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Promoter Methylation and Silencing of PTEN in Gastric Carcinoma

Young-Hwa Kang, Hye Seung Lee, and Woo Ho Kim

Cancer Research Institute (Y-HK, WHK) and Department of Pathology (HSL, WHK), Seoul National University College of Medicine, and BK21 Project for Medicine, Dentistry and Pharmacy (Y-HK), Seoul, Korea

SUMMARY: The *PTEN/MMAC1/TEP1* gene (phosphatase and tensin homolog deleted on chromosome 10/mutated in multiple advanced cancers/TGF-β regulated and epithelial cell enriched phosphatase 1), which regulates the signaling pathways of Akt, is a novel tumor suppressor gene implicated in multiple cancers. Because a number of tumor suppressor genes are known to be silenced by aberrant promoter methylation, we examined the methylation status of the 5' CpG islands of *PTEN* using methylation-specific PCR. The altered expression of *PTEN* in 310 gastric carcinomas was analyzed by immunohistochemical staining using tissue-array and clinicopathologic profiles related to *PTEN* expression were characterized. Of 310 consecutive gastric carcinomas, 62 cases (20%) showed expression loss of *PTEN*. Altered *PTEN* expression was significantly associated with tumor depth and size, lymphatic invasion, advanced stage, pTNM stage, and patient survival ($p < 0.001$). The promoter methylation frequency of *PTEN* was found to be present in 26 (39%) of 66 cases examined, and 19 (73%) of 26 gastric cancer tissues showing promoter methylation exhibited the loss of *PTEN* expression. Abnormalities in the expression of *PTEN* significantly correlated with promoter methylation ($p < 0.001$). In conclusion, silencing of the *PTEN* gene occurs frequently in gastric carcinoma and aberrant promoter methylation is a major mechanism of silencing of the *PTEN* gene. The abnormalities of the *PTEN* gene are associated with tumor progression, metastasis, and survival. (*Lab Invest 2002, 82:285–291*).

The recently identified *PTEN/MMAC1/TEP1* gene is a novel tumor suppressor gene located on chromosome 10q23.3 (Myers et al, 1997). This gene was identified by three research groups in 1997 and named *PTEN* (phosphatase and tensin homolog deleted on chromosome 10), *MMAC1* (mutated in multiple advanced cancers), or *TEP1* (TGF-β regulated and epithelial cell enriched phosphatase 1), respectively (Li and Sun, 1997; Li et al, 1997; Steck et al, 1997). PTEN/MMAC1/TEP1 (hereafter, PTEN) is a dual-specificity phosphatase with homology with the adhesion molecules tensin and auxillin. PTEN dephosphorylates and inactivates phosphatidylinositol (3, 4, 5)-triphosphate (PI-3,4,5-P3), an intracellular second messenger, which activates serine/threonine kinase, Akt, through phosphoinositide-dependent kinase (PDK1) (Dahia, 2000). Activated Akt inactivates forkhead family transcription factor (FKHR), glycogen synthase kinase (GSK3), and the proapoptotic proteins, Bad and caspase-9 (Datta et al, 1997). The phosphatase activity of PTEN down-regulates the signaling of Akt, which suppresses apoptosis and promotes cell survival (Sun et al, 1999). PTEN induces G1 cell cycle arrest (Li and Sun, 1998). PTEN inhibits focal adhesion, spreading, and migration by dephosphorylating focal adhesion kinase (Tamura et al, 1998). PTEN regulates tumor-induced angiogenesis (Wen et al, 2001).

Gene silencing by genetic and epigenetic alteration of the *PTEN* gene was recently reported in a subset of cancers. The loss of heterozygosity and mutations of *PTEN* have been observed in a variety of cancers, including breast cancer, kidney cancer, bladder cancer, melanoma, glioblastoma, and lung cancer (Birck et al, 2000; Tashiro et al, 1997). Germline mutations of the *PTEN* genes cause Cowden's syndrome, which is characterized by multiple hamartomas and an increased risk of breast and thyroid cancer (Liaw et al, 1997). Autosomal dominant diseases, such as juvenile polyposis syndrome and Bannayan-Zonana syndrome, were found to result from germline mutation of *PTEN* (Marsh et al, 1997). *PTEN* plays a role in the pathogenesis of hematologic malignancy (Dahia et al, 1999). Altered expression of PTEN was reported in endometrial cancer, prostate cancer, and melanoma (Whang et al, 1998). Aberrant promoter methylation was associated with inactivation of the *PTEN* gene in endometrial cancer, prostate cancer, and melanoma (Salvesen et al, 2001; Zhou et al, 2000).

DNA promoter methylation has been recently established as an alternative mechanism of transcriptional inactivation of tumor suppressor genes (Herman, 1999). Inactivation of tumor suppressor genes has been known to contribute to the abnormal proliferation, transformation, and progression in human cancers. Aberrant promoter methylation of cancer-related key genes, such as *p16, E-cadherin, TIMP-3, APC, THBS1,* and *hMLH1*, has been observed frequently in

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Address reprint requests to: Dr. Woo Ho Kim, Department of Pathology, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Korea. E-mail: woohokim@snu.ac.kr

gastric carcinoma (Kang et al, 2001; Leung et al, 2001).

To our knowledge, the role of the *PTEN* gene in gastric carcinoma has not been studied to date. In this study, the methylation status of the promoter CpG islands and altered expression were examined to address the involvement of *PTEN* gene alteration in gastric carcinogenesis. The relationships between *PTEN* gene inactivation and clinicopathologic parameters were also analyzed.

Results

Altered PTEN *Expression Occurs Frequently in Gastric Carcinoma*

To address the involvement of *PTEN* in gastric carcinoma, the abnormalities of PTEN protein expression were examined immunohistochemically in 310 consecutive gastric carcinomas. The tissue-array slide containing 60 tissue specimens was used for immunohistochemical staining. Tumor tissues and normal tissues were simultaneously stained on the same tissue-array slide. Immunohistochemical staining exhibited cytoplasmic localization of PTEN protein in normal as well as tumor cells (Fig. 1). Expressional loss of PTEN was observed in 62 (20%) of the 310 consecutive gastric carcinomas. This finding demonstrates that alteration in PTEN expression occurs frequently in gastric carcinoma.

Clinicopathologic Profiles

The clinicopathologic parameters were characterized and their relationship with PTEN expression was analyzed. Table 1 shows the correlation between clinicopathologic parameters and PTEN expression status. Gastric carcinomas showing expressional loss of PTEN were most common in Borrmann's type III carcinoma ($p = 0.001$). The expressional loss of PTEN was significantly associated with advanced stage compared with early stage ($p < 0.001$). Fifty-seven (92%) of 62 cases showing the expressional loss of PTEN were in the advanced stage. The altered expression of PTEN was significantly associated with tumor depth and tumor size ($p < 0.01$), lymph node metastasis, lymphatic invasion ($p < 0.001$), and pTNM stage ($p < 0.001$). However, no correlation was found between PTEN expression phenotype and distant-organ metastasis or vascular invasion. This indicates that the altered expression of PTEN is involved in the progression and lymph node metastasis of gastric carcinoma.

The immunohistochemical staining results of p53, MUC1, MUC2, and Bcl2 proteins were analyzed to address the relationships between PTEN and the expressions of the other proteins (Table 2). The loss of PTEN expression was significantly associated with p53 overexpression ($p = 0.04$) and positive Bcl2 expression ($p = 0.04$). Mucin phenotypes of gastric carcinoma were also significantly correlated with PTEN expression status.

Survival Analysis

The survival of patients showing expressional loss of PTEN was remarkably worse than that of patients showing positive PTEN expression ($p = 0.001$) (Fig. 2). When survival curves were stratified according to disease progression using the Kaplan-Meier method, PTEN expression phenotype tended to correlate with patient survival in a subgroup of advanced carcinoma, but without significance ($p = 0.08$). Of the other clinicopathologic parameters, tumor location, tumor size, lymphatic invasion, and pTNM stage were found to correlate significantly with patient survival ($p < 0.01$). By multivariate Cox regression model, pTNM stage was found to be significantly and independently associated with patient survival ($p < 0.001$), but PTEN expression status ($p = 0.36$) and Lauren's classification ($p = 0.12$) were not.

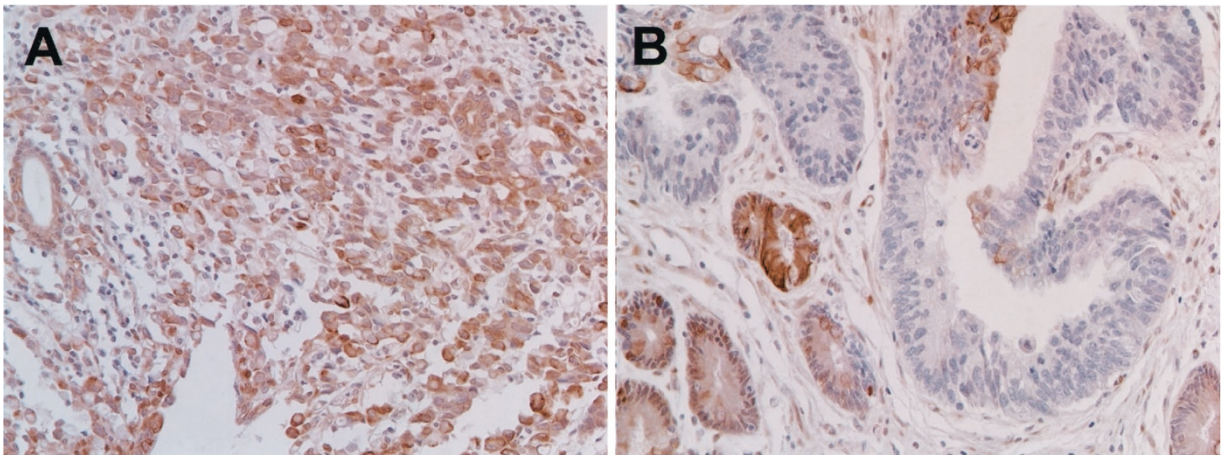

Figure 1.
Immunohistochemical staining of PTEN protein. A, Diffuse positive signals on cytoplasm of tumor cells are shown. B, PTEN protein was absent in the tumor cells, whereas adjacent noncancerous glands showed positive staining. Original magnification, ×200.

Table 1. Clinicopathologic Characteristics of PTEN Expression in Gastric Cancers

Characteristics	Total (n = 310)	PTEN negative (n = 62)	PTEN positive (n = 248)	p value[a]
Sex				N.S.
Male	212	47	165	
Female	98	15	83	
Mean age (yr)	54.4 ± 13.1	56.8 ± 13.4	53.7 ± 12.9	N.S.
Location				N.S.
Antrum	166	30	136	
Body & Cardia	144	32	112	
Tumor size (cm)	5.2 ± 3.7	6.5 ± 3.4	4.8 ± 2.9	0.003
WHO classification				N.S.
W/D and M/D	115	24	91	
P/D	131	30	101	
Mucinous	17	0	17	
Signet ring cell	47	8	39	
Lauren's classification				N.S.
Intestinal	119	23	96	
Diffuse	161	36	125	
Mixed	30	3	27	
Lymphatic invasion				<0.001
Absent	223	31	192	
Present	87	31	56	
Depth of invasion				<0.001
EGC	98	5	93	
AGC	212	57	155	
Lymph node metastasis				<0.001
Absent	120	11	109	
Present	190	51	139	
Distant-organ metastasis				N.S.
Absent	294	56	238	
Present	16	6	10	
PTNM stage[b]				<0.001
I	138	13	125	
II	60	10	50	
III	68	23	45	
IV	44	16	28	

N.S., not significant.
[a] The χ^2 test (unpaired t-test) or Fisher's exact test were used to compare all variables.
[b] Pathological stage was classified according to the criteria of the American Joint Committee on Cancer.

Table 2. Correlation Between Expression of PTEN and Expression of Other Proteins

	PTEN expression		
	Negative	Positive	p value
p53 overexpression	27/59 (46%)	75/237 (32%)	0.04
Bcl2 expression	2/58 (3%)	34/241 (14%)	0.04
MUC1 expression	21/60 (35%)	51/241 (21%)	0.03
MUC2 expression	7/59 (12%)	73/233 (31%)	<0.01

Promoter Methylation of PTEN is a Major Alternative Mechanism of Gene Silencing

The status of promoter methylation of PTEN in 66 gastric cancer tissues was determined using methylation-specific PCR (MSP). Aberrant promoter methylation of PTEN was detected in 26 (39%) of 66 gastric carcinomas. Seven (27%) of 26 primary gastric cancers with promoter methylation expressed PTEN protein, whereas 33 (83%) of 40 gastric cancers without methylation expressed PTEN protein. Promoter methylation of PTEN was significantly correlated with the loss of PTEN protein expression ($p < 0.001$, Fisher's exact test), but this correlation was not completely consistent (Table 3). These findings suggest that the silencing of the PTEN gene can be caused by promoter methylation as well as by other inactivating mechanisms. The unmethylated form was consistently found in tumor tissue because of normal cell contamination (Fig. 3).

Discussion

The role of *PTEN* identified as a novel tumor suppressor gene has been studied in a variety of cancers. Loss of heterozygosity and mutation of *PTEN* have been reported in melanoma, glioblastoma, kidney

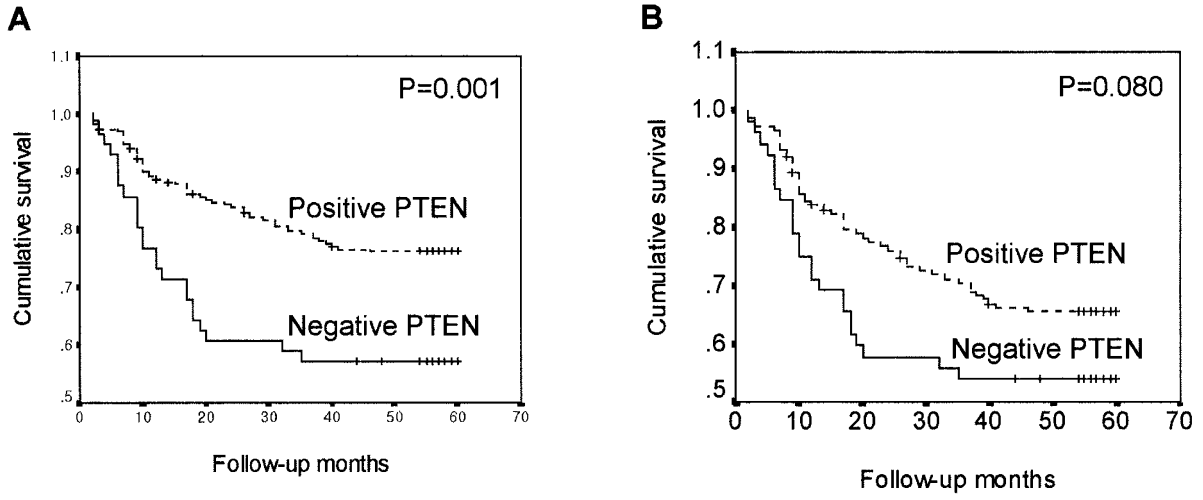

Figure 2.

The effects of PTEN expression on patient survival. A, Survival curves by the Kaplan-Meier method. PTEN-negative tumors have an unfavorable prognosis as compared with PTEN-positive tumors ($p = 0.001$). B, Survival curves of advanced stage cancers by the Kaplan-Meier method. PTEN expression is correlated with patient survival in advanced cancers, but without significance ($p = 0.080$). p was determined using the log-rank test.

cancer, lung cancer, and breast cancer (Rhei et al, 1997; Risinger et al, 1998; Teng et al, 1997). In particular, the mutation of *PTEN* appears with a frequency of up to 50% in endometrial cancer (Tashiro et al, 1997) compared with other cancers, including glioblastoma (28%), prostate cancer (30%), and melanoma (15%) (Dahia, 2000). Abnormalities in the expression of the *PTEN* gene have been shown in esophageal cancer, melanoma, and prostate cancer (Cairns et al, 1997; Whang et al, 1998). Gene silencing by promoter methylation of *PTEN* has been reported in endometrial and prostate cancer (Salvesen et al, 2001). Aberrant promoter methylation has been suggested as a potential mechanism of *PTEN* inactivation.

DNA methylation plays an important role in the gene regulation of mammals (Razin and Riggs, 1980) and transcriptional inactivation is a recognized major mechanism for the loss of function in the tumor suppressor gene. Promoter methylation is a representative example of the transcriptional silencing of tumor suppressor genes (Baylin et al, 1998; Herman, 1999). Cancer-related genes, such as *APC, p16, p15, E-cadherin, hMLH1,* and *TIMP3,* have been inactivated by aberrant promoter methylation in gastric carcinoma (Leung et al, 2001; Tsuchiya et al, 2000**)**. The role of *PTEN* in gastric carcinogenesis has not been reported to date. To address the involvement of alterations of the PTEN gene in gastric carcinoma, we examined the promoter methylation and the altered expression of the *PTEN* gene. Aberrant promoter methylation of PTEN was detected in 26 (39%) of 66 primary gastric cancers. Promoter methylation of *PTEN* was significantly correlated with the expressional loss of the PTEN protein ($p < 0.001$, Fisher's exact test). Nineteen (73%) of 26 gastric cancer tissues showing promoter methylation in MSP revealed the loss of PTEN expression, whereas 7 (18%) of 40 gastric cancers showing no methylation exhibited the expressional loss of PTEN. This may indicate that the gene inactivation of *PTEN* is caused by promoter methylation as well as by other inactivating mechanisms, such as mutation or loss of heterozygosity.

Since the *PTEN* gene was recently identified as a tumor suppressor gene, study of the biological and biochemical roles of the *PTEN* gene has intensified. It has been reported that PTEN controls cellular processes, such as cell cycling, apoptosis, and cell death, as a regulator of phosphatidylinositol 3-kinase (PI3K) (Dahia, 2000). Although many studies on PTEN have already been reported, the role of the *PTEN* gene in cancer is unclear and many controversial results have been presented. Several articles on endometrial car-

Table 3. Correlation Between PTEN Expression and Promoter Methylation

	Methylation-specific PCR	
PTEN expression	Unmethylated	Methylated
Positive	33	7
Negative	7	19

$p < 0.001$.

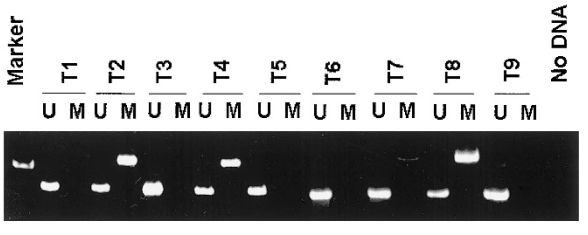

Figure 3.

Methylation-specific PCR of PTEN in gastric cancer tissue. The unmethylated (162 bp) and methylated (206 bp) PCR products were designated as *U* or *M*, respectively. T2, T4, and T8 exhibited PCR products by methylated primers. All tumor samples showed the unmethylated form because of normal cell contamination.

cinoma have shown that altered PTEN expression is associated with an early stage in endometrial cancer (Mutter et al, 2000) and that inactivation of PTEN is associated with nonmetastatic disease and favorable clinical characteristics (Risinger et al, 1998). In contrast, other papers have shown that gene silencing of PTEN is associated with advanced stage and metastasis in endometrial and prostate carcinoma (Rasheed et al, 1997; Whang et al, 1998). It was reported that mutation of the *PTEN* gene correlated with tumor progression and poor outcome in cervical cancer, breast cancer, and melanoma (Depowski et al, 2001; Poetsch et al, 2001).

In this study, clinicopathologic parameters related to PTEN expression were characterized. The expressional loss of PTEN increased remarkably according to disease stage (pTNM Stage I, 9%; Stage II, 16%; Stage III, 34%; Stage IV, 36%). Lymph node metastasis was observed in 82% of PTEN-negative tumors compared with 52% of PTEN-positive tumors. Our data show that *PTEN* gene alteration is associated with advanced stage and lymph node metastasis. Our results suggest that *PTEN* may play an important role in the regulation of tumor progression and metastasis during the development of gastric carcinoma. Gastric carcinoma patients showing expressional loss of PTEN showed poor prognosis. This result suggests that PTEN is a potential prognostic factor in gastric carcinoma.

Our previous study showed that MUC1, MUC2, and p53 were prognostic markers in gastric carcinoma (Lee et al, 2001). In the present study, the loss of PTEN expression was significantly associated with p53 overexpression and a similar association was reported in glioblastoma (Kraus et al, 2000). A recent study demonstrates that loss of PTEN leads to up-regulation of the Bcl2 gene, thus contributing to survival of cancer cells in prostate cancer (Huang et al, 2001). Our results also show the strong association of altered PTEN expression with Bcl2-positive expression ($p = 0.04$) in gastric carcinoma.

In conclusion, our findings demonstrate that aberrant promoter methylation and the altered expression of the PTEN gene occur frequently in gastric carcinoma. Promoter methylation of CpG islands of PTEN presents a major alternative mechanism of gene silencing. Moreover, the expressional loss of PTEN correlates significantly with tumor progression, lymph node metastasis, and poor survival. Our results suggest that PTEN probably plays an important role in the progression of gastric carcinoma and that the expressional phenotype of PTEN may be a potential prognostic factor in gastric carcinoma.

Materials and Methods

Patients and Samples

Three hundred and ten specimens of gastric cancer tissues, resected surgically at the Seoul National University College of Medicine, were used for this study. The age, sex, tumor location, tumor size, lymphatic invasion, and pTNM stage were evaluated by reviewing medical charts and pathologic records. The mean age of the patients was 54.4 years. No patient had received preoperative chemo- or radiotherapy. Glass slides were reviewed for histologic classification (according to the World Health Organization's and Lauren's classification). The clinical outcome of the patients was followed from the date of surgery (1995) to the date of death or to December 1, 1999. The follow-up period was 1 month to approximately 60 months (mean, 42 months). Cases lost to follow-up and deaths from causes other than gastric cancer were regarded as censored data (3.8%) for the analysis of survival rates. The DNA of 66 cancerous tissues selected randomly from 310 consecutive gastric carcinomas was obtained from formalin-fixed, paraffin-embedded surgical blocks. DNA was extracted by the proteinase K digestion and phenol/chloroform/isoamyl alcohol (25:24:1) procedure.

Immunohistochemical Staining

The protein expression of PTEN was assessed by an immunohistochemical staining method using a tissue array (Superbiochips Laboratories, Seoul, Korea). Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded gastric tumors and seeded in a new recipient paraffin block (tissue-array block). Each tissue-array block consisted of 60 samples. In total, 6 tissue-array slides of 310 cases were used in the immunohistochemical study. Each tissue-array block contained normal gastric mucosa from body, antrum, and intestinal metaplasia as an internal control. Sections 4 μm thick were cut from each tissue-array block. These were then deparaffinized and dehydrated. Immunohistochemical staining against PTEN (mouse monoclonal antibody; AG Science, San Diego, California) was performed using a streptavidin peroxidase procedure. Anti-PTEN antiserum was diluted to 1:200 with PBS. Antigen-bound primary antibody was detected using a standard avidin-biotin immunoperoxidase complex (ABC) method (Vectastain Elite ABC peroxidase kit; Vector Laboratories, Burlingame, California). Those cases with less than 10% of cytoplasmic staining in tumor cells were considered to have loss of PTEN expression. Immunohistochemical staining against p53 (mouse monoclonal antibody; DAKO, Glostrup, Denmark), Bcl2 (mouse monoclonal antibody; DAKO), MUC1 (mouse monoclonal antibody NCL-MUC-1: Novocastra Laboratories, Newcastle, United Kingdom), and MUC2 (mouse monoclonal antibody NCL-MUC-2; Novocastra) was performed and the relationships between the expression of PTEN and the other proteins were examined.

Bisulfite Modification and MSP

Genomic DNA was modified with sodium bisulfite as described previously (Herman et al, 1996). Briefly, 1 ug of genomic DNA was denatured with 0.2 M NaOH for 10 minutes at 37° C. Then 10 mM of hydroquinone and

3 M of sodium bisulfite (pH 5.0) were added and mixed. Samples were incubated at 50° C for 16 hours. DNA was purified using the Wizard DNA purification kit (Promega, Madison, Wisconsin). This procedure resulted in the conversion of unmethylated cytosine to thymine, but methylated cytosine remained unchanged. Then 1 ul of bisulfite-modified DNA was amplified using primers that specifically amplify methylated or unmethylated DNA (gene bank accession number AF067844). The primers used were as described previously (Salvesen et al, 2001). Primer sequences of PTEN were as follows: unmethylated reaction, sense primer, 5'-GTGTTGGTGGAGGTAGTTGTTT-3', antisense primer, 5'-ACCACTTAACTCTAAACCACAACCA-3'; methylated reaction, sense primer, 5'- TTCGTTCGTCGTCGTCGTATTT-3', antisense primer, 5'-GCCGCTTAACTCTAAACCGCAACCG-3'. PCR was performed as follows: one cycle of 95° C for 5 minutes and 35 cycles of 94° C for 45 seconds, 61° C for 30 seconds, and 72° C for 30 seconds, and then one cycle of 72° C for 10 minutes. PCR products were analyzed using nondenaturing 6% polyacrylamide gel electrophoresis and ethidium bromide staining. Normal lymphocytes were used as a negative control for unmethylated form in MSP.

Statistical Analyses

The χ^2 test or Fisher's exact test (two sided) was used to determine the correlation between PTEN expression phenotype and the clinicopathologic parameters. Survival curves were estimated using the Kaplan-Meier product-limit method, and differences between the survival curves were tested using the log-rank test. A multivariate survival analysis was conducted according to the Cox proportional hazards model. Results were considered to be statistically significant when *P* values were less than 0.05. All statistical analyses were conducted using the SPSS 9.0 statistical software program (SPSS, Chicago, Illinois).

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