapies.

MATERIALS AND METHODS

Patients and samples

Analyses were performed on two data sets:

1. The KMC consisted of 158 patients with melanoma, diagnosed from January 2005 to January 2012, at Yonsei University College of Medicine, Severance Hospital and Yonsei Cancer Hospital in Seoul, Korea. Clinical data including age, sex, tumor-node-metastases stage, Breslow's thickness, ulceration, and survival (follow-up persisted until patients were lost to follow-up) were collected (Supplementary Table S1). The staging was determined according to the American Joint Committee on Cancer guidelines for melanoma at the time of diagnosis (Balch et al., 2009). This study protocol was approved by the Institutional Review Board of Severance Hospital and was conducted according to the

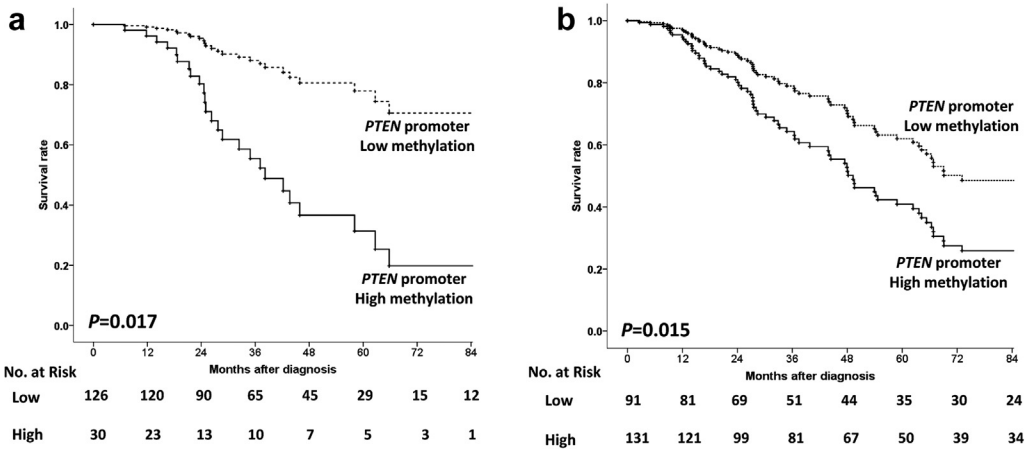

Figure 3. Adjusted Kaplan-Meier curves for overall survival according to *PTEN* promoter methylation status in the Korean melanoma cohort (KMC) and TCGA melanoma (TCGA-MEL) cohort. *PTEN* promoter high methylation status was independently associated with poorer survival in both (**a**) KMC ($P = 0.017$) and (**b**) TCGA-MEL cohort ($P = 0.015$). KMC, Korean melanoma cohort; *PTEN*, phosphatase and tensin homolog; TCGA-MEL, The Cancer Genome Atlas melanoma.

Declaration of Helsinki Principles. Written informed consent was acquired before inclusion in this study.

2. The TCGA-MEL cohort is available for public access and includes genomic, mRNA, and DNA methylation data, as well as corresponding clinical information for 234 patients with melanoma (Supplementary Table S3 online). All somatic mutation and normalized RNAseq data for TCGA-MEL (http://cancergenome.nih.gov) were downloaded from the BROAD Firehose pipeline management system via the R package RTCGA-Toolbox (Samur, 2014). Normalized RNAseq Version 2 data were made available via the Broad Firehose pipeline, using MapSplice (Wang et al., 2010) to perform alignment and RSEM (Li and Dewey, 2011) to perform quantification. DNA methylation level 2 data for TCGA-MEL samples were obtained from the TCGA-MEL data portal (https://TCGA-mel-data.nci.nih.gov/TCGA-mel/). Processed methylation data from March 2012 were used for this analysis. Somatic and RNAseq data were downloaded from the October 2014 run date. Some samples were not available across all platforms. After merging, a total of 234 samples were available for analysis. All data that were available across all three platforms were used, and no other selection/inclusion criteria were used. Among the 234 patients, 230 patients were Caucasian, 1 patient was African American, 1 patient was Asian, and 2 patients were of unknown race.

DNA preparation and mutation analyses for the KMC

Formalin-fixed, paraffin-embedded tissue blocks were retrieved and independently confirmed as malignant melanoma by two pathologists. Tumor-rich areas (>80%) were extracted from five paraffin sections of 10 μm thickness containing a representative portion of each tumor block, using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany). To detect hotspot mutations, we amplified exon 15 (codon 600) of *BRAF* and exons 1 and 2 (codon 12, 13, 61) of *NRAS* by PCR in at least two separate preparations of genomic DNA. The primer sequences are listed in Supplementary Table S4 online. We performed pyrosequencing using PyroMark Q24 (Qiagen, Valencia, CA) at room temperature with PyroMark Gold Q24 reagents (Qiagen) following the manufacturer's instructions. Sequencing analysis was performed using PyroMark Q24 software (Version 1.0.10; Qiagen) (Jin et al., 2013; Si et al., 2012). All mutations were confirmed by repeat bidirectional sequencing on the ABI sequencer.

Analysis of DNA methylation for the KMC

One microgram of DNA isolated from paraffin-fixed tissue was used for bisulfite treatment done by the EZ DNA methylation kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. The Gene2Promoter software (Genomatix Software, Munich, Germany) allows the automated selection of promoters from a list of accession numbers or gene IDs. We analyzed five potential promoter regions spanning 1,333 basepairs upstream and 1,297 basepairs downstream around the transcription start site of the *PTEN* gene. Subsequently, CpG islands were identified within this core promoter region. For primer design, the DNA sequences were converted in silico to the methylated form of CpG as follows: CG motifs were converted to YG with Y equaling either C/T or G/A, and subsequently, C was converted to T. Using this converted sequence, methylation-specific primers for quantitative sequencing (pyrosequencing) of *PTEN* CpGs were designed using the Biotage Assay Design software (PyroMark Assay Design 2.0) and pyrosequencer PyroMark Q24 version 1.0.10 software (Qiagen, Germantown, MD) as follows: forward primer, GGATGTGGGTGTTTGTGTAATTA; reverse primer, Biotin-AATTCCCACTCCCCAATAATAAC (reverse complementary); sequencing primer, TTTGTGTAATTAGTTTTTTA; sequence to analyze, AGYGTTAGTTTYGATAGYGTTTTTTYGGG AGGTTGGTTYG. For pyrosequencing, 50 ng of bisulfite treated DNA was used in the PCR reaction with 200 nmol/l forward and reverse primers. PCR conditions for *PTEN* were 1 × 95 °C for 15 minutes (95 °C for 40 seconds, 55 °C for 40 seconds, and 72 °C for 40 seconds), 50 cycles and 1 × 72 °C for 10 minutes using 0.5U of Amplitaq Gold (Applied Biosystem, TX). The percentage methylated fraction (C/T ratio) is automatically calculated. Each site is analyzed as a C/T polymorphism where a 100% C-reading denotes a fully methylated C in the original genomic DNA sample and a 100% T-reading denotes that this C was unmethylated in the gDNA. Intermediate C/T percentages denote partial methylation at the level of the sample. Then the value of methylation was calculated as the peak height methylated/(peak height methylated + peak height unmethylated) × 100.

Analysis of DNA methylation for TCGA-MEL

DNA methylation level 2 data for TCGA-MEL samples were obtained from the TCGA-MEL data portal (https://TCGA-mel-data.nci.nih.gov/TCGA-mel/). The Illumina Infinium DNA methylation platform HumanMethylation 450 (Illumina, San Diego, CA) was used to obtain DNA methlyation profiles of 234 melanoma samples. The DNA methylation score for each locus is presented as a β-value ($\beta = M/(M+U)$), in which M and U indicate the mean methylated and unmethylated signal intensities for each locus, respectively. To be able to compare Illumina I and II chemical assays, we applied a peak-based correction method (Dedeurwaerder et al., 2011). For each assay of each sample we estimated the β-value positions of the unmethylated and methylated peaks using an Epanechnikov kernel. The unmethylated peak was moved to β-value = 0 and the methylated peak to β-value = 1 by linear scaling, stretching β-values in between accordingly. Values below 0 were set to 0, and those above 1 were set to 1. The *PTEN* gene region contained 63 CpGs. We used standard deviation > 0.1 to reduce the data to 38 CpGs with considerable variation of DNA methylation across samples. We removed four paired samples and three samples with conflicting primary/metastatic annotations between sample barcode and clinical annotation file. For a parallel comparison of methylation sites, we restricted the methylation CpG sites corresponding to KMC promoter regions resulting in 10 CpG sites.

Analysis of *PTEN* mRNA level for TCGA-MEL

Normalized RNAseq data for TCGA-MEL (http://cancergenome.nih.gov) were downloaded from the BROAD Firehose pipeline management system via the R package RTCGA-Toolbox (Samur, 2014). For *PTEN* mRNA expression level, we segregated the TCGA-MEL cohort into two groups by using the median expression level. We compared the upper and lower halves for our survival analysis, labeling them *PTEN* $mRNA_{high}$ versus *PTEN* $mRNA_{low}$.

Cluster analysis

Ward's agglomerative hierarchical clustering using Euclidian distance was performed using the function hclust of the R statistical package (R Development Core Team, 2010; version 3.1.1).

Statistical analysis

Association of* PTEN *promoter methylation status with clinical and pathologic variables. Categorical data are described using frequencies and percentages, and continuous data are described

using means ± standard deviations or median (range) for normally distributed data. The chi-squared test or Fisher's exact test was used to differentiate the rates of different groups, and differences in measurement data of two groups were evaluated by the unpaired *t*-test or the Mann-Whitney test. We used univariate logistic regression analyses to explore associations of *PTEN* promoter methylation status with available clinical and pathologic variables, including age, sex, stage, *BRAF* and *NRAS* mutation status, anatomical distribution of primary tumor, Breslow thickness, and ulceration.

Association of* PTEN *promoter methylation status and clinicopathologic variables with overall survival. We investigated association between clinicopathologic factors, *PTEN* promoter methylation status, and oncogene mutation status with overall survival, defined as the interval from time of diagnosis of primary melanoma to death. Cases in which the endpoint was not reached at the time of the last follow-up were censored. Univariate results were displayed by the Kaplan-Meier method, and hazard ratio estimates and *P*-values were derived from a Cox proportional hazard model. Multivariable analyses were performed on variables with a *P*-value of 0.20 or less in univariate analyses. CIs were calculated with coverage of 95%. All reported *P*-values are nominal and two sided. We applied a significance level of 5%. All statistical analyses were performed using SPSS Statistics software (version 18.0; SPSS Chicago, IL) or R 3.1.1.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

For correspondence about integrative data analysis including TCGA data, please contact HT. For correspondence about clinical and experimental results of Korean melanoma cohort, please contact SYR.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.01.024

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