

Mutation analysis of tumor suppressor gene *PTEN* in patients with gastric carcinomas and its impact on PI3K/AKT pathway

YU-GANG WEN^{1*}, QUAN WANG^{1*}, CHONG-ZHI ZHOU¹, GUO-QIANG QIU¹,
ZHI-HAI PENG¹ and HUA-MEI TANG²

Departments of ¹General Surgery and ²Pathology, Affiliated First People's Hospital,
College of Medicine, Shanghai Jiao Tong University, Shanghai 200080, P.R. China

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Abstract. The aim of this study was to clarify the participation of *PTEN* mutation in gastric carcinogenesis and its impact on PI3K/AKT pathway. All nine exons of *PTEN* were screened for mutations by direct sequencing in 144 patients with pathologically proven gastric carcinoma and their corresponding normal mucosae, followed by Western blotting to detect the changes in PI3K/AKT pathway. Direct sequencing indicated there were 27 cases with mutations among 144 patients consisting of 15 cases (55.6%) of missense mutation, nine nonsense mutations (33.3%), two 1-bp deletion (7.4%), and a mutation within intron 6 (3.7%). The mutation hot spots at codons 36, 75, 232 and 393 had not been observed previously, and the mutation sites in exons 3, 5, 6 and 8 were not found, suggesting that there might be some unique characteristic of *PTEN* inactivation mechanism in the Shanghai population. The *PTEN* mutation rate was significantly higher at pTMN stages III and IV than that at stages I and II ($P < 0.005$), and it was higher in poorly differentiated gastric cancer than in well or moderately differentiated types ($P < 0.05$). *PTEN* and E-cadherin protein expression in gastric cancer was significantly down-regulated comparing with that in paracancerous tissues, while the PI3K, AKT, MMP-2, MMP-9 and NF- κ Bp65 protein were overexpressed in cancer tissues. Our results implicated that the mutations of *PTEN* did not occur at a significant rate in gastric carcinoma in Shanghai, but might play a role in tumorigenesis. The mutation status of *PTEN* was significantly relevant to pTNM

staging and degree of cell differentiation, hinting that *PTEN* might be a prognostic biomarker of gastric cancer. The decreased expression of *PTEN* and E-cadherin, together with the overexpression of PI3K, AKT, MMP-2, MMP-9 and NF- κ Bp65, contributed cooperatively to the accelerated progress of gastric cancer.

Introduction

Despite the declining incidence of gastric cancer over the past decade, it is still the second most common fatal malignancy worldwide (1). In Shanghai, gastric cancer is the second major cause of cancer-related death among males and the third major cause of cancer-related death among females. There are about 5000 new cases diagnosed every year, with over 4000 patients died annually from this disease (2,3). The genetic bases underlying gastric tumorigenesis and progression are largely unknown. Recently, much progress has been made in identifying genes involving in the development of gastric cancer, which is useful for understanding the pathogenesis of gastric cancer and defining its molecular signature.

In the past ten years, we have used some high-throughput methods to fully examine the gene expression patterns in gastric cancer, including LOH (loss of heterozygosity) and wide-genome expression microarray. A candidate tumor suppressor gene, *PTEN* (phosphatase and tensin homologue deleted on chromosome ten/mutated in multiple advanced cancers 1), has been screened. The *PTEN* gene locates on human chromosome band 10q23.31, contains nine exons and encodes a 403-amino acid, dual-specificity phosphatase with homology to the cytoskeleton proteins chicken tensin and bovine auxilin, cytoskeletal proteins that interact with adhesion molecules (4-8). Mutations of *PTEN* gene have been found in a variety of human cancer cell lines and primary tumors of the endometrium, brain, prostate, breast and kidney in different frequencies (9-17), indicating the gene might play a role in the tumorigenesis of some cancers. Furthermore, it was reported that the *PTEN* gene might regulate tumor cell invasion and metastasis (8,18,19). Thus, this gene is probably correlated with some human cancer progression (11,20,21). However, the role of *PTEN* mutation in the development of human gastric cancer and the molecular mechanisms under-

Correspondence to: Dr Hua-Mei Tang or Professor Zhi-Hai Peng, Department of Pathology, Affiliated First People's Hospital, College of Medicine, Shanghai Jiao Tong University, 85 Wujin Road, Shanghai 200080, P.R. China
E-mail: tanghuamei@gmail.com
E-mail: pengzh@hotmail.com

*Contributed equally

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Table I. Oligonucleotide primers for *PTEN* analysis.

Exon	Sequence	Annealing temperature (°C)	Product size (bp)
1	Forward: 5'-CTCCTCCTTTTTCTTCAGCC-3' Reverse: 5'-TATGACCTAGCAACCTGACCA-3'	56	253
2	Forward: 5'-TGACCACCTTTTATTACCA-3' Reverse: 5'-AAGTATCTTTTTCTGTGGCTTA-3'	48	242
3	Forward: 5'-ATAGAAGGGGTATTTGTTGGA-3' Reverse: 5'-ACCTCACTCTAACAAGCAGATA-3'	56	293
4	Forward: 5'-TTCAGGCAATGTTTGTTA-3' Reverse: 5'-CTCGATAATCTGGATGACTCA-3'	48	225
5	Forward: 5'-GCAACATTTCTAAAGTTACCTA-3' Reverse: 5'-TCTGTTTTCCAATAAATTCTC-3'	48	376
6	Forward: 5'-GAGTAACTATTCCCAGTCAGA-3' Reverse: 5'-TAATTTGTTCAAATGCTTCAGA-3'	54	286
7	Forward: 5'-ATCGTTTTTGACAGTTTG-3' Reverse: 5'-CCAATGAAAGTAAAGTACA-3'	48	260
8	Forward: 5'-AGGTGACAGATTTTCTTTTTTA-3' Reverse: 5'-TAGCTGTACTCCTAGAATTA-3'	52	384
9	Forward: 5'-GTTCATCTGCAAAATGGA-3' Reverse: 5'-TGGTAATCTGACACAATGTCCTA-3'	50	397

lying the roles of the *PTEN*/*PI3K*/*AKT* pathway during gastric tumorigenesis remain poorly defined.

In the present study, we examined the mutation frequency and pattern of the *PTEN* gene in primary gastric cancer specimens by direct sequencing of all exons and flanking regions, then identified its associated gene expression using Western blot analysis. We observed mutations of *PTEN* in 27 of 144 (18.75%) patients with gastric cancer, showing some hot spot mutation sites, suggesting that the *PTEN* gene might have a critical role in the pathogenesis of human gastric cancer.

Materials and methods

Tissue samples and DNA extraction. Between January 2007 and April 2008, 144 consecutive patients with gastric carcinoma that underwent surgical resection at the Department of Surgery of Shanghai Jiao Tong University Affiliated First People's Hospital were enrolled in this study. There were 79 males, 65 females (average age, 65 years; ranging from 31-85 years), including all of the stages and histological types of gastric cancer. All patients were proven to have a primary adenocarcinoma of the stomach with no evidence of any other malignancy. None of the patients received preoperative chemotherapy and/or radiation therapy. Clinicopathological descriptions were carried out according to the Japanese Research Society for Gastric Cancer (JRS GC) standards (22). The cancer tissues with their matched normal tissues were collected immediately after surgical resection, frozen instantly in liquid nitrogen, and then stored at -80°C until analyzed. Genomic DNA was isolated from frozen primary gastric cancer and the paired normal tissues using the proteinase-K (Stratagene, La Jolla, CA) digestion and phenol/

chloroform extraction procedures according to the method by Sambrook *et al.* (23). The protocols used in the studies were approved by the Hospital's Protection of Human Subjects Committee. Patients whose surgical tissue was studied gave their written informed consent.

PCR amplification. Tumor DNAs were subjected to PCR amplification. All nine exons of the *PTEN* gene were amplified using primers designed and synthesized by Invitrogen (Shanghai) Corp. (Table I). PCR amplification was performed within a reaction mixture containing 10X PCR Buffer 2.5 μ l (Mg²⁺ free, Promega Corp., Madison, WI), Mg²⁺ 0.8 μ l (50 mM), dNTP 0.5 μ l (10 mM, Promega), primer F 0.5 μ l (10 μ M), primer R 0.5 μ l (10 μ M), Taq polymerase 0.2 μ l (5 U/ μ l, Promega), genomic DNA 1.0 μ l and ddH₂O 19.0 μ l. A programmable thermocycler (GeneAmp PCR System 9700, Applied Biosystems Inc., USA) was used to perform amplifications. The PCR condition for each exon was as follows: pre-denaturation 4 min at 95°C, 35 cycles of 30 sec denaturation at 95°C, 40 sec annealing at temperature shown in Table I, 50 sec extension at 72°C, and 10 min final extension at 72°C. The PCR products were loaded onto polyacrylamide gels of GeneGel Excel 12.5/24 kit (Pharmacia Biotech Inc., San Francisco, CA) using the Genephore electrophoresis unit (Pharmacia Biotech). Gels were run for 3 h at 38 V.

DNA sequencing. The PCR products were purified using the QIAEX II Gel Extraction kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instruction. Then mixed 1 μ l PCR gel recycling product, 1 μ l BigDye Mix (Applied Biosystems), 1 μ l sequencing primer, 2 μ l ddH₂O into 0.2 ml PCR tube, centrifuged briefly, and amplified again: 98°C

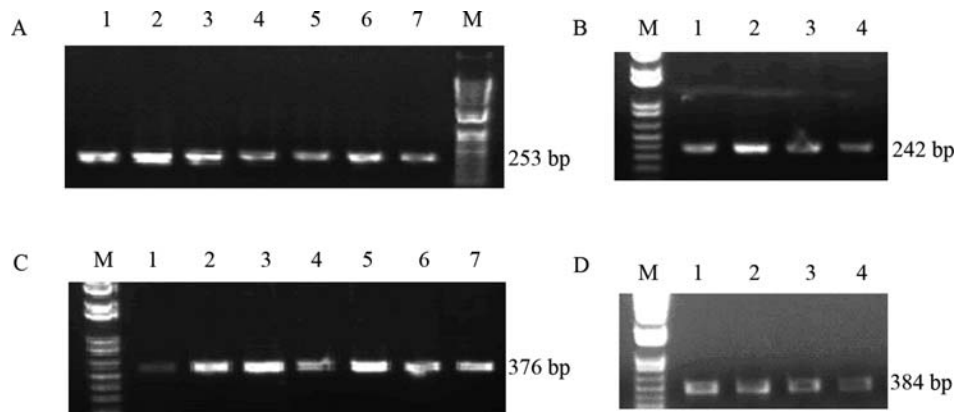


Figure 1. Part of the PCR amplification products detected by electrophoresis gel. (A) exon 1 at 253 bp. (B) exon 2 at 242 bp. (C) exon 5 at 376 bp. (D) exon 8 at 384 bp. M, marker; 1-7, samples.

denaturation 2 min, 25 cycles of 96°C 10 sec, 50°C 5 sec, 60°C 4 min, and ended at 4°C. The products were purified one more time with 25 μ l sodium acetate/ethanol, vortexing, put on ice 10 min, 4°C 12000 g centrifuged 30 min, the supernatant was discarded, followed by 50 μ l 70% (V/V) ethanol washing of sediment twice, 4°C 12000 g centrifuged for 5 min, discarding supernatant, and vacuum drying for 10-15 min. Then 12 μ l TSR was added, vortexed thoroughly to dissolve the DNA sediment, centrifuged briefly, and the sample was heated to 95°C for 2 min, snap-cooled on ice and loaded onto the sequencing gel.

An automated DNA electrophoresis system (Model 3730, Applied Biosystems) with a laser diode emitting at 785 nm and fluorescence detection between 815 and 835 nm was used to detect and analyze the sequencing ladder. Electrophoresis was performed on a 41 cm x 25 cm x 0.2 mm gel consisting of 6% Long Ranger gel matrix (AT Biochem; Malvern, PA) with 7 M urea and a running buffer consisting of 133 mM Tris base, 44 mM boric acid and 2.5 mM EDTA, pH 9 at 50°C. Following the loading of samples, electrophoresis was carried out at 1.2 kV constant voltage 25 min, 7.5 kV 2 h, with the gel heated to 50°C. The PCR products were sequenced directly from both forward and reverse directions. Data collection and image analysis utilized the software supplied with the model 3730 DNA sequencer.

Western blotting. The tissues of gastric cancer or matched normal mucosa were cut into pieces, then homogenized in lysis buffer (50 mM Tris-HCl, 0.1% SDS, 150 mM NaCl, 100 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1% NP-40 and 0.5% sodium orthovanadate) 200 μ l/20 mg tissue sample, and incubated at 4°C for 30 min, centrifuged at 12000 g for 20 min. Concentration of total proteins in the supernatant was quantified by Bradford assay. Proteins (50 μ g/lane) were electrophoresed on 10% SDS-polyacrylamide gels, then transferred onto PVDF membrane (0.45 mm, Millipore, USA) in 25 mM Tris-base, 190 mM glycine and 20% methanol using a wet blotter (Bio-Rad, USA). Followed by blocking with 5% fat-free milk in PBS for 2 h, the membrane was incubated with anti-PTEN (Abcam, 1:500), anti-Akt (Santa Cruz, 1:200), anti-p-Akt (Santa Cruz, 1:100), anti-E-cadherin (Santa Cruz, 1:200), anti-MMP-2 (Santa Cruz, 1:500), anti-MMP-9 (Santa Cruz, 1:500) and anti-GAPDH (Santa Cruz,

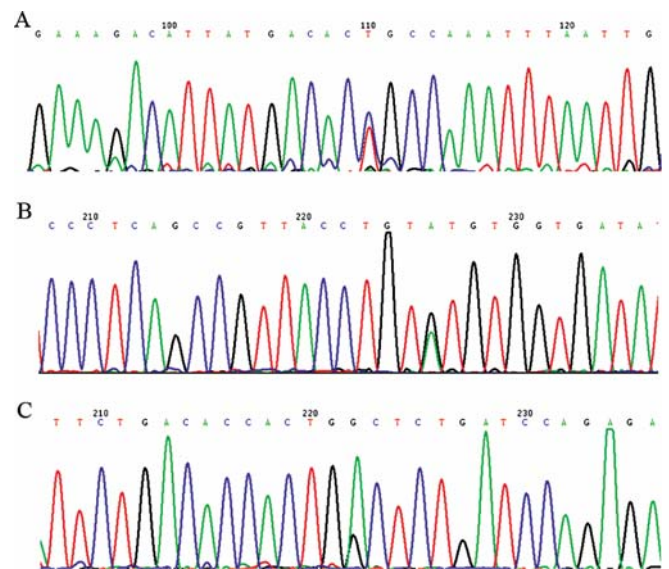


Figure 2. The chromatograms of part of the mutant exons. (A) exon 4, base C→T at 110. (B) exon 7, base G→A at 226. (C) exon 9, base A→G at 222.

1:2000) at 4°C overnight, respectively. After binding of horseradish peroxidase (HRP)-coupled goat anti-mouse or goat anti-rabbit IgG (Santa Cruz, 1:2000) at room temperature for 2 h with gently shaking, antigens were visualized by enhanced chemiluminescence (ECL-kit, Millipore Biotechnology). All results are representatives of three independent experiments.

Statistical analysis. The χ^2 test for proportion was used, as appropriate, to analyze the relationship between *PTEN* gene mutation rate and clinicopathological parameters. P<0.05 was considered to be statistically significant. Analyses were performed using the SPSS statistical software program version 13.0 (SPSS Inc., Chicago, IL).

Results

All of the gastric cancers and their adjacent normal gastric tissues were screened for mutations of the *PTEN* gene in nine

Table II. Mutations of *PTEN* in gastric cancer and clinicopathological features.

Case no.	Gender	Age (years)	pT	pN	Stage ^a	Histology	Exon	Mutated codon	Base substitution	Amino acid change
4	Male	65	T3	N3	III	PD	9	Codon 380	GAC→GGC	Asp→Gly
17	Female	72	T3	N3	III	PD	4	Codon 77	1 bp deletion	Ile→Phe
20	Male	74	T2	N3	III	Mucinous	7	Codon 232	ACA→ACG	Nonsense mutation
24	Male	69	T3	N3	III	MD	9	Codon 341	GCT→GCG	Nonsense mutation
28	Male	60	T3	N2	III	Signet	7	Codon 249	GTG→GTA	Nonsense mutation
31	Female	58	T2	N2	II	WD	2	Codon 36	AGA→AAA	Arg→Lys
36	Male	63	T2	N1	I	PD	9	Codon 380	GAC→GGC	Asp→Gly
51	Female	64	T2	N2	II	PD	9	Codon 393	GCA→ACA	Ala→Thr
60	Male	61	T4	N3	IV	MD	2	Codon 36	AGA→AAA	Arg→Lys
64	Male	67	T3	N1	III	WD	7	Codon 232	ACA→ACG	Nonsense mutation
67	Male	59	T3	N3	III	MD	9	Codon 393	GCA→ACA	Ala→Thr
70	Female	62	T4	N2	III	PD	4	Codon 75	CGC→TGC	Arg→Cys
72	Male	66	T2	N3	III	WD	2	Codon 36	AGA→AAA	Arg→Lys
79	Male	68	T2	N2	II	PD	9	Codon 393	GCA→ACA	Ala→Thr
82	Female	67	T4	N3	IV	PD	2	Codon 36	AGA→AAA	Arg→Lys
85	Male	68	T2	N2	II	WD	7	Codon 249	GTG→GTA	Nonsense mutation
87	Male	78	T2	N2	III	PD	7	Codon 232	ACA→ACG	Nonsense mutation
95	Female	69	T3	N1	III	PD	1	Codon 11	AGA→AAA	Arg→Lys
96	Male	73	T2	N2	II	WD	1	Codon 17	CAA→CAG	Nonsense mutation
101	Male	58	T3	N2	III	PD	9	Codon 341	GCT→GCG	Nonsense mutation
106	Female	68	T4	N1	III	PD	4	Codon 77	1 bp deletion	Ile→Phe
116	Male	69	T2	N2	II	MD	4	Codon 75	CGC→TGC	Arg→Cys
120	Male	66	T3	N2	III	PD	4	Codon 75	CGC→TGC	Arg→Cys
126	Female	55	T3	N2	III	PD	7	Codon 232	ACA→ACG	Nonsense mutation
135	Male	77	T2	N2	II	MD	4	Codon 75	CGC→TGC	Arg→Cys

^aStaging according to Japanese Research Society for Gastric Cancer. pT, pathological depth of tumor invasion; pN, pathological status of lymph node metastasis; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; Signet, signet ring cell.

exons using the PCR amplification, purification and direct sequencing analysis. The PCR amplification products were detected by electrophoresis gel (Fig. 1). The chroma-tograms of mutant exons are shown in Fig. 2. Twenty-seven patients (18.75%) showed an apparent mutation between the cancer and its paired normal tissue. Of 27 such differences, 2 were detected in exon 1 (cases 95 and 96), 4 in exon 2 (cases 31, 60, 72 and 82), 6 in exon 4 (cases 17, 70, 106, 116, 120 and 135), 6 in exon 7 (case 20, 28, 64, 85, 87 and 126), 8 in exon 9 (cases 4, 24, 36, 51, 55, 67, 79 and 101), and 1 in intron 6 (Table II).

Direct sequencing analysis of the *PTEN* gene mutations revealed 15 cases of missense mutation, nine nonsense mutations, two 1-bp deletion and one mutation within the

splice donor site of intron 6 (Table II). The results indicated that the mutation site in exon 1 were found to be at codon 11 (AGA→AAA) in one case, leading to arginine to lysine substitution; at codon 17 (CAA→CAG) in one cases, leading to nonsense mutation. Exon 2 was found to be at codon 36 (AGA→AAA) in four cases, all leading to arginine to lysine substitution. The mutation sites in exon 4 were found to be at codon 75 (CGC→TGC) in four cases, leading to arginine to cysteine substitution; at codon 77 (1 bp deletion) in two cases, resulting in a change from isoleucine to phenylalanine substitution. The mutation sites in exon 7 were found to be at codon 232 (ACA→ACG) in four cases, resulting in nonsense mutations, at codon 249 (GTG→GTA) in two cases, resulting in nonsense mutations. The mutation site in exon 9 was found

Table III. Correlation between *PTEN* gene mutation and clinicopathological parameters in gastric cancer patients.

Clinicopathological parameters	Total cases	Cases of mutation	P-value
Differentiation extent			
Well and moderate differentiation	78	10	<0.05
Poor differentiation	55	13	
pTNM staging			
I, II	69	8	<0.005
III, IV	75	18	

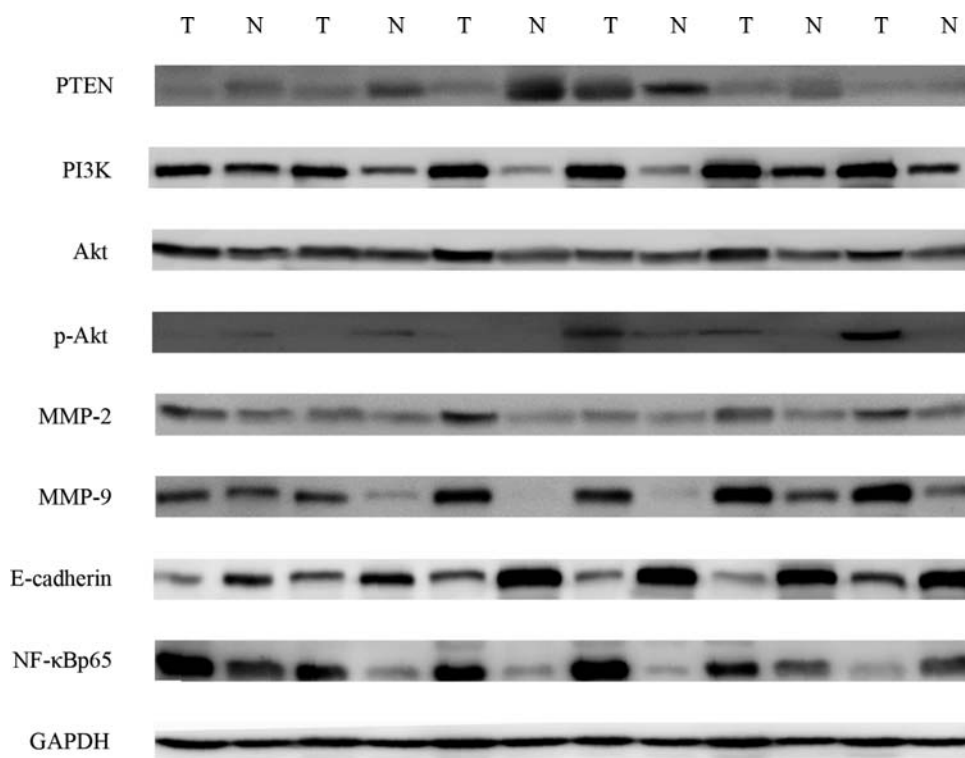


Figure 3. The protein expression of PTEN/PI3K/AKT pathway in gastric cancer detected by Western blot. With reduced expression of PTEN in gastric cancer, the overexpression of PI3K and AKT might inhibit E-cadherin expression, and promote expression of MMP-2, MMP-9 and NF-κBp65. T, tumor; N, normal mucosa.

to be at codon 341 (GCT→GCG) in two cases, resulting in nonsense mutations; at codon 380 (CAC→GGC) in two cases, leading to aspartic acid to glycine substitution; at codon 393 (GCA→ACA) in four cases, leading to alanine to threonine substitution. The mutation site in intron 6 was found at 7 bp upstream of 5' lateral exon 6 (T→G) in one case. The mutation sites in exon 3, 5, 6, 8 were not found. The *PTEN* gene mutation rate was significantly higher at pTMN stages III and IV than that at stages I and II ($P<0.005$), and it was higher in poorly differentiated gastric cancer than in well and moderately differentiated pathological types ($P<0.05$) (Table III).

Furthermore, Western blot detection showed a significantly reduced expression of PTEN and E-cadherin protein in the mutant gastric carcinoma tissues compared with normal mucosa tissues (Fig. 3). There were significant differences of

PTEN, PI3K and AKT proteins expressions between gastric carcinoma and normal gastric mucosa epithelium. A negative correlation was found between the expression of PTEN and PI3K or AKT proteins respectively, and there was a highly positive correlation between PI3K, AKT, MMP-2, MMP-9 and NF-κBp65 protein expression.

Discussion

A series of genetic alterations, including activation of proto-oncogenes and inactivation of tumor suppressor genes, are an important process in human carcinogenesis. At present, research on structure alteration of tumor suppressor genes in tumor tissues and cancer cell lines, including point mutation, deletion, insertion, and cut point, indicates that the mutation rate of tumor suppressor genes is 33-50% in endometrial

cancer, 25% in glioblastomas, 21% in ovarian cancer, 13% in prostate cancer, less than 5% in breast and thyroid cancer (9-17).

The *PTEN* gene was identified as a tumor suppressor gene, involved in cell survival, apoptosis prevention and growth stimulation. In addition, it regulates cell migration, modulates angiogenesis (7,8) and, more recently, has been reported to be essential for maintaining chromosomal integrity through a physical association to centromere proteins (24). In fact, mutations and deletions of this gene have been described in a wide range of human cancers such as endometrial carcinomas, glioblastomas, and prostate cancers (10,14,17). Germ-line mutations have also been identified in several rare autosomal dominant cancer predisposing conditions such as Cowden's disease and Bannayan-Zonana syndrome (25,26). Recent studies indicate that mutations of the *PTEN* gene seem to be infrequent in gastric cancer (27-30), but the sample size analyzed was small in these investigations.

The mutations of *PTEN* gene are correlated with tumor progression and poor outcome in breast cancer and melanoma (10,14). Therefore, the analysis of the *PTEN* gene mutations in gastric cancer might provide some information regarding tumor development and progression. In the current study, we examined the mutation status of the *PTEN* gene in gastric cancer in Shanghai, with a large number of samples, by direct sequencing all the 9 exons of *PTEN*, and found somatic mutations in 27 of 144 (18.75%) primary tumor specimens, of which the mutation frequency is lower than some published reports (27). The exact reason remains unclear. As our tumor samples included all the histological subtypes, an environmental carcinogen prevailing in the geographic region might be responsible. Kang *et al.* (31) pointed out that gastric carcinoma patients with silencing of the *PTEN* gene showed poor prognosis, and inactivation of the *PTEN* gene may play a vital role in the development and progression of gastric cancer. Also, the aberrant promoter methylation is suggested to be a major mechanism in the silencing of this gene (31,32). Moreover, we found that mutational patterns of the *PTEN* gene were scattered on exon 1-9, which is different from a former study resulting in clustering on exon 2-6 (27); with the mutation hot spots at codons 36, 75, 232 and 393, leading to missense mutations on codons 36, 75 and 393, and nonsense mutations on codon 232. Herein, mutations at codons 36, 75 and 393 might have a crucial role in the carcinogenesis or progression of gastric cancer. The result showed that there might be some unique characteristic of *PTEN* gene inactivation mechanism in the Shanghai population.

The status of *PTEN* gene mutation was significantly relevant to the pTNM staging and the degree of cell differentiation. *PTEN* mutation rate was significantly higher at pTMN stages III and IV than at stages I and II ($P < 0.005$). Furthermore, the mutation rate was higher in poorly differentiated gastric cancer than in well and moderately differentiated pathological types ($P < 0.05$). These results suggest that *PTEN* may play an important role in regulation of infiltration and metastasis of gastric cancer, and *PTEN* gene might be a prognostic biomarker of gastric cancer (5,33,34).

In addition, we validated the regulation pathway of *PTEN* gene by immunoblotting. The mutations of *PTEN* gene really

reduced *PTEN* protein expression in gastric cancer. In the development and progress of gastric carcinoma, PI3K and *PTEN* may counteract each other (19,30), and the loss of *PTEN* protein expression may be related with the overexpression of PI3K, AKT, MMP-2, MMP-9 and NF- κ Bp65 protein. PI3K and AKT protein may cooperate to promote malignant progress of gastric carcinoma. E-cadherin protein was down-regulated in tumor tissues, which was negatively related with PI3K and AKT. With reduced expression of *PTEN*, the overexpression of PI3K and AKT might inhibit E-cadherin expression, highly promote expression of MMP-2, MMP-9 and NF- κ Bp65, thereby participated in the progress, invasion and metastasis of gastric cancer. It was reported that inactivation of *PTEN* induces infiltration and metastasis of tumors (35-39).

We, therefore, suggest that in gastric cancers inactivation of the *PTEN* gene by mutation may contribute to its down-regulation besides hypermethylation of the *PTEN* promoter (7,31). Nevertheless, the actual role of *PTEN* expression in patients with gastric cancer needs further investigation for confirmation. In contrast to most identified mutations of *PTEN* within exons 5-9, however, the specific mutation spectrum from our analysis is located within exons 1, 2, 4, 7 and 9. Consequently, these controversial results concerning the mechanism of the *PTEN* gene alterations and need further investigation to elucidate the viewpoint. In addition, we also found a mutation in intron 6 in one of 144 gastric cancers, and whether this mutation in the intron affects transcriptional or post-transcriptional modulation has still to be investigated.

Collectively, these results indicated that the mutations of the *PTEN* gene did not occur at a significant rate in gastric cancer in Shanghai, but the mutation hot spots suggested *PTEN* might have an important role in the pathogenesis and development of the tumor. Inactivation of *PTEN* induces infiltration and metastasis of gastric cancer through the overexpression of PI3K, AKT, MMP-2, MMP-9 and NF- κ Bp65. Thus, clarifying the role of *PTEN* in the development and progression of gastric cancer will be helpful to comprehend the mechanism of gastric tumorigenesis. *PTEN* gene status might be considered as an indicator for the pathological behavior of gastric cancer.

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