

# Aberrant promoter 2 methylation-mediated downregulation of protein tyrosine phosphatase, non-receptor type 6, is associated with progression of esophageal squamous cell carcinoma

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**Abstract.** The human protein tyrosine phosphatase, non-receptor type 6 (PTPN6) gene is located on chromosome 12p13 and encodes an Mr 68,000 non-receptor type protein-tyrosine phosphatase. The PTPN6 gene has been considered as a candidate tumor suppressor in hematological and solid malignancies, and promoter methylation may be an epigenetic modification silencing its expression. However, the detailed role of PTPN6 and its promoter methylation status in the pathogenesis of esophageal squamous cell carcinoma (ESCC) has not been fully elucidated. The aim of the present study was to investigate PTPN6 expression in ESCC tissues and esophageal cancer cell lines, detect the effect of CpG hypermethylation on the activity of PTPN6, and additionally elucidate the role and prognostic significance of PTPN6 in ESCC tumorigenesis and progression. The expression of PTPN6 was identified to be significantly downregulated in esophageal cancer cell lines and ESCC tissues. Marked upregulation of PTPN6 was detected in 5-aza-2'-deoxycytidine-treated esophageal cancer cells, and frequent hypermethylation of the CpG sites within the P2 promoter (P2) was detected in ESCC tissues and esophageal cancer cell lines. The expression and methylation status of PTPN6 was associated with tumor node metastasis stage, pathological differentiation and lymph node metastasis in patients with ESCC. Aberrant hypermethylation of the P2 exhibited marked tumor specificity and was identified to be associated

with the expression level of PTPN6. Downregulation and hypermethylation of PTPN6 were identified to be associated with poor ESCC patient survival. Furthermore, upregulation of PTPN6 inhibited the proliferation and invasion of esophageal cancer cells *in vitro*. The results of the present study suggest that PTPN6 may serve as a tumor suppressor in ESCC, and it may serve as a potential target for antitumor therapy.

## Introduction

Esophageal cancer is a malignant tumor associated with poor prognosis and high mortality rates (1). The incidence of esophageal cancer exhibits marked geographic variation, appearing to be low in Western Africa, and high in Japan, Southeastern Africa and Northern China (2). Esophageal squamous cell carcinoma (ESCC) is the dominant histological type of esophageal cancer in China and it is associated with a particularly high morbidity in certain areas, including the south of Hebei in Northern China (3). The 5-year survival rate of patients with ESCC with advanced-stage or metastatic disease is <20% (4). Tobacco and alcohol use have been demonstrated to be independent risk factors for ESCC, but the exact pathogenetic mechanism remains to be elucidated. An improved understanding of the mechanisms underlying ESCC pathogenesis may identify promising molecular biomarkers for the early diagnosis and prevention of this malignancy.

Aberrant protein phosphorylation is a prerequisite for the occurrence and progression of several tumors, and it is one of the hallmarks of cancer cells (5). Protein kinases and protein phosphatases serve key roles in regulating cellular signal transduction. Protein phosphatases include protein serine/threonine phosphatases, protein tyrosine phosphatases (PTPs), tumor-suppressive metal-dependent protein phosphatases, tumor-suppressive phosphoprotein phosphatases, tumor-suppressive PTPs, receptor type and tumor-suppressive PTPs, non-receptor type (PTPN). Protein phosphatases were initially considered to be tumor suppressors, but several have been demonstrated to serve purely as oncogenes, whereas others may serve as tumor suppressors and oncogenes, according to the cellular environment or other unidentified factors (6). PTPNs are represented by 17 members and are

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**Abbreviations:** P2, promoter 2; ESCC, esophageal squamous cell carcinoma; PTPs, protein tyrosine phosphatases; PTPN6, protein tyrosine phosphatase, non-receptor type 6

**Key words:** protein tyrosine phosphatase, non-receptor type 6, esophageal squamous cell carcinoma, expression, methylation

absolutely specific to phospho-tyrosine. They consist of a highly conserved catalytic domain and variable regulatory domain arrays, acting by subcellular targeting or directly regulating phosphatase activity (7). A number of PTPNs serve as tumor suppressors by inhibiting Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling, receptor tyrosine kinase-mediated growth factor signaling, or cell motility/invasion. Furthermore, certain PTPNs promote tumor-suppressive Hippo signaling and inhibit tyrosine-protein kinase-ABL1-mediated transformation through Yes-associated protein 1 (8).

Protein tyrosine phosphatase, non-receptor type 6 (PTPN6) (also known as HCP, HCPH or SHP1), is encoded by 17 exons and has 2 Src homology 2 domains, is primarily expressed in hematopoietic cells, and serves as a key regulator of multiple signaling pathways in hematopoietic cells. PTPN6 has been demonstrated to interact with and dephosphorylate a variety of phospho-proteins involved in hematopoietic cell signaling pathways. Plutzky *et al* (9) first identified that PTPN6 is located in a chromosomal region that is frequently damaged in childhood leukemia. Oka *et al* (10) observed the loss of mRNA and protein expression of PTPN6 in natural killer T-cell lymphomas and 95% of several other types of malignant lymphomas, while only 60% of less malignant forms were negative. The loss of expression of PTPN6 is likely associated with malignant transformation and increased invasiveness. In subsequent studies, promoter hypermethylation of PTPN6 was identified in several hematological malignancies (11,12) and in solid tumors, including nasopharyngeal carcinoma (13) and breast ductal carcinoma (14). The PTPN6 gene has two promoter regions that are 7 kb apart, and has 3 different transcripts. The longer transcript, driven by the P1 promoter, is expressed primarily in non-hematopoietic cells, whereas the shorter transcript, driven by the P2 promoter (P2), is only expressed in cells of the hematopoietic lineage (15). PTPN6 expression driven by P1 in non-hematopoietic cells is low compared with the expression regulated by P2 in hematopoietic cells (16). It has been suggested that downregulation of PTPN6 is primarily due to DNA hypermethylation of CpG islands in the PTPN6 P2 (10,11). However, the mechanism and methylation status of PTPN6 in ESCC have not yet been fully elucidated. The aims of the present study were to investigate the expression of PTPN6 in ESCC tissues and esophageal cancer cell lines, elucidate the role of CpG hypermethylation in the inactivation of PTPN6, and improve the understanding of the functional and prognostic significance of PTPN6 in ESCC tumorigenesis and progression.

## Materials and methods

**Cell culture and treatment.** A total of 5 human esophageal cancer cell lines (Eca109, Kyse150, Kyse170, Yes-2 and TE1) and a human normal esophageal epithelial cell (HEEPiC) line were purchased from American Type Culture Collection (Manassas, VA, USA). All the cell lines were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>, and were assessed by reverse transcription-quantitative polymerase chain

reaction (RT-qPCR) analysis for mycoplasma contamination. All the cell lines were seeded prior to drug treatment. Cells (1.5x10<sup>5</sup>/ml) were treated with 5 μM DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-Aza-dC; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for the first 48 h and, subsequently, the medium containing 5-Aza-dC was changed every 24 h. Control cells were cultured in RPMI-1640 medium with no drug treatment.

**Patients and specimens.** A total of 71 primary ESCC samples and corresponding adjacent normal tissues were collected by surgical resection between January 2008 and January 2011 at the Department of Thoracic Surgery of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China). The study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University, and conformed to all relevant ethical regulations for human research subjects in accordance with Declaration of Helsinki. All the participants signed a written informed consent form. The patients comprised 51 males and 20 females, with a median age of 62 years (range, 39-78 years) (Table I). Freshly removed ESCC and paired adjacent non-cancerous esophageal tissues were divided into two groups, one of which was fixed in formalin at room temperature and embedded in paraffin, and the other was frozen and stored at -80°C for DNA and RNA isolation. Clinical data and clinicopathological characteristics were collected from medical records. The subjects were interviewed for information on demographic and exogenous risk factors, including smoking, alcohol consumption and family history.

**RT-qPCR analysis.** Total RNA was extracted from cell lines and frozen tumor tissues using TRIzol<sup>®</sup> reagent (Thermo Fisher Scientific, Inc.). The RT-for-PCR kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to synthesize single-stranded cDNA according to the protocol of the manufacturer. The mRNA expression levels were quantified using primers, cDNA template and Power SYBR-Green PCR Master Mix (Promega Corporation, Madison, WI, USA), according to the protocol of Power SYBR-Green PCR Master Mix. The primers used for PTPN6 are listed in Table II. The PCR cycle conditions were: 94°C for 30 sec, followed by 40 cycles of 94°C for 10 sec, 60°C for 30 sec and 72°C for 1 min. The data were analyzed by the 2<sup>-ΔΔC<sub>q</sub></sup> method (17) and the human GAPDH gene was used as an endogenous control.

**Western blot analysis of PTPN6 protein expression in ESCC cell lines.** Total protein from cultured cell lines was extracted using radioimmunoprecipitation assay reagent supplemented with protease inhibitors (Thermo Fisher Scientific, Inc.). The protein was quantified using BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China). Protein samples (20 μg/lane) were prepared for western blot analysis with 15% SDS-PAGE gels and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). To detect the expression of PTPN6, the membranes were incubated at 4°C overnight with the specific primary antibodies (1:1,000; rabbit anti-human monoclonal antibody; cat. no. ab32559; Abcam, Cambridge, UK). Subsequently, the secondary antibody (goat anti-rabbit IgG-HRP; 1:2,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) incubated with

Table I. Clinicopathologic characteristics of esophageal squamous cell carcinoma cases.

Group	N (%)
Age, years	
<62	30 (42.3)
≥62	41 (57.7)
Sex	
Male	51 (71.8)
Female	20 (28.2)
TNM stage	
I	4 (5.6)
II	24 (33.8)
III	38 (53.5)
IV	5 (7.0)
Pathological differentiation	
Well	7 (9.9)
Moderate	30 (42.3)
Poor	34 (47.9)
Depth of invasion	
T1/2	28 (39.4)
T3/4	43 (60.6)
LN metastasis	
Negative (N0)	22 (31.0)
Positive (N1/2/3)	49 (69.0)
Family history of UGIC	
Negative	47 (66.2)
Positive	24 (33.8)

TNM, tumor-node-metastasis; LN, lymph node; UGIC, upper gastrointestinal cancer.

the membrane at room temperature for 1 h. To ensure equal loading in all the lanes, anti-β actin (1:1,000; cat. no. ab119716; Abcam) was used as the control. Consequently, the protein bands were analyzed by the Image Lab software version 4.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Immunohistochemical staining for the PTPN6 protein in ESCC tissues.** PTPN6 protein expression was determined by immunostaining using the streptavidin-peroxidase method in tumor samples and corresponding adjacent normal sections. Specimens were embedded in paraffin and cut into 4-μm sections. Then descending alcohol series were used to deparaffinization and rehydration were used by descending alcohol series. Antigen retrieval was performed in a pressure cooker at 100°C for 5 min in Tris-EDTA buffer (pH 9.0). Rabbit anti-human monoclonal antibody for PTPN6 (1:100 dilution; cat. no., ab32559; Abcam) was applied to investigate the protein expression of PTPN6 at 4°C overnight. Following an overnight incubation, specimens were subjected to the streptavidin-peroxidase (SP) method using a standard SP kit (cat. no. PV-9001; OriGene Technologies, Inc., Beijing, China) according to the manufacturer's protocol. PBS (pH 9.0) was

used as negative control of the primary antibody. The slides were examined using a light microscope (Olympus BX41; Olympus Corporation, Tokyo, Japan; magnification, x200 and x400) and scored by experienced pathologists in a double-blinded manner.

**DNA extraction and sodium bisulfite treatment.** Genomic DNA was isolated from esophageal cancer cell lines, frozen ESCC tumor samples and corresponding normal tissues using a DNA extraction kit (Shanghai Generay Biotech Co. Ltd., Shanghai, China). To assess the DNA methylation patterns, DNA was bisulfite-modified using an Epitect Fast Bisulfite Conversion kit (Qiagen GmbH, Hilden, Germany), which converts unmethylated cytosine residues to thymine, whereas methylated cytosine residues remain unaffected.

**Methylated CpG site distribution via bisulfite genomic sequencing (BGS) assay.** To analyze the DNA methylation pattern of the PTPN6 P2, a BGS assay was used to detect the methylated CpG site distribution in the esophageal cancer cell lines. Subsequently, the online MethPrimer program was used to detect the distribution of CpG islands (URL: <http://www.urogene.org/methprimer/>). A pair of primers (from-167 to-326 bp) was designed by Sangon Biotech Co., Ltd. (Shanghai, China) to recognize sodium bisulfite-converted genomic DNA. The primer sequence for BGS: Sense 5'-AGGGTTGTGGTGAGAAATTAATTAG-3', and antisense 5'-TTACACACTCCAAACCCAAATAATAC-3'. The PCR products were purified using the QIAEXII Gel Extraction kit (Qiagen GmbH) and cloned into pGEM-T vectors (Promega Corporation). Up to 10 clones for each specimen were analyzed by bisulfite sequencing.

**Methylation analysis of PTPN6 via bisulfite conversion-specific and methylation-specific polymerase chain reaction (BS-MSP) assay.** The PTPN6 P2 was analyzed by the BS-MSP method as described above using bisulfite-treated genomic DNA. According to the distribution of the primary methylated CpG sites by the BGS assay, the MSP primers were designed by Sangon Biotech Co., Ltd., and the reaction conditions were summarized in Table II. According to the manufacturer's recommendations, genomic DNA methylated *in vitro* by CpG methyltransferase (Sss I) (New England BioLabs, Inc., Ipswich, MA, USA) and water blanks were applied as positive and negative controls, respectively. The BS-MSP products were analyzed on 2% agarose gel with ethidium bromide staining. All reactions were performed in duplicate for each of the samples.

**Cell transfection.** To determine the overexpression of PTPN6, Eca109 and Yes-2 cells in the logarithmic growth phase were cultured in 6-well plates. When the density of Eca109 and Yes-2 cells reached to 80%, the cells were transfected with PTPN6 expression plasmid (pcDNA3.1-PTPN6) or the empty vector (pcDNA3.1-EV) (Sangon Biotech Co., Ltd.) as control at a final concentration of 2.5 μg/μl using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following transfection, the cells were incubated in RPMI-1640 medium for 4-6 h, followed by replacement with RPMI-1640

Table II. Primer sequences and reaction conditions of PTPN6 used.

PCR types	Gene	Primer sequence	Annealing temperature, °C	Product size, bp
RT-qPCR	PTPN6	F: 5'-GGCCTGGACTGTGACATTGA-3' R: 5'-ATGTTCCCGTACTCCGACTC-3'	56	188
	GAPDH	F: 5'-AGGTGAAGGTCGGAGTCAACG-3' R: 5'-AGGGGTCATTGATGGCAACA-3'	56	104
BGS	PTPN6	F: 5'-AGGGTTGTGGTGAGAAATTAATTAG-3' R: 5'-TTACACACTCCAAACCCAAATAATAC-3'	58	222
BS-MSP	Methylation	F: 5'-GAACGTTATTATAGTATAGCGTTC-3' R: 5'-TCACGCATACGAACCCAAACG-3'	60	158
	Unmethylation	F: 5'-GTGAATGTTATTATAGTATAGTGTGG-3' R: 5'-TTCACACATACAAACCCAAACAAT-3'	59	158

RT-qPCR, reverse transcription polymerase chain reaction; BGS, bisulfite genomic sequencing; BS-MSP, bisulfite conversion-specific and methylation-specific polymerase chain reaction; PTPN6, protein tyrosine phosphatase, non-receptor type 6.

supplemented with 10% FBS. After 24 h, the transfected cells were extracted for subsequent experimentation.

**Cell proliferation.** The cells ( $1.0 \times 10^5$ ) were seeded into 96-well plates for the cell proliferation assays. The proliferation of Eca109 and Yes-2 cells transfected with PTPN6 was determined by MTS assay. The absorbance was measured at a wavelength of 492 nm, followed by incubation for 4 h in a humidified incubator containing 5% CO<sub>2</sub> at 37°C. The proliferation rates were determined at 0, 24, 48, 72 and 96 h after transfection. All the experiments were performed in triplicate.

**Colony formation assay.** For the colony formation assay, 2,500 cells were seeded in 6-well plates and incubated with RPMI-1640 medium containing 10% FBS for 1 week. Colonies (>50 cells) were fixed in methanol for 15 min (at room temperature) and dyed with 0.5% crystal violet solution for 20 min, and the colony number was counted under an inverted microscope (DWI40CCB; Leica, Wetzlar, Germany; magnification, x100).

**Wound healing assay.** Cells ( $5.0 \times 10^5$ ) in the logarithmic growth phase were inoculated in 6-well plates. Following transfection for 24 h, scratch wounds were created using a 200  $\mu$ l pipette tip. The detached cells were removed by washing with PBS 3 times. RPMI-1640 medium was then added to the plates and images were observed after culture for 0, 12 and 24 h. The inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany; magnification, x100) was applied to measure the relative migration distance.

**Cell invasion assay.** The invasion of PTPN6-transfected Eca109 and Yes-2 cells was measured in 24-well Transwell chambers (Corning Incorporated, Corning, NY, USA). The Transwell chambers were coated with 20  $\mu$ l Matrigel at 4°C and incubated at 37°C for 4 h. After 24 h transfection, 5,000 cells/well were seeded in the upper chambers, and the lower chambers were filled with RPMI-1640 medium

supplemented with 10% FBS. Following incubation at 37°C for 24 h, invading cells located in the lower chamber were fixed in 4% paraformaldehyde at room temperature for 15 min and stained with 0.1% crystal violet at room temperature for 30 min. The number of cells that had invaded through the membrane to the lower surface was observed in 5 microscopic fields per filter under the inverted microscope (Leica Microsystems GmbH; magnification, x100). The experiments were performed in triplicate.

**Statistical analysis.** Statistical analysis was performed with SPSS 22.0 software package (IBM Corp., Armonk, NY, USA). The RT-qPCR results are presented as the mean  $\pm$  standard deviation. Student's t-test was applied to compare the expression means between different continuous variables. Pearson's  $\chi^2$  test was applied to assess the status of gene methylation. For prognostic analysis of PTPN6 protein expression and methylation, survival curves were constructed using the Kaplan-Meier method and the log-rank or the Breslow tests. One-way analysis of variance was adopted to measure the comparison of multiple groups (the function of PTPN6 in esophageal cancer cell lines), and within-group variations were performed by Student Newman-Keuls test. All statistical tests were two-sided and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**mRNA and protein expression of PTPN6 is decreased in esophageal cancer cell lines and ESCC tissues.** The mRNA expression of PTPN6 was first detected in 5 esophageal cancer cell lines and normal human esophageal epithelial cells. As demonstrated in Fig. 1A, the mRNA expression of PTPN6 was markedly decreased in the ESCC TE1, Eca109, Kyse150, Kyse170 and Yes-2 cell lines compared with that in the normal esophageal epithelial HEEpiC cells. This result was additionally confirmed by the results of western blot analysis (Fig. 1B).



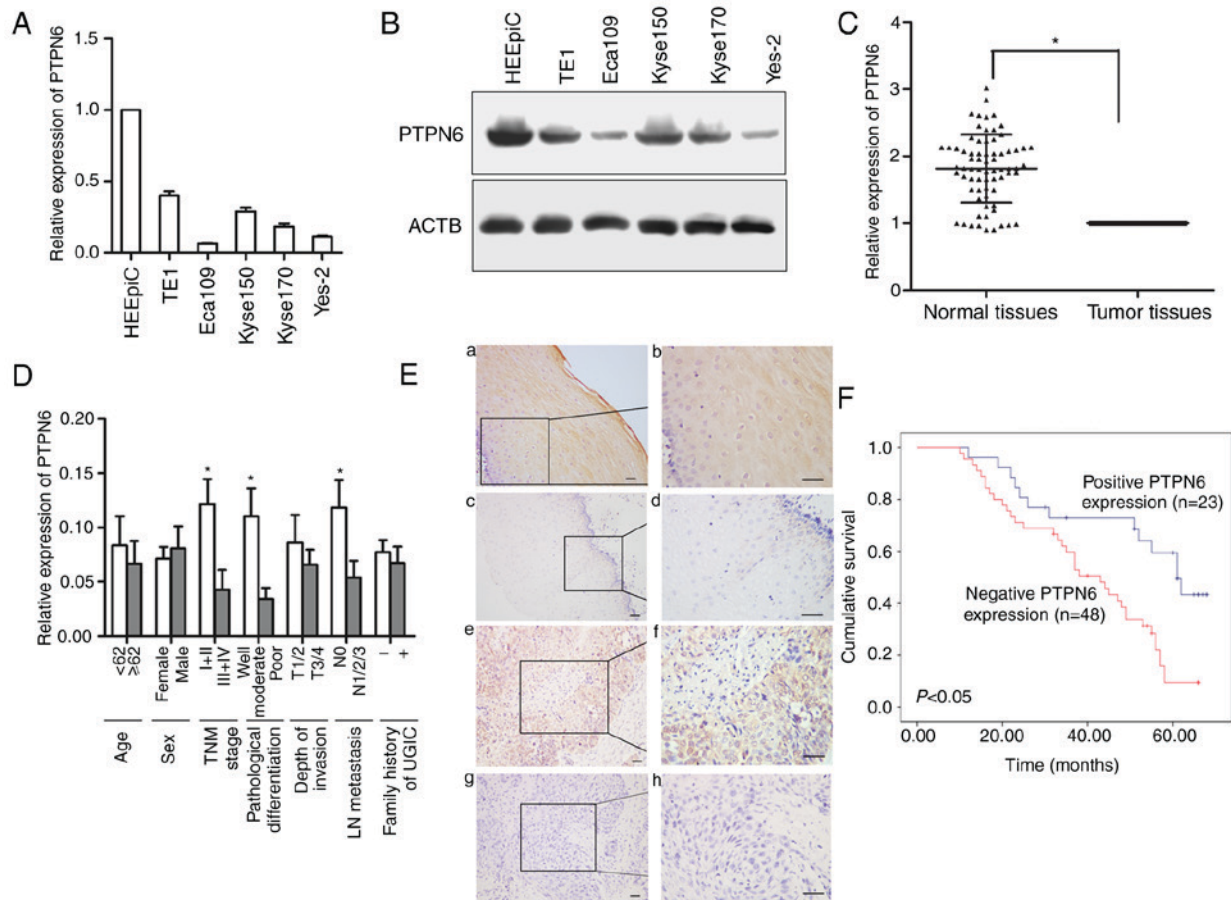


Figure 1. Expression of PTPN6 in human esophageal cancer cell lines and ESCC tissues. (A) Relative mRNA expression of PTPN6 in 5 esophageal cancer cell lines and human normal esophageal epithelial cells detected by reverse transcription-quantitative polymerase chain reaction analysis. (B) Protein expression of PTPN6 in esophageal cancer cell lines and human normal esophageal epithelial cells detected by western blot analysis. (C) Relative mRNA expression of PTPN6 in normal tissues and corresponding ESCC tumor tissues; \* $P<0.05$ . (D) Relative mRNA expression of PTPN6 in different subgroups. \* $P<0.05$  vs. TNM: I+II stage vs. III+IV stage; Well + moderate differentiation vs. poor differentiation; Positive lymph node metastasis vs. negative lymph node metastasis. (E) Streptavidin-peroxidase immunohistochemical staining of PTPN6 in ESCC tumor tissues and corresponding normal tissues (SP9001): (a and b) Positive staining of PTPN6 in normal tissues at (a) magnification, x200 and (b) magnification, x400; (c and d) negative staining of PTPN6 in normal tissues at (c) magnification, x200 and (d) magnification, x400; (e and f) positive staining of PTPN6 in ESCC tissues at (e) magnification, x200 and (f) magnification, x400; (g and h) negative staining of PTPN6 in ESCC tissues at (g) magnification, x200 and (h) magnification, x400. (F) Direct association between negative PTPN6 protein expression and poor patient survival. PTPN6, protein tyrosine phosphatase, non-receptor type 6; ESCC, esophageal squamous cell carcinoma; TNM, tumor node metastasis; LN, lymph node; UGIC, upper gastrointestinal cancer.

The mRNA expression of PTPN6 in ESCC tumor tissues was significantly decreased compared with that in the corresponding normal tissues ( $P<0.01$ ; Fig. 1C). PTPN6 mRNA expression was associated with Tumor Node Metastasis stage (18), pathological differentiation and lymph node (LN) metastasis (Fig. 1D). Immunohistochemical staining was used to assess the protein expression of PTPN6 in tumor tissues and corresponding normal tissues. Protein expression of PTPN6 was detected primarily in the cytoplasm and the nucleus of tumor or normal cells (Fig. 1E). The protein expression of PTPN6 in tumor tissues (32.4%, 23/71) was markedly decreased compared with that in corresponding normal tissues (77.5%, 55/71;  $P<0.01$ ; Table III). When stratified for clinicopathological characteristics, PTPN6 protein expression was identified to be significantly associated with tumor-node-metastasis (TNM) stage, pathological differentiation and LN metastasis ( $P<0.05$ ; Table IV).

**Downregulation of PTPN6 is associated with poor ESCC patient survival.** The 5-year survival rate in the positive and negative PTPN6 expression ESCC groups was 47.8 and 20.8%,

respectively ( $P<0.05$ ; log-rank test). As presented in Fig. 1F, patients with ESCC negative for protein expression of PTPN6 exhibited poor survival.

**Upregulation of PTPN6 by 5-Aza-dC treatment in esophageal cancer cell lines.** As demonstrated in Fig. 2A, the online MethPrimer program was used to detect the distribution of CpG islands in the PTPN6 promoter region and genomic sequence. A total of 1 CpG island was identified to be located in the promoter region. The Eca109, Kyse170 and Yes-2 cell lines, which exhibited a relatively low PTPN6 expression, were subsequently treated with 5-Aza-dC. As indicated in Fig. 2B, the mRNA expression level of PTPN6 was markedly upregulated in these 3 esophageal cancer cell lines following treatment with 5-Aza-dC, suggesting that aberrant methylation may be one of the mechanisms leading to PTPN6 silencing in esophageal cancer cell lines.

**Methylation analysis of PTPN6 in esophageal cancer cell lines and tumor tissues.** The methylation status of the CpG

Table III. Protein expression and methylation status of PTPN6 in ESCC tumor tissues and corresponding normal tissues.

Group	N	Protein expression		Methylation frequency	
		n (%)	P-value	n (%)	P-value
Normal tissues	71	55 (77.5)	<0.001	12 (16.9)	<0.001
Tumor tissues	71	23 (32.4)		45 (63.4)	

PTPN6, protein tyrosine phosphatase, non-receptor type 6; ESCC, esophageal squamous cell carcinoma.

Table IV. Immunohistochemical staining characteristics and methylation status of PTPN6 in ESCC tissues.

Group	N	Protein expression		Methylation frequency	
		n (%)	P-value	n (%)	P-value
Age, years					
<62	30	12 (40.0)	0.241	17 (56.7)	0.315
≥62	41	11 (26.8)		28 (68.3)	
Sex					
Male	51	15 (29.4)	0.391	35 (68.6)	0.143
Female	20	8 (40.0)		10 (50.0)	
TNM stage					
I+II	28	16 (57.1)	<0.001	12 (42.9)	0.004
III+IV	43	7 (16.3)		33 (76.7)	
Pathological differentiation					
Well/moderate	37	21 (56.7)	<0.001	18 (48.6)	0.007
Poor	34	2 (5.9)		27 (79.4)	
Depth of invasion					
T1/2	28	11 (39.3)	0.317	14 (50.0)	0.059
T3/4	43	12 (27.9)		31 (72.1)	
LN metastasis					
Negative (N0)	22	11 (50.0)	0.034	9 (40.9)	0.008
Positive (N1/2/3)	49	12 (24.5)		36 (61.0)	
Family history of UGIC					
Negative	47	17 (36.2)	0.341	30 (63.8)	0.912
Positive	24	6 (25.0)		15 (62.5)	

PTPN6, protein tyrosine phosphatase, non-receptor type 6; ESCC, esophageal squamous cell carcinoma; TNM, tumor-node-metastasis; LN, lymph node; UGIC, upper gastrointestinal cancer.

Table V. Association between PTPN6 protein expression and methylation status in patients with esophageal squamous cell carcinoma.

Group	Protein expression		P-value
	+	-	
Methylation status			<0.001
M	6	39	
U	17	9	

PTPN6, protein tyrosine phosphatase, non-receptor type 6; M methylated, U unmethylated.

sites in the promoter region of PTPN6 was first verified by BGS assay in esophageal cancer cell lines, and frequent hypermethylation of the CpG sites in the promoter region of PTPN6 was detected in Eca109, Kyse170 and Yes-2 cells (Fig. 2C). In particular, fully methylated PTPN6 in the Eca109 and Yes-2 cell lines was detected by the BS-MSP assay (Fig. 2D). Following treatment with 5-Aza-dC, the aberrant methylation status of the cells was reversed in the 3 cell lines. The frequency of PTPN6 methylation in ESCC tumor tissues (63.4%, 45/71) was significantly higher compared with that in corresponding normal tissues (16.9%, 12/71;  $P<0.05$ ; Table III and Fig. 2D). When stratified for clinicopathological characteristics, the methylation frequency of PTPN6 was associated with TNM stage, pathological differentiation and LN metastasis ( $P<0.05$ ).

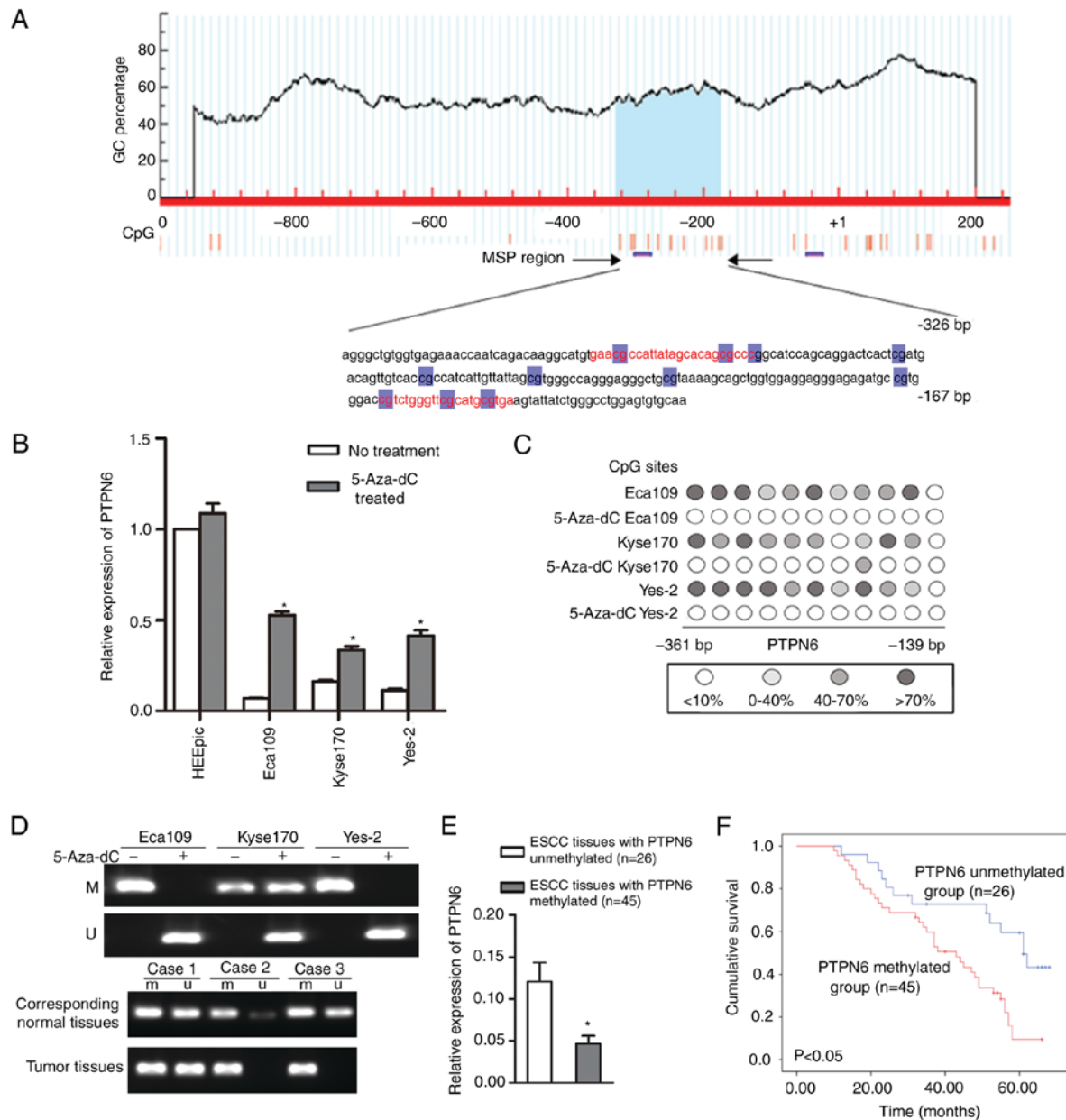


Figure 2. Methylation analysis of PTPN6 in esophageal cancer cell lines and ESCC tissues. (A) CpG islands are included and the MSP region analyzed is indicated. (B) Relative mRNA expression of PTPN6 in 3 esophageal cancer cell lines and human normal esophageal epithelial cells treated or not treated with 5-Aza-dC. \*P<0.05 vs. untreated cells. (C) Methylation status of each CpG site in the PTPN6 promoter region detected by BGS in 3 ESCC cell lines. Each CpG site is presented at the top row as an individual number. Percentage methylation was determined as percentage of methylated cytosines from 10 sequenced colonies. The color of circles for each CpG site represents the percentage of methylation. (D) Methylation status of PTPN6 detected by bisulfite conversion-specific-MSP analysis in various cancer cell lines and ESCC tissues. (E) Relative mRNA expression of PTPN6 in the tumor tissues with or without methylation; \*P<0.05. (F) A direct association between PTPN6 methylation and poor patient survival was demonstrated. PTPN6, protein tyrosine phosphatase, non-receptor type 6; BGS, bisulfite genomic sequencing; ESCC, esophageal squamous cell carcinoma; 5-Aza-dC, 5-aza-2'-deoxycytidine; MSP region, methylation specific region; M, methylated; U, unmethylated.

However, the methylation status of PTPN6 in ESCC tumor tissues was not associated with age or sex (P>0.05; Table IV).

**Association between PTPN6 expression and methylation status.** As demonstrated in Fig. 2E, the mRNA expression level of PTPN6 in ESCC tissues with PTPN6 methylation was significantly decreased compared with that in ESCC tissues with unmethylated PTPN6 (P<0.05). Similarly, the protein expression of PTPN6 in ESCC tissues with PTPN6 methylation was significantly decreased compared with that in ESCC tissues with unmethylated PTPN6 (P<0.05; Table V).

**Promoter hypermethylation of PTPN6 is associated with poor ESCC patient survival.** As demonstrated in Fig. 2F, PTPN6 methylation was identified to be negatively associated with ESCC patient survival. In patients with ESCC with hypermethylation of PTPN6, the 5-year survival rate was 17.8% compared with 50.0% in patients with ESCC with unmethylated PTPN6 (P<0.05; log-rank test).

**Upregulation of PTPN6 inhibits esophageal cancer cell proliferation and invasion in vitro.** The function of PTPN6 was then investigated in esophageal cancer cell lines. The

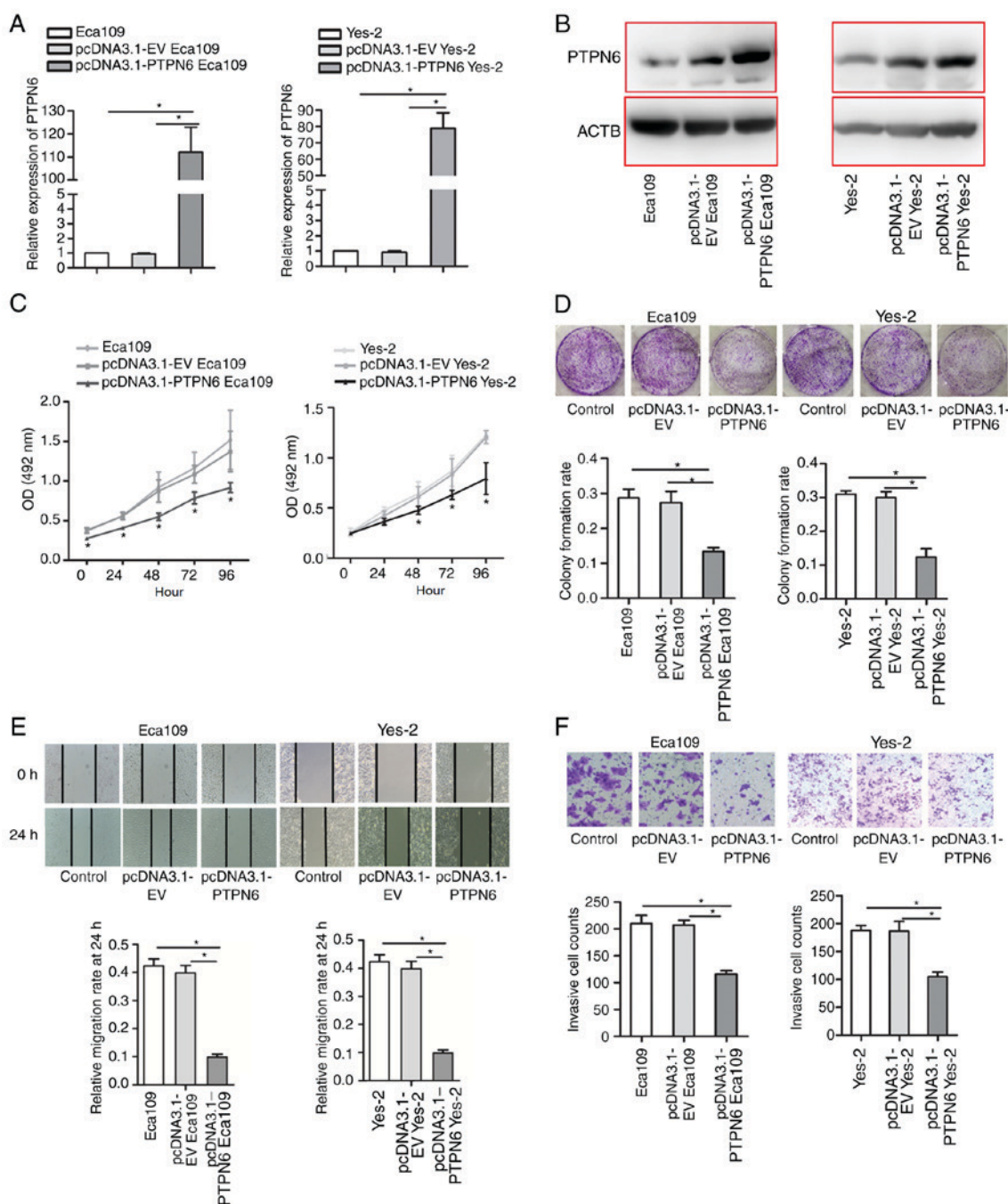


Figure 3. Functional analysis of PTPN6 in human esophageal cancer cell lines. (A) Overexpression of PTPN6 was detected by reverse transcription-quantitative polymerase chain reaction in PTPN6-transfected Eca109 and Yes-2 cells compared with empty vector-transfected cells.  $^*P < 0.05$ . (B) Overexpression of PTPN6 was detected by western blot analysis in PTPN6-transfected Eca109 and Yes-2 cells compared with empty vector-transfected cells. (C) Overexpression of PTPN6 inhibited Eca109 and Yes-2 cell proliferation, as detected by the MTS assay;  $^*P < 0.05$  vs. empty vector. (D) Colony formation assay of PTPN6 cells transiently overexpressing PTPN6.  $^*P < 0.05$ . (E) Upregulation of PTPN6 inhibited Eca109 and Yes-2 cell migration, as detected by the wound healing assay.  $^*P < 0.05$ . Magnification,  $\times 200$ . (F) Overexpression of PTPN6 inhibited Eca109 and Yes-2 cell invasiveness, as detected by the Transwell invasion assay. The results were determined by counting the cells that had penetrated through the Matrigel-coated Transwell chambers ( $8\text{-}\mu\text{m}$  pore size).  $^*P < 0.05$  vs. EV. Magnification,  $\times 100$ . PTPN6, protein tyrosine phosphatase, non-receptor type 6; ACTB,  $\beta$ -actin; OD, optical density; EV, empty vector.

construct containing PTPN6 transcripts (pcDNA3.1-PTPN6) was transfected into Eca109 and Yes-2 cells. As indicated in Fig. 3A and B, significant upregulation of PTPN6 was detected in pcDNA3.1-PTPN6-transfected Eca109 and Yes-2 cells. Transfection of PTPN6 led to a marked inhibition of Eca109 and Yes-2 cell proliferation, as detected by the MTS assay (Fig. 3C). The results were additionally verified with the colony formation assay (Fig. 3D). Furthermore,

the wound healing assay was performed, starting 24 h after pcDNA3.1-PTPN6 transfection. Overexpression of PTPN6 effectively decreased the area of the scratch covered (Fig. 3E). Similarly, the Transwell assay confirmed a decrease in the migration ability of pcDNA3.1-PTPN6-transfected cells (Fig. 3F). These results indicate that PTPN6 inhibited the proliferation, migration and invasion of Eca109 and Yes-2 cells *in vitro*.



## Discussion

The PTPN6 gene is located on human chromosome 12p13 and encodes a Mr 68,000 non-receptor type protein-tyrosine phosphatase. The PTPN6 gene has been considered as a candidate tumor suppressor in hematological and solid malignancies, and promoter methylation may be an important epigenetic mechanism involved in silencing its expression. However, the detailed roles of PTPN6 and its promoter methylation status in the pathogenesis of primary ESCC remain elusive. In the present study, significant downregulation of PTPN6 and frequent hypermethylation of the CpG sites within the P2 were detected in esophageal cancer cell lines and ESCC tissues. The mRNA expression level of PTPN6 was significantly upregulated in 5-Aza-dC-treated esophageal cancer cells. In addition, the methylation status and expression of PTPN6 were associated with TNM stage, pathological differentiation and LN metastasis in patients with ESCC. Additional study verified that aberrant hypermethylation of the P2 exhibited higher tumor specificity and was associated with the expression level of PTPN6. Survival analysis demonstrated that downregulation and hypermethylation of PTPN6 were associated with poor ESCC patient survival. Furthermore, upregulation of PTPN6 inhibited the proliferation and invasion of esophageal cancer cells *in vitro*.

Genomic DNA methylation is an important epigenetic event in humans, and the alterations of methylation patterns may serve important roles in tumorigenesis (19-21). As aberrant DNA methylation is one of the earliest molecular changes during the transformation process from normal to cancerous cells (22), detection of an aberrant DNA methylation pattern may have potential applications in the early detection of malignancies. Transcriptional silencing of PTPN6 due to promoter hypermethylation has been previously demonstrated in several hematopoietic cell lines, leukemia and lymphoma (10,23,24). A high frequency of promoter hypermethylation was also observed in endometrial carcinoma, and was identified to be associated with patient age and tumor differentiation. PTPN6 promoters were completely methylated in endometrial carcinoma cell lines, and this methylation status was reversed by 5-Aza-dC treatment (25). In the present study, downregulation of PTPN6 was detected in esophageal cancer cell lines and ESCC tissues, and P2 hypermethylation may be one of the important epigenetic mechanisms silencing this gene in ESCC. Furthermore, the expression and methylation status of PTPN6 were associated with TNM stage, pathological differentiation and LN metastasis in patients with ESCC, and were associated with patient survival, indicating that detection of the P2 methylation status may be a promising biomarker for predicting the prognosis of ESCC.

It has been suggested that epigenetic silencing of PTPN6 in myeloproliferative neoplasms and K562 cells causes constitutive activation of JAK/STAT signaling (26). The reversal of PTPN6 expression by 5-Aza-dC treatment caused decreased expression levels of p-STAT3, p-JAK3 and JAK3, but not of the STAT3 protein (27). The JAK/STAT signaling pathway is one of the most important signaling cascades that regulate immune response, cell growth, differentiation and other cellular biological activities (28). The silence of PTPN6 may result in JAK or STAT activation in cancer cells (11,24). However, the role of PTPN6 in ESCC has not been fully elucidated. In the

present study, it was confirmed that upregulation of PTPN6 inhibited the proliferation and invasion of esophageal cancer cells *in vitro*, indicating that PTPN6 may serve as a tumor suppressor gene by inhibiting the proliferation and invasion of cancer cells. However, the specific regulated pathway of PTPN6 in ESCC requires additional investigation.

In summary, PTPN6 may serve as tumor suppressor gene in ESCC and inhibit esophageal cancer cell proliferation and invasion. The P2 is frequently methylated in esophageal cancer cells and ESCC tissues, and this may be one of the epigenetic mechanisms implicated in PTPN6 silencing in ESCC. Furthermore, PTPN6 may serve as a potential prognostic marker for predicting survival in patients with ESCC.

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## Availability of data and materials

All data generated and analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

LL conducted the analyses, participated in the overall conceptualization of the study, wrote the final manuscript and performed the computational analyses. JL conceptualized and supervised the study. SZ and XL participated in the analysis of results. All authors have read and approved the final version of this manuscript.

## Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Fourth Hospital of Hebei Medical University, and conformed to all relevant ethical regulations for human research subjects. All the participants signed a written informed consent form.

## Patient consent for publication

All the participants signed a written informed consent form.

## Competing interests

The authors declare that they have no competing interests.

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