Epigenetically silenced PTPRO functions as a prognostic marker and tumor suppressor in human lung squamous cell carcinoma

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Abstract. Protein tyrosine phosphatase receptor-type O (PTPRO), a member of the PTP family, has been frequently reported as potential tumor suppressor in many types of cancer. However, the exact function of *PTPRO* in lung squamous cell carcinoma (LSCC) remains unclear. Bisulfite sequencing and methylation specific polymerase chain reaction (PCR) were used to identify the methylation status of *PTPRO* in LSCC cells, and quantitative methylation specific PCR was used to evaluate the methylation levels of *PTPRO* in LSCC patients. Stably expressing *PTPRO* vectors were constructed and transfected into H520 and SK-MES-1 cells, followed by MTT and colony formation assays, and analysis of tumor weight and volume in *in vivo* mouse xenograft models. The present study demonstrated that the CpG island of *PTPRO* exon 1 was obviously hypermethylated in LSCC cells and tissues. The mRNA expression of *PTPRO* could be restored by treatment with a demethylation agent. Increased methylation and decreased mRNA levels of *PTPRO* were observed in LSCC samples compared with adjacent healthy tissues, and were associated with poor prognosis of patients. The mRNA expression of *PTPRO* was negatively correlated with its methylation level in tumors. Functionally, ectopic *PTPRO* expression in LSCC cells significantly inhibited the proliferation rates, and colony formation, in comparison with control and non-transfected cells. *In vivo* assays confirmed the inhibitory effect of *PTPRO* on LSCC cell growth. In conclusion, these data provided evidence that epigenetic regulation of PTPRO impairs its tumor suppressor role in LSCC, and restoration of *PTPRO* may be a potential therapeutic strategy.

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Introduction

Lung cancer is the most common malignancy and the leading cause of cancer-associated mortality worldwide, primarily in the form of squamous cell carcinoma (1,2). Many cases are diagnosed at an advanced stage, which may be responsible for the poor prognosis in lung cancer patients. Thus, determining novel and effective therapeutic targets is indispensable for lung squamous cell carcinoma (LSCC) treatment, and may provide novel insights into carcinogenesis.

Carcinogenesis is a multistep progress. Tumor-suppressor genes or oncogenes serve critical roles in regulating tumor-associated biological process, including proliferation, apoptosis, migration and invasion (3-5). Previous studies have identified many classic tumor suppressor genes, including reversion-inducing-cysteine-rich protein with kazal motifs *(RECK)*, breast cancer 1 *(BRCA1)* and Ras association domain family 1 isoform A *(RASSF1A)* (6-8). These genes were hypermethylated in various types of tumors and were involved in tumor-associated biological processes. For example, promoter methylation of *RECK* contributes to metastasis of osteosarcoma (9). *BRCA1* negatively mediates cell proliferation, and its mRNA levels were downregulated by methylation (10). The epigenetically inactivated *RASSF1A* gene was associated with poor prognosis and advanced tumor stage (11). Therefore, revealing the potential underlying mechanism and function of deregulated tumor-associated genes may have great benefit for the understanding of carcinogenesis. Protein tyrosine phosphatase receptor-type O (PTPRO) is a candidate tumor suppressor belonging to the protein tyrosine phosphatase (PTP) family, and is highly conserved in different species (12). Previous studies have reported that DNA methylation is involved in the regulation of *PTPRO* in hepatocellular carcinomas (13), lung cancer (14), chronic lymphocytic leukemia (15), esophageal squamous cell carcinoma (16) and colorectal cancer (17). Overexpression of PTPRO inhibits cell proliferation and promotes apoptosis in hepatocellular carcinoma and lymphoma (18,19), while downregulation of *PTPRO* is associated with metastases in breast cancer (20). *PTPRO* regulates mammary epithelial transformation via directly targeting the receptor tyrosine kinase ErbB2/human epidermal growth factor receptor 2 (21). Although these studies suggested the candidate tumor suppressor role of *PTPRO*, the expression and biological function of *PTPRO* in LSCC remains to be fully elucidated.

The present study assessed the methylation and expression of *PTPRO* in LSCC cells and tissues, and the effect of over-expression of *PTPRO* on tumor growth. The CpG island of *PTPRO* exon 1 was hypermethylated in H520 and SK-MES-1 cells. In LSCC patients, the significantly higher methylation levels of *PPTPRO* was correlated with its decreased mRNA levels. Furthermore, upregulation of *PTPRO* significantly inhibited cell proliferation and colony formation *in vitro*, and the tumorigenicity of H520 cells *in vivo*. These data suggested that epigenetic regulation of *PTPRO* expression is likely to be involved in the progression of LSCC.

Materials and methods

Tissue samples. Primary tumors and corresponding adjacent healthy tissues were obtained from 65 patients, including 40 men and 25 women, with a mean age 61.7 years, who were diagnosed in Department of Thoracic Surgery, Hubei Cancer Hospital (Wuhan, China) between March 2010 and July 2011. All the tumors used in this study were squamous cell carcinoma, and tumor stages were confirmed by pathologists according to the criterion of Union for International Cancer Control. The clinical characteristics were obtained from medical records. This study was approved by the ethical committees of Hubei Cancer Hospital and written informed consent was obtained prior to surgery. All tissue specimens were surgically resected and immediately flash-frozen in liquid nitrogen, and stored at -80˚C.

Cell lines and cDNA transfection. The H520 and SK-MES-1 LSCC cell lines and the BEAS-2B healthy human bronchial epithelial cell line were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in the conditions as recommended (22). The cells were maintained in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum and 100 U/ml penicillin sodium at 37˚C in an humidified atmosphere of 5% CO_2. To construct a vector stably expressing *PTPRO*, a pcDNA 3.1-Hemagglutinin A (HA)-tagged vector (Invitrogen; Thermo Fisher Scientific, Inc.) was purchased and used in this study. The cDNA encoding the complete coding region of human *PTPRO* cDNA was obtained from GeneBank (NM_030667.2). The HA label was introduced to protein C in the vector, and the *E.coli* strain of DH5a was also preserved in a laboratory at Hubei Cancer Hospital. A pcDNA-PTPRO-HA expression vector was established using a traditional method (23).

DNA extraction and methylation analysis. Total amounts of DNA (2 μg) were extracted from cells and tissues using a DNeasy Blood & Tissue kit (Qiagen GmbH, Germany) according to manufacturer's protocol. The quantity of DNA was tested by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.), and stored at -80˚C until use. All DNA samples were treated using an EpiTect Bisulfite kit (Qiagen GmbH), and converted-DNA was used as a template in next step analysis. For bisulfite sequencing-polymerase chain reaction (BSP-PCR) analysis, the PCR reaction was conducted in 50 μl solution containing converted-DNA (200 ng), dNTP (200 nM for each), forward and reverse primers (50 pM), and *Taq* DNA Polymerase (2.5 U; Thermo Fisher Scientific, Inc.). The 4 μl PCR products (0.1 μg/μl) were ligated into the PMD18T vector. Recombinant vectors were then transformed to *E. coli* and the positive colonies were selected for sequencing. As inactivation of tumor suppressor genes may occur via hypermethylation of CpG islands upstream of the transcription start site, the present study selected a target region spanning from -405 to -74 (containing 23 CpG sites) in the BSP analysis, and the primers for PTPRO (forward: 5'-GAG GTTGTTGTTATTTTATGGG-3'; reverse: 5'-TAAAACTAC AACCTCAAACCCT-3') were used. Methylation specific PCR (MSP) assays were performed using a Techne-512 system (Techne, Staffordshire, UK) and included an initial incubation at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 56˚C for 20 sec, extension at 72˚C for 20 sec and a final extension step at 72˚C for 10 min. One pair of primers for methylated PTPRO (forward: 5'-TGT TGTTAGAGGATTACGGC-3'; reverse: 5'-CAAAAACGT ACCAAACGCTA-3') and unmethylated PTPRO (forward: 5'-TTTTGTTGTTAGAGGATTATGGT-3'; reverse: 5'-TCC AAAAACATACCAAACACTAC-3') were used to amplify methylation and unmethylation alleles of PTPRO, respectively. Quantitative (Q) MSP was performed to detect the methylation levels of *PTPRO* in tumors and matched healthy tissue, and the quantity of methylated *PTPRO* was normalized to β-actin. Briefly, 10 ng bisulfite-coverted DNA was used in the QMSP assay in 384-well plates with a LightCycler480 system (Roche Diagnostics, Basel, Switzerland). The PCR reaction included an initial incubation at 95˚C for 5 min, followed by 45 cycles of denaturation at 95˚C for 30 sec, 58˚C for 10 sec, 72˚C for 20 sec and 80˚C for 1 sec. Each plate consisted of clinical samples, water blanks and a positive control. Serial dilutions of the H520 *PTPRO* methylation-positive cell line were used for constructing the calibration curve. QMSP analyses yield values are expressed as ratios between two absolute measurements (*PTPRO*:β-actin x100) (24). Each sample was analyzed in duplicate.

5-Aza-2'-deoxycytidine (5-AZA) treatment. For the demethylation assay, $1x10^5$ H520 and SK-MES-1 cells were seeded into 6-well plates, cultured for 24 h and treated with 0, 2.5 or 5 μM 5-AZA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Fresh medium containing 5-AZA was changed every 24 h for 3 days and the treated cells were harvested for reverse transcription-quantitative (RT-q) PCR analysis.

RNA extraction and RT-qPCR analysis. Total RNA was isolated from cells using TRIzol® regent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA, and this procedure was performed once using the PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan), which was subsequently used for RT-qPCR analysis using an ABI 7500 fast Sequence Detector (ABI, Carlsbad, CA, USA). The reaction conditions were as follows: an initial predenaturation step at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 20 sec and extension at 72˚C for 30 sec. β-actin served as the endogenous control for detection of mRNA expression levels (25). Relative quantification analysis was performed using the $2^{-\Delta\Delta Cq}$ method (25). The following

primers for *PTPRO* (NM_030667.2, transcript variant 1) were used: Forward, 5'-ACTGCCCCTTATCCACCTCA-3' and reverse, 5'-TGTTGCCCGAGGGAATTTCA-3'.

Cell proliferation and colony formation assays. Cell proliferation was assessed by MTT assay. H520 and SK-MES-1 cells were seeded into 96-well culture plates at a density of $1.5x10^3$ per well. after 1-6 days, cells were incubated with 20 μl MTT (5 mg/ml, Sigma-Aldrich; Merck KGaA) for 4 h at 37˚C. The cell medium was removed and 150 μl dimethyl sulfoxide was added to each well. The absorbance was measured at a wavelength of 490 nm using a microtiter plate reader (Tecan Schweiz AG, Männedorf, Switzerland). To investigate clonogenic ability, cells were transfected with *PTRPO* or an empty vector, and subsequently seeded into 6-well (200 cells per well) plates. The culture medium was replaced every 3 days. After 2 weeks, the medium was removed and the plates were washed twice using PBS. The colonies were fixed in methanol at -20˚C for 5 min, stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA), and counted using an inverted microscope (Nikon Corporation, Tokyo, Japan) in five random fields.

Tumorigenicity analysis. Xenograft experiments were performed to evaluate the tumorigenicity of H520 cells transfected with *PTPRO* or an empty vector. Briefly, $1x10^7$ H520 cells resuspended in 200 μl PBS were subcutaneously injected into the flanks of athymic nude male mice (n=5; age, 4 weeks), which were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Animal experiments were conducted in accordance with guidelines approved by the Institutional Animal Care and Use Committee of Hubei Cancer Hospital. The mice were maintained at a temperature of 18-22˚C and humidity of 50-60% under 12:12 h light-dark cycle with had free access food and water. Each mouse was injected in left flank with the *PTPRO* vector, and in the right flank with the empty vector. A total of 28 days after injection, tumors were harvested, weighed and assayed for mRNA expression.

Western blot analysis. Equal amount of protein extracts were lysed using radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich; Merck KGaA). Cells were centrifuged in a microcentrifuge at 12,000 x g for 15 min at 4˚C to collect the supernatant. Protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 30 μg protein were separated by 10% SDS-PAGE and then transferred onto polyvinyl fluoride membranes (Merck KGaA). The membranes were blocked with 5% fat-free milk in Tris-buffered saline for 1 h, and incubated with anti-PTPRO (1:1,000; catalog no. sc-365354; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-β-actin (1:2,000; catalog no. 4970, Cell Signaling Technology, Inc., Danvers, MA, USA) primary antibodies at 4˚C overnight. The membranes were washed three times with Tris-buffered saline containing 0.1% Tween and incubated for 2 h at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit (catalog no. 7074; 1:1000; Cell Signaling Technology, Inc.) or anti-mouse secondary antibody (catalog no. sc-516102, 1:2000, Santa Cruz Biotechnology). Proteins were visualized using a Bio-Rad ChemiDoc Imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical analysis was performed using Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard error of three independent experiments. Two-way ANOVA followed by Bonferroni correction was used to determine the statistical significance when the number of groups was more than three. The methylation and expression levels of *PTPRO* in tumors and healthy controls were compared using paired-samples t-test. The overall survival of LSCC patients were analyzed using the Log-rank test. All tests were two sided and $P<0.05$ was considered to indicate a statistically significant difference.

Results

CpG island of PTPRO exon 1 is hypermethylated in LSCC cells and tissues. As the methylation status of *PTPRO* in LSCC cells is unclear, a BSP assay was performed in H520 and SK-MES-1 cells, and BEAS-2B cells served as a healthy control. As presented in Fig. 1A and B, the CpG island in the 1st exon was hypermethylated in H520 and SK-MES-1 cells, while partially methylated in BEAS-2B cells. Following this, the methylation status of *PTPRO* in LSCC tissues was assessed using the MSP method. The intensity of methylated alleles was noticeably increased compared with unmethylated alleles (Fig. 1C). To assess if a demethylation agent could restore transcriptional activity, LSCC cells were treated with 0, 2.5 or 5 μM 5-AZA for 72 h. The mRNA expression levels of *PTPRO* were significantly increased following 5-AZA treatment in all groups except the 2.5 μM treatment group in SK-MES-1 cells (Fig. 1D). These data demonstrated that the CpG island of *PTPRO* exon 1 was hypermethylated in LSCC cells and tissues, suggesting that the epigenetic regulation of *PTPRO* may serve a role in LSCC tumorigenesis.

PTPRO is epigenetically downregulated in LSCC tissues. To understand the methylation and mRNA levels of *PTPRO*, QMSP and RT-qPCR analyses were performed in LSCC and matched healthy tissues. The methylation levels of *PTPRO* were significantly increased in LSCC tissues compared with healthy controls (0.0438±0.0263 vs. 0.0381±0.0264; P=0.0005; Fig. 2A). The mean methylation level of *PTPRO* in tumors was used as cut-off to divide cases into two groups (high- or low-methylation). A low level of *PTPRO* methylation was significantly associated with high overall survival probability in LSCC patients (P=0.027; Fig. 2B). Furthermore, the mRNA expression levels of *PTPRO* were markedly reduced in tumors compared with healthy tissues (66.87±12.11 vs. 68.24±11.81; P=0.005; Fig. 2C), and low expression of *PTPRO* (<the mean of mRNA levels of *PTPRO* in tumors) was associated with poor prognosis of patients (P=0.002; Fig. 2D). Pearson correlation coefficient analysis identified an inverse correlation between methylation and mRNA levels of *PTPRO* in tumors (Pearson r=-0.409; P=0.0007; Fig. 2E).

Subsequently, the present study verified whether the methylation or expression of *PTPRO* was associated with clinicopathological features of patients. As presented Table I, mRNA expression levels of *PTPRO* were significantly reduced in advanced tumors compared with early-stage tumors (P=0.042). The methylation or mRNA levels of *PTPRO* were not associated

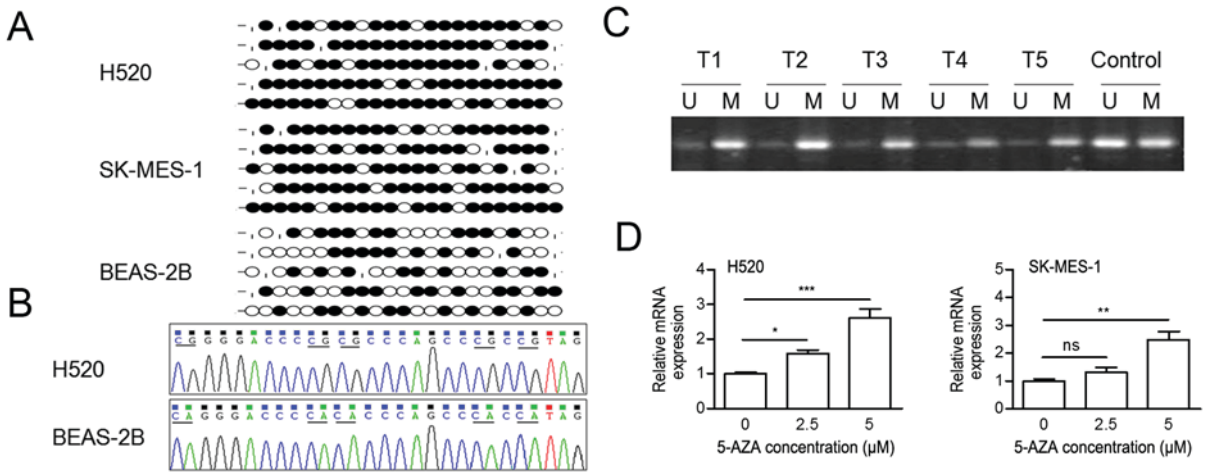

Figure 1. CpG island of the PTPRO promoter (from -405 to -74) is hypermethylated in LSCC cells. (A) Dot graph of BSP data in H520 and SK-MES-1 LSCC cells and BEAS-2B healthy control cells. The BSP-tested region contained 23 CpG sites. Black dot, methylated; white dot, unmethylated; stub, not available. (B) Representative sequences of BSP in H520 and BEAS-2B cells. (C) Methylation-specific polymerase chain reaction of PTPRO in five LSCC tissues. M, methylation alleles; U, unmethylation alleles. (D) Reverse transcription-quantitative polymerase chain reaction analysis of PTPRO mRNA expression levels after 0, 2.5 or 5 μM 5-AZA treatment for 72 h. Data are presented as the mean ± standard deviation. *P<0.05; **P<0.01; ***P<0.001. ns, non-significant; 5AZA, 5-Aza-2'-deoxycytidine; BSP, bisulfite sequencing; LSCC, lung squamous cell carcinoma, PTPRO, protein tyrosine phosphatase receptor-type O.

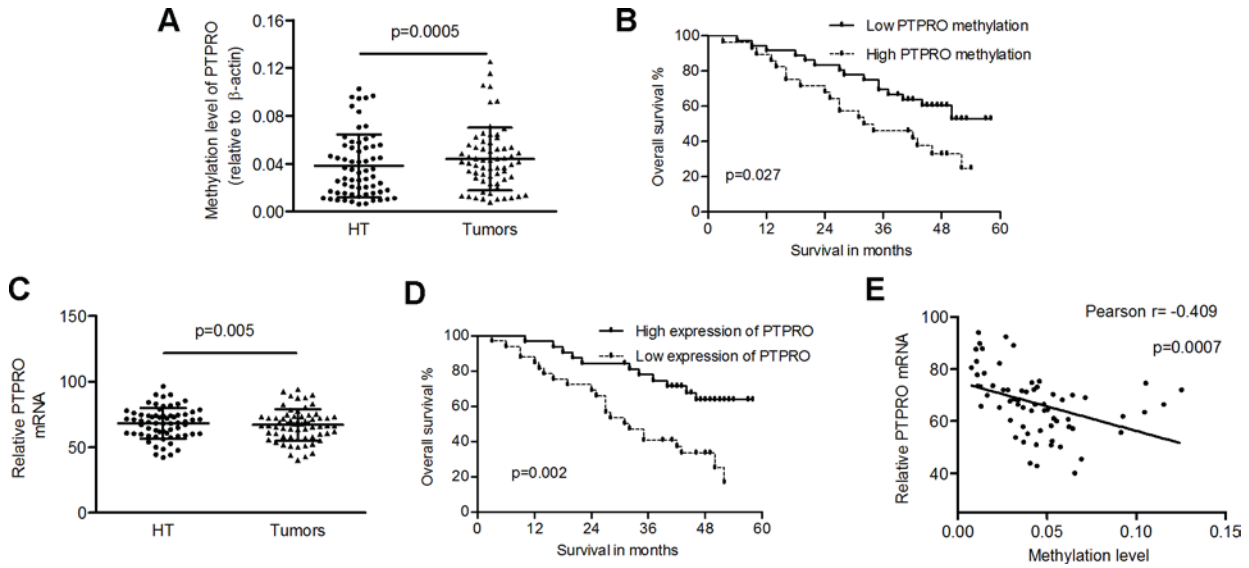

Figure 2. Methylation and expression levels of PTPRO in 65 LSCC tissues and adjacent healthy tissues. (A) Quantitative methylation-specific polymerase chain reaction analysis of PTPRO demonstrated that the methylation level of PTPRO was significantly increased in tumors. (B) Higher PTPRO methylation levels in patients predicted poor prognosis. (C) The mRNA expression levels of PTPRO were detected in tumors or matched healthy tissue by reverse transcription-quantitative polymerase chain reaction. (D) Overall survival analysis indicated that reduced PTPRO mRNA expression levels in patients was associated with poor prognosis. (E) Expression of PTPRO was inversely correlated with its methylation in tumors. Data are presented as the mean ± standard deviation. HT, healthy tissues; LSCC, lung squamous cell carcinoma, PTPRO, protein tyrosine phosphatase receptor-type O.

with other clinical parameters (Table I). Univariate analysis demonstrated that the high methylation of *PTPRO*, low *PTPRO* mRNA, smoking, advanced tumor stage, higher T stage and lymph node metastasis were predictors of poor prognosis for patients, whereas only mRNA expression levels of *PTPRO* (P=0.005) and higher TNM stage (P=0.001) were identified as significantly independent prognostic factors in multivariate analysis, with relative risks of 2.826 and 3.714, respectively (Table II). Taken together, these data suggested that epigenetically down-regulated PTPRO may be involved in LSCC development and may be a potential prognostic marker. Detailed clinical and molecular data of the patients are presented in Table III.

PTPRO inhibits cell viability in vitro. The expression levels of *PTPRO* in transfected cells and control cells were detected. The mRNA (Fig. 3A) and protein (Fig. 3B) expression levels of *PTPRO* were upregulated in H520 and SK-MES-1 cells transfected with stably expressing *PTPRO* vectors in comparison with control cells and non-transfected cells. The results of MTT demonstrated that when compared with the empty vector group (control) and non-transfected group (untreated), the proliferation of LSCC cells was significantly inhibited in over-expressing *PTPRO* cells (Fig. 3C). Colony formation assay was performed to evaluate the effect of *PTPRO* on LSCC cells. As a result, the number of colonies were significantly reduced in

Table I. Associations between clinicopathological features and methylation or mRNA expression levels of PTPRO in lung squamous cell carcinoma.

Variable	Total (n=65)	PTPRO Methylation	P-value	PTPRO mRNA	P-value
Gender			0.322		0.249
Female	25	0.0471±0.0254		64.69±12.13	
Male	40	0.0417±0.0269		68.23±12.04	
Age (years)			0.126		0.133
<60	28	0.0389±0.0264		69.11±13.26	
≥60	37	0.0475±0.0259		65.17±11.04	
Smoking			0.896		0.788
Never	31	0.0411±0.0201		67.33±12.51	
Past, current	34	0.0462±0.0309		66.44±11.90	
TNM stage			0.237		0.042
I, II	34	0.0396±0.0233		70.55±12.49	
III, IV	31	0.0484±0.0289		62.82±10.42	
pT stage			0.703		0.603
T1-2	43	0.0429±0.0261		67.56±13.13	
T3-4	22	0.0455±0.0271		65.51±9.955	
pN stage			0.263		0.286
N0	17	0.0374±0.0222		70.64±3.001	
N1-3	48	0.0461±0.0274		65.53±1.711	

Never, no smoking history; Past, stopped smoking for <1 year; current, current smoker; SD, standard deviation; TNM, tumor node metastasis; PTPRO, protein tyrosine phosphatase receptor-type O.

Table II. Clinical characteristics of lung squamous cell carcinoma patients correlates with overall survival.

	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
PTPRO methylation (low/high)	2.108	1.068-4.163	0.032			
PTPRO mRNA (high/low)	2.971	1.442-6.121	0.003	2.826	1.364-5.853	0.005
Gender (female/male)	1.15	0.569-2.326	0.696			
Age (<60 y/≥60 y)	1.037	0.991-1.085	0.119			
Smoking (never/past, current)	2.113	1.042-4.286	0.038			
TNM stage (I, II/III, IV)	4.145	1.998-8.061	0.000	3.714	1.771-7.792	0.001
T stage (T1-2/T3-4)	2.288	1.149-4.555	0.018			
N stage (N0/N1-3)	2.743	1.060-7.101	0.038			

HR, hazard ratio; CI, confidence interval; TNM, tumor node metastasis; PTPRO, protein tyrosine phosphatase receptor-type O.

H520 and SK-MES-1 cells transfected with *PTPRO* expressing vectors, compared with control and untreated cells (Fig. 3D). These *in vitro* analyses demonstrated the inhibitory effect of *PTPRO* on cell viability.

PTPRO impairs the tumorigenicity of H520 cells in vivo. The inhibitory role of *PTPRO* was further confirmed using a xenograft model of H520 cells in nude mice. As expected, there was a significant reduction in tumor volume and weight in the *PTPRO* overexpression group compared with the empty vector group (Fig. 4A and B). In addition, mRNA expression levels of *PTPRO* were upregulated in tumors injected with *PTPRO* vectors (Fig. 4C).

Discussion

Many LSCC patients are already in the advanced stages when diagnosed, rendering treatment difficult. The initiation and progression of squamous cell carcinoma is a complex process involving the abnormalities of a variety of oncogenes and tumor suppressors (26,27). The present study focused on the novel tumor suppressor *PTPRO*.

Table III. Detailed clinical and molecular data of cases.

IID	Methy (H)	Methy (T)	mRNA (H)	mRNA (T)	Gender	Age	Smoking	TNM	Survival	Months
1	0.0416	0.0445	78.02	73.09	M	57	current	T3N2M0	No	16
2	0.0253	0.0384	71.13	71.283	M	66	current	T1N2M0	No	32
3	0.0553	0.0623	56.214	57.89	M	45	past	T3N0M0	No	52
4	0.0363	0.0487	67.083	64.097	F	61	never	T2N0M0	Yes	52
5	0.00652	0.0149	79.032	78.37	M	55	current	T1N1M0	Yes	50
6	0.0272	0.0368	57.734	51.98	M	59	never	T2N1M0	No	50
7	0.0966	0.1252	76.04	72.01	M	58	current	T1N2M0	Yes	54
8	0.0357	0.0427	74.32	72.094	M	72	never	T3N0M0	Yes	41
9	0.0623	0.0619	52.77	60.67	F	64	never	T3N0M0	Yes	41
10	0.0422	0.0534	74.04	70.32	M	62	never	T2N1M0	Yes	43
11	0.0342	0.0415	68.32	74.89	M	59	never	T2N1M0	No	44
12	0.0164	0.0268	96.43	92.384	M	60	current	T1N1M0	Yes	51
13	0.1026	0.1154	69.05	66.391	M	66	current	T4N1M0	No	19
14	0.0949	0.1045	60.21	63.42	F	68	current	T1N1M0	No	42
15	0.0713	0.0643	70.14	70.06	M	72	past	T2N2M0	No	34
16	0.0532	0.0503	60.69	64.382	M	68	current	T3N2M0	No	13
17	0.0106	0.0483	66.985	66.45	F	55	never	T2N2M0	Yes	52
18	0.0214	0.0312	84.376	89.103	F	53	never	T1N0M0	Yes	47
19	0.0123	0.0164	62.165	70.01	F	62	never	T2N2M0	Yes	57
20	0.0226	0.0324	58.54	53.75	M	64	current	T1N1M0	No	12
21	0.0351	0.0456	81.23	75.376	F	59	never	T2N1M0	Yes	49
22	0.0556	0.0575	44.01	50.09	F	77	never	T3N1M1	No	16
23	0.0434	0.0537	62.93	61.067	M	62	past	T1N2M0	No	24
24	0.0153	0.0292	72.71	67.651	M	69	never	T3N1M0	Yes	49
25	0.0441	0.0439	59.92	51.06	M	68	current	T3N1M0	No	25
26	0.0532	0.0528	54.04	52.39	F	72	never	T2N0M0	Yes	9
27	0.0503	0.0645	60.87	57.09	F	75	never	T1N1M0	No	48
28	0.0413	0.0392	58.36	55.09	F	55	never	T1N0M0	Yes	46
29	0.0096	0.0126	74.09	73.213	M	67	current	T2N0M0	Yes	47
30	0.0634	0.0714	73.98	69.08	M	63	past	T2N1M0	No	46
31	0.00921	0.0105	85.362	82.99	M	62	current	T2N1M0	Yes	42
32	0.04632	0.0443	50.093	42.78	F	63	never	T1N2M0	No	27
33	0.02752	0.03648	59.986	57.853	M	67	current	T3N2M0	Yes	39
34	0.0264	0.0336	70.84	67.895	F	77	never	T1N1M0	Yes	52
35	0.0144	0.0293	63.468	60.432	M	57	past	T2N2M0	Yes	49
36	0.01123	0.01293	64.35	65.783	M	65	current	T2N3M0	No	9
37	0.0261	0.0359	67.94	71.67	M	53	current	T1N0M0	No	40
38	0.0292	0.0366	68.17	65.332	F	73	never	T3N1M0	No	35
39	0.0061	0.0077	81.653	80.56	M	58	past	T2N0M0	Yes	48
40	0.04493	0.0523	60.41	58.24	F	70	never	T3N1M1	No	3
41	0.0236	0.0413	68.56	64.02	F	52	never	T2N1M0	Yes	46
42	0.0958	0.0914	59.054	55.67	F	64	current	T1N2M0	No	14
43	0.0142	0.0317	71.23	68.541	M	66	current	T2N2M0	No	22
44	0.01116	0.01157	90.01	94.03	M	55	past	T1N0M0	Yes	58
45	0.08357	0.01094	78.55	77.83	F	59	never	T3N1M0	Yes	48
46	0.0607	0.0555	61.64	60.098	M	51	never	T3N1M0	No	32
47	0.0578	0.0586	71.62	68.154	M	53	current	T1N1M0	Yes	31
48	0.0545	0.0523	57.43	50.82	M	64	current	T1N1M0	Yes	43
49	0.0945	0.1054	76.841	74.54	F	69	never	T3N2M0	Yes	10
50	0.0582	0.0693	48.68	45.469	M	60	never	T3N2M0	No	9
51	0.00843	0.01128	74.71	73.52	M	55	past	T3N1M0	Yes	45
52	0.0446	0.0431	73.12	66.376	F	68	never	T1N0M0	No	35

Table III. Continued.

IID	Methy (H)	Methy (T)	mRNA (H)	mRNA (T)	Gender	Age	Smoking	TNM	Survival	Months
53	0.0882	0.0925	64.89	61.87	M	56	current	T2N0M0	Yes	46
54	0.00948	0.01237	88.56	89.82	F	59	never	T1N1M0	Yes	46
55	0.01512	0.02538	73.02	72.02	M	78	past	T3N2M0	No	18
56	0.0197	0.0336	69.332	66.365	M	63	never	T3N0M0	No	27
57	0.0703	0.0655	42.24	40.08	F	58	current	T1N2M0	No	31
58	0.01692	0.02335	83.01	82.32	M	64	never	T1N0M0	Yes	52
59	0.01808	0.04049	44.33	43.9	M	47	current	T2N1M1	No	6
60	0.0156	0.01964	74.841	73.546	F	57	never	T3N2M0	Yes	37
61	0.00938	0.01015	90.091	87.569	M	50	never	T2N1M0	Yes	53
62	0.02085	0.02322	71.841	65.323	F	52	past	T3N0M0	No	28
63	0.0203	0.02813	73.841	72.04	F	59	current	T2N2M0	No	20
64	0.03256	0.0463	47.65	56.33	M	52	current	T2N3M0	No	27
65	0.01014	0.01379	83.01	87.83	M	62	never	T3N0M0	Yes	49

Methy (H), methylation levels in healthy tissues; Methy (T), methylation levels in tumors; mRNA (H), mRNA levels in healthy tissues; mRNA (T), mRNA levels in tumors; F, female; M, male; Months, survival in months; TNM, tumor node metastasis.

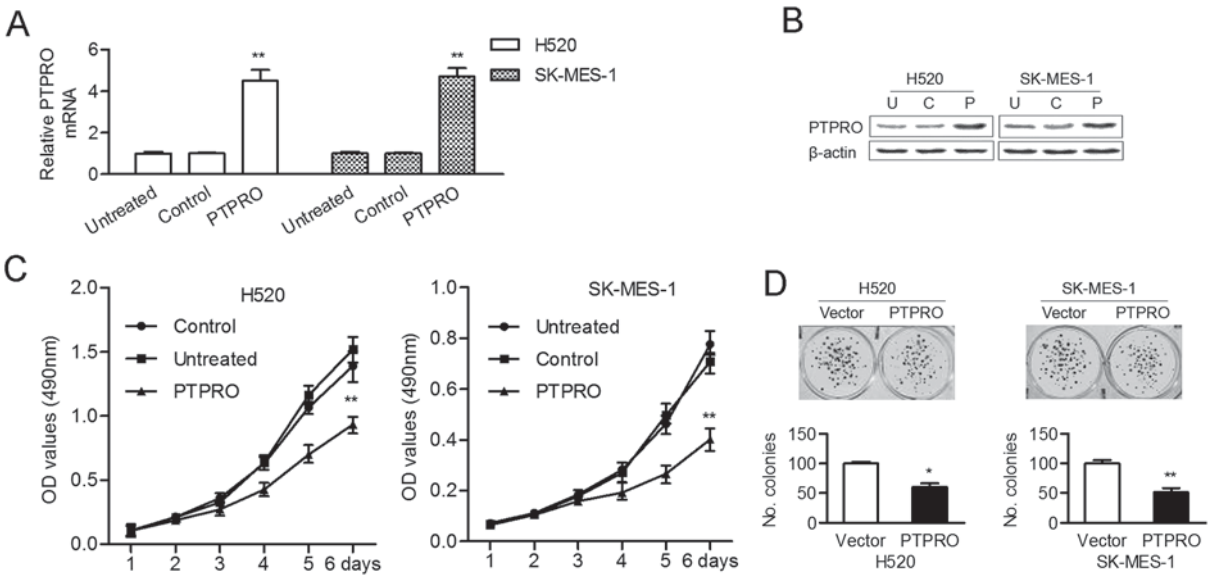

Figure 3. Overexpression of PTPRO inhibits LSCC cell growth *in vitro*. (A) The mRNA expression levels of PTPRO were markedly increased following transfection with a pcDNA-PTPRO vector. (B) The protein expression levels of PTPRO were detected by western blotting. (C) The effect of PTPRO on cell proliferation was measured by MTT assay. (D) Representative micrographs and quantification of stained H520 and SK-MES-1 cell colonies. Data are presented as the mean ± standard deviation. *P<0.05; **P<0.01. LSCC, lung squamous cell carcinoma, PTPRO, protein tyrosine phosphatase receptor-type O; OD, optical density; U, untreated; C, control; P, PTPRO..

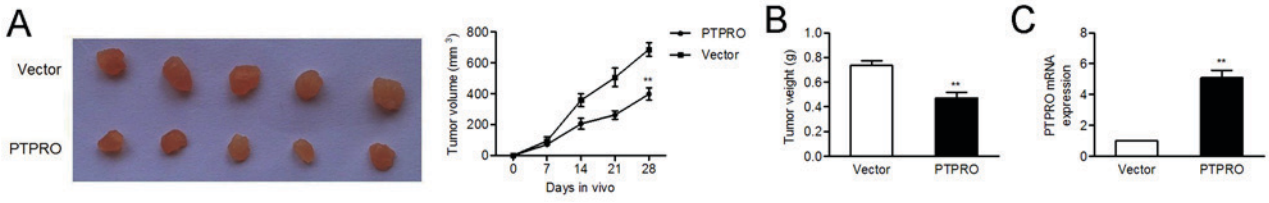

Figure 4. PTPRO inhibits tumor growth *in vivo*. (A) pcDNA-PTPRO- and control vector- transfected H520 cells were injected subcutaneously into the flanks of nude mice. Tumor volumes were measured every 7 days. Tumor volumes was significantly reduced in tumors stably expressing PTPRO. (B) Tumor weight was calculated at 4 weeks post injection. (C) mRNA expression levels of PTPRO in xenograft tumors were confirmed by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation. **P<0.01. PTPRO, protein tyrosine phosphatase receptor-type O.

It is well known that the cell tyrosine phosphorylation levels are co-regulated by PTP and protein tyrosine kinase (PTK); dysfunction of tyrosine phosphatase is closely associated with the occurrence of a variety of human tumors (28). Previous studies have demonstrated that overexpression of PTP in cancer cells may reverse the malignant transformation induced by PTK (29). *PTPRO* is a member of the PTP family that has been reported to be frequently methylated, and is characterized as a tumor suppressor gene in the occurrence and development of many malignancies (13-16). The present study examined H520 and SK-MES-1 LSCC cells and LSCC cases for analysis. The CpG island of *PTPRO* exon1 was revealed to be hypermethylated, which was consistent with previous studies (14). Additionally, in the present study, high methylation or low expression of *PTPRO* were associated with poor prognosis. Similar results have been reported in breast (21,30) and colorectal (31) cancer. Li *et al* (30) reported that methylation of *PTPRO* was an independent predictor for survival. However, mRNA expression of *PTPTO*, rather than methylation, was an independent factor in the present study. All these data strongly suggested that *PTPRO* is involved in tumorigenesis and may serve as a valuable prognostic marker in cancers.

Although the function and underlying mechanism of *PTPRO* has been documented in former studies (18-20), the biological effect of *PTPRO* in LSCC remains unclear. The present study demonstrated that ectopic *PTPRO* expression significantly inhibited the proliferation rate and colony formation ability of cells. Previous investigations observed a similar effect in lung adenocarcinoma and lymphoma (14,19). *PTPRO* was also reported to be involved in other critical biological processes including angiogenesis, metastasis and apoptosis (18,20,21). Therefore, *PTPRO* may serve as a multi-functional regulator in tumorigenesis. In the present study, tumorigenicity analysis confirmed the tumor suppressive effect of *PTPRO in vivo*. Taken together, these findings expanded current knowledge of *PTPRO* in LSCC, suggesting the potential value of *PTPRO* as a therapeutic target.

In conclusion, the present study demonstrated that *PTPRO* inhibits tumor growth *in vitro* and *in vivo*, indicating the tumor suppressive function of *PTPRO* in LSCC. This study highlights PTPRO as an epigenetically silenced gene, and a candidate tumor-suppressor of LSCC.

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