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ORIGINAL ARTICLE

PPM1D Functions as Oncogene and is Associated with Poor Prognosis in Esophageal Squamous Cell Carcinoma

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Abstract

Mounting evidence has demonstrated that PPM1D participates in the development and progression of a wide variety of tumors. However, its precise roles in esophageal squamous cell carcinoma (ESCC) remain under investigation. Here, UALCAN, an interactive web-portal to perform the expression analyses of PPM1D using TCGA gene expression data, and PPM1D high expression was exhibited in primary esophageal cancer. Further investigation revealed that PPM1D expression was obviously higher in ESCC tissues than in normal tissues ($P < 0.01$), which was consistent with the results from real-time qPCR and Western blotting in ESCC tissues and paired normal esophageal tissues. Besides, PPM1D expression was closely correlated with TNM staging, tumor differentiation and lymph node metastasis ($P < 0.01$), but not related to the patients' gender and age ($P > 0.05$). Notably, PPM1D expression in metastatic ESCC patients was markedly higher than that in non-metastatic ESCC patients ($P < 0.01$), and the ESCC patients with high PPM1D expression predicted poor prognosis. Multivariate assay demonstrated that PPM1D and lymph node metastasis were considered as independent prognostic factors for the ESCC patients. These findings suggest PPM1D plays a pivotal important role in onset and progression of ESCC, and may be a new biomarker for metastasis and prognosis of the ESCC patients.

Keywords Protein phosphatase Mg^{2+}/Mn^{2+} dependent 1D · Esophageal squamous cell carcinoma · Tumor metastasis · Prognosis

Introduction

Esophageal cancer (EC) is the eighth most frequently occurring form of all tumors and the sixth leading cause of tumor related deaths [1], harboring approximately 455,800 new diagnosed cases and 400,200 deaths occurred in 2012 in the world. There are two main histological types for EC: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) [2]. At present, ESCC, the most common type of EC, with the highest incidence is mainly distributed in Asia (Lin Xian of the china [3], Kashmir of India [4] and Golestan province of Iran [5]) or Africa [6]. Despite recent progress in diagnosis and therapy of ESCC, the actual 5-year survival rate of the patients is only 20%–40% [7, 8]. Consequently, it is in dire need to seek for novel biomarker for diagnosis, therapy and prognosis of the ESCC patients.

The p53 gene, an important transcription factor, is implicated in the manipulation of multiple cell biological processes, including cell cycle, cell growth, apoptosis, senescence and metabolism, etc. [9, 10], and is closely associated with the occurrence, development and progression of a large number of tumors [11]. Protein phosphatase Mg^{2+}/Mn^{2+} dependent 1D (PPM1D) (a wild type p53-induced phosphatase 1, WIP1) negatively regulates some proteins related to DNA damage, such as p53, p38MAPK and ATM [12]. PPM1D, as one of members of the protein phosphatase type 2C (PP2C) and p53 target gene families [13, 14], was verified to be involved in tumorigenesis, which was from the evidence that PPM1D overexpression relieved the senescence of human primary fibrolbasts induced by oncogenic ras [15–17]. More recently, more and more evidence has revealed that PPM1D participates in many tumor development and progression, including pancreatic cancer [18], colorectal cancer [19], non-small cell lung cancer [20], bladder carcinoma [21], and renal

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cell carcinoma [22], etc. Most notably, PPM1D may be an underlying therapeutic and prognostic target in many tumors [20, 23, 24]. These findings highlight the potential value of PPM1D in these tumors, and may provide new directions toward further understanding of the detailed roles and molecular mechanisms of PPM1D in these tumors.

However, to date, it is not available about the data of the roles of PPM1D in ESCC. Therefore, in this study, we detected PPM1D expression in ESCC tissues and corresponding normal esophageal tissues, investigated the association of its expression with clinicopathological features, and eludicated its role in metastasis and prognosis of the ESCC patients. Our findings suggest that PPM1D may function as oncogene and be a novel therapeutic and prognostic target for ESCC.

Materials and Methods

Tissue Samples

One hundred and one cases of ESCC samples and paired normal esophageal samples were obtained from our hospital with informed consent form signed by each patient before surgery. This study was approved by the Ethics Committee of Henan Cancer Hospital. None of the patients with ESCC received any chemotherapy, radiotherapy or other therapies. All the tissues were approved by H&E staining. Fresh ESCC samples and paired normal esophageal samples from patients were immediately frozen in liquid nitrogen for real-time quantitative RT-PCR (real-time qPCR) and Western blotting assay.

TCGA Data Assay

TCGA assay was performed using website http://ualcan.path.uab.edu/index.html according to previous publication [25]. PPM1D gene was submitted to website, and related expression information was displayed.

Immunohistochemistry Assay

Immunohistochemistry (IHC) assay for PPM1D protein expression was carried out according to previous publication [26]. Briefly, the tissue slides were pretreated in 0.01 M citrate buffer for antigen retrieval in a microwave oven for 20 min after deparaffinization and hydration. Subsequently, the tissues slides were incubated with PPM1D primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted using 1: 100 at 4 °C overnight. Signal intensity was developed using DAB staining kit (Beyotime Institute of Biotechnology, Beijing, China). Normal horse serum in place of PPM1D primary antibody was employed as negative control.

Evaluation of Staining

The staining of the slides was performed according to previous publication [27]. Briefly, the staining was blindly scored by two independent investigators based on the percentage of positive staining cells and staining intensity. The percentage of positive staining cells was performed described below: 0 (≤5%), 1 (5%-≤25%), 2 (25%-≤50%), 3 (50%-≤70%) and 4 (>75%). Staining density was considered as the following: 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). The final staining scores were obtained using the percentage of positive staining cells multiplied by staining density. Scores more than or equal than 1 were considered to be positive, and the other cases were regarded as negative.

Real-Time qPCR Assay for PPM1D mRNA Level

Real-time qPCR detection for PPM1D mRNA level was performed according to manufacturer's protocol. Briefly, total RNAs were isolated from ESCC tissues and paired normal esophageal tissues based on standard procedure from manufacturer using Trizol reagent (Invitrogen, CA, USA). Subsequently, reverse transcription reaction and real-time qPCR assay were carried out using FastFire qPCR PreMix (SYBR Green) (TIANGEN Biotech, Beijing, China). PPM1D (NM_003620) and β-actin (NM_001101) primers were designed using NCBI Primer-BLAST: PPM1D forward primer, 5'-GATCCATGGCCAAGGGTGAA-3′, and reverse primer, 5'-AGTCAGGGCTTTAGCGCAAT-3′ (product length: 177 bp); β-actin forward primer, 5'-AACTGGGACGACATGGAGAAAA-3′, and reverse primer, 5′-GGATAGCACAGCCTGGATAGCA-3′, (product length: 192 bp). The final relative expression level was counted according to the formula "$2^{-\Delta\Delta Ct}$", in which β-actin was used as internal control.

Semi-Quantitative RT-PCR Detection for PPM1D mRNA Level

Semi-quantitative RT-PCR assay for PPM1D mRNA level from paraffin-embedded tissue sections using RNA extraction kit (TIANGEN Biotech, Beijing, China) was performed according to manufacturer's instructions. PPM1D primers were designed as follows: Forward primer, 5′- CTAAGGTTTGCGCTGCCATC-3′, and Reverse primer, 5′-AAGTGCTCTTGCTACTGCCA-3′ (Product length: 419 bp), and β-actin primer was consistent with the primers used in real-time qPCR. The relative PPM1D level was counted based on the ratio of PPM1D expression level to β-actin level.

Western Blotting Assay

Western blotting assay for PPM1D protein expression was carried out as described previously [28], using antibodies against PPM1D and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). β-actin was used as a loading standard.

Statistical Assay

All experimental data (means ± SD) were investigated by the SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) using χ^2 test, One-way ANOVA, Log-rank test and Cox proportional hazards model. A $P < 0.05$ was regarded to be statistically significant.

Results

Relative Expression of PPM1D Detected in Esophageal Cancer Using TCGA Database

To investigate the expression pattern of PPM1D gene in ESCC, here, UALCAN, an interactive web-portal to perform the in-depth analyses of TCGA gene expression data. We found the relative expression of PPM1D in primary esophageal cancer was significantly higher than that in normal tissues ($P = 0.027$) (Fig. 1a). Further investigation revealed that the relative expression of PPM1D in adenocarcinoma and ESCC patients was dramatically higher than that in normal patients (both $P = 0.0025$ and $P = 0.036$), besides, the relative expression of PPM1D in adenocarcinoma patients was markedly higher than that in ESCC patients ($P < 0.0001$) (Fig. 1b). Furthermore, PPM1D expression in esophageal cancer patients' gender, age, grade and stage were exhibited in this study (Fig. 1c-f).

Increased PPM1D Expression in ESCC Tissues

To investigate the PPM1D expression in ESCC tissues, IHC was utilized to detect the PPM1D expression in 101 cases of ESCC tissues and paired normal esophageal tissues, which was confirmed by H&E staining (Fig. 2). Besides, we found that PPM1D was mainly localized in cell nucleus of ESCC tissues (Fig. 2), and positive ratio of PPM1D protein expression in ESCC tissues (69.3%, 70/101) was significantly higher than that in normal esophageal tissues (14.9%, 15/101), and the difference was statistical significance ($X^2 = 61.443$, $P = 0.000$) (Table 1). These data suggest that PPM1D may participate in the development and progression of ESCC.

Expressions of PPM1D mRNA and Protein in ESCC Tissues and Corresponding Normal Esophageal Tissues

To verify the validation of the results of IHC, real-time qPCR and Western blotting was employed to investigate the expressions of PPM1D mRNA and protein in randomly selected 6 cases of ESCC tissues and paired normal esophageal tissues. We found that expressions of PPM1D mRNA and protein in ESCC tissues were all significantly higher than those in normal esophageal tissues ($P < 0.05$) (Fig. 3). These findings suggest that PPM1D may be an important regulator in occurrence and development of ESCC.

The Correlations of PPM1D Expression with Clinicopathological Features of ESCC

To further dissect the underlying roles of PPM1D in occurrence and development of ESCC, SPSS17.0 software was employed to investigate the associations of PPM1D protein expression with clinicopathological features of ESCC. The results revealed that PPM1D protein expression was tightly associated with TNM staging, tumor differentiation and lymph node metastasis ($P < 0.01$), but not related to the patients' gender and age ($P > 0.05$) (Table 2). These data suggest that PPM1D may play an essential important role in progression of ESCC.

High PPM1D Expression Predicts Metastasis and Poor Prognosis of the Patients with ESCC

To further explore the roles of PPM1D in metastasis and prognosis of ESCC, semi-quantitative RT-PCR and Kaplan Meier survival assay were utilized to investigate the correlations of PPM1D with metastasis and prognosis of ESCC. We found that the level of PPM1D mRNA in metastatic group (0.971 ± 0.064) is significantly higher than that in non-metastatic group (0.621 ± 0.039) ($t = 4.950$, $P < 0.001$) (Fig. 4a). Stepwise survival assay revealed that the patients with negative PPM1D expression exhibited longer survival time than those with positive PPM1D expression ($P = 0.007$) (Fig. 4b). These findings suggest that PPM1D may be a novel predictor for metastasis and prognosis of ESCC.

Multivariate Analysis

To determine the independent prognostic factors for ESCC, Cox proportional hazards model was used to investigate all significant prognostic factors for the patients with ESCC. The results revealed that lymph node metastasis and PPM1D were independent prognostic factors for the patients with ESCC (Table 3).

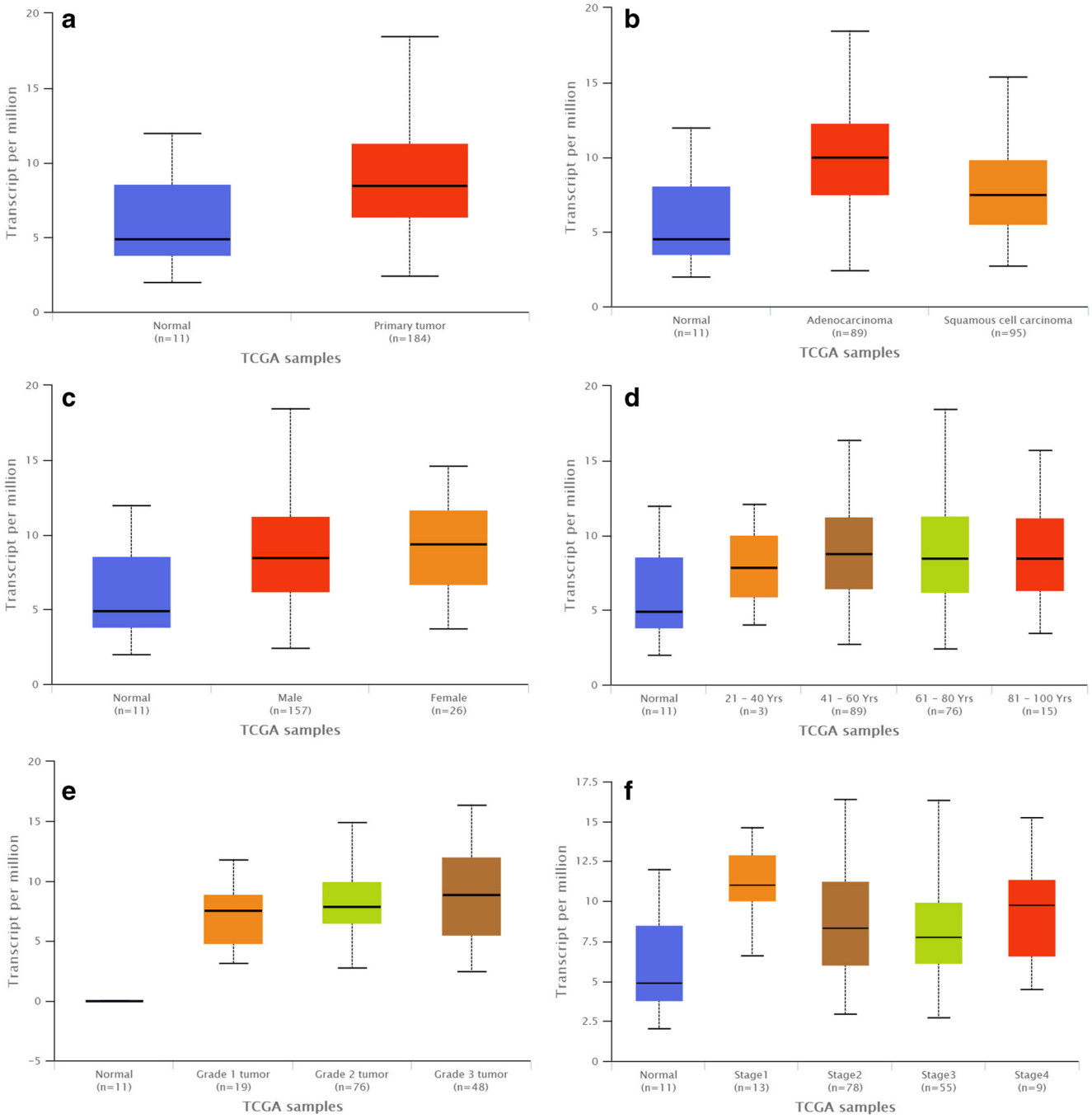

Fig. 1 TCGA database analysis for PPM1D expression and its association with patients' gender, age, tumor grade and stage in ESCC. UALCAN, an interactive web-portal to perform the expression analyses of PPM1D using TCGA gene expression data. **a**: PPM1D expression in normal tissues and primary esophageal cancer tissues; **b**: PPM1D expression in normal tissues, esophageal adenocarcinoma tissues and esophageal squamous cell carcinoma; **c**: PPM1D expression in normal tissues and esophageal cancer tissues with various gender; **d**: PPM1D expression in normal tissues and esophageal cancer tissues with various ages; **e**: PPM1D expression in normal tissues and esophageal cancer tissues with various tumor grade; **f**: PPM1D expression in normal tissues and esophageal cancer tissues with various tumor stage

Discussion

Increasing evidence has demonstrated that PPM1D, a serine/threonine phosphatase, has been verified to be overexpressed in a wide variety of tumors [18, 21, 29]. However, its expression patterns and clinical outcomes in ESCC remain to be elucidated to date. To clarify the expression pattern and clinical significance of PPM1D in ESCC, in the current study, we found increased expression of PPM1D in ESCC tissues by IHC, real-time qPCR and Western blotting. PPM1D expression was tightly associated with TNM staging,

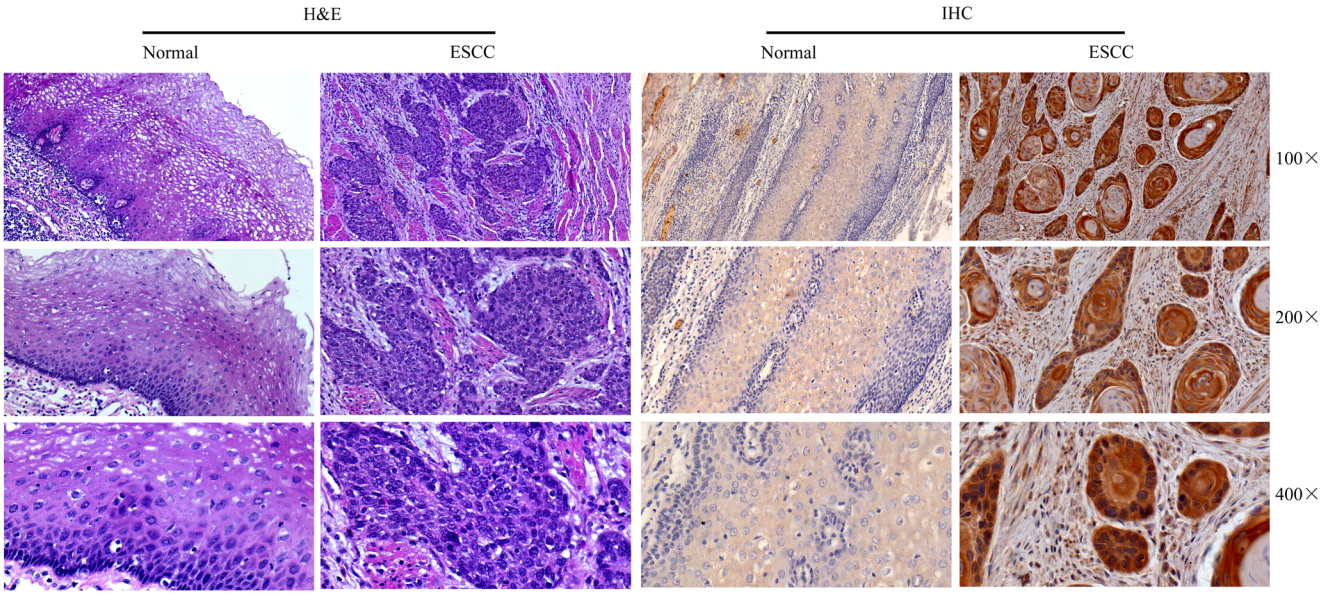

Fig. 2 Expression of PPM1D protein in ESCC tissues and normal esophageal tissues. Sections from formalin-fixed and paraffin-embedded ESCC tissues and normal esophageal tissues were investigated using H&E staining and IHC antibody, representative figures was exhibited using 100×, 200× and 400×magnification

tumor differentiation and lymph node metastasis ($P < 0.01$), but not related to the patients' gender and age ($P > 0.05$). Importantly, PPM1D expression level in metastatic group was significantly higher than that in non-metastatic group, and high PPM1D predicts poor prognosis of the patients with ESCC. Multivariate assay revealed that lymph node metastasis and PPM1D were independent prognostic factors for the patients with ESCC. These findings suggest that PPM1D may participate in the development and progression of ESCC, and may be a novel molecular target for diagnosis and prognosis of the patients with ESCC.

It is well known that PPM1D functions as oncogene in several different tumor types, plays a critical role in tumor development and progression, and thus may be a novel molecular target for many tumors. Lambros MB, et al. revealed that PPM1D was overexperssed and amplified in breast cancers, and overexpression and amplification of PPM1D were tightly related to tumor displaying luminal or phenotypes of HER2 [30], suggesting PPM1D may be a novel diagnostic and therapeutic target for the patients with breast carcinoma. Further investigation from ovarian carcinoma showed that PPM1D mRNA had significantly higher level in ovarian cell carcinoma cell lines harboring amplifications of 17q23.2, PPM1D expression and phosphatase activity were selectively required for the survival of ovarian cell carcinoma cell lines with 17q23.2 amplification, and PPM1D expression is tightly associated with tumor histology [31], implying that PPM1D is a potential therapeutic target for ovarian carcinoma. These findings suggest that PPM1D overexpression may be a potential biomarker in these tumors. To further confirm the expression of PPM1D in ESCC tissues, IHC, real-time qPCR and Western blotting were used to examine the PPM1D expression in ESCC tissues. The results of IHC revealed that PPM1D protein expression in ESCC tissues was significantly higher than that in normal esophageal tissues, which was further supported by real-time qPCR and Western blotting. In addition, we found that PPM1D protein expression was tightly associated with TNM staging, tumor differentiation and lymph node metastasis ($P < 0.01$), but not related to the patients' gender and age ($P > 0.05$). Our findings indicate that PPM1D may function as oncogene in ESCC, and thus may be a diagnostic marker for the patients with ESCC.

Despite rapid development of treatment strategies, the prognosis of the patients with ESCC remains dismal in recent years [32, 33], which may be mainly due to the high prevalence of invasion and metastasis of ESCC

Table 1 Immunohistochemistry assay for PPM1D protein expression in ESCC tissues and normal esophageal tissues

Tissues	N	PPM1D protein expression		X^2	P
		+	−		
ESCC	101	70	31	61.443	0.000
Normal	101	15	86		

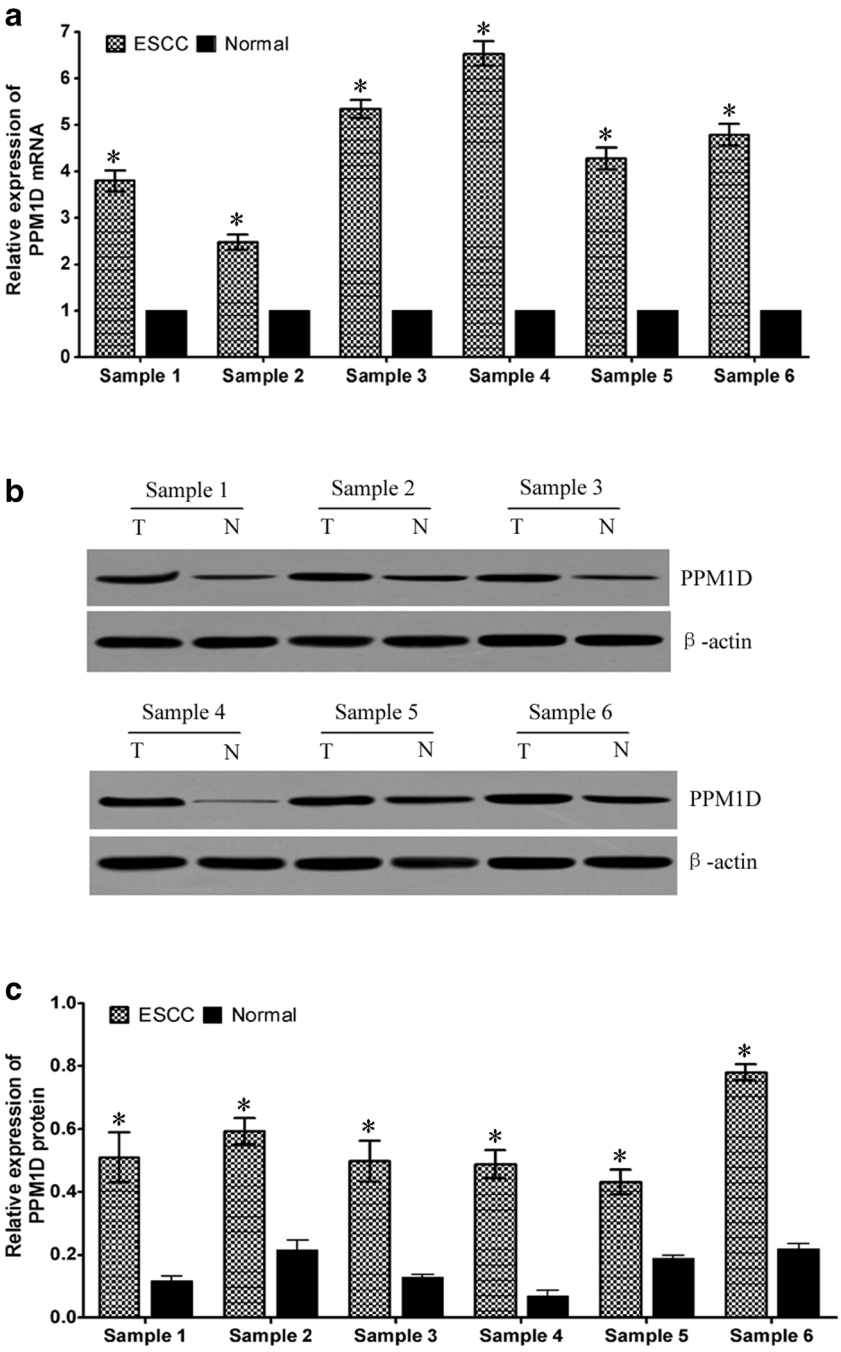

Fig. 3 Real-time qPCR and Western blotting assay for PPM1D mRNA and protein expression in ESCC tissues and normal esophageal tissues. **a**: Real-time qPCR assay for PPM1D mRNA expression in randomly selected 6 cases of ESCC tissues and paired normal esophageal tissues. **b**: Western blotting assay for PPM1D protein expression in randomly selected 6 cases of ESCC tissues (T) and paired normal esophageal tissues (N), and β-actin was utilized as an internal control. **c**: The relative level of PPM1D protein was counted according to the ratio of PPM1D level to β-actin level. * $P < 0.05$, compared with normal esophageal tissues

[34]. Therefore, it is very necessary to identify the factors related to metastasis and prognosis of ESCC. Several investigation has demonstrated that PPM1D overexpression may be a novel prognostic factor in several various tumors, including non-small cell lung carcinoma [20], colorectal cancer [24], hepatocellular carcinoma [35], and ovarian clear cell adenocarcinomas [36], etc. Importantly, PPM1D promotes cell migration and invasion of pancreatic cancer, which potentiates the Wnt/β-catenin pathway through downregulation of apoptosis-stimulating of p53 protein 2 (ASPP2) [18], implying the essential role of PPM1D in the regulation of metastasis of pancreatic cancer. Besides, PPM1D upregulation was tightly correlated with depth of invasion, distant metastasis and lymph node status, and further investigation found increased PPM1D expression

Table 2 The association of PPM1D protein expression with clinicopathological features in ESCC

Characteristics	n	PPM1D protein expression		X^2	P
		+	–		
Gender					
Male	66	47	19		
Female	35	23	12	0.325	0.569
Age					
≥60	60	43	17		
<60	41	27	14	0.387	0.534
TNM staging					
I and II	73	43	30		
III and IV	28	27	1	13.396	0.000
Tumor differentiation					
Well	50	28	22		
Moderate	28	20	8		
Poor	23	22	1	11.726	0.003
Lymph node metastases					
Yes	37	35	2		
No	64	35	29	17.553	0.000

Table 3 Multivariate analysis of ESCC patients by Cox model

Variables	X^2	P value	RR	95% CI
Gender	0.160	0.690	1.156	0.568–2.350
Age	1.234	0.267	0.643	0.295–1.401
Histologic grade	1.067	0.302	1.013	0.989–1.037
Clinical stage	3.452	0.063	1.872	0.966–3.627
Lymph node metastases	4.549	0.033	0.468	0.233–0.940
PPM1D	9.550	0.002	4.036	1.666–9.778

promoted the growth and aggressive phenotype in human clear cell renal cell carcinoma [22], which also implies the important connections between PPM1D and metastasis in human clear cell renal cell carcinoma. To further elucidate the roles of PPM1D in metastasis and prognosis of ESCC, we found that PPM1D expression in metastatic group was markedly higher than that in non-metastatic group, importantly, high PPM1D expression displayed lower survival rate in the patients with ESCC. Further investigation from Cox proportional hazards model revealed that lymph node metastasis and PPM1D were independent prognostic factors for the patients with ESCC. These findings suggest that PPM1D may be an ideal predictor for metastasis and prognosis of the patients with ESCC.

In conclusion, our current data support oncogenic role of PPM1D in development and progression of ESCC. High PPM1D expression is tightly correlated with TNM staging, tumor differentiation and lymph node metastasis. Importantly, the ESCC patients with high PPM1D expression predicts metastatic and poor prognosis. Most notably, PPM1D may be an independent prognostic factor for the patients with ESCC. Our findings provided herein suggest that PPM1D may a novel biomarker for diagnosis, therapy and prognosis of ESCC.

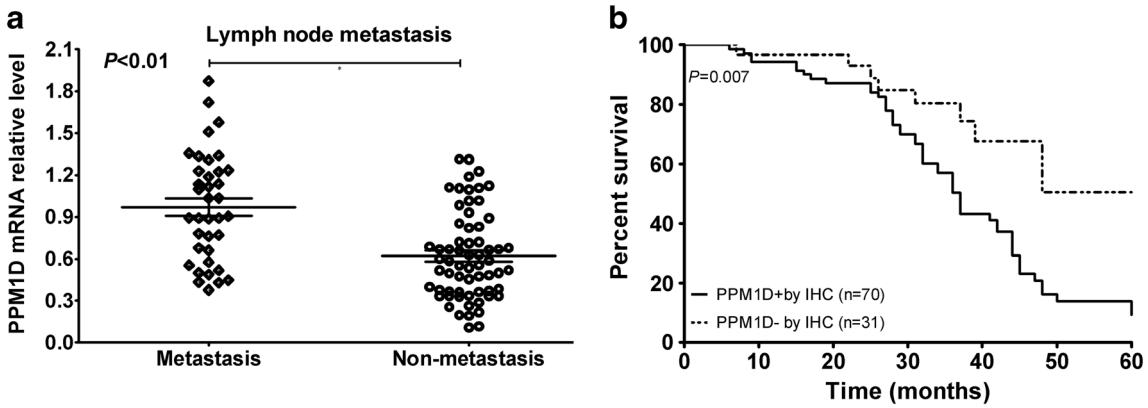

Fig. 4 Predictive value of PPM1D in metastasis and prognosis of ESCC. **a**: Semi-quantitative RT-PCR was employed to detect PPM1D mRNA level in the patients with lymph node metastasis and non-lymph node metastasis, and there was a significant difference in PPM1D mRNA level between metastatic group and non- metastatic group ($P < 0.01$). **b**: High PPM1D level predicts poor prognosis of the patients with ESCC, and Kaplan-Meier survival assay was used to detect the survival rate of the patients with ESCC, $P < 0.05$, indicating a significant difference

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