

Full Length Research Paper

Exploring the Lack of Correlation Between PTEN Gene Promoter Methylation and Urocytic Tumor Development

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Accepted 1 February, 2025

Tumor suppressor gene *PTEN* plays an important role in cell cycle. Disorder of *PTEN* protein can cause cell growth and division in an uncontrolled way, which can lead to the formation of tumors. It has been proven that epigenetic mechanisms, such as promoter hypermethylation, may account for inactivation of *PTEN* in a subset of tumors. *PTEN* promoter hypermethylation has been found to be involved in many kinds of cancers. Up to date, no report about the relationships between methylation of *PTEN* promoter region and bladder cancer has been found. To investigate the methylation pattern of *PTEN* gene transcriptional regulation region (TRR), bisulfite-specific (BSP) polymerase chain reaction (PCR)-based sequencing analysis was performed among 15 bladder cancer tissues and five normal bladder tissues. Analysis of *PTEN* gene TRR methylation showed that the methylation level in bladder cancer had no significant difference with that of normal ($P = 0.4307$, by unpaired Student's *t* test).

Key words: *PTEN*, promoter methylation, bladder cancer.

INTRODUCTION

PTEN also called MMAC1/TEP1 is a tumor suppressor gene which encodes a protein with protein tyrosine phosphatase activity. *PTEN* is found in almost all tissues of the body. It plays roles in cell cycle, which can prevent cells from growing and dividing rapidly and cause apoptosis when necessary (Chu and Tarnawski, 2004), so abnormal function of *PTEN* can cause tumor. *PTEN* plays its biological action mainly by PI3K-AKT signaling pathway. It can specifically dephosphorylate the phospholipid phosphatidylinositol 3,4,5-trisphosphate (PI [3,4,5]P3) to produce phosphatidylinositol 4,5-bisphosphate (PI [4,5]P2) and negatively control the downstream biological events such as cell proliferation and survival caused by AKT (Dahia, 2000). *PTEN* is somatically mutated or deleted in several types of tumors, however, the frequencies of mutations and deletions are very low.

PTEN transcriptional regulation region (TRR) hypermethylation is the main molecular mechanism that inactivated the gene without mutations or deletions in many types of tumors (Alvarez-Nunez et al., 2006). Since the frequency of *PTEN* suppression in tumors exceeds that of *PTEN* mutations or deletions, it is very likely that epigenetic mechanisms, such as promoter hypermethylation, may account for its inactivation in a subset of tumors (Alvarez-Nunez et al., 2006). Epigenetic abnormalities and genetic alterations can cause cancer. An explosion of evidences indicates the role of epigenetic processes in the stages of neoplasia by silencing key regulatory genes such as tumor suppressor genes, which led to the realization that genetics and epigenetics cooperate at all stages of cancer development (Jones and Baylin, 2007). Up to date, frequent inactivation of *PTEN* by TRR hypermethylation of different level has been found to be involved in many kinds of cancers, such as colorectal cancer (Goel et al., 2004), endometrial carcinoma (Salvesen et al., 2001), gastric carcinoma (Kang et al., 2002), glioblastoma (Baeza et al., 2003), breast cancer (Garcia et al., 2004; Khan et al., 2004), ovarian cancer (Schondorf et al., 2004), cervical

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neoplasm (Cheung et al., 2004), lung cancer (Marsit et al., 2005), pancreatic cancer (Asano et al., 2004), thyroid cancer (Frisk et al., 2002; Alvarez-Nunez et al., 2006), soft tissue sarcomas (Kawaguchi et al., 2005), melanoma (Mirmohammadsadegh et al., 2006). Many reports that methylation is the important reason for the decreased expression of protein (Chen et al., 2005) suggest that *PTEN* TRR methylation plays an important role in the progression of the early gestational trophoblast and in the pathogenesis of hydatidiform mole, though no report about the relationships between methylation of *PTEN* TRR and bladder cancer has been found.

MATERIALS AND METHODS

The tissue samples from bladder cancer patients

All tissue samples were obtained from Daping Hospital Research Institute of Surgery, Third Military Medical University. Bladder cancer tissue samples from 15 patients and the corresponding normal tissue samples adjacent to the tumor from the same patients were collected from surgery in 2008. A total of 15 resected normal samples were sent for histopathological analysis. Five out of them were then confirmed as normal control after histopathological analysis. The cancer tissue samples were all WHO grade II. Informed written consent was obtained from each patient. The tumor and normal tissue samples were preserved in liquid nitrogen immediately after being resected. Patients' clinical features were recorded, including sex, age of onset, clinical stage, lymph node metastasis and histological grade. All procedures described in the present study were reviewed and approved by the Ethical Committee of Qingdao Agricultural University, China.

In silico screening for transcription factors binding sites and CpG islands

The CpG islands in *PTEN* TRR were analyzed by EMBOSS CpGPlot/CpGReport/Isochore online tool (<http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html>) with default settings (window: 100; step: 1; observed to expected ratio of C plus G:0.6; minimum average percentage of G plus C:50; minimum length of reported CpG island: 200 bp) and online tool MethPrimer (<http://www.urogene.org/methprimer/index1.html>) with default settings (window: 100; shift:1; Obx/Exp: 0.6; GC%:50%) with the genomic position of the 5' end of the sense primer at -473 and the antisense primer at -197 relative to the start codon of the *PTEN* gene. The sequence of 277bp within the genomic position of the 5' end region from -473 to -197 bp to the start codon of the *PTEN* gene that contained 27 CpG sites was chosen for bisulfite sequencing PCR (BSP).

DNA isolation

DNA isolated from tissue samples were pulverized for subsequent DNA isolation by using micro DNA isolation kit (Tiangen, Beijing, China), according to the manufacturer's instructions. Briefly, after digestion by proteinase K, nucleic acid released from tissues were adsorbed on a silica column, and the lysate was passed through the adsorption column using centrifugal force supplied by a high speed centrifuge. After several washing cycles, DNA was dissolved in the supplied elution buffer and collected into a clean 1.5 ml collection tube and stored at -80°C.

Bisulfite-specific (BSP) PCR and DNA sequencing

The isolated DNA was treated with sodium bisulfite with Methylamp™ DNA modification kit (Epigentek, USA) according to the manufacturer's instructions. Briefly, 1 µg of DNA was denatured using NaOH and treated with sodium bisulfite for 8 h at 55°C. The bisulfite-treated DNA was amplified by PCR for TRR of the *PTEN* gene with BSP specific primer pair (PTENF: TAGTTTTA-GGGAGGGGGTTT; PTENR: ACTTCTCCTCAACAACCAAAA) with the genomic position of the 5' end of the sense primer at -473 and the antisense primer at -197 relative to the start codon of the *PTEN* gene. The PCR condition is as follows: 94°C denaturation for 5 min, 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C elongation for 5 min.

The PCR products were separated by electrophoresis in 1% agarose gel with ethidium bromide. Correct sized bands were isolated from the gel and purified with the Wizard SV Gel and PCR Clean-Up System (Promega, USA). Purified PCR products were then subcloned into a PMD18-T Vector (TaKaRa, Dalian, China) according to the manufacturer's instructions. Positive clones were obtained by ampicillin antibiotic selection then followed by PCR colony screening. Eight positive clones were randomly collected for sequencing at GeneScript company (Nanjing, China) and JIE LI Biology (Shanghai, China).

Statistical analysis

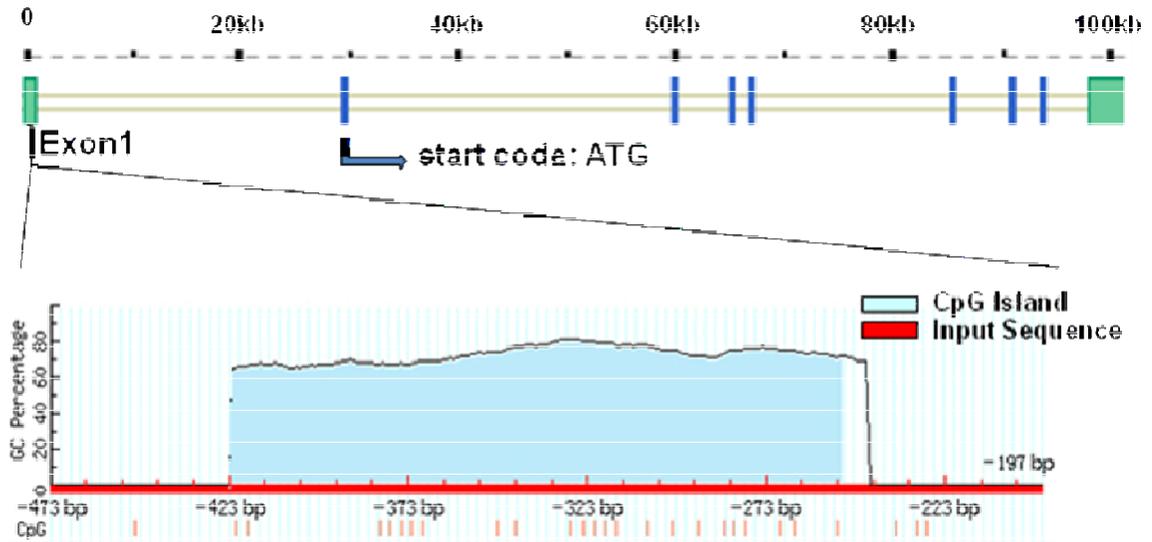
The percentage of methylation for tumor and normal samples was calculated from the number of methylated CpG (black squares in figures) divided by total CpG loci (all squares in figures). Following arcsine transformation, the percentage of DNA methylation was analyzed by ANOVA and differences between tumor and normal samples were analyzed with unpaired Student's t test. All analyses were carried out with the statistical analysis system.

RESULTS

With the online tools of Methprimer (<http://www.urogene.org/methprimer>), and p-match (<http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi>), the promoter region of *PTEN* gene was characterized. The first exon, first intron, GC-rich sites and putative transcription factor binding sites were identified. Results show that CpG loci were prevalent at -473 to -197 bp, which were then selected for cloning (Figures 1A and B). Meanwhile, genomic DNA from normal bladder and bladder cancer tissues were treated with bisulfite and subjected to sequence analysis.

Sequencing of individual bisulfite-converted genomic DNAs revealed that the CpG loci of *PTEN* gene from normal bladder tissues and bladder cancer tissues were highly demethylated (Figures 2A, B, 3A and B). Methylation percentage of bladder cancer tissues and normal bladder tissues was 1.1396 and 1.0528%, respectively. Methylation percentage of five normal bladder tissues was 0, 0, 0.7407, 1.8519 and 2.4691%, respectively. In bladder cancer tissues, seven of 15 (46.7%) were 0, two (13.3%) were 1.2346%, one (6.7%) was 1.8519% and five (33.3%) were 3.7037%. Methylation percentage of 27 CpG island sites in *PTEN* TRR region in all tested tumor and normal tissues showed no

A



B

-473 bp
CAGCTCCAGGGAGGGGGTCTGAGT**CG**CCTGTCACCATTTCCAGGGCTGGGAA**CGCCGGAG**
TAGTTTTAGGGAGGGGGTTTGAGTCGTTTGTTATTTTATTTTAGGGTTGGGAACGTCGGAG

AGTTGGTCTCTCCCCTTCTACTGCCTCCAACA**CGGCGGCGGCGGCGG**CTGGCACATCCAG
 AGTTGGTTTTTTTTTTTTTTTATTTGTTTTTAATACGGCGGCGGCGGCGGTTGGTATATTTAG

GGACC**CGGGCCGG**GTTTTTAAACCTCC**CGTGCGCCGCGCCG**CACCCCC**CGTGGCCCGGGCT**
 GGATTCGGGTCGGTTTTTAAATTTTTCGTGCGTCGTCGTCGTAATTTTTCGTGGTTCGGGTT

CCGGAGGCCCGCCGGCGGAGGCAGCCGTTCCGGAGGATTATTCGTCTTCTCCCCATT**CCGCT**
 TCGGAGGTCGTCGGCGGAGGTAGTCGTTCCGGAGGATTATTCGTTTTTTTTTTTATTTGCTT

-197
G**CCGCGC**CTGCCAGGCCTCTGGCTGCTGAGGAGAAGC
GTCGTCGTTGTTAGGTTTTTGGTTGTTGAGGAGAAGT

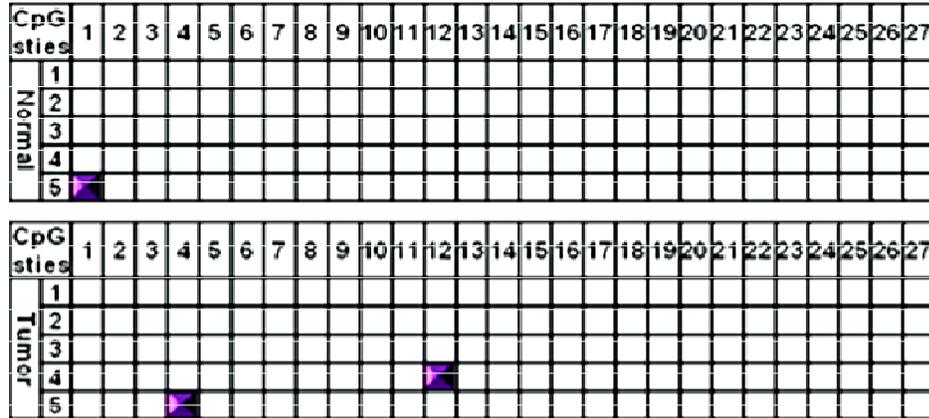
Figure 1. Methylation dynamics of *PTEN* gene. (A) CpG-pattern rich regions. The red horizontal line represents the input sequence. The red vertical lines represent the positions of the 27 CpG sites within the 277 bp fragment. (B) The detailed *PTEN* sequence (-473 to -197 bp). The top sequence and bottom sequence correspond to the bisulfite sequencing and original sequence, respectively. The black horizontal lines indicate the location of primers. The CpG sites in the sequence are marked in red.

significant difference between tumor and normal samples ($P = 0.4307$) (Figure 4). The unpaired Student's t test analysis showed no significant relationships between tumor and normal samples of all 27 CpG sites. P values of different CpG sites are listed in Table 1, and unlisted CpG sites are all unmethylated in all tested bladder cancer tissues and normal tissues.

DISCUSSION

The *PTEN* protein is a phosphatase, which can remove phosphate groups from key intracellular phosphoinositide signaling molecules, such as PI [3,4,5]P₃, an important intracellular second messenger (Sansal and Sellers, 2004). *PTEN* can negatively regulate PI3K-Akt signaling

A



B

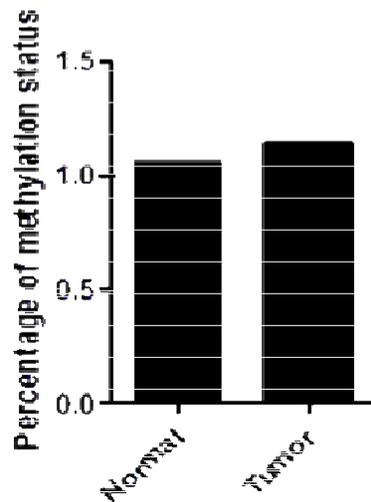


Figure 2. Comparison of *PTEN* TRR methylation levels between tumor and normal samples. (A) Representative data of the *PTEN* gene TRR in 2 different groups of bladder tissues obtained using BSP PCR-based sequencing analysis. Upper part shows the representation of normal sample, and lower part, the representation of bladder cancer sample. Each line represents an individually sequenced clone, and each square represents a CpG residue. White and black squares stand for unmethylated and methylated cytosines, respectively. (B) Percentage of methylated CpG loci in all successful sequencing of normal tissues or bladder cancer tissues. The data was calculated from the number of all methylated CpG loci of all individual clone from 5 normal samples or 15 tumor samples divided by total CpG loci of all individual clone from 5 normal samples or 15 tumor samples, respectively.

pathway which is involved in cell growth and survival, by antagonizing PI [3,4,5]P3 (Yamada and Araki, 2001). Abnormal function of PTEN can cause tumor. DNA methylation has shown a principal epigenetic mechanism regulating gene expression and normal development of mammals, which is often associated with the stable repression of certain genes (Pan et al., 2011). Aberrant DNA methylation of some genes, especially some onco-genes and tumor suppressor genes, appears to be a frequent epigenetic event for tumorigenesis and metastasis (Sato et al., 2006; Adrien et al., 2006; Torng et al., 2009). It has been proven that tumor suppressor gene *PTEN* promoter hypermethylation is the main molecular mechanism that inactivated the gene without mutations or

deletions in many types of tumors (Alvarez-Nunez et al., 2006).

In this study, we have demonstrated no significant difference by unpaired Student's t test in the methylation pattern of tumor suppressor gene *PTEN* promoter between bladder cancer and normal bladder tissues. Sequencing of individual bisulfite-converted genomic DNAs revealed that CpG loci of *PTEN* TRR were almost unmethylated both in bladder cancer tissues (1.1396%) and normal bladder tissues (1.0528%). The data were calculated from the number of all methylated CpG loci divided by total CpG loci of all individual clone from five normal samples or 15 tumor samples, respectively. The difference has no statistical significance. Though, the

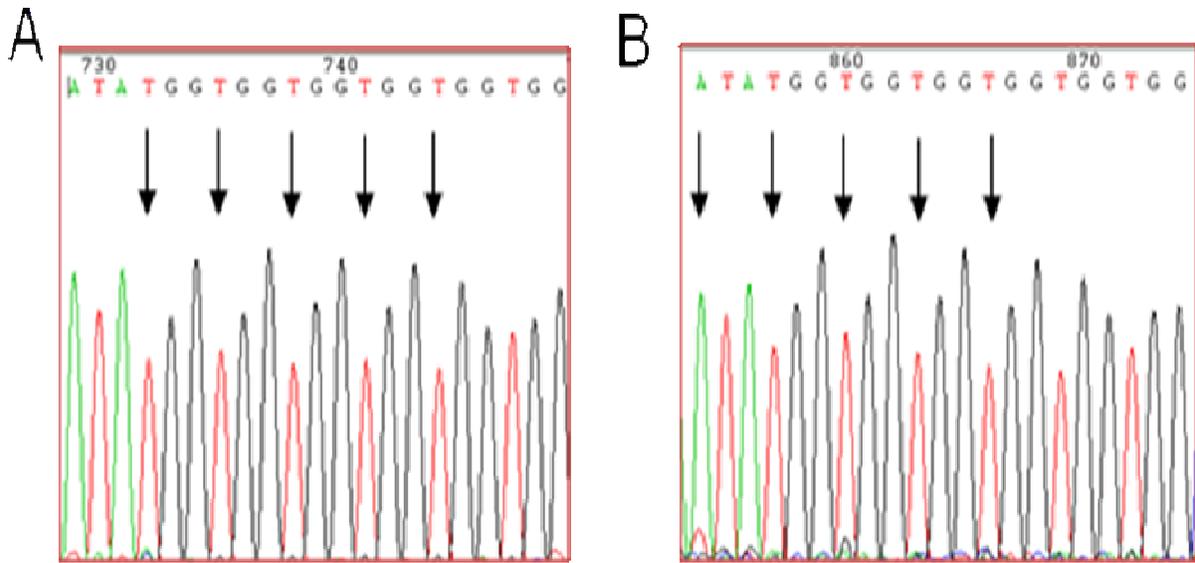


Figure 3. Examples of direct sequencing chromatograms. Bisulfite treated DNA was amplified and sequenced on ABI automated sequencer. The sequences representing CpG sites are marked by arrows. Both normal sample (A) and tumor sample (B) show complete conversion of cytosine to thymine (non-methylated).

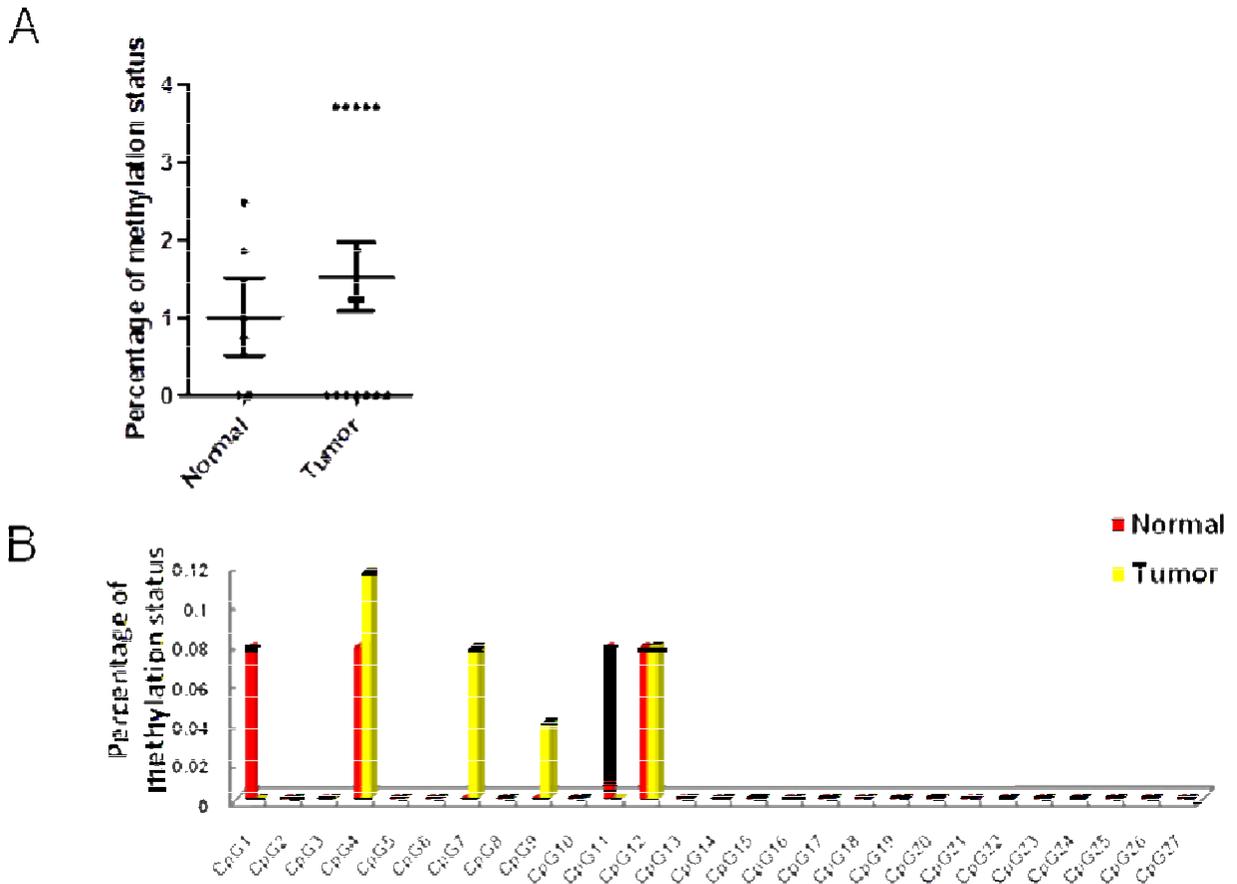


Figure 4. (A) Methylation percentage of 27 CpG island sites in PTEN promoter region in all tested tumor and normal tissues. (B) Comparison of methylation percentage of each CpG site in PTEN promoter region in tumor and normal tissues.

Table 1. Relationship between methylation pattern and bladder cancer.

| <i>CpG site</i> | <i>P value</i> | <i>P value summary</i> |
|-----------------|----------------|------------------------|
| 1 | 0.082761 | ns |
| 4 | 0.73456 | ns |
| 7 | 0.416283 | ns |
| 9 | 0.577753 | ns |
| 11 | 0.082761 | ns |
| 12 | 0.804134 | ns |

sequence we amplified partially overlapped with the sequence analyzed by Baeza et al. (2003), the results are different. They found that *PTEN* methylation occurred frequently in glioblastomas, which was association with focal loss of *PTEN* expression in glioblastomas. Furthermore, many evidences that CpG islands of *PTEN* TRR region are methylated in tumors described above have proved that tumor suppressor gene *PTEN* has been considered to be a hallmark of cancer, though our results show no statistical difference in *PTEN* TRR methylation between bladder cancer tissues and normal bladder tissues. Different results suggest that *PTEN* promoter region methylation may be tissue specific, but the results remain to be confirmed. Generally speaking, there would be more significant statistics when more samples are used, but it is difficult to collect the clinical samples. So, our analysis and conclusion are just based on our limited clinical samples (15 bladder cancer tissues and five normal tissues). On the other hand, these relationships between *PTEN* methylation and tumor may be due to the complexity of cancer progression where other modes of epigenetic modifications such as micro RNA also play a role. Moreover, it is well known that mutations and deletions, which is inherited through the germ line or, more commonly, arising in somatic tissues later in life can cause cancer, which may be an important mechanism in tumorigenesis of bladder cancer.

ACKNOWLEDGEMENTS

This work is supported by National Nature Science Foundation (31001010 and 31101716), Foundation of Distinguished Young Scholars (JQ201109), Doctoral Foundation (BS2010NY010), Foundation of Education Department (J11LC20) and Taishan Scholar Foundation of Shandong Province, and Doctoral Foundation (630917) of Qingdao Agricultural University.

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