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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Nuclear PROX1 is Associated with Hypoxia-Inducible Factor 1α Expression and Cancer Progression in Esophageal Squamous Cell Carcinoma

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ABSTRACT

Background. Transcription factor prospero homeobox 1 (PROX1) has been identified as a master regulator of lymphangiogenesis associated with metastasis. Although PROX1 expression has been investigated in several cancers, its clinical significance remains controversial and needs further validation. In this study, we investigated the clinical and functional significance of PROX1 and PROX1 regulator hypoxia-inducible factor 1α (HIF1α) in esophageal squamous cell carcinoma (ESCC).

Methods. A total of 117 samples from ESCC patients were analyzed for PROX1, HIF1α, and E-cadherin expression by immunohistochemistry; correlation with clinicopathological characteristics was determined. PROX1 function was evaluated in PROX1 small interfering RNA (siRNA)-transfected human ESCC cells in vitro by assessing cell proliferation and migration.

Results. PROX1 expression was higher in ESCC than in normal tissues. Patients with higher PROX1 expression ($n = 26$) had increased nuclear accumulation of HIF1α ($p = 0.004$) and more advanced metastasis, both lymph node (N factor; $p = 0.09$) and hematogenous (M factor; $p = 0.04$), than those with lower PROX1 expression ($n = 91$). In addition, high PROX1 and HIF1α expression correlated with low levels of E-cadherin, an epithelial cell marker. Analysis of overall and cancer-specific survival indicated that elevated PROX1 expression was significantly correlated with poor prognosis ($p = 0.0064$). PROX1 downregulation in ESCC cells inhibited cellular proliferation and migration ($p < 0.05$). Hypoxia restored PROX1 levels that were reduced by *PROX1*-specific siRNA.

Conclusion. Our data suggest that high expression of PROX1 in ESCC could be used as an indicator of poor prognosis, and that PROX1 is a promising candidate molecular target for ESCC treatment.

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Esophageal squamous cell carcinoma (ESCC) is a cancer with poor prognosis because of early lymph node metastasis and hematogenous metastasis.[1–3] Therefore, patients with ESCC tend to relapse more frequently compared with those with other gastrointestinal cancers, even after radical resection. To provide optimal treatments and postoperative surveillance for high-risk ESCC patients, a reliable diagnostic and prognostic biomarker for the prediction of cancer recurrence in these patients is required. Moreover, such a marker might be a promising molecular target to control ESCC progression.

Lymphangiogenesis is very important for the establishment of lymph node metastases.[4] Therefore, lymphatic endothelial cell markers such as prospero homeobox 1 (PROX1), vascular endothelial growth factor receptor 3 (VEGFR3), and forkhead box protein C2 (FOXC2) appear to be essential for lymphatic vessel growth and have been investigated in relation to metastatic potential in several cancers.[5,6] In particular, PROX1 has been identified as a master regulator of lymphangiogenesis via induction of VEGFR3 and FOXC2 expression.[7,8] The clinical significance of PROX1 in human solid cancers is controversial, and PROX1 expression has been associated with cancer progression and poor prognosis in hepatocellular carcinoma,[9] colon cancer,[10,11] and malignant astrocytic gliomas.[12] In addition, PROX1 has been reported as a tumor suppressor candidate in hepatocellular carcinoma,[13] neuroblastoma,[14] breast cancer,[15] and pancreatic cancer,[16] suggesting that the oncogenic potential of PROX1 is cancer type-dependent.

In ESCC, the clinical significance of PROX1 is even less clear. A study on human esophageal cancer cells found a loss-of-function RNA mutation in PROX1;[17] it has also been shown that PROX1 suppresses interferon-γ-induced antiproliferative activity in these cells.[18] Thus, the role of PROX1 in ESCC has not been established.

A previous study has demonstrated that PROX1 functions as a stimulator of expression and protein stability of hypoxia-inducible factor 1α (HIF1α).[9] In turn, HIF1α and HIF2α have been reported to activate PROX1 expression.[19] HIF1α, a transcription factor playing an essential role in cellular responses to hypoxia, has been found to regulate epithelial-mesenchymal transition (EMT) and cancer stem cell properties, and to be associated with the recurrence and poor prognosis in several cancers.[20] Therefore, HIF1α has been viewed as a potential target in the treatment of refractory cancers. However, the relationship between HIF1α and PROX1 expression and clinicopathological parameters in ESCC has not yet been investigated.

The purpose of this research was to study the function and clinical significance of PROX1 in ESCC. We examined PROX1 and HIF1α expression in ESCC tissues using immunohistochemistry to evaluate prognostic potential of PROX1 expression in ESCC. Moreover, we inhibited PROX1 expression in ESCC cells in vitro to determine whether PROX1 can be a treatment target in ESCC.

MATERIALS AND METHODS

Clinical Samples and Cell Lines

Surgical specimens were obtained from 117 ESCC patients (103 males and 14 females) who had undergone potentially curative surgery at the Department of General Surgical Science, Gunma University, between 1991 and 2009. Informed consent was obtained according to the Ethical Guidelines for Medical and Health Research Involving Human Subjects (Ministry of Health, Labor and Welfare, and Ministry of Education, Culture, Sports, Science and Technology [MEXT], Japan, 2015). Protocol approval was obtained from an independent review board at Gunma University Hospital. The mean patient age was 63.1 years (range 42–80 years), and the median follow-up period for survivors was 30.3 months (range 1–113 months). ESCC pathological stage was determined based on the 6th edition of the TNM classification of the Union for International Cancer Control (UICC). None of the patients had received irradiation or chemotherapy prior to surgery, or had hematogenous metastases at the time of surgery.

Human ESCC cell lines KYSE140,[21] TE1, TE8, and TE15 were provided by the Japanese Collection of Research Bioresources (JCRB) cell bank and RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. TE8 cells were cultured in RPMI-1640 supplemented with penicillin/streptomycin, and 10 % fetal bovine serum (FBS) in a humidified 5 % CO_2 incubator at 37 °C. Cells were subjected to hypoxia (1 % O_2) using the BIONIX-1 hypoxic culture kit (Sugiyama-Gen, Tokyo, Japan), and were not cross-contaminated with other cell lines as confirmed by short tandem repeat polymerase chain reaction (STR-PCR) in the JCRB cell bank and RIKEN BioResource Center.

Immunohistochemistry

An ESCC tissue microarray was prepared using surgically resected specimens, including noncancerous tissues. Immunohistochemistry was performed as previously described.[22] Two sequential 2-μm slides of each sample were treated with primary antibodies against PROX1 (1:100) and HIF1α (1:300) (both from Abcam, Tokyo, Japan), for 24 h at 4 °C. Negative control slides were incubated without the primary antibodies, and no detectable staining was observed.

Nuclear levels of PROX1 and HIF1α were evaluated as follows: low expression (0, no staining; 1, weak staining) and high expression (2, moderate-to-strong nuclear staining) (Electronic Supplementary Fig. 1).

Fluorescent Immunohistochemistry

After endogenous peroxidase was blocked, the sections were boiled in citrate buffer (pH 6.4) for 15 min in a microwave. Nonspecific binding sites were blocked by incubation with Protein Block Serum-Free Reagent for 30 min, and the sections were incubated with the primary antibodies against PROX1 (1:400), HIF1α (1:1000), and E-cadherin (1:500; Cell Signaling Technology, Tokyo,

Japan) for 1 h at room temperature. Multiplex covalent labeling (PROX1, cyanine 3; HIF1α and E-cadherin, fluorescein) with tyramide signal amplification (Opal™ 3-Plex Kit; Perkin Elmer, Waltham, MA, USA) was performed according to the manufacturer's protocol. All sections were counterstained with DAPI and examined under an All-in-One BZ-X710 fluorescence microscope (Keyence Corporation, Osaka, Japan).

PROX1 RNA Interference

PROX1-specific small interfering RNA (siRNA) [Silencer Pre-Designed siRNA] purchased from Bonac Corporation (Kurume, Fukuoka, Japan) was mixed with HilyMax transfection reagent (Dojindo Laboratories, Kumamoto, Japan) in six-well flat-bottom microtiter plates. TE8 cells were seeded in 2 mL RPMI-1640 in the microtiter plates and incubated with siRNAs at 37 °C for 24 h before analysis using Western blot.

Protein Extraction and Western Blotting

Total proteins were extracted from ESCC cells using PRO-PREP (iNtRON Biotechnology, Kyungki-Do, Korea), separated by SDS-PAGE using 10 % gels, and transferred to membranes which were incubated overnight at 4 °C with the antibodies against PROX1 (1:2000), HIF1α (1:1000), and β-actin (1:4000) [Sigma, St. Louis, MO, USA], followed by horseradish peroxidase-conjugated secondary antibodies. Specific signals were detected using the ECL Prime Western Blotting Detection System (GE Healthcare, Tokyo, Japan) and quantified using an Image Quant LAS 4000 instrument (GE Healthcare).

In Vitro Proliferation Assay

TE8 cells transfected for 48 h were plated in 96-well plates (3.0×10^4 cells/well) in 10 % FBS–RPMI-1640. After 48 h, 10 μL of CCK-8 solution (CCK-8; Dojindo Laboratories) was added to each well for 2 h at 37 °C, and the absorbance was detected at 450 nm using an xMark™ Microplate Absorbance Spectrophotometer (Bio Rad, Hercules, CA, USA).

In Vitro Migration Assay

Cell migration was analyzed by chemotaxis assay using 24-well Falcon Cell Culture Inserts (pore size, 8 μm). TE8 cells (8.0×10^4) were seeded in the upper chamber, and the lower chamber was filled with 750 μL of 10 % FBS–RPMI-1640. After 24 h incubation at 37 °C, the cells that migrated to the lower surface of the filter were fixed with 4 % paraformaldehyde, stained with Giemsa Stain Solution (Wako, Osaka, Japan), and then counted under a microscope. A total of six randomly chosen fields were evaluated in duplicate assays.

Statistical Analysis

For continuous variables, the data were expressed as mean ± SD. The association between PROX1 and HIF1α expression and clinicopathological factors, as well as the in vitro data, was analyzed using Student's t test, Chi square test, and analysis of variance (ANOVA). Overall and cancer-specific survival were measured from the day of surgery and plotted according to the Kaplan–Meier method; the log-rank test was used for comparison. Differences were considered statistically significant at $p < 0.05$. Cox proportional hazards regression was used to test PROX1 independent prognostic contribution. All statistical analyses were performed using the JMP software package (SAS Institute Inc., Cary, NC, USA).

RESULTS

Immunohistochemical Analysis of (Prospero Homeobox 1 (PROX1) and Hypoxia-Inducible Factor 1α (HIF1α) Expression in Esophageal Squamous Cell Carcinoma (ESCC) Tissues

PROX1 and HIF1α nuclear expression in ESCC specimens was evaluated by immunohistochemistry. PROX1 expression was scored high in 26 (22.2 %) ESCC samples and low in 91 (77.8 %) ESCC samples, while HIF1α expression was scored high in 42 (35.9 %) ESCC samples and low in 75 (64.1 %) ESCC samples; representative images are shown in Fig. 1. Our analysis revealed a direct correlation between HIF1α and PROX1 nuclear expression, i.e. patients with high PROX1 demonstrated higher HIF1α levels than those with low PROX1 expression (Fig. 1).

In normal esophageal epithelium, PROX1 and HIF1α immunofluorescence was almost undetected (Fig. 1b), but in ESCC tissues, both transcription factors were significantly upregulated (Fig. 1c). Expression of the epithelial marker E-cadherin was examined to validate the relationship between EMT, PROX1, and HIF1α in ESCC. The results indicate that in almost all ESCC cells, E-cadherin expression was not co-localized with that of PROX1 and HIF1α (Electronic Supplementary Fig. 2a, b, respectively).

Association Between PROX1 Expression and Clinicopathological Features of ESCC

Correlations between PROX1 expression, patients' clinicopathological characteristics (age, sex, tumor location

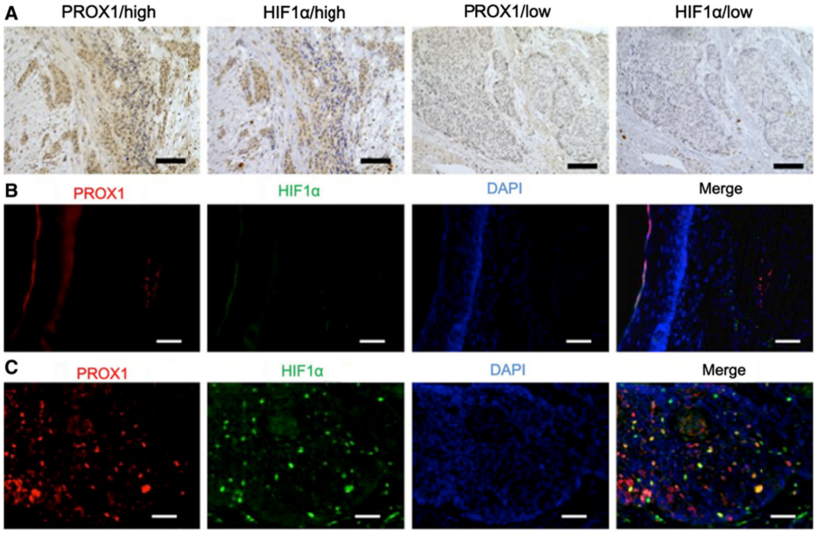

FIG. 1 Immunostaining analysis of PROX1 and HIF1α expression in ESCC tissue samples. **a** Deparaffinized sections of ESCC tumors were stained with anti-PROX1 and anti-HIF1α antibodies and counterstained with hematoxylin. *Left panels*: ESCC patients with high levels of PROX1 expression in tumors demonstrated enhanced HIF1α expression. *Right panels* ESCC patients with low levels of PROX1 expression in tumors demonstrated decreased HIF1α expression. Original magnification: ×100; scale bar, 100 μm. **b**, **c** Noncancerous squamous epithelial cells (**b**) and ESCC cells (**c**) were immunostained with the antibodies against PROX1 (*red*) and HIF1α (*green*). All sections were counterstained with DAPI (*blue*). *Scale bar* 50 μm. *PROX1* prospero homeobox 1, *HIF1α* hypoxia-inducible factor 1α, *ESCC* esophageal squamous cell carcinoma

and histology, T, N and M factors, lymphatic and venous invasion, and pathological stage), and HIF1α levels are shown in Table 1. The results indicate that patients with high and low PROX1 expression in tumors differed in HIF1α levels and metastasis: the first group had increased HIF1α nuclear accumulation ($p = 0.004$) and a higher degree of metastasis, both in regional lymph nodes (N factor; $p = 0.09$) and distant tissues (M factor; $p = 0.04$). However, no significant differences were observed in age, sex, tumor location and histology, T factor, lymphatic and venous invasion, or pathological stage.

Prognostic Significance of PROX1 Expression in ESCC Patients

Overall survival and cancer-specific survival of ESCC patients with high PROX1 tumors was significantly lower than that of patients with low PROX1 tumors ($p = 0.01$ and $p = 0.005$, respectively) (Fig. 2a, b). Multivariate analysis indicated that high PROX1 expression in ESCC tissues was an independent prognostic marker of poor survival, similar to T and N factors ($p = 0.0064$) (Electronic Supplementary Table 1).

The results were consistent with overall and cancer-specific survival of ESCC patients with high HIF1α expression in tumors, which were significantly lower than that of patients with low HIF1α expression ($p = 0.0022$ and $p = 0.0155$, respectively) (Electronic Supplementary Fig. 3a, b).

PROX1-Specific Small Interfering RNA (siRNA) Inhibits Cancer Cell Proliferation and Migration In Vitro

PROX1 was found to be highly expressed in all analyzed ESCC cell lines (Fig. 3a). We chose two *PROX1*-specific siRNAs (siRNA1 and siRNA2) to investigate the effect of PROX1 inhibition on functional parameters in TE8 cells. Both species of *PROX1* siRNA significantly reduced PROX1 levels compared with control siRNAs; however, the effect was abrogated when TE8 cells were subjected to hypoxia, which restored PROX1 expression to control levels (Fig. 3b and Electronic Supplementary Fig. 4a). In normoxic conditions, HIF1α protein was not detected in *PROX1* siRNA-transfected TE8 cells (data not shown); however, it was elevated in hypoxic cells (Fig. 3b and Electronic Supplementary Fig. 4b). The reduction in

TABLE 1 Clinicopathological characteristics and HIF1α nuclear levels in 117 ESCC patients stratified by PROX1 expression

Factors	Nulclear PROX1 expression		*p* value
	Low $n = 91$	High $n = 26$	
Age	62.5 ± 8.1	65.2 ± 6.8	0.12
Gender			
Male	80	23	1
Female	11	3	
Location			
Cervical	0	1	0.4
Upper thoracic	11	4	
Middle thoracic	38	10	
Lower thoracic	37	9	
Abdominal	5	2	
Histology			
Well	21	2	0.16
Moderate	50	19	
Poorly	20	5	
T factor			
Tl	19	4	0.36
T2	10	6	
T3	58	14	
T4	4	2	
N factor			
Absent	22	2	0.09**
Present	69	24	
M factor			
M0	61	17	0.04*
M1a	9	0	
Mlb	15	9	
Lymphatic invasion			
Absent	2	1	0.53
Present	88	25	
Venous invasion			
Absent	11	5	0.34
Present	79	21	
Stage			
I, II	35	7	0.35
III, IV	56	19	
HIF-1α nuclear expression			
Low	65	10	0.004*
High	26	16	

* $p < 0.05$; ** $p < 0.1$

PROX1 expression significantly inhibited ESCC cell proliferation (Fig. 3c) and migration (Fig. 3d), indicating the involvement of PROX1 in the ability of cancer cells to metastasize.

DISCUSSION

In this study, we demonstrated that high PROX1 expression correlated with the increase in HIF1 and decrease in E-cadherin levels, and also with cancer progression, and that PROX1 expression is an independent prognostic factor for ESCC patients. These results are supported by the findings in vitro showing that PROX1 regulates proliferation and migration of ESCC cells.

PROX1 expression in ESCC tumors was higher than that in noncancerous tissues. PROX1 is known to be regulated by the hypoxia-induced transcription factors HIF1α and HIF2α,[19] COUP-TFII,[23] NF-κB,[24] SOX18,[25] and HOXD8,[7] as well as by Kaposi sarcoma herpes virus[26] in vascular endothelial cells and cancer cells. In ESCC tumors, the expression of HIFs[27] and NF-κB[28] was higher than in noncancerous tissues, and was associated with cancer progression, therapeutic resistance, and poor outcome. In this study, we showed that hypoxia induced PROX1 expression in *PROX1* siRNA-transfected cancer cells, suggesting that in ESCC, PROX1 may be one of the important downstream target genes regulated by hypoxia-induced transcription factors and NF-κB in promoting cancer progression.

PROX1 expressed in lymphatic endothelial cells is considered to play an important role in lymphangiogenesis.[29,30] In cancer patients, high PROX1 expression was correlated with lymphatic invasion and lymph node metastasis (N factor).[31,32] In our study, no significant association of high PROX1 expression with lymphatic invasion was found; however, it tended to correlate with N factor. The reason might be that, among the 117 ESCC patients, only three did not have lymphatic invasion. To investigate the relationship between PROX1 and lymphatic invasion in ESCC, it may be important to evaluate PROX1 expression in early ESCC.

Cancer development depends on the metastatic cascade characterized by the induction of EMT in migrating cancer cells originating from both primary tumors and metastatic sites.[33–35] EMT is induced by many cancer-related signaling molecules, including HIF1, NF-κB, FOXC2, TGF-β, integrins, Wnt/β-catenin, receptor tyrosine kinases, Notch, matrix metalloproteinases, and microRNAs.[33,36] Previous reports indicate that PROX1 regulates some of these EMT inducers, including HIF1,[37] FOXC2,[7] integrin α9,[38,39] β-catenin/TCF,[10] and microRNA-9.[11] In this study, ESCC patients with high PROX1 expression also demonstrated increased HIF1α nuclear accumulation, reduced expression of epithelial marker E-cadherin, and increased lymph node and hematogenous metastases, while cancer cells with inhibited PROX1 expression demonstrated slower proliferation and migration. These findings indicate that in

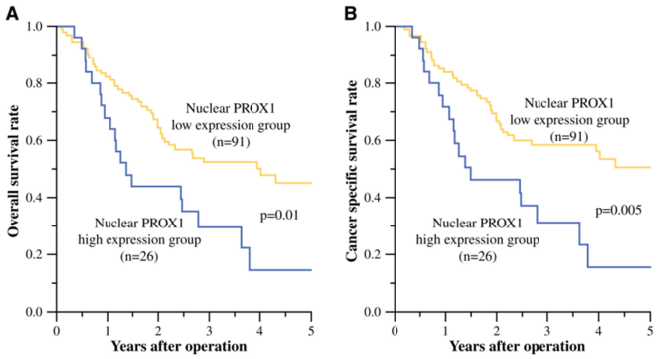

FIG. 2 Correlation of postoperative survival with PROX1 expression. **a** Overall survival of ESCC patients according to PROX1 expression in the nuclei. ESCC patients with high PROX1 expression in tumors ($n = 26$) had significantly lower overall survival compared with those with low PROX1 expression ($n = 91$; $p = 0.01$). **b** Cancer-specific survival of ESCC patients according to PROX1 expression in the nuclei. ESCC patients with high PROX1 expression had significantly lower cancer-specific survival compared with those with low PROX1 expression ($p = 0.005$). *PROX1* prospero homeobox 1, *ESCC* esophageal squamous cell carcinoma

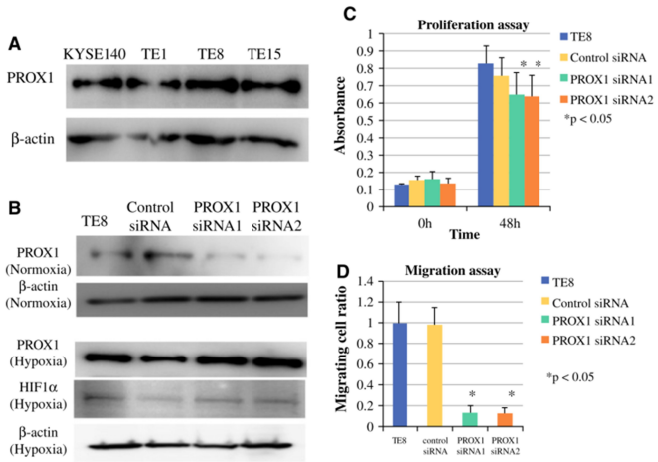

FIG. 3 Proliferation and migration of ESCC cells transfected with *PROX1*-specific siRNA. **a** PROX1 expression in the ESCC cell lines KYSE140, TE1, TE8, and TE15 was analyzed using Western blotting; β-actin was used as a loading control. **b** TE8 cells were transfected with *PROX1*-specific siRNA (PROX1 siRNA1 and 2), and protein expression was analyzed using Western blotting; β-actin was used as a loading control. In normal conditions (normoxia, *upper panels*), PROX1 expression was reduced in *PROX1* siRNA-transfected TE8 cells. In the cells subjected to hypoxic conditions, the expression of PROX1 and HIF1α was induced (*lower panels*). **c** Proliferation of TE8 cells was analyzed using the CCK-8 assay. **d** Migration of TE8 cells was analyzed using the chemotaxis assay. TE8 cells transfected with *PROX1*-specific siRNA showed significantly reduced proliferation and migration compared with control siRNA-transfected and wild-type TE8 cells. Data are expressed as mean ± SD. *ESCC* esophageal squamous cell carcinoma, *PROX1* prospero homeobox 1, *siRNA* small-interfering RNA, *CCK-8* Cell Counting Kit-8, *HIF1α* hypoxia-inducible factor 1α, * indicates $p < 0.05$

ESCC, PROX1 regulates metastatic potential by inducing EMT via HIF1α.

Importantly, our data also suggest that PROX1 may be a promising prognostic biomarker and a new candidate for targeted therapy in ESCC. PROX1 has been shown to have dual functions as a tumor suppressor and tumor promoter in several cancers.[9,16–18] In search of the explanation for functionally diverse roles of PROX1 in cancer, we noted a report that PROX1 inhibited proliferation of hepatocellular carcinoma cells by inducing p53-dependent senescence-like phenotype, suggesting a relationship of PROX1 with cell proliferation.[40] Using next-generation sequencing analysis, other studies have reported that the frequency of p53 mutations in ESCC (92 %) is much higher than that in hepatocellular carcinoma (20.8 %).[41,42] PROX1 functions as a tumor suppressor in hepatocellular carcinoma and neuroblastoma, where p53 somatic mutations are rare.[43] These findings suggest that p53 status may regulate PROX1 function in cancer cells. In this study, PROX1 knockdown in TE8 cells harboring p53 mutations inhibited cell proliferation and migration. Although PROX1 has been demonstrated to act as a tumor suppressor, our results strongly suggest that PROX1 expression is associated with poor prognosis and can be a new therapeutic target in patients with p53-mutated ESCC tumors.

CONCLUSIONS

Elevated PROX1 expression is a factor contributing to shorter survival in ESCC patients, and may be used as a prediction biomarker for poor prognosis. PROX1 expression was associated with both local lymph node and distant metastases in ESCC patients, and with the ability for proliferation and migration of ESCC cells. Our results suggest that PROX1 may be a promising molecular target in ESCC.

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