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From Cell Biology to Tissue Engineering

Expression and clinical relations of protein tyrosine phosphatase receptor type S in esophageal squamous cell carcinoma

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Summary. Protein tyrosine phosphatase receptor type S is a tumor suppressor gene, located at chromosome 19p13.3, frequently inactivated through deletions or epigenetic mechanisms in many types of cancers. In this study, we investigate protein tyrosine phosphatase receptor S (PTPRS) expression level, clinicopathological and prognostic significance in 205 cases of esophageal squamous cell carcinoma (ESCC). Paraffin embedded tissue with immunohistochemistry methods was adopted to exam PTPRS expression in ESCC and paired normal esophageal mucosa tissues on Tissue Microarrays (TMAs). The protein tyrosine phosphatase receptor S was significantly down-regulated in ESCC (58.0%) relative to normal tissues (43.9%) (P=0.006). Statistical analysis revealed that reduced PTPRS expression was significantly associated with TNM stage (P=0.013), invasion depth (P<0.001), local lymph node metastasis (P=0.042) and tumor differentiation (P=0.001). Furthermore, Kaplan-Meier survival analysis revealed that low expression of PTPRS significantly correlated with poor survival of ESCC patients (P=0.002). Cox regression analysis confirmed PTPRS expression as an independent predictor of the overall survival of ESCC patients (HR=1.573, P=0.049). The 5-year overall survival rates in patients with high and low PTPRS expression were 50.6% and 37.2%, respectively. PTPRS deficiency is independently associated with shorter survival and increased recurrence in patients. Our data offer convincing evidence that loss of PTPRS expression may predict an aggressive clinical course in ESCC patients. PTPRS may function as a tumor suppressor and play an important role in ESCC growth and metastasis.

Key words: Esophageal squamous cell carcinoma (ESCC), Immunohistochemistry, PTPRS, Tissue microarrays

Introduction

Esophageal squamous cell carcinoma (ESCC) is currently the eighth most common malignancy and the sixth leading cause of cancer mortality globally (Ke, 2002). Hebei province in north-central China has one of the highest rates of esophageal cancer in the world. Squamous cell carcinoma is the predominant esophageal cancer occurring in this region (Li, 1982). Although surgical techniques, chemotherapy and radiotherapy treatment methods have improved, the prognosis for patients with ESCC remains poor. However, a key obstacle to improving ESCC patient survival is a lack of sensitive early detection methods. There is no accurate

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Abbreviations. PTPRS, protein tyrosine phosphatase receptor S; TMAs, tissue microarrays; ESCC, esophageal squamous cell carcinoma; IHC, immunohistochemistry; PTPs, protein tyrosine phosphates; RPTPs, receptor protein tyrosine phosphatases; non-RPTPs, non-receptor protein tyrosine phosphatases; AJCC, American Joint Committee on Cancer; OS, overall survival

clinical method for ESCC screening. Molecular events associated with the initiation and progression of ESCC remain poorly understood (Dong et al., 2012). Therefore, understanding of ESCC progression and metastasis is of paramount importance to identify new, effective treatment targets.

Protein tyrosine phosphatases (PTPs) are signaling molecules that regulate a variety of cellular processes, including cell growth, differentiation, cell cycle and oncogenic transformation (Takekawa et al., 1994). The constitutive activation of PTPs signaling pathways is a biochemical hallmark of cancer (Fischer, 1999). PTPs, fulfilling either tumor suppressors or oncogenic roles, consist of non-receptor protein tyrosine phosphatases (non-RPTPs) and receptor protein tyrosine phosphatases (RPTPs) (Alonso et al., 2004; Tabernero et al., 2008). The protein encoded by the PTPRS gene (protein tyrosine phosphatase, receptor type, S) is one of 38 known human receptor-type PTPs, a group of proteins that are increasingly thought to be important in human neoplasia and cancer progression (Meathrel et al., 2002). Along with PTPRF and PTPRD, they form the type IIa subgroup of PTPRs, whose extracellular regions are composed of multiple immunoglobulin-like and fibronectin type III-like domains (Alonso et al., 2004). Meanwhile, whole-genome sequencing approaches have uncovered several mutations in PTPRS, such as V224M in colorectal cancer (Wood et al., 2007), A1384T in cholangiocarcinoma (Gao et al., 2014), and P141S in malignant melanoma (Solomon et al., 2008). Early study of involvement of PTPRS in cancer came from evidence in head and neck squamous cell carcinoma, where frequent deletion of PTPRS was detected (Morris et al., 2011). A recent study showed PTPRS expression is significantly down-regulated in nearly 80% of hepatocellular carcinomas (Wang et al., 2015). These researches suggested that PTPRS might be one of a select group of tumor suppressor genes that are inactivated in a wide range of common human tumor types. However, the role of PTPRS in human esophageal squamous cell carcinoma has not yet been investigated.

In the present study, we investigated the clinical relevance and biological impacts of PTPRS in human ESCC. We detected PTPRS expression level in esophageal squamous cell carcinoma using immunohistochemistry. Meanwhile, prognostic and clinicopathological features of PTPRS were investigated in 205 esophageal squamous cell carcinoma tissue samples.

Materials and methods

Patients and tissue specimens

Human primary ESCC were collected from 205 patients who were diagnosed and treated at the Fourth Hospital of Hebei Medical University, (Shijiazhuang, China) from July 2004 to August 2014, and the diagnosis was confirmed by pathological examination. None of the patients received preoperative treatment, such as radiation or chemotherapy. After obtaining informed consent, patients were interviewed to obtain information on demographic and lifestyle cancer risk factors (e.g. smoking habit, alcohol drinking, and family history of cancer) and clinicopathological data. Among 205 patients, normal esophageal mucosa tissues (5 cm from the tumor) were randomly selected as normal control. All esophageal cancer samples and normal controls were established into tissue microarray (TMA) for further assessments. The median age of the patients was 60 years (range, 34 to 76 years), and 132 cases (64.4%) were men. Of 205 ESCC patients, 128 (62.4%) received a left thoracotomy, and 77 (37.6%) underwent a video assisted thoracic surgery (VATS) abdomino-thoraco-cervical incision. The tumor median size of the patients was 40 mm (range, 10 to 90 mm). The distribution tumor location was 18 of cervical patients (8.8%), 40 of upper thoracic patients (19.5%), 107 of middle thoracic patients (52.2%), and 40 of lower thoracic patients (19.5%).

The pathological features were evaluated by independent pathologists according to the TNM staging system of the American Joint Committee on Cancer (AJCC 7th edition). All patients were followed-up after primary treatment at intervals increasing from 3 months to 1 year until death or the end of the study. The follow-up period ranged from 2 months to 10 years (median: 27 months) for esophageal cancer patients. Routine examinations, including systemic review, tumor marker testing, computed tomography (CT), and endoscopic examination, were performed to evaluate the outcome of the disease, which was classified as relapse or death according to the WHO criteria for clinical response. Clinicopathological and follow-up data were stored in a database in accordance with hospital privacy rules. The study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University, and informed consent was obtained from all participants under institutional reviewer board protocols.

Construction of the tissue microarray

The tissue microarray was constructed according to methods described previously (Kononen et al., 1998). The tissues (ESCC tissues and normal esophageal tissues) from the tumor bank were collected, fixed in ethanol, and embedded in paraffin. Hematoxylin and eosin stained sections from a single random block from each patient were reviewed by a senior pathologist to define representative tumor regions. Two targeted core samples of each specimen were obtained using a tissue array instrument. Briefly, 10 mm tissue cylinders were punched and arrayed on a recipient paraffin block. Sections (5 μm) of the tissue array (recipient) block were cut and placed on glass slides. After exclusion of cores with inadequate tissue following sectioning and tissue

transfer, the final immunohistochemical analysis included cores from ESCC and normal esophageal tissues.

Immunohistochemistry analysis

Slides from both the tumor and biopsy tissue microarrays were stained according to manufacturer's protocols for PTPRS. In brief, tissue microarray sections were rehydrated through graded alcohol. After deparaffinization with xylene, the tissue sections were rehydrated through 100%, 85% and 75% ethanol. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 15 min. After three washes in PBS (phosphate-buffered saline), the tissue sections were boiled in antigen retrieval buffer containing 0.1% sodium citrate-hydrochloric acid (pH 6.0) for 15 min using a microwave oven. After rinsing with PBS, the sections were incubated with primary antibody and then rinsed in 3% peroxidase quenching solution to block endogenous peroxidase. The sections were then incubated with a rabbit polyclonal antibody against PTPRS (diluted into 1:100, provided by Sigma-Aldrich, St Louis, MO, USA) at 4°C overnight. After washing with PBS, the sections were incubated with a biotinylated secondary antibody (provided by Outdo Biotech Company, Shanghai, China) at room temperature for 30 min. The visualization signal of the slides was treated with 3-diaminobenzidine (DAB) solution, and all of the slides were counterstained with hematoxylin for 15 min. As negative control, adjacent sections were processed as described above except incubating at 4°C overnight in blocking solution without the primary antibody.

Semi-quantitative evaluation

The PTPRS protein expression level was assessed by immunostaining score. Two investigators who were unaware of the clinicopathologic data independently evaluated PTPRS staining under a light microscope. PTPRS protein expression in cellular membrane was evaluated according to the percentage of positively stained cells (median, 60%; range, 0% to 100%) and staining intensity: low staining, light yellow; intermediate staining, yellow brown; and high staining, brown. The PTPRS expression index is as follows: 1, 60% or fewer cells positive with low intensity; 2, more than 60% of cells positive with low intensity or 60% or fewer cells with intermediate intensity; 3, more than 60% of cells positive with intermediate intensity or 60% or fewer cells positive with high intensity; and 4, more than 60% of cells positive with high intensity. Tumors with absent immunostaining were classified as negative expression 0. We defined an expression index of 0, 1 and 2 as low expression of PTPRS, and an expression index of 3 to 4 was considered high expression. In this study, a minimum of 500 epithelial cells were counted for each normal or tumor case. To reach a conclusive judgment, discordant cases were reviewed.

Statistical analysis

The statistical analyses were performed using the SPSS software (version 18.0, Chicago, IL, USA). The Pearson's χ^2 test was used to analyze the relationships between PTPRS expression and various clinicopathological parameters. Recurrence and overall survival (OS) were defined as the time from the date of surgery to the date of regional recurrence or distant metastasis and death or final clinical follow-up, respectively. Survival curves were calculated using the Kaplan-Meier method and compared by the log-rank test. Univariate and multivariate analyses were performed to detect PTPRS expression and the clinicopathological variables by using Cox proportional hazards regression model. A two-sided P value less than 0.05 was considered to be statistically significant.

Results

Expression and localization of PTPRS

Among the primary ESCC tumor and paired normal esophageal tissue specimens were investigated. As a result, high expression PTPRS were 56.1% (115/205) in normal tissue and 42.0% (86/205) in ESCC tissues. The difference was statistically significant (P=0.006, Table 1). Positive expression of PTPRS in esophageal squamous cell carcinoma tissue and normal esophageal epithelial tissue cells displayed a primarily cytomembrane pattern (Fig. 1). Normal esophageal mucosa tissues showed the strongest PTPRS positive staining (Fig. 1B). Immunostaining of esophageal carcinoma sample showed a sharp contrast in PTPRS staining intensity (Fig. 1C-E).

PTPRS expression and correlation with clinicopathologic characteristics

We analyzed the correlation between the PTPRS expression and multiple clinicopathological features in 205 ESCC patients. Results indicated that high and low expression of PTPRS were in 86 of 205 (42.0%) and 119 of 205 (58.0%), respectively, of the ESCCs in the whole cohort. There was no significant correlation between

Table 1. Expression of PTPRS in ESCC tissues and paired normal esophageal mucosa tissues.

Group	N	PTPRS expression (%)		χ^2	P-value
		Low	High		
ESCC	205	119 (58.0)	86 (42.0)	8.208	0.006
normal tissues	205	90 (43.9)	115 (56.1)		

ESCC, esophageal squamous cell carcinoma; PTPRS, protein tyrosine phosphatase receptor S.

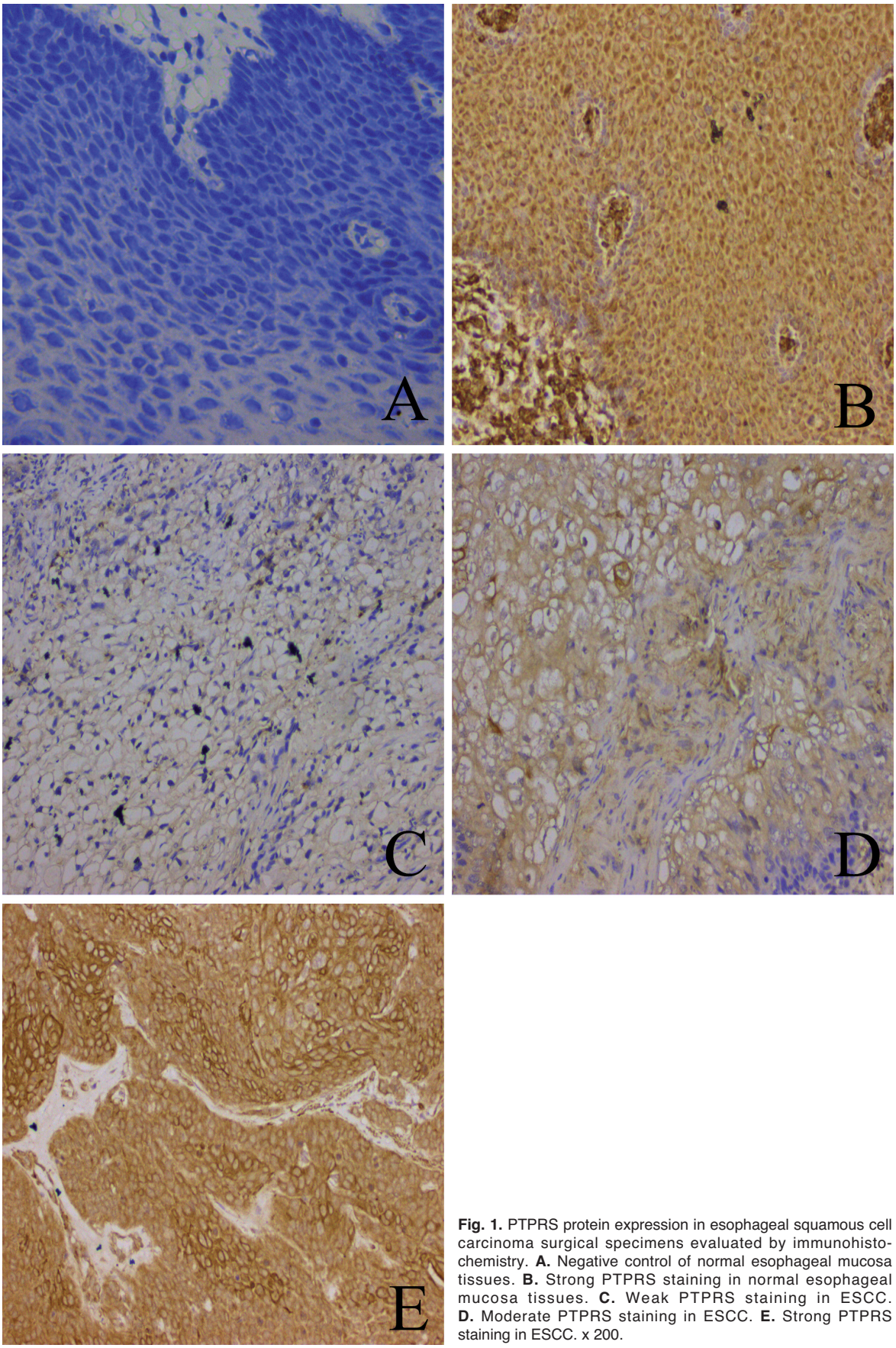

Fig. 1. PTPRS protein expression in esophageal squamous cell carcinoma surgical specimens evaluated by immunohistochemistry. **A.** Negative control of normal esophageal mucosa tissues. **B.** Strong PTPRS staining in normal esophageal mucosa tissues. **C.** Weak PTPRS staining in ESCC. **D.** Moderate PTPRS staining in ESCC. **E.** Strong PTPRS staining in ESCC. x 200.

PTPRS expression and several clinicopathological variables including age, gender, smoking, drinking, tumor history, tumor location, tumor size and operative method. In contrast, low expression of PTPRS was significantly correlated with invasion depth (P<0.001), local lymph node metastasis (P=0.042), tumor differentiation (P=0.001), and TNM stage (P=0.013) in ESCC (Table 2).

Relationship between PTPRS expression, clinicopathologic variables, and ESCC patient survival

The prognostic value of PTPRS for esophageal squamous cell carcinoma patients overall survival was evaluated between patients with high and low PTPRS protein levels. The 5-year overall survival rates in patients with high and low PTPRS expression were 50.6% and 37.2%, respectively. Low PTPRS expression correlates with aggressive clinicopathological characteristics and poor prognosis in ESCC patients (P=0.002, Fig. 2). Kaplan-Meier curve assessment showed that the recurrence rate of patients with low PTPRS expression was significantly higher than that of high PTPRS patients (P=0.01, Fig. 3).

To identify the potentially significant prognostic variables in all the patients with esophageal squamous cell carcinoma, univariate Cox regression analysis of each variable was performed in relation to the survival time. Data showed that tumor invasion depth, local lymph node metastasis, tumor differentiation, TNM stage, and PTPRS expression were significantly associated with overall survival (Table 3). Furthermore, to determine independent variables among these prognostic factors, we performed a multivariate analysis using Cox proportional hazard models. The analysis confirmed local lymph node metastasis and PTPRS expression as independent predictors of overall survival of ESCC patients. The relative risk of death in patients with high PTPRS tumors was significantly lower than that of patients with low PTPRS tumors.

Table 2. Correlation between PTPRS expression and clinicopathologic characteristics in esophageal squamous cell carcinoma.

Characteristics	PTPRS expression			χ^2	P-value
	Total (n=205)	Low (n=119)	High (n=86)		
Gender					
male	132	77	55	0.012	0.912
female	73	42	31		
Age (years)					
≤60	107	63	44	0.063	0.801
>60	98	56	42		
Tumor history					
yes	54	37	17	3.300	0.069
no	151	82	69		
Smoking					
yes	116	70	46	0.578	0.447
no	89	49	40		
Drinking					
yes	110	67	43	0.797	0.372
no	95	52	43		
Tumor location					
cervical/upper	58	33	25	0.044	0.834
middle/lower	147	86	61		
Tumor depth					
pT1/2	72	26	46	21.932	<0.001
pT3/4	133	93	40		
Lymph node metastasis					
negative	119	62	57	4.121	0.042
positive	86	57	29		
Tumor differentiation					
well	57	23	34	10.155	0.001
moderate/poor	148	96	52		
Tumor stage					
I/II	125	64	61	6.170	0.013
III/IV	80	55	25		
Tumor size (mm)					
<40	93	48	45	2.895	0.089
≥40	112	71	41		
Operative method					
thoracoscope	77	47	30	0.453	0.501
thoracotomy	128	72	56		

PTPRS, protein tyrosine phosphatase receptor S.

Discussion

It is well known that receptor protein tyrosine phosphatase S (PTPRS) is a member of the highly conserved family of receptor protein tyrosine

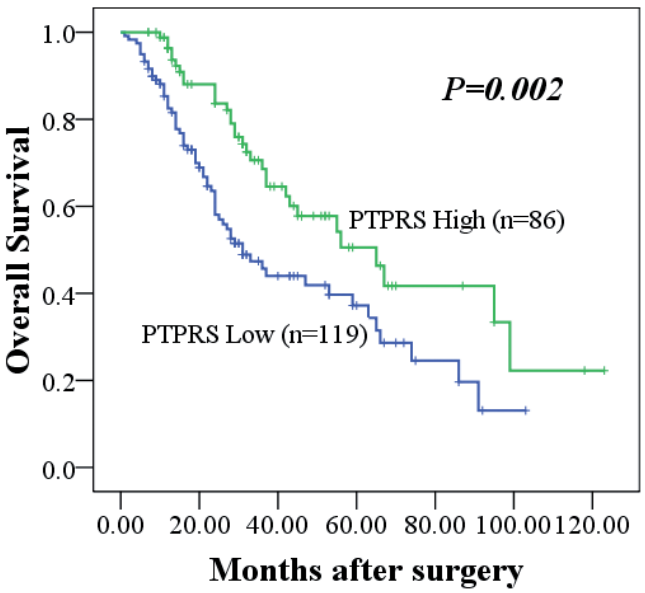

Fig. 2. Kaplan-Meier survival curves for esophageal squamous cell carcinoma patients by PTPRS expression (n=205). The survival rate of patients in high PTPRS group was significantly higher than that of the patients in the low PTPRS group (P=0.002).

phosphatases (PTPs) (Jacob and Motiwala, 2005). PTPs are signaling molecules that regulate a variety of cellular processes, including cell growth, differentiation, mitotic cycle and oncogenic transformation (Takekawa et al., 1994). Recently, several classical PTPs have been identified as potential tumor suppressors, including receptor PTPs such as PTPRO, PTPRG, PTPRD, and PTPRT (Motiwala et al., 2003; Doorn et al., 2005; Veeriah et al., 2009; Zhao et al., 2010). This group of genes is increasingly thought to be important in cancer development and progression.

PTPRS, encoding protein tyrosine phosphatases receptor type S, belongs to a tumor suppressor gene, is located at chromosome 19p13.3 (Wagner et al., 1996), a loci frequently lost in many types of tumors. Loss of tumor suppressor function leads to the initiation and progression of cancer (Futreal et al., 2004). Inactivation of tumor suppressor genes can result from genetic mechanisms such as mutation and deletion or epigenetic mechanisms such as DNA hypermethylation (Chan et al., 2008). PTPRS has been demonstrated to have a vital role in colitis with evidence that PTPRS knockout (KO) mice spontaneously developed mild colitis and became severe when challenged with colitis inducers (Muise et al., 2007). Recently, Morris et al. (2011) described PTPRS expression was frequently deleted (26%) in head and neck squamous cell carcinoma and first proposed PTPRS as a tumor suppressor. Meanwhile, an independent dataset found PTPRS expression was down-regulated in lung adenocarcinomas by Morris LG (Morris and Chan, 2011). Reduced PTPRS expression of lung adenocarcinomas patients has significantly poorer survival than similar patients with normal PTPRS-expressing tumors. A recent study showed promoter hypermethylation and mutations of PTPRS in human hepatocellular carcinomas cell lines were first identified by Wang and colleagues in 2015 (Wang et al., 2015). PTPRS expression is significantly down-regulated in nearly 80% of hepatocellular carcinomas. These researches suggested that PTPRS might have a growth suppressive role in many types of human cancer. However, thus far, the expression, clinical significance and biological functions of PTPRS in esophageal squamous cell carcinoma have not been explored.

To our knowledge, for the first time, we demonstrated a potential tumor suppressor role of

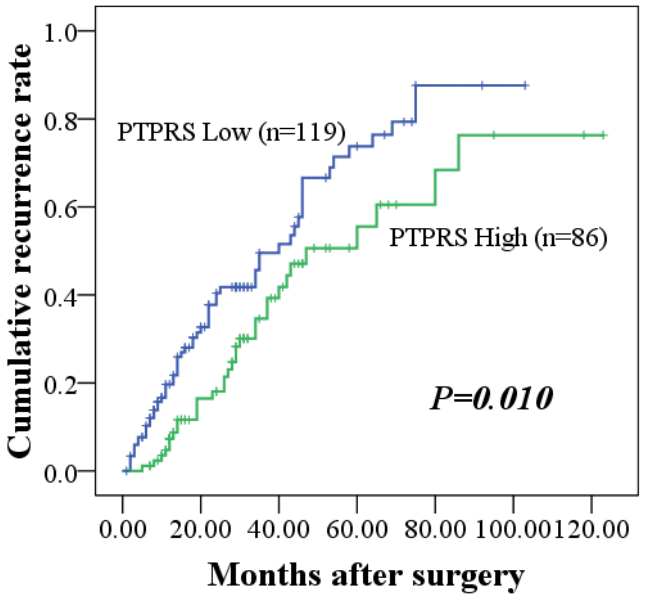

Fig. 3. Kaplan-Meier survival curves for recurrence rate according to PTPRS expression in training cohort (n=205). The recurrence of the low-expression of PTPRS group was significantly higher than the high-expression group (P=0.01).

Table 3. Univariate and multivariate analysis for overall survival in 205 patients with esophageal squamous cell carcinoma.

Factor	Overall Survival					
	Univariate analysis			Multivariate analysis		
	HR	(95% CI)	P-value	HR	(95% CI)	P-value
Gender	1.210	(0.801-1.827)	0.365	---	---	---
Age	0.776	(0.519-1.161)	0.217	---	---	---
Smoking	0.974	(0.647-1.466)	0.899	---	---	---
Drinking	0.925	(0.617-1.388)	0.707	---	---	---
Tumor history	1.083	(0.680-1.726)	0.737	1.075	(0.659-1.753)	0.772
Tumor location	1.331	(0.857-2.067)	0.204	1.025	(0.645-1.630)	0.916
Operative method	0.791	(0.519-1.208)	0.278	0.862	(0.561-1.325)	0.499
Tumor size	0.746	(0.491-1.32)	0.169	1.119	(0.707-1.771)	0.632
Tumor depth	0.570	(0.365-0.891)	0.014	0.781	(0.457-1.335)	0.336
Lymph node metastasis	0.305	(0.199-0.465)	<0.001	0.314	(0.149-0.663)	0.002
Tumor differentiation	0.558	(0.334-0.932)	0.026	0.719	(0.424-1.218)	0.220
Tumor stage	0.355	(0.235-0.536)	<0.001	1.112	(0.510-2.427)	0.789
PTPRS	1.950	(1.271-2.992)	0.002	1.573	(1.002-2.468)	0.049

CI, confidence interval; HR, hazard ratio; PTPRS, protein tyrosine phosphatase receptor S.

PTPRS in ESCC. Compared with the matched normal esophageal mucosa tissues, the protein expression of PTPRS in ESCC specimens were significantly reduced. These results indicated that PTPRS might be a candidate tumor suppressor in ESCC and may be a potential therapeutic target. Our observation is in agreement with a series of studies revealing that PTPRS expression is frequently lost or reduced in a number of human cancer tissues, an independent dataset of non-small cell lung cancer and adenocarcinoma of the gastroesophageal junction.

In this study, the ESCC series investigated here for PTPRS expression is well characterized and conventional pathological indicators, including nodal involvement, tumor differentiation, depth of tumor infiltration, and TNM stage, all show the expected prognostic significance. Recent studies indicate that a strong correlation between PTPRS expression and tumor stage, low expression of PTPRS was more frequent in primary advanced-stage ESCC tumors (III/IV) than in earlier stages (I/II) (P=0.013). Reduced expression of PTPRS was significantly correlated with tumor local lymph node metastasis (P=0.042). Our results are consistent with those reported for other human cancers and suggest that PTPRS may be involved in promoting the metastasis of ESCC. Furthermore, we also observed PTPRS expression was significantly correlated with depth of tumor infiltration (P<0.001), and tumor differentiation (P=0.001), and which suggest that PTPRS expression may be involved in esophageal squamous cell carcinoma invasion. Thus, PTPRS expression in ESCC may have a critical role in tumor growth. In contrast, no correlations were observed between PTPRS expression and tumor size, location. This could be due to differences in sample size or to differences in sample processing. These observations demonstrate growth-suppressor characteristics of PTPRS that are typical of a classical tumor suppressor gene.

Our studies reveal that the relationship of low PTPRS expression with poor prognosis of ESCC patient and aggressive tumor characteristics. Kaplan-Meier survival analysis revealed that low expression of PTPRS significantly correlated with poor survival of ESCC patients (P=0.002). Furthermore, Cox regression analysis confirmed PTPRS expression as an independent predictor of the overall survival of esophageal squamous cell carcinoma patients. These results indicated that PTPRS is a candidate tumor suppressor in ESCC. Given these aforementioned correlations, it is not surprising that low PTPRS expression served as a prognostic indicator of worse outcome. In summary, a univariate model including strong prognostic factors such as nodal status, tumor differentiation, tumor depth infiltration, and TNM stage, low PTPRS expression of overall tumors was found to be predictive of poorer outcome for ESCC.

In conclusion, we demonstrated reduced PTPRS expression in esophageal squamous cell carcinoma and its correlation with a more malignant phenotype and poorer prognosis in a large number of clinical samples. In addition, the data generated in the current study represent a valuable report correlating the presence of PTPRS with clinicopathological characteristics and the overall survival of esophageal carcinoma patients. Further studies are needed to fully evaluate the molecular mechanism of PTPRS and ESCC. Thus, PTPRS may play an important role in esophageal squamous cell carcinoma development and progression.

Author Contributions. Conceived and designed the experiments: Rui Wang. Performed the experiments: Qikun Zhu, Tao Wang, Xinyang Liu. Analyzed the data: Hui Zeng. Contributed reagents/materials/analysis tools: Jingjing Wu. Wrote the paper: Guoliang Zhang.

Conflict of interests. The authors declare there is no conflict of interests.

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