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Report

Infrequent hypermethylation of the *PTEN* gene in Korean non-small-cell lung cancers

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CpG islands (CGIs) hypermethylation is implicated in the pathogenesis of many cancers, including lung cancer. The phosphate and tension homolog (PTEN) is a tumor suppressor that controls a variety of biological processes including cell proliferation, growth, migration, and death. The defects in PTEN regulation have a profound impact on carcinogenesis. Herein, we have examined the methylation status of the human *PTEN* gene in 137 primary non-small-cell lung cancers (NSCLCs) by using a methylation-specific PCR and correlated the results with clinicopathological features. Promoter methylation of the *PTEN* gene was observed in 5.1%, 2.9%, and 0.0% of three different CpG regions, which were localized at −1460 to −1263, −984 to −848, and −300 to −128 nucleotides upstream of the translation start site, respectively. Reverse transcription-PCR and immunohistochemical analysis showed the methylation of the CGI region at −984 to −848 correlated more accurately with *PTEN* expression. In addition, no significant correlation was found between *PTEN* methylation and clinicopathological factors, including the survival rates. These findings suggest that promoter methylation is not an important mechanism for *PTEN* deregulation in NSCLCs from Koreans. (*Cancer Sci* 2010; 101: 568–572)

The tumor suppressor phosphate and tension homolog (PTEN) is a phosphatase that antagonizes the phosphoinositol-3-kinase (PI3K)/AKT signaling pathway and suppresses cell survival, as well as cell proliferation.[1] Importantly, it is the second-most frequently mutated in human cancer after p53, and its deregulation is also implicated in several other diseases.[2,3] The functions and mechanisms of PTEN have become more diverse and are currently under intensive investigation. Recent study demonstrates that the loss of PTEN leads to massive alterations of chromosomes.[2] Although mutation and homozygous deletion represent the most common mechanism underlying *PTEN* inactivation,[3] the promoter methylation frequently occurs in certain types of cancers, such as thyroid cancer, melanoma, and breast cancers.[4–6] Notably, reduction of PTEN protein expression has been reported in ~70% of NSCLCs.[7] However, a genetic analysis of *PTEN* in non-small-cell lung cancers (NSCLCs) has demonstrated a *PTEN* alteration in 8–16% of the examined NSCLC cell lines.[8,9] Epigenetic-mediated gene silencing constitutes an alternative or complementary mechanism to genetic alterations in tumorigenesis.[10,11] Actually, methylated gene profiles have been widely studied in lung cancer among Western populations.[12,13] Moreover, smokers are predisposed to an acquisition of multiple epigenetic alterations in key cellular regulatory genes within the respiratory tract.[14] In this study, we investigated the methylation status of the promoter regions of the human *PTEN* gene in surgically resected primary NSCLCs by using a methylation-specific PCR (MSP), and correlated the results with the clinicopathological characteristics.

Materials and Methods

Patients and tissue samples. All tumor and corresponding nonmalignant lung tissue specimens were obtained from 137 Korean NSCLC patients who underwent curative resection at the Kyungpook National University Hospital (Daegu, Korea) from January 2002 to July 2006. None of these patients received chemotherapy and radiotherapy before the surgery. Informed consent was obtained from each patient before surgery. This study was approved by the institutional review board of the Kyungpook National University Hospital. The clinicopathological characteristics of the patients are summarized in Table 1. Ninety-three males and 44 females, with a mean age of 63.1 ± 8.5 years, were included in this study. There were 41 never-smokers and 96 ever-smokers (current- or former-smokers) with mean pack-years of 27.4 ± 25.2. Histologic type of NSCLS was 59 cases of squamous cell carcinomas (SCC) and 78 adenocarcinomas (AC). The pathologic stages were 86 cases at stage I, and 51 cases at stages II–IIII. All of the tumor and macroscopically normal lung tissue samples were obtained at the time of surgery, and were rapidly frozen in liquid nitrogen and stored at −80°C until genomic DNA preparation. The macroscopically normal lung tissues were confirmed by hematoxylin–eosin staining. All AC patients had been examined for epidermal growth factor receptor (*EGFR*) (exon 18-21) mutations. *EGFR* mutations was found in 18 (23.1%) of 78 AC cases.

Cell culture and 5-aza-2′-deoxycytidine (5-AzadC) treatment. Twelve human lung cancer cell lines, five AC (H23, H522, H1373, H1793, and H2009), three SCC (H157, SW900 and H226), two LC (H460 and H1299), and two small-cell lung cancer lines (H187 and H2108) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were propagated under the instructions of ATCC. H157, H1299, and H2108 cells were treated with 20 μM 5-AzadC for 3 days and the culture media were changed daily.

Total RNA isolation and reverse transcription–PCR. Total RNA was extracted from either the cultured cells or tumor tissues of the patients by using TRIzol (Invitrogen, Mt Waverley, Vic., Australia) according to the manufacturer's instructions. Residual genomic DNA was digested with RNase-free DNase (Invitrogen). First strand cDNA was reverse-transcribed from 2 μg of total RNA in a total volume of 20 μL using oligo(dT) and a SuperScript preamplification kit (Invitrogen). The resulting cDNA was amplified by the forward (5′-AACCCACCACAGCTAGAACT-3′) and reverse (5′-ATACACATAGCGCCTCTGAC-3′) primers

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Table 1. Primer set for MSP

Primers	Sequences	Size (bp)	Ta (°C)
Methylated alleles			
Region 1 MF	5′-GTTTGGGGATTTTTTTTTCGC-3′	178	57.5
Region 1 MR	5′-AACCCTTCCTACGCCGCG-3′		
Region 2 MF	5′-TTTTTTTTCGGTTTTTCGAGGC-3′	132	59
Region 2 MR	5′-CAATCGCGTCCCAACGCCG-3′		
Region 3 MF	5′-GGTTTCGGAGGTCGTCGGC-3′	155	58
Region 3 MR	5′-CAACCGAATAATAACTACTACGACG-3′		
Unmethylated alleles			
Region 1 UMF	5′-TATTAGTTTGGGGATTTTTTTTTTGT-3′	184	57.5
Region 1 UMR	5′-CCCAACCCTTCCTACACCACA-3′		
Region 2 UMF	5′-TTTTGAGGTGTTTGGGTTTTTGGT-3′	124	59
Region 2 UMR	5′-ACACAATCACATCCCAACACCA-3′		
Region 3 UMF	5′-TGGGTTTTGGAGGTTGTTGGT-3′	173	56
Region 3 UMR	5′-ACTTAACTCTAAACCACAACCA-3′		

bp, base pairs; MSP, methylation-specific PCR; Ta, annealing temperature.

under the same conditions as described previously.[15] All amplified products were separated on 2% agarose gels, visualized using ethidium bromide, and photographed.

Genomic DNA isolation and methylation analysis. Genomic DNA was extracted by using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). The methylation status of the *PTEN* gene was analyzed with bisulfite-treated genomic DNA by an MSP.[16] Three primer sets were used to amplify specifically unmethylated or methylated forms of the *PTEN* gene, as previously reported.[7,17] The primer sequences, annealing temperatures, and expected PCR product length are summarized in Table 1. All PCR amplifications were carried out by using reagents supplied in a GeneAmp DNA Amplification Kit with AmpliTaq Gold as the polymerase (PE Applied Biosystems, Foster City, CA, USA) on PTC-100 (MJ Research, Watertown, MA, USA). CpGenome Universal methylated and unmethylated DNA (Chemicon, Temecula, CA, USA) was used as a positive control for the methylated and unmethylated genes, respectively. Negative control samples without DNA were included for each set of PCR. PCR products were analyzed on 2% agarose gel, stained with ethidium bromide, and visualized under UV light. Each MSP was repeated at least once to confirm the results.

Immunohistochemistry. Tissue sections (3 μm) were collected on gelatin-coated slides. The sections were deparafinized in xylene, hydrated, and endogenous peroxidase was blocked by incubating with 0.3% H_2O_2 for 10 min. Antigen retrieval was carried out by microwave-heating for 10 min in citrate buffer (pH 6.0). After blocking with 10% goat non-immune serum, section was incubated with rabbit polyclonal antibody against PTEN (1:100 dilution; Invitrogen) at 4°C for overnight. The UltraTech HRP Streptavidin-Biotin Detection system (Immunotech SAS, Marseille, France) was used to visualize the antibody binding, and the sections were counterstained with hematoxylin. Immunohistochemical staining in each section was estimated by a pathologist unaware of the clinical data.

Statistical analysis. The relationship between the methylation and clinicopathological characteristics was analyzed by using the χ^2 or Fisher's exact test for categorical variables. A *P*-value of <0.05 was considered to be statistically significant. A logistic regression test was conducted to estimate the relationship between methylation and the covariates of age, gender, exposure to tobacco smoke, and histology. The overall survival rate of NSCLC patients diagnosed, with or without *PTEN* methylation, was compared by using the Kaplan–Meier method and the log-rank test.

Results and Discussion

The *PTEN* methylation status was analyzed in 137 primary NSCLCs and their corresponding nonmalignant lung tissues by

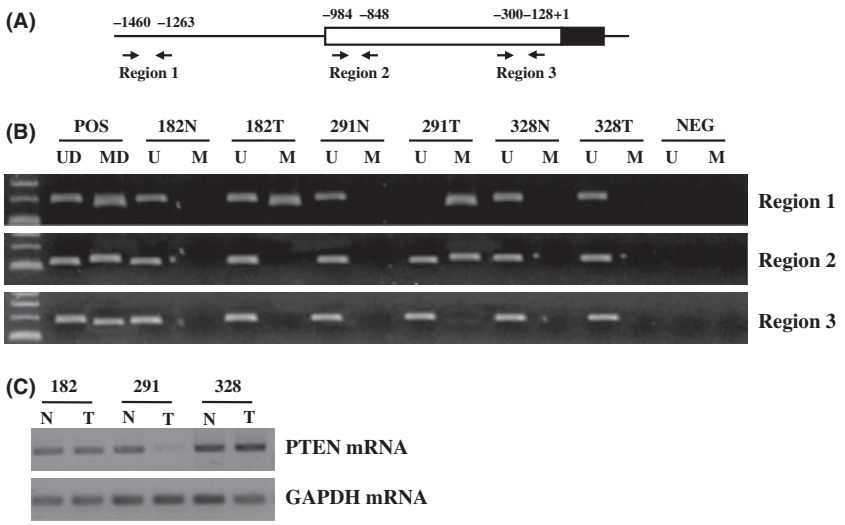

Fig. 1. Representative results of methylation-specific PCR (MSP) and RT-PCR analysis of the phosphate and tension homolog (*PTEN*) gene in non-small-cell lung cancer (NSCLC) patients. (A) Map of the *PTEN* promoter area. Open and closed boxes indicate 5′-untranslated regions and protein coding domain of the first exon *PTEN* gene, respectively. +1 indicates the translation start site and the location of the three primer sets is shown. (B) The methylation status of *PTEN* gene was analyzed by MSP. CpGenome Universal methylated or unmethylated DNA (Chemicon, Temecula, CA, USA) was used as a positive control for the methylated or unmethylated products, respectively. Water was used as a negative control. N, non-malignant tissue; T, tumor tissues; U and M, amplified product with primers that recognize the unmethylated or methylated sequences. (C) Expression of *PTEN* mRNA was performed on primary tissues by RT-PCR. Amplified products were run on 2% agarose gel and appeared at positions corresponding to base pair lengths. GAPDH was used as an internal loading control.

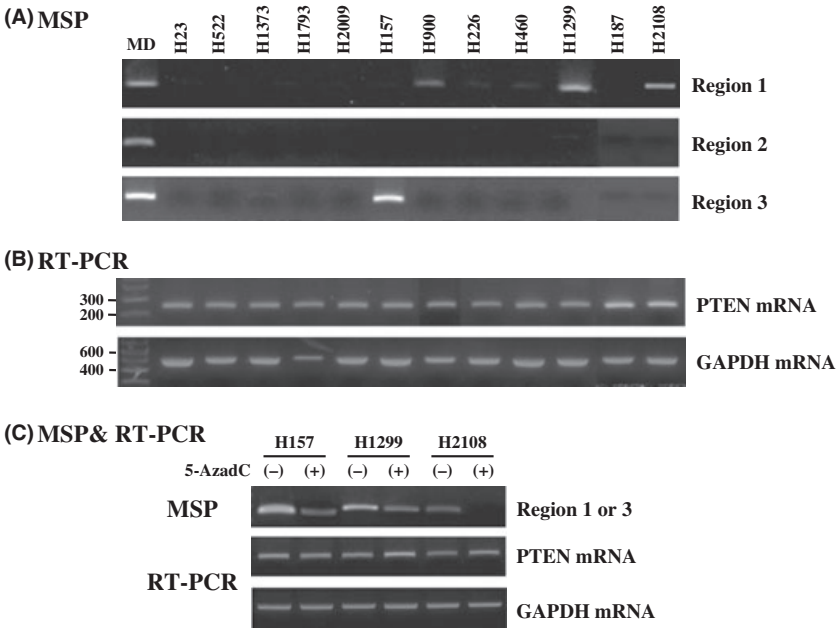

Fig. 2. Methylation-specific PCR (MSP) and RT-PCR analysis of the phosphate and tension homolog (*PTEN*) gene in 12 human lung cancer cell lines. The methylation status (A) and mRNA expression (B) of the *PTEN* gene was analyzed for 12 cell lines by MSP and RT-PCR, respectively. (C) Methylation status of region 1 or 3 CGIs and expression of *PTEN* mRNA after demethylating agent 5-aza-2′-deoxycytidine (5-AzadC) treatment were examined by MSP and RT-PCR analysis. (–) indicates vehicle alone treatment; (+) the 20 μM 5-AzadC treatment for 3 days. The legend is the same as for Fig. 1.

using MSP. A sequence examination of the *PTEN* and highly conserved processed *PTEN* pseudogene indicates that the high degree of homology extends to −841 nucleotides upstream of the *PTEN* translational start site. We designed three primer sets which covered different CpG islands (CGIs), localized upstream of the translation start site of the human *PTEN* gene (Fig. 1A). Both region 1 and 2 primers (nucleotides −1460 to −1263 and nucleotides −984 to −848, respectively) amplified a region of the promoter specific *PTEN* that is not homologous to the pseudogene. The region 3 primer (nucleotides −300 to −128) was localized within the *PTEN* sequence, which shares over 98% of its identity with the pseudogene. Each region specific primer set yielded a single band of expected size, and representative examples of the MSP analysis are illustrated in Figure 1B. Unmethylated (U) bands were detected in most of the nonmalignant and malignant tissues, thus confirming the integrity of the DNA in these samples. As the tumor specimens represented macroscopically isolated samples containing both tumor and nonmalignant tissue, this finding was expected. MSP analysis with *PTEN* gene-specific region 1 and 2 primers showed the methylation frequencies of 5.1% and 2.9%, respectively, in the tumor tissues. However, through using region 3 primers, none of the tumors and matched normal tissues was methylated (Fig. 1B). The bisulfite-sequencing analysis of the representative PCR products confirmed their methylation status, and showed that all cytosines at non-CpG sites were converted to thymine (data not shown), ruling out the possibility of incomplete bisulfite conversion. From these results, it is concluded that *PTEN* methylation is likely to be an rare event, being apparently different from the previous finding in which an aberrant methylation of the region 1 CGI in the *PTEN* promoter focused was detected in seven of 20 (35%) PTEN-negative NSCLCs, and in 39 of 151 (26%) primary lung cancers.[7,18] Of 12 lung cancer cell lines, three cell lines (H900, H1299, and H2108) exhibited methylation at the region 1 CGI, and one cell line (H157) exhibited methylation at the region 3 CGI. However, no methylation was detected at the region 2 CGI (Fig. 2A). Although the reported frequencies of certain genes are highly variable,[13] several factors may account for this discrepancy. One, this difference might depend on the MSP assay sensitivity and the position of the CpG sites examined. However, the standard MSP with the same primers was used to determine the methylation status of the *PTEN* promoter in both studies. Alternatively, the discrepant results might also have been due to genetic or environmental differences of the study population. Intriguingly, mutations in the kinase domain of the *EGFR* gene are more frequent in never-smokers, females, and East Asian populations, whereas Kristen rat sarcoma viral oncogene homolog (*KRAS*) mutations are more frequent in smokers, males, and Western populations.[19,20] Moreover, the methylation rates of O^6-methylguanine DNA methyltransferase (*MGMT*) and Glutathione *S*-transferase pi 1 (*GSTP1*) are significantly higher in Western cases than in those from East Asia; conversely, *p16* methylation more frequently occurs in Chinese patients.[21,22] Furthermore, recent studies have shown that the susceptibility of gene-specific methylation also varies according to the type of carcinogen exposure, including cigarette smoking, dietary factors, and occupational exposure.[23] Therefore, further studies of the cause and biological implications of this discrepancy remain to be elucidated in large numbers of patients.

To examine the methylation of which CGIs correlated with *PTEN* expression, we examined the expression levels of *PTEN* mRNAs in tumor tissues, as well as a panel of 12 lung cancer cell lines. As shown in Figure 1C, RT-PCR analysis showed undetectable *PTEN* mRNA expression in the tumor (sample, 291T) with methylation at the region 2 CGI, whereas *PTEN* mRNA was present in the tumor (sample, 182T) with methylation at the region 1 CGI. In addition, four cell lines with methylation at the region 1 or 3 CGI had expressed *PTEN* mRNA (Fig. 2B). Moreover, in the cell lines (H157, H1299, and H2108) that exhibited methylation at the region 1 or 3 CGI, the demethylating agent 5-AzadC treatment did not increase the *PTEN* mRNA expression level (Fig. 2C). These findings suggest that methylation at the region 2 CGI may correlate with loss of *PTEN* expression. In contrast to our finding, Soria *et al.*[7] reported that *PTEN* mRNA expression level was low in H1299 cells with methylation at the region 1 CGI and after treatment with 5-AzadC, its mRNA expression level was increased, thereby suggesting that methylation at the region 1 CGI correlates with *PTEN* expression. In order to verify whether our finding of no increased *PTEN* mRNA expression in H1299 cells after 5-AzadC treatment was due to incomplete demethylation, we performed a bisulfite sequencing analysis of the region 1 CGI containing 17 CpG dinucleotides on clones of H1299 cells treated with or without 5-AzadC. In 5-AzadC untreated H1299

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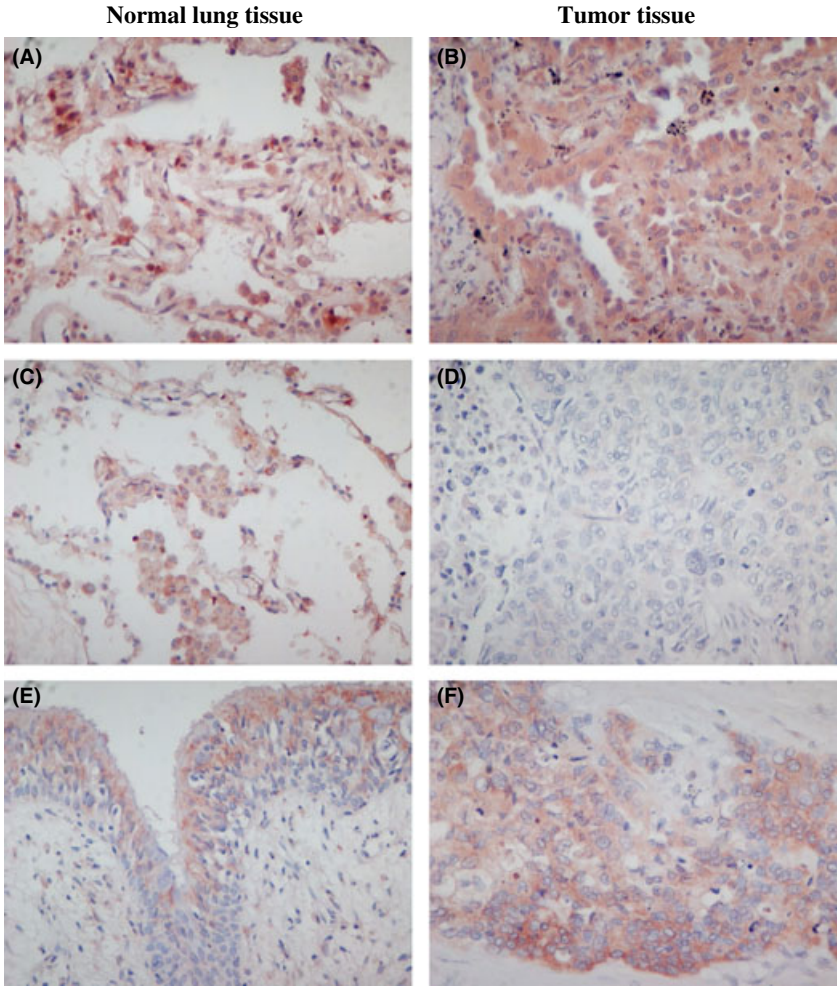

Fig. 3. Phosphate and tension homolog (PTEN) expression by immunohistochemistry in tumor and matched nonmalignant lung tissues. Representative photomicrographs of sections of lung tissue stained with a 1:100 dilution of polyclonal anti-PTEN antibody. Tumor and matching nonmalignant lung tissues with unmethylated region 2 (sample 182 [A,B], sample 328 [E,F]) exhibited strong PTEN staining in the cytoplasm, but PTEN protein was undetectable in tumor tissue with methylation at the region 2 CGI (sample 291 [D]). PTEN expression was also detected in tumor tissue with methylation at the region 1 CGI (sample 182 [B]). Magnification, ×400.

Table 2. Correlation between *PTEN* methylation and the clinicopathological features

	Methylation frequency (%)		
	Region 1	Region 2	Region 3
All subjects (*n* = 137)	7 (5.1)	4 (2.9)	0
Age			
≤63 years (*n* = 64)	4 (6.3)	3 (4.7)	0
>63 years (*n* = 73)	3 (4.1)	1 (1.4)	0
Gender			
Male (*n* = 93)	4 (4.3)	3 (3.2)	0
Female (*n* = 44)	3 (6.8)	1 (2.3)	0
Smoking status			
Ever (*n* = 96)	5 (5.2)	3 (3.1)	0
Never (*n* = 41)	2 (4.9)	1 (2.4)	0
Histologic types			
Squamous cell carcinoma (*n* = 59)	2 (3.4)	2 (3.4)	0
Adenocarcinoma (*n* = 78)	5 (6.4)	2 (2.6)	0
Adenocarcinoma			
EFGR mutation (–) (*n* = 60)	3 (5.0)	1 (1.7)	0
EFGR mutation (+) (*n* = 18)	2 (11.1)	1 (5.5)	0
Pathologic stages			
Stage I (*n* = 86)	5 (5.8)	2 (2.3)	0
Stages II–IV (*n* = 51)	2 (3.9)	2 (3.9)	0

PTEN, phosphate and tension homolog.

cells, six clones showed methylation at the region 1 CGI and four clones showed no methylation. In 20 μM 5-AzadC treated H1299 cells, eight clones revealed a lack of methylation, but methylation was still found in two clones. Therefore, it is possible that lack of significant increase of *PTEN* mRNA expression after 5-AzadC treatment in the present study may be due to incomplete demethylating agent treatment. To resolve this limitation, we performed the same experiments on the H2108 cell line which was methylated at the region 1 CGI. In the case of the H2108 cell line, although all the clones were converted to unmethylated status after 5-AzadC treatment, no increase of *PTEN* mRNA was observed after the treatment (Fig. 2C), which is in agreement with our suggestion. Furthermore, PTEN protein expression was evaluated by immunohistochemical staining in 34 representative tissue samples. In all the tumor and matched nonmalignant lung tissues with unmethylated alleles at the region 2 CGI, PTEN protein was abundantly expressed (Fig. 3A,B [sample 182] and Fig. 3E,F [sample 328]). However, PTEN protein was low or undetectable in the tumor and nonmalignant lung tissues with methylation at the region 2 CGI. The representative figures of immunohistochemical staining for the case (sample 291) in which methylation at the region 2 CGI was detected in the tumor tissue but not in the nonmalignant lung tissue are shown in Figure 3C,D. In addition, PTEN protein was strongly expressed in the tumor and nonmalignant lung tissues with methylation at the region 1 CGI (Fig. 3A,B). These results further support our suggestion. Interestingly, the region 2 CGI

closely localized to the transcription start site/minimal promoter associated with expression, suggesting that it therefore may act as a regulatory CGI.

An aberrant methylation in NSCLCs had no significant correlation with age, gender, histological subtype, pathological stage, and *EGFR* mutational status (Table 2). Additionally, there was no influence of the *PTEN* methylation on the survival outcomes of the patients (data not shown).

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