Aberrant CpG island methylation of PTEN is an early event in nasopharyngeal carcinoma and a potential diagnostic biomarker

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Abstract. The inactivation of phosphatase and tensin homolog (PTEN) due to its CpG island hypermethylation has been observed in some types of tumors except nasopharyngeal carcinoma (NPC). In the present study, we focused on the aberrant methylation of PTEN CpG islands in NPC. The mRNA expression of PTEN was detected by quantitative PCR in 45 NPC and 22 non-tumor nasopharyngeal epithelial (NP) tissues. The methylation status of PTEN was examined by methylation-specific polymerase chain reaction and sequencing. The mRNA expression of PTEN in three NPC cell lines treated with 5-aza-2'-deoxycytidine (5-aza-dC) was also examined. PTEN was downregulated in both NPC tissues and NPC cell lines and a relatively higher methylation level of PTEN was found in NPC specimens (82.2%) relative to NP tissues (5.3%). The PTEN mRNA expression was restored in NPC cell lines by treatment with 5-aza-dC. These results first reveal an epigenetic alteration, aberrant methylation of PTEN, in NPC, which is probably an early event and may be regarded as a novel candidate biomarker for early stage of NPC detection and prevention.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy particularly prevalent in the southern Chinese population of Guangdong, in the Inuit population of Alaska and in native Greenlanders (1,2). The 5-year survival rates of stage I and II NPC range from 72 to 90%; however, the 5-year survival rates of stage III and IV drop to 55 and 30% (3). NPC detection rate and early diagnosis are major factors adversely affecting the effect of treatment (4). Finding a biomarker detecting early stage of NPC remains the most promising to facilitate the early NPC diagnosis and therapy, probably improving the long-term survival of patients.

Phosphatase and tensin homolog (PTEN) has been identified and mapped to chromosome 10q23 (5). Considerable attention has been paid to the tumor suppressor gene PTEN, since it may suppress tumor cell growth by antagonizing protein tyrosine kinases and regulate the first step of tumor cell invasion and metastasis through its interaction with focal adhesions (5,6). The loss of function of PTEN leads to increases in cellular proliferation, survival and growth in many types of cancer (7). Recombinant PTEN is able to dephosphorylate phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate as well as antagonize the phosphoinositide 3-kinase pathway (8). Notably, CpG island hypermethylation has been identified as an alternative mechanism of PTEN inactivation in cancers including lung cancer, endometrial carcinoma, prostate cancer, brain tumors, EBV-associated gastric carcinoma, cervical neoplasm, malignant melanoma and hematologic malignancies (9-17). However, to date, this mechanism of PTEN inactivation has not been reported in NPC.

In the present study, we evaluated the PTEN expression in NPC specimens and control samples by quantitative RT-PCR (qPCR) and examined the CpG island methylation status of PTEN using methylation-specific polymerase chain reaction (MSP) and sequencing. 5-aza-dC treatment can lead to DNA demethylation via inhibition of DNA methyltransferase activity (18). We also treated NPC cell lines (HONE1, CNE1 and 6-10B) with 5-aza-dC and then examined PTEN expression. Thus, we preliminarily demonstrated the role of PTEN methylation in relation to the mechanism of PTEN inactivation in NPC.

Materials and methods

Methylation-specific PCR. The methylation status of the PTEN promoter region was determined by MSP using bisulfite-modified DNA. The targets of the promoter regions were one site.

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Table I. Summary of the primers used in the present study.

Primer	Sequence	Sequence product size (bp)	Annealing temperature (˚C)
qRT-PCR			
GAPDH-forward	5'-CATGGGTGTGAACCATGAGA-3'	165	60
GAPDH-reverse	5'-GTCTTCTGGGTGGCAGTGAT-3'		
PTEN-forward	5'-TGCAGAAGAAGCCCCGCCA-3'	208	60
PTEN-reverse	5'-ACGCCTTCAAGTCTTTCTGCAGG-3'		
Methylated specific PCR assay			
Methylated			
PTEN-forward	5'-TTCGTTCGTCGTCGTCGTATTT-3'	207	58
PTEN-reverse	5'-GCCGCTTAACTCTAAACCGCAA-3'		
Unmethylated			
PTEN-forward	5'-GTGTTGGTGGAGGTAGTTGTTT-3'	163	58
PTEN-reverse	5'-ACCACTTAACTCTAAACCACAACCA-3'		
PCR primers for 6 PTEN exons			
Exon 1-forward	5'-TTCTGCCATCTCTCTCCTCC-3'	194	60
Exon 1-reverse	5'-ATCCGTCTACTCCCACGTTC-3'		
Exon 2-forward	5'-GTTTGATTGCTGCATATTTCA-3'	201	50
Exon 2-reverse	5'-TCTAAATGAAAACACAACATGAA-3'		
Exon 3-forward	5'-AGCTCATTTTTGTTAATGGTGG-3'	178	60
Exon 3-reverse	5'-CCTCACTCTAACAAGCAGATAACTTTC-3'		
Exon 4-forward	5'-AAAGATTCAGGCAATGTTTGTTAG-3'	200	60
Exon 4-reverse	5'-TGACAGTAAGATACAGTCTATCGGG-3'		
Exon 5-1-forward	5'-TTTTTTCTTATTCTGAGGTTATC-3'	184	50
Exon 5-1-reverse	5'-TCATTACACCAGTTCGTCC-3'		
Exon 5-2-forward	5'-TCATGTTGCAGCAATTCAC-3'	176	50
Exon 5-2-reverse	5'-GAAGAGGAAAGGAAAAACATC-3'		
Exon 6-forward	5'-ATGGCTACGACCCAGTTACC-3'	284	60
Exon 6-reverse	5'-AAGAAAACTGTTCCAATACATGG-3'		

The sequences of the methylated and unmethylated primer pairs are listed in Table I with the other primer sequences used in the study. DNA was modified by the bisulfite reaction using an EpiTect Bisulfite kit (Qiagen). Methylated and unmethylated genomic regions can be distinguished by PCR using each sequence-specific pair of primers. MSP experiments were performed at least in duplicate.

Patients and tissue samples. All NPC and NP samples were collected from the Department of Otorhinolaryngology, Head and Neck Surgery, Nanfang Hospital, Affiliated Hospital of Southern Medical University, Guangzhou, China. For the use of these clinical materials for research purposes, prior written informed consent and ethics approval were obtained from all participants and the Ethics Committees of the Nanfang Hospital, respectively.

Cells and culture conditions. NPC cell lines (5-8F, CNE1, CNE2, 6-10B, SUNE1 and HONE1) were available from the Cancer Institute of Southern Medical University (Guangzhou, China). All cell lines used in the present study were maintained in DMEM medium (Invitrogen) with 10% FBS (HyClone), 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen), and incubated at 5% CO_2 at 37˚C.

Quantitative RT-PCR (qRT-PCR). Total RNA of tissues and cells was reverse transcribed using PrimeScript® RT reagent kit (Takara). Quantitative real-time PCR was performed using SYBR® Premix Ex Taq™ II (Takara) on a StrataGene Mx3005P System. The sequences of the qPCR primer pairs are listed in Table I.

Cell viability assay. Cell viability was analyzed using an MTT assay (Sigma, St. Louis, MO, USA). Briefly, $1x10^3$ cells were seeded into a 96-well plate with quadruplicate repeat for each condition. After 24 h of incubation, MTT reagent was added to each well and incubated for 4 h. The formazan crystals formed by viable cells were then solubilized in DMSO and measured at 490 nm for the absorbance values. Each experiment was performed in triplicate. The 5-aza-dC concentration required to inhibit cell growth by 50% (IC_{50}) was calculated from survival curves. Survival percent was calculated using the following formula: Survival percent (%) = (mean experimental absorbance/mean control absorbance) x 100%.

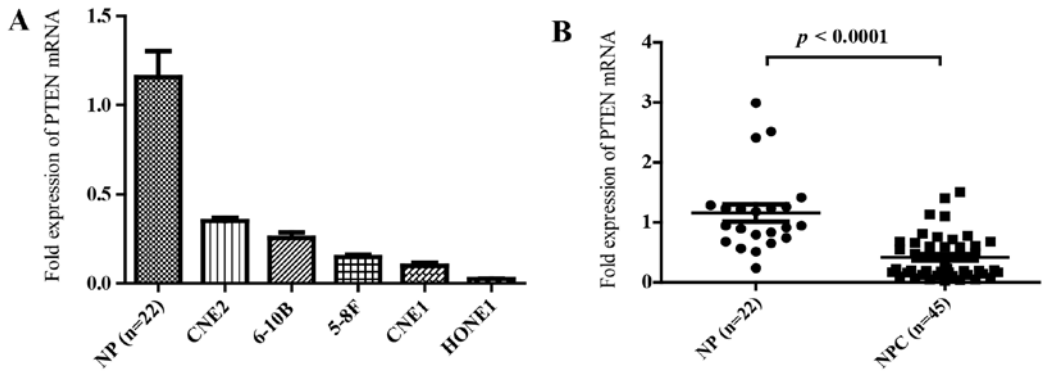

Figure 1. The expression of PTEN is reduced in NPC cell lines and clinical specimens. (A) Expression of PTEN in 5 NPC cell lines. (B) Average expression level of PTEN in human NPC specimens (n=45) and non-tumor nasopharyngeal epithelial (NP) tissues (n=22). mRNA abundance was normalized to GAPDH mRNA. Each bar represents the means ± SD of three experiments.

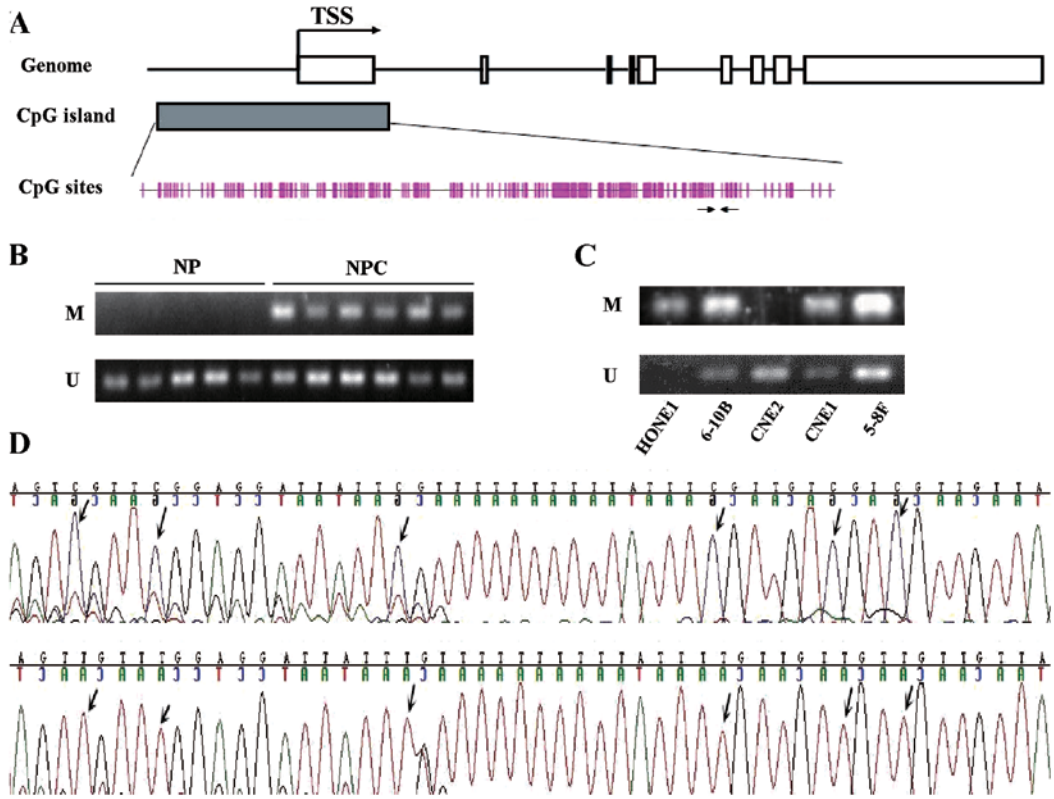

Figure 2. Hypermethylation of the PTEN gene in human NPC cell lines and clinical specimens. (A) Schematic representation of the human PTEN promoter. The CpG island of PTEN extends from -2011 to +1390 from TSS. Each pink tick mark represents one CpG site. The arrows indicate the TSS. Methylation-specific PCR (MSP) assay to determine methylation status was carried out on 8 CpG sites in its primer region, as underlined by the arrows. TSS, transcriptional start site. (B) Detection of hypermethylation of PTEN in nasopharyngeal epithelial (NP) and NPC tissues, respectively. (C) Detection of hypermethylation of PTEN in NPC cell lines. MSP analysis was performed using primers specifically for methylated (M) or unmethylated (U) CpG sites of PTEN gene. PCR products were visualized after electrophoresis on a 2% agarose gel. (D) Sequencing analysis of M and U products of MSP for PTEN in NPC tissues. For M products, all cytosines were changed to thymines, except those in CpG dinucleotides. For M products, all cytosines were changed to thymines, with those in CpG dinucleotides. Arrows show the cytosines in CpG dinucleotides.

Statistical analysis. The data are presented as mean ± SEM, unless otherwise indicated, of at least three independent experiments. Statistical analysis was performed using an SPSS 13.0 package system. Statistical significance was assessed by the Student's t-test, the Pearson's Chi-square test, Fisher's exact test or one-way ANOVA analysis ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$).

Results

PTEN is downregulated in clinical NPC specimens and human NPC cell lines. We first examined the expression level of PTEN in 45 NPC specimens and 22 non-tumor nasopharyngeal epithelial (NP) tissues. The average expression level of PTEN was significantly lower in NPC specimens compared

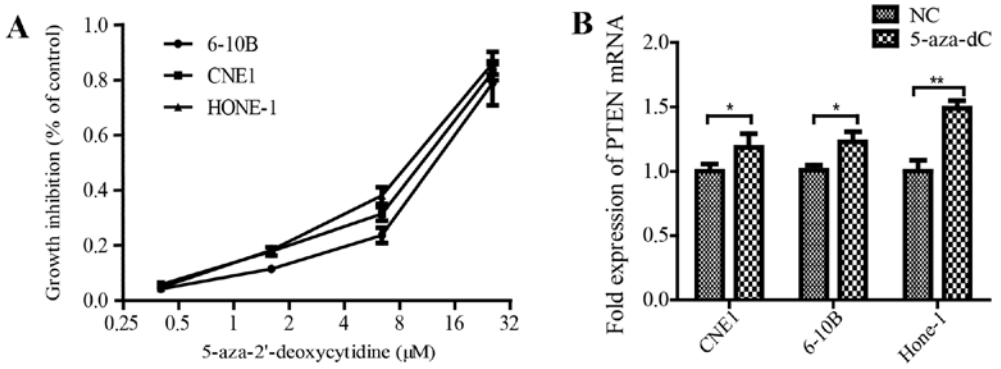

Figure 3. Re-expression of PTEN in NPC cell lines after 5-aza-dC treatment. (A) Effect of various concentrations of 5-aza-dC on the growth of NPC cell lines. The 5-aza-dC concentration required to inhibit cell growth by 50% (IC_{50}) was calculated from survival curves. (B) MSP/USP results of NPC cell lines treated with either vehicle or 5-aza-dC for 72 h; 5-aza-dC treatment leads to DNA demethylation. Data are presented as the average of triplicate measurements from duplicate experiments. qRT-PCR in NPC cell lines showed detection of PTEN mRNA following treatment with 5-aza-dC. Each bar represents the means ± SD of three experiments. *P<0.05; **P<0.01.

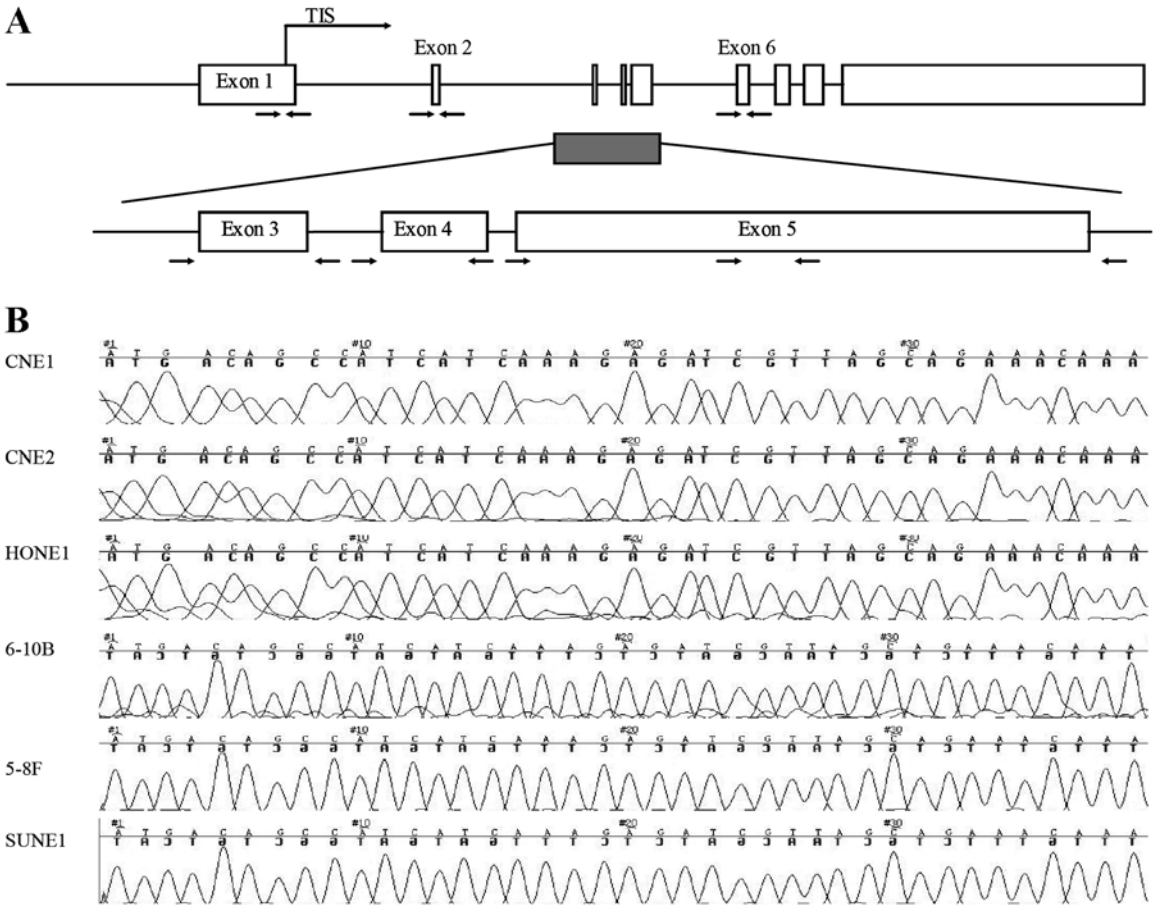

Figure 4. DNA sequencing from exon 1 to exon 6 of the PTEN gene in 6 NPC cell lines. (A) Schematic representation of the human PTEN exons. The arrows indicate the TIS. TIS, transcriptional initiation site. PCR primers to detect mutation status are underlined by the arrows. (B) Sequence analysis of the PCR product from exon 1 TIS region of the PTEN gene in 6 NPC cell lines.

with non-tumor NP tissues (Fig. 1B; P<0.0001). A panel of human NPC cell lines was also analyzed for the expression level of PTEN. Similarly, the expression level of PTEN was observed to be decreased in all 5 NPC cell lines compared with the non-tumor NP tissues (Fig. 1A). These data supported that PTEN was downregulated in NPC.

Hypermethylation of PTEN in clinical NPC specimens and human NPC cell lines. In order to explore the potential role of CpG island methylation in the transcriptional silencing of the PTEN gene, we investigated the methylation status of PTEN in clinical specimens and NPC cell lines. As the 5' region of PTEN contained many CpG islands spanning ~3 kb, we

Table II. Association between PTEN methylation and clinicopathological parameters of NPC.

		PTEN methylation		
	Total no.	Positive n/total (%)	Negative n/total (%)	P-value[a]
Gender				
Male	36	30/36 (83.3)	6/36 (16.7)	0.65
Female	9	7/9 (77.8)	2/9 (22.2)	
Age (years)				
<60	35	28/35 (80)	7/35 (20)	0.66
≥60	10	9/10 (90)	1/10 (10)	
T grade[b]				
1, 2	31	27/31 (87.1)	4/31 (12.9)	0.23
3, 4	14	10/14 (71.4)	4/14 (28.6)	
N stage[b]				
0	7	6/7 (85.7)	1/7 (14.3)	1
1, 2, 3, 4	38	31/38 (81.6)	7/38 (18.4)	
Stage[b]				
I, II	20	18/20 (90)	2/20 (10)	0.27
III, IV	25	19/25 (76)	6/25 (24)	
Histology				
Keratinizing squamous cell carcinoma	45	37/45 (82.2)	8/45 (17.8)	
Non-keratinizing carcinoma	0			

[a]Comparisons were made by Pearson's Chi-square test or Fisher's exact test (SPSS 13). [b]Staging according to the International Union Against Cancer (UICC).

focused only on its promoter region (Fig. 2A) in the present study.

Our MSP analysis showed that CpG islands in the PTEN promoter region were methylated in 82.2% (37/45) of NPC tissues, whereas the methylated PTEN appeared in only 5.3% (1/19) of the non-tumor NP tissues (Fig. 2B) and in 80% (4/5) of NPC cell lines (Fig. 2C). The difference in the hypermethylation level between NPC tissues and non-tumor NP tissues was statistically significant (P<0.0001).

To validate MSP results, we sequenced M-MSP and U-MSP products amplified from two NPC tissues using either M or U primers of PTEN. The sequencing results showed that all the cytosine residues in the M-MSP product were converted to thymines, except for those in CpG dinucleotides, indicating the presence of methylated cytosines in these CpG dinucleotides. The representative sequencing results of M- and U-MSP products of PTEN are shown in Fig. 2D.

Restoration of PTEN in NPC cell lines after 5-aza-dC treatment. To directly test the effect of promoter methylation on PTEN inactivation, we treated 3 NPC cell lines (HONE-1, CNE1 and 6-10B) with 5-aza-dC for 3 days (Fig. 3A), and then examined PTEN mRNA expression changes. We observed that PTEN mRNA expression was clearly upregulated in all 3 NPC cell lines after 5-aza-dC treatment (Fig. 3B). These results suggested that methylation of promoter region plays a regulatory role in silencing the expression of PTEN in NPC cells.

PTEN mutation in NPC cell lines. To detect the mutation of tumor suppressor PTEN in NPC, DNA sequencing was used to detect the mutation from exon 1 to exon 6 (Fig. 4A) of the PTEN gene in 6 NPC cell lines (5-8F, CNE1, CNE2, 6-10B, SUNE1 and HONE1). None of the NPC cell lines showed mutation in exon 1 to exon 6 of the PTEN gene (Fig. 4B).

Discussion

NPC is one of the most common types of cancer in southern China. In contrast to other head and neck malignancies, NPC is highly sensitive to radiation and chemotherapy. High survival rates are reported for early stage (stages I and II) of diseases, but the prognosis for advanced stage (stages III and IV) remains poor; in patients with advanced NPC at the local site or with distant metastases, the disease will subsequently recur in 30-40% (19). Unfortunately, the majority of NPCs are diagnosed at an advanced stage due to the difficulty of a thorough nasopharyngeal exam, as well as non-specific presenting symptoms (cervical nodal enlargement, headache, nasal and aural dysfunction). In light of these, the molecular targets for early diagnosis of NPC need to be clarified.

In NPC, gene silencing by deletion, insertion and mutation of tumor suppressor genes (TSGs), such as p53 (20), are uncommon events in carcinogenesis. The present study demonstrated that no mutation was found in functional DNA regions of PTEN by PCR sequencing, which is in accordance

Table III. Summary of the PTEN methylation in human tumor/normal tissues and cell lines.

Tumor type (Ref.)	Testing method	Histology	Methylation rate	Tumor cell lines	Methylation rate
Lung cancer (9)	MSP	PTEN-negative NSCLC	7/20 (35)	NSCLC cell lines	11/16 (69)
	MSP	PTEN-positive NSCLC	0/10 (0)		
Endometrial carcinoma (10)	MSP	Endometrial carcinoma	26/138 (19)		
Prostate cancer (11)	MSP	Primary prostate tumor	0/6 (0)		
Brain tumor (12)	MSP	Non-tumor brain	0/13 (0)		
		Gliomas	44/90 (49)		
EBV-associated gastric carcinoma (13)	MSP	EBV-negative gastric cancer	26/87 (30)		
		EBV-associated gastric cancer	18/28 (64)		
Malignant melanoma[a] (15)	MSP	Malignant melanoma		Melanoma cell lines	3/13 (23)
Gastric cancer (14)	MSP	Gastric cancer	26/66 (39)		
		PTEN-negative gastric cancer	19/26 (73)		
Cervical neoplasm (17)	MSP	CIN-Hb	4/10 (40)		
		Squamous cell carcinoma	36/62 (58)		
Nasopharyngeal carcinoma (present study)	MSP	Nasopharyngeal epithelial	1/19 (5.3)	NPC cell lines	4/5 (80)
		Nasopharyngeal carcinoma	37/45 (82.2)		

[a]PTEN did not show complete methylation in malignant melanoma tissues. [b]CIN-H, high-grade cervical intraepithelial neoplasia. The methylation rate is expressed as n/total (%).

with the results by PCR-SSCP in NPC. Downregulation of TSG expression by aberrant methylation is increasingly emerging as an important mechanism of nasopharyngeal tumorigenesis (21-24).

Phosphatase and tensin homolog (PTEN) deleted on chromosome 10 was originally cloned as a tumor suppressor. It has been confirmed that PTEN is deleted or inactivated in many tumor types, including renal (25), melanoma (26), endometrial (27), breast (5), prostate (5), lung (9), bladder (28), thyroid (29) and NPC (30), identifying PTEN as an important tumor suppressor. On the other hand, promoter methylation is widely considered to be an important epigenetic mechanism in the carcinogenesis of NPC, and has been proven promising for early diagnosis of multiple types of tumors (31-33).

To the best of our knowledge, this is the first study to demonstrate an essential role of PTEN methylation in the carcinogenesis of NPC. We found that 82.2% (37/45) of NPC specimens and 80% (4/5) of NPC cell lines present PTEN CpG island DNA methylation, compared with 5.3% (1/19) of non-tumor NP tissues; PTEN methylation was not related to age, gender, lymphatic node metastasis and tumor local grade (Table II). However, our present study did not demonstrate a significant difference between methylation frequency in early and late stage NPC cases. Considering PTEN expression in tumor specimens and cell lines (Table III), PTEN methylation may be an early event in the carcinogenesis of NPC. As to whether the epigenetic change of PTEN is one of the potential markers for early diagnosis, a profound study is currently in progress in the local high risk population and preclinical disease.

In brief, our more recent studies indicated that PTEN methylation and loss of PTEN expression are early events in the development of NPC and may serve as a biomarker for early diagnosis.

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