Oncogenic role of protein tyrosine kinase 7 in esophageal squamous cell carcinoma

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Esophageal squamous cell carcinoma (ESCC) is a common subtype of esophageal cancer that is particularly prevalent in East Asian countries. Our previous expression profile analysis showed that the gene encoding protein tyrosine kinase 7 (PTK7) is upregulated in ESCC tissues. Here, we aimed to validate PTK7 as a prognostic factor and a candidate target for molecular treatment of ESCC. Both RT-PCR and Western blot analysis of tissues from ESCC patients revealed that PTK7 was significantly upregulated in tumor tissue samples of ESCC. Immunohistochemical staining of PTK7 showed that increased expression of PTK7 was inversely correlated with overall survival ($P = 0.021$). In vitro knockdown of PTK7 inhibited proliferation, survival, wound healing, and invasion of ESCC cells. In addition, PTK7 knockdown decreased phosphorylation of Akt, Erk, and focal adhesion kinase (FAK), important determinants of cell proliferation, survival, and migration. Therefore, our findings suggest that PTK7 has potential as a prognostic marker for ESCC and might also be a candidate for targeted therapy in the treatment of ESCC. (Cancer Sci 2013; 104: 1120–1126)

Esophageal cancer is one of the most devastating malignancies. Despite recent developments in its management, advanced esophageal cancer is associated with high rates of local recurrence and distant metastasis.[1,2] Although there has been improvement in clinical outcome through multidisciplinary treatment strategies,[3–5] such as total mediastinal lymph node dissection,[6] and new diagnostic modalities such as positron emission tomography,[7] these developments have not proven satisfactory in preventing the high recurrence rates. Currently, the prognosis is only promising if the tumor is detected early and resected completely.

Esophageal squamous cell carcinoma (ESCC) is the most common histologic subtype of esophageal cancer in East Asian countries.[8] It has a unique profile of clinical and anatomical characteristics that is distinct from those of esophageal adenocarcinoma, which is more prevalent in North America, UK, and Australia.[8] To develop prognostic diagnosis and effective therapeutics for ESCC, the identification of biomarkers is of vital importance. In a gene expression profiling analysis to pursue biomarkers, we identified protein tyrosine kinase 7 (PTK7; also known as colon carcinoma kinase-4 or CCK-4), as a candidate biomarker for ESCC.[9]

Protein tyrosine kinase 7 is a receptor tyrosine kinase-like molecule containing an extracellular domain with seven immunoglobulin-like loops, a transmembrane domain, and a defective tyrosine kinase domain that resembles a catalytic domain but lacks catalytic activity.[10–12] Mice expressing a truncated form of PTK7 protein die perinatally, revealing a defect in neural tube closure and stereociliary bundle orientation. These findings implicate PTK7 as a regulator of planar cell polarity

(PCP).[13] It has been shown that PTK7 recruits RACK1, which affects Dsh recruitment by interaction with PKCδ1.[14] Interaction between PTK7 and Dsh at the plasma membrane activates non-canonical Wnt signaling, which then directs PCP.[15] Also, PTK7 interacts with β-catenin, enhancing β-catenin-dependent transcriptional events.[16] These studies suggest that PTK7 plays a role in the canonical Wnt pathway, the non-canonical Wnt pathway, and the PCP signaling pathway.

Increased PTK7 expression has been reported in several types of cancer, including colon cancer,[11,17] gastric cancer,[18] lung cancer,[19] acute myeloid leukemia,[20,21] and liposarcoma.[22] Expression of PTK7 in leukemia cells enhances cell migration and survival.[20] In addition, PTK7 knockdown has been shown to inhibit proliferation and invasion of liposarcoma cells,[22] as well as proliferation and antiapoptotic activity of colon cancer cells.[23] Moreover, treatment with the entire extracellular domain of PTK7 (soluble PTK7) acting as a decoy receptor or knockdown of PTK7 prevented vascular endothelial growth factor-induced migration, invasion, and tube-formation of HUVECs, and angiogenesis in vivo.[24] However, PTK7 expression has been shown to be decreased in metastatic melanoma[25] and clear cell renal cell carcinoma.[26] In addition, chromosome 6p,[27] on which the PTK7 gene is located, was found to be deleted in breast carcinoma[28] and malignant melanoma.[29]

Although the role of PTK7 in different cancers has not been studied comprehensively, PTK7 is likely to have a tumor-promoting role in general. To evaluate the prognostic value of PTK7 in ESCC, we first analyzed expression of PTK7 by RT-PCR and Western blotting using tumor and adjacent normal tissue samples from ESCC patients. Second, we examined the expression pattern of PTK7 in surgical specimens of 157 ESCC patients by immunohistochemical (IHC) staining and investigated the correlation between expression of PTK7 and parameters of clinical outcome. Finally, we analyzed the oncogenic properties of PTK7 and PTK7-mediated signaling pathways by PTK7 knockdown in ESCC cell lines.

Materials and Methods

Patients and preparation of tissue samples. This study was approved by the institutional review board of Korea Cancer Center Hospital. For RT-PCR and western blot analysis, frozen samples of tumor and adjacent healthy esophageal tissue were obtained from patients who underwent surgery for ESCC in Korea Cancer Center Hospital (Seoul, Korea). For IHC staining, paraffin blocks were obtained from 157 patients who underwent curative surgical resection for thoracic ESCC from 1999 to 2006 at Korea Cancer Center Hospital and were followed up

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until January 2012. Surgical resection was carried out with total mediastinal, lesser curvature, and common hepatic lymph node dissection, followed by evaluation of the resection margins. Pathologic staging was based on the 7th edition of the American Joint Committee on Cancer's Cancer Staging Manual.

RNA isolation and RT-PCR. Total RNA was isolated from frozen esophageal samples (ESCC tissue and adjacent normal epithelium) using an RNeasy Mini Kit (Qiagen, Cambridge, MA, USA). The RT-PCR was carried out as described previously.[24]

Western blot analysis. Protein extraction from frozen tissues was carried out using tissue extraction reagent II (Invitrogen, Carlsbad, CA, USA) containing protease inhibitors (Roche, Basel, Switzerland). Cultured cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM NaF, 1 mM Na_3VO_4) supplemented with protease inhibitor cocktail I (Calbiochem, La Jolla, CA, USA) for 10 min at 4°C. Lysates containing 50 µg protein were subjected to SDS-PAGE and transferred onto a nitrocellulose or PVDF membrane. Western blotting was carried out using: anti-phospho-Erk, anti-Erk, anti-focal adhesion kinase (FAK), and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phospho-Akt (Ser473) and anti-Akt (Cell Signaling Technology, Beverly, MA, USA); anti-phospho-FAK (Tyr397) (Millipore, Billerica, MA, USA); and HRP-conjugated secondary antibodies (KOMA Biotech, Seoul, Korea and Santa Cruz Biotechnology). Rabbit anti-PTK7 anti-serum, which recognizes the extracellular domain of both human and murine PTK7, was used as described previously.[24] The immune reactions were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and a LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

Immunohistochemical staining. The IHC staining was carried out using a tissue array technique. Paraffin blocks with a diameter of 3 mm were punched out and arranged into 6 × 4 block tissue arrays, which were sectioned at 4-µm thickness. After paraffin removal and dehydration, the sections were quenched with 3% H_2O_2 in methanol for 20 min and transferred to warm 10 mM citrate buffer solution (pH 6) for 10 min. After washing with PBS (137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, and 2 mM KH_2PO_4; pH 7.4), the sections were incubated with primary antibody in a humid chamber at room temperature for 1 h. The sections were then washed with PBS, incubated with HRP polymer conjugate for 15 min at room temperature, and DAB Chromogen (ScyTek Laboratory, West Logan, UT, USA) was added for 5 min at room temperature. After staining, micrographs were taken and evaluated for intensity of staining and percentage of stained cells by a pathologist who was blind to the clinical information. The intensity of staining was scored in a 0–10 scale, with the following intensity score ranges: 0–3, weak; 4–7, moderate; and 8–10, strong.

Cell culture. Human ESCC cell lines TE-5, TE-9, TE-10, TE-11, and TE-14 were obtained from the RIKEN BioResource Center (Tsukuba, Japan). Human colon cancer SW480 cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). The HEK293 cell line expressing SV40 T antigen (HEK293T) was kindly provided by Professor Jang-Hee Hahn (Kangwon National University, Chuncheon, Korea). These cells were grown in DMEM (HyClone, South Logan, UT, USA) supplemented with 5% FBS (HyClone), 100 U/mL penicillin, and 100 µg/mL streptomycin. All cells were grown at 37°C in 5% CO_2 and 95% air.

Generation of PTK7-knockdown lentivirus and infection of ESCC cells. Lentiviruses were produced in HEK293T cells cotransfected with: 10 µg pLKO.1-Control, pLKO.1-shRNA-PTK7-6433, or pLKO.1-shRNA-PTK7-6434 plasmid (Sigma-

Aldrich, St. Louis, MO, USA); 7.4 µg packaging vector psPAX2 (Addgene, Cambridge, MA, USA); and 2.6 µg envelope vector pMD2.G (Addgene). The ESCC cells were infected with lentiviruses according to the manufacturer's recommendations (Addgene). The infected cells were cultured with 2.5 µg/mL puromycin for 14 days, to produce puromycin-resistant colonies as a mixed culture.

Cell proliferation, survival, wound healing, and invasion assays. For cell the proliferation assay,[30] cells (0.1 mL, 4×10^4 cells/mL) in a 96-well plate were grown in DMEM supplemented with 5% FBS over a 7-day period. For the cell survival assay,[31] cells (0.1 mL, 5×10^4 cells/mL) were plated in 96-well plates, allowed to attach for 4 h, and treated with 0–80 µM 5-fluorouracil (5-FU) for 48 h. Viable cells were measured with the colorimetric MTT assay.

For the wound healing assay, a wound was introduced by scraping the monolayer with a micropipette tip. The cells were incubated for 24 h in DMEM supplemented with 5% FBS, fixed with 3.7% formamide, and evaluated by light microscopy.

The invasion assay was carried out using a Transwell system (Corning-Costar, Lowell, MA, USA) as described previously.[24] Briefly, detached cells (0.1 mL, 5×10^5 cells/mL) were loaded into the upper chamber which was coated with 10 µL of 0.1% gelatin and 80 µL of 0.3 µg/mL growth-factor reduced Matrigel (BD Biosciences, Bedford, MA, USA) on the lower and upper surfaces, respectively. DMEM (0.6 mL) supplemented with 5% FBS as a chemoattractant was added to the lower chamber. After 24 h, invaded cells on the lower surface of the filter were stained with H&E and analyzed under a light microscope.

Statistical analysis. The IHC grade and various survival parameters (sex, age, pathologic stage, differentiation, and adjuvant and neoadjuvant therapy) were analyzed with respect to recurrence-free survival and overall survival using a multivariate Cox proportional hazard model. The software SPSS version 14.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

All data were obtained from at least three independent experiments and expressed as the mean ± standard deviation. Statistical significance was analyzed by Student's t-test. A P-value less than 0.05 was considered significant.

Results

Expression of PTK7 increased in ESCC samples. Expression levels of PTK7 mRNA and protein in tumor and normal tissue pairs from 20 and 10 ESCC patients, respectively, were determined by RT-PCR and Western blot analysis. The ESCC tissue showed significantly increased expression of PTK7 at both the mRNA and protein levels compared with normal esophageal epithelial tissue (Fig. 1).

Characteristics of ESCC patients analyzed for IHC staining. Among the 157 ESCC patients analyzed for IHC staining, the mean age was 62.4 years and 150 patients were male. All patients underwent a trans-thoracic esophagectomy, after which the esophagus was reconstructed by interposition of the stomach or colon. There were 19 patients with pathologic stage I disease, 36 with stage II, 40 with stage IIIA, 26 with stage IIIB, and 36 with stage IIIC (Table 1). The mean duration of follow-up was 44.5 months.

Most ESCC tissues stained for PTK7. Each specimen was subjected to IHC staining of PTK7. Most normal epithelia were not stained, with a few exceptions showing stained benign epithelia or stroma. In contrast, most tumor cell nests stained positive for PTK7 expression (Fig. 2). Both the intensity of staining and the percentage of stained tumor cells were semiquantitatively scored. Examples for the lowest (Score 0) and

highest (Score 10) intensity of staining are shown in Figure 2(a,b), respectively. The specimens were classified according to intensity and number of stained tumor cells on a
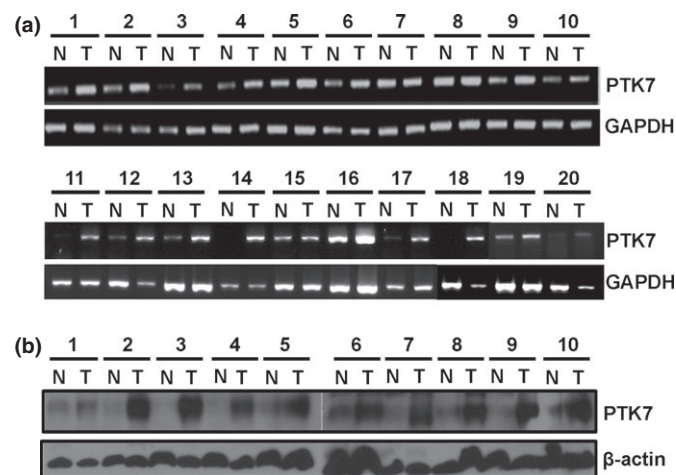

Fig. 1. Expression of protein tyrosine kinase 7 (PTK7) in tumor samples from 10 patients with esophageal squamous cell carcinoma. (a) Levels of PTK7 mRNA determined by RT-PCR. The GAPDH mRNA levels are shown for normalization. Numbers 1–20 are patient identifiers. (b) Western blot showing PTK7 protein levels. β-Actin levels are shown for normalization. Numbers 1–10 are patient identifiers. N, healthy esophageal epithelium, adjacent to the tumor; T, tumor tissue sample.

3-point scale (Fig. 2c). The number of specimens with each staining grade was 67 for grade 1, 61 for grade 2, and 29 for grade 3 (Fig. 2d–f). Approximately 60% of specimens showed moderate to strong staining for PTK7 (grade 2 or 3). Among the 67 cases classified as grade 1, three specimens were completely negative for PTK7 whereas another 17 specimens showed staining in fewer than 10% of tumor cells. Apart from these exceptions, over 80% of the total cases displayed PTK7 expression. In most specimens classified as grade 2 or 3, PTK7 staining mainly localized to the cell membranes of tumor cells. Notably, in well-stained tumor regions, unstained portions of tumor cell nests were sporadically observed (Fig. 2g). In addition, in fewer than 10% of the specimens, several cells within the stromal tissue surrounding a tumor cell nest stained positive for PTK7 (Fig. 2h). Intraepithelial neoplastic lesions around tumor cells which were found in some specimens were usually stained for PTK7. These findings suggest that PTK7 expression is upregulated even at the early stages of tumor development.

High expression of PTK7 correlates with poor prognosis in patients with ESCC. Using the follow-up data of the 157 patients in conjunction with the results from the PTK7 IHC staining experiments, we analyzed which tumor characteristics affected recurrence-free survival and overall survival. The mortality rate during the follow-up period was 71% ($n = 113$). Analysis of recurrence-free survival showed that pathologic stage was the only significant parameter ($P = 0.001$) when analyzed with a multivariate Cox proportional hazard model (Table 1). The PTK7 staining grade determined by IHC did

Table 1. Clinical parameters and recurrence-free and overall survival after surgery in patients with esophageal squamous cell carcinoma ($n = 157$)

Prognostic factor	No. (%)	Recurrence-free survival			Overall survival		
		HR	95%CI	P-value	HR	95%CI	P-value
Gender				0.710			0.800
Female	7 (4.5)	1.00			1.00		
Male	150 (95.5)	1.22	0.44–3.35	0.710	1.14	0.41–3.15	0.800
Age, years (>65)	62.4 ± 7.6			0.610			0.150
<65	96 (61.1)	1.00			1.00		
65	61 (38.9)	1.37	0.89–2.22	0.610	1.37	0.89–2.22	0.150
Pathologic stage				0.001*			0.005*
Stage I	19 (12.1)	1.00			1.00		
Stage II	36 (22.9)	1.02	0.73–1.49	0.760	1.17	0.98–1.35	0.570
Stage IIIA	40 (25.5)	1.75	1.10–2.95	0.029*	1.68	1.11–3.31	0.025*
Stage IIIB	26 (16.6)	2.07	1.12–3.21	0.015*	1.92	1.13–3.98	0.210
Stage IIIC	36 (22.9)	2.68	1.39–5.20	0.003*	2.98	1.51–5.85	0.002*
Neoadjuvant chemotherapy				0.216			0.089
No	150 (95.5)	1.00			1.00		
Yes	7 (4.5)	1.22	0.72–4.10	0.216	2.03	0.89–4.60	0.089
Adjuvant chemotherapy				0.520			0.560
No	129 (82.2)	1.00			1.00		
Yes	28 (17.8)	0.85	0.51–1.41	0.520	0.86	0.52–1.43	0.560
Adjuvant radiotherapy				0.890			0.880
No	134 (85.4)	1.00			1.00		
Yes	23 (14.6)	0.89	0.60–1.81	0.890	1.04	0.60–1.81	0.880
Degree of differentiation				0.910			0.890
Well	36 (22.9)	1.00			1.00		
Moderate	103 (65.6)	1.14	0.74–1.74	0.840	1.10	0.71–1.69	0.820
Poor	18 (11.5)	1.39	0.51–2.84	0.900	1.19	0.69–1.99	0.880
IHC staining grade				0.131			0.163
Grade 1	67 (42.7)	1.00			1.00		
Grade 2	61 (38.9)	1.23	0.80–1.79	0.360	1.16	0.75–1.79	0.510
Grade 3	29 (18.5)	1.61	0.97–2.65	0.064	1.81	1.09–2.99	0.021*

*$P < 0.05$. Immunohistochemical (IHC) staining: grade 1, weak staining or fewer than 40% of tumor cells stained; grade 2, moderate to strong staining and 40–70% of tumor cells stained; grade 3, moderate to strong staining and more than 70% of tumor cells stained. CI, confidence interval; HR, hazard ratio.
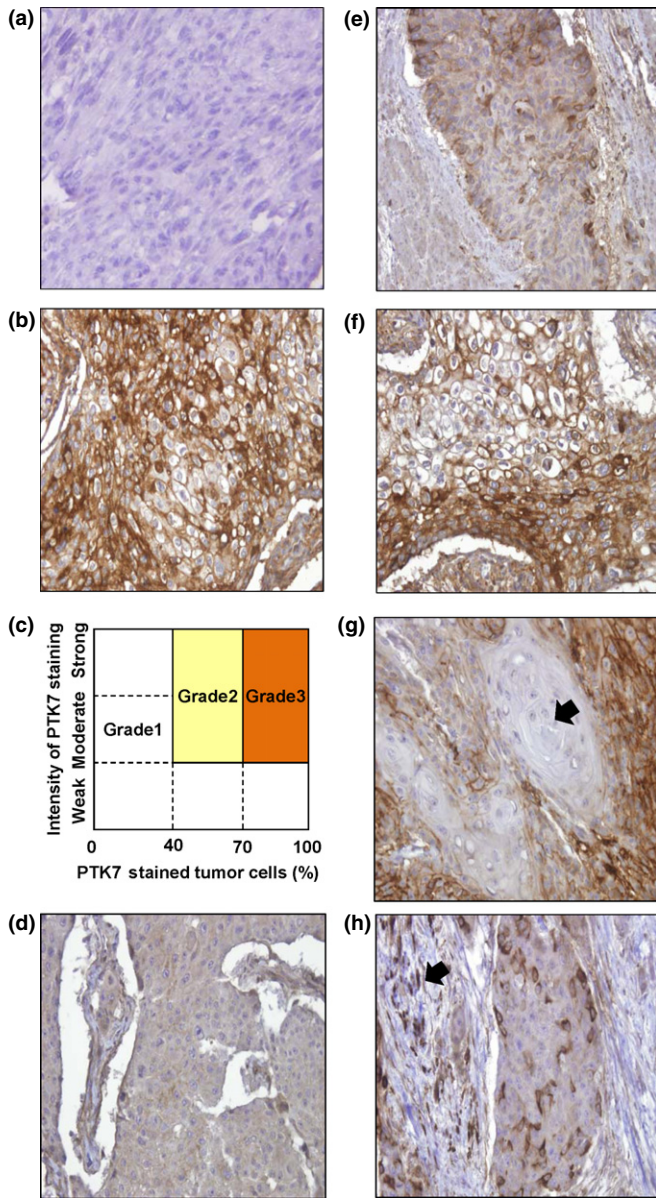
Fig. 2. Immunohistochemical (IHC) analysis of esophageal squamous cell carcinoma tissue stained for protein tyrosine kinase 7 (PTK7). Representative images of poorly stained tumor cells (Score 0) (a) and heavily stained tumor cells (Score 10) (b). (c) Classification of IHC staining grade. Grade 1, weak staining or fewer than 40% of tumor cells stained; grade 2, moderate to strong staining and 40–70% of tumor cells stained; grade 3, moderate to strong staining and more than 70% of tumor cells stained. (d–f) Representative images of PTK7 IHC grades 1–3, respectively. (g) PTK7-negative tumor cell nest. Some differentiated tumor cell nests (arrow) were found to be negative for PTK7 expression. (h) PTK7-positive stromal cells. Some stromal cells (arrow) around a tumor cell nest were positive for PTK7. Magnification, ×200.

Fig. 3. Analysis of survival rates of patients with esophageal squamous cell carcinoma according to protein tyrosine kinase 7 (PTK7) immunohistochemical staining grade. (a) Kaplan–Meier curves for recurrence-free survival of each grade. (b) Kaplan–Meier curves for overall survival of each grade. Grade 1, weak staining or fewer than 40% of tumor cells stained; grade 2, moderate to strong staining and 40–70% of tumor cells stained; grade 3, moderate to strong staining and more than 70% of tumor cells stained.

not correlate significantly with recurrence-free survival, although patients with IHC staining classified as grade 3 tended to have poorer recurrence-free survival than patients with grade 1 or 2 staining (Fig. 3a). However, analysis of overall survival showed that the IHC staining grade clearly correlates with prognosis of ESCC (Fig. 3b). Multivariate analysis of overall survival also showed that grade 3 staining was associated with worse prognosis than grade 1 ($P = 0.021$) and indicated a correlation between prognosis and the
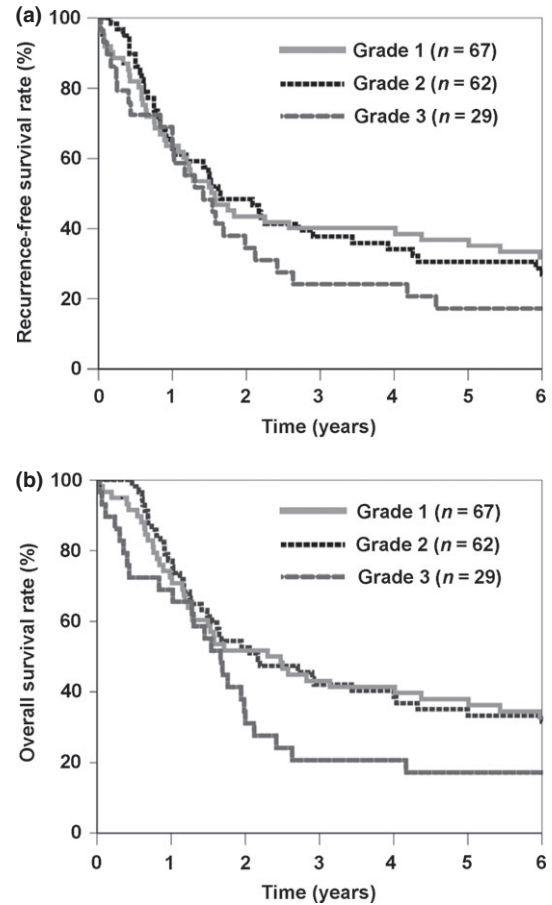
pathologic stage of the tumor (Table 1). However, there was no significant association between the IHC staining grade and clinical parameters (Table S1). In conclusion, our analysis reveals that PTK7 IHC staining grade and pathologic stage were significant factors influencing overall survival, whereas age, gender, and neoadjuvant and adjuvant therapy did not affect prognosis of ESCC patients significantly.

Knockdown of PTK7 decreases the oncogenic potential of ESCC cells *in vitro*. To investigate the oncogenic role of PTK7 in ESCC, a knockdown model for the PTK7 gene was applied in ESCC cell lines. Among ESCC cell lines TE-5, TE-9, TE-10, TE-11, and TE-14, the expression level of PTK7 was highest in TE-10 and TE-11 cells (Fig. S1a). Therefore, PTK7 knockdown was carried out in TE-10 and TE-11 cells by infection with one of two different PTK7-silencing lentiviruses, shRNA-PTK7-6433 and shRNA-PTK7-6434 (Fig. S1b).

Using the PTK7 knockdown ESCC cells, we analyzed the effect of PTK7 on cell proliferation and survival using an MTT assay. In cell proliferation assay, the number of TE-10 and TE-11 cells infected with control lentiviral vector increased to 313% and 306% of the original number, respectively, after 7 days of culture in the presence of serum (Fig. 4a). However, in the same cell lines infected with PTK7-knockdown lentiviruses, the number of cells was
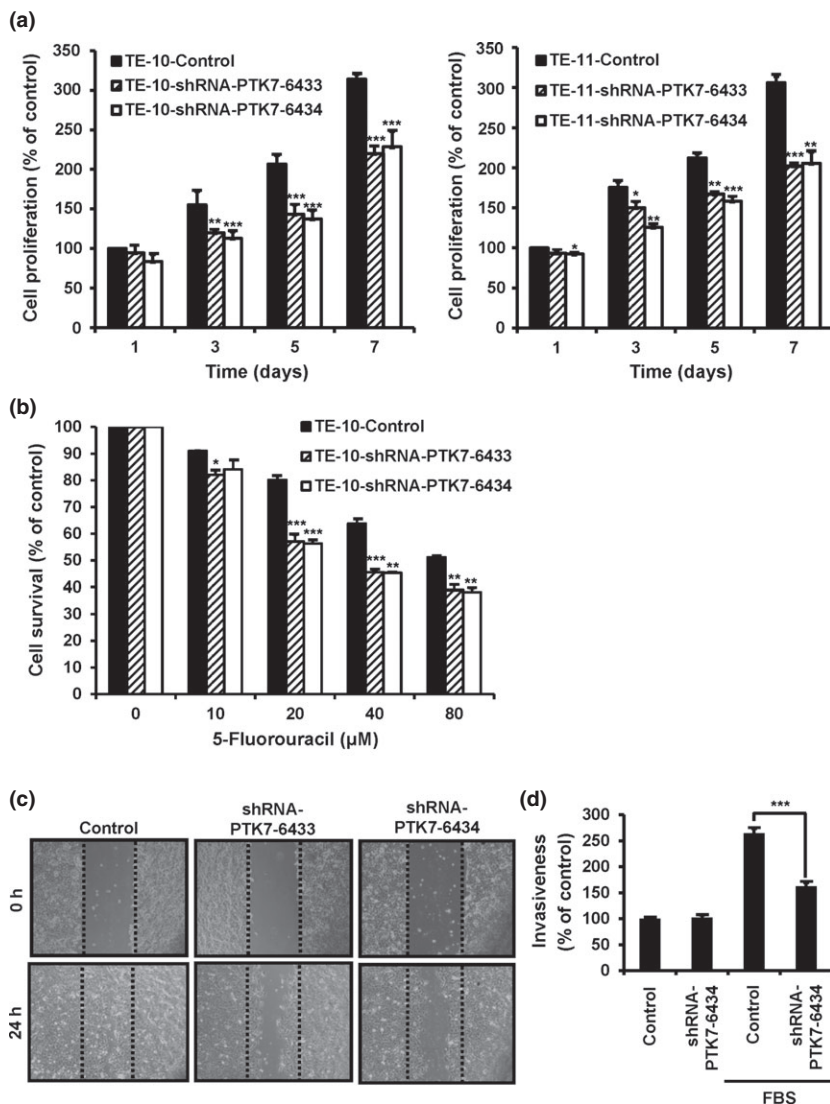
Fig. 4. Effects of protein tyrosine kinase 7 (PTK7) knockdown on oncogenic properties of esophageal squamous cell carcinoma (ESCC) cell lines. The ESCC cells were infected with lentivirus harboring a puromycin resistance gene (control *vs* shRNA-PTK7-6433 *vs* shRNA-PTK7-6434) and mixed cultures of puromycin-resistant colonies were used. (a) Effect of PTK7 knockdown on cell proliferation in TE-10 (left panel) and TE-11 (right panel) cells. (b) Effect of PTK7 knockdown on cell survival of 5-fluorouracil (5-FU)-treated TE-10 ESCC cells. (c) Effect of PTK7 knockdown on wound healing in TE-10 ESCC cells. Light micrographs (×100) showing wound healing in monolayers of TE-10 cells with or without PTK7 knockdown. (d) Effect of PTK7 knockdown on Matrigel invasion of TE-10 ESCC cells. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *vs* control.

approximately 65–75% that of the control cells at 5 and 7 days of culture. In the survival assay of 5-FU-treated TE-10 cells, treatment with 20 μM 5-FU, at which the most significant statistical difference was observed, showed 80% survival of control cells but 55% survival of PTK7-knockdown cells (Fig. 4b).

We next investigated the role of PTK7 on wound healing and invasion of TE-10 cells. In the wound healing assay, control cells repaired the wound in 24 h, whereas PTK7-knockdown cells showed significantly delayed wound repair (Fig. 4c). Regarding invasiveness, in the absence of serum the invasion of control cells was similar to that of PTK7-knockdown cells. When serum was added as an attractant, invasion of control cells increased 2.6-fold, whereas invasion of PTK7-knockdown cells was approximately 60% of that shown by the control cells (Fig. 4d).

Knockdown of PTK7 inhibits activation of Akt, MAPKs, and FAK in ESCC cells. Activation of PI3-Kinase/Akt and MAPK pathways is an important process during tumorigenesis and is closely linked to cell proliferation and survival.[32] In addition, FAK activation is involved in cell migration and invasion.[33] Therefore, we analyzed the role of PTK7 in the activation of Akt, MAPKs, and FAK. In both TE-10 and TE-11 cells, PTK7 knockdown significantly decreased the phosphorylation levels

of Akt, Erk, JNK, p38 MAPK, and FAK (Fig. 5). This result suggests that PTK7 modulates signaling pathways involving Akt, Erk, JNK, p38 MAPK, and FAK to enhance the proliferation, survival, migration, and invasion of ESCC cells.

Discussion

Esophageal squamous cell carcinoma is the most prevalent subtype of esophageal cancer in East Asian countries, accounting for over 90% of total cases, whereas esophageal adenocarcinoma is more prevalent in North America, UK, and Australia. The carcinogenic mechanisms and biological and clinical characteristics of ESCC differ from those seen in adenocarcinoma. In ESCC, smoking, alcohol abuse, lower socioeconomic status, and consumption of extremely hot beverages have been reported as significant predisposing factors. For adenocarcinoma, known risk factors are gastroesophageal reflux disease and obesity.[34]

In our preliminary study, we detected upregulation of the PTK7 mRNA level by gene expression profiling in ESCC, but not in normal esophageal tissues or in other thoracic squamous cell carcinoma specimens (data not shown). In the present study, we confirm that PTK7 is significantly upregulated compared with adjacent normal tissue at both the mRNA and
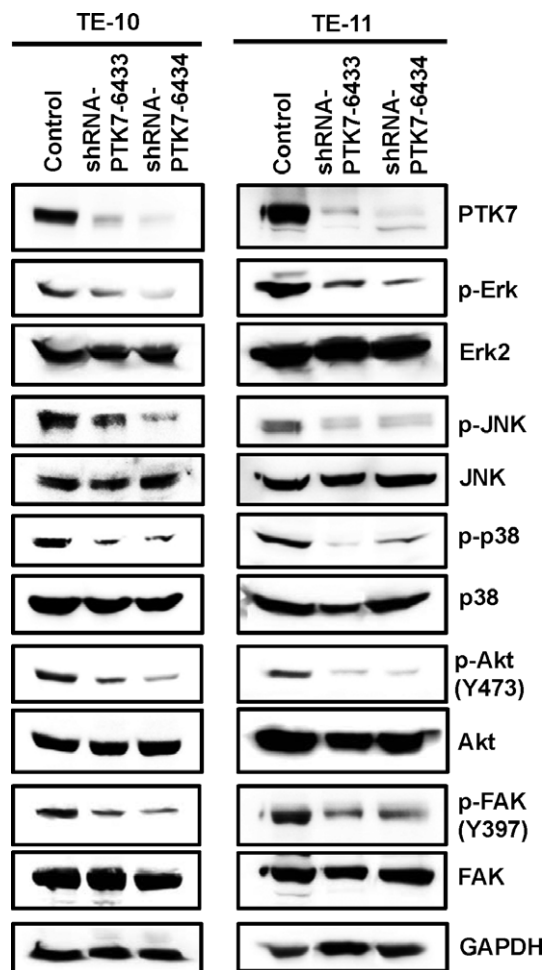
Fig. 5. Effect of protein tyrosine kinase 7 (PTK7) knockdown on activation of signal molecules in TE-10 and TE-11 esophageal squamous cell carcinoma cells. Expression of signaling proteins Akt, Erk, JNK, p38 MAPK, and focal adhesion kinase (FAK) was examined by Western blot analysis in TE-10 (left panel) and TE-11 (right panel) cells with or without PTK7 knockdown. The GAPDH levels are shown for normalization.

protein levels in tumor samples of ESCC patients. Moreover, we found moderate to strong staining for PTK7 through IHC in over 60% of ESCC specimens. Multivariate analysis correlating clinical outcome parameters of ESCC patients with tumor characteristics yielded pathologic stage as the only statistically significant determinant of recurrence-free survival. However, when correlating tumor characteristics with overall survival, PTK7 IHC staining grade and pathologic stage were identified as independent prognostic factors. Thus, we propose

that PTK7 IHC staining grade could be used as a prospective prognostic determinant of ESCC.

Although previous studies have reported a decrease in PTK7 expression levels in metastatic melanoma[25] and clear cell renal cell carcinoma,[26] PTK7 has been shown to be upregulated in many types of human malignancy, including colon cancer, lung carcinoma, liposarcoma, gastric cancer, and acute myeloid leukemia.[11,17–20,22] Prebet *et al.*[20] reported that increased expression of PTK7 is correlated with poor clinical outcome in acute myeloid leukemia, a finding that was linked to increased cell migration and resistance to apoptosis. Meng *et al.*[23] showed that knockdown of PTK7 in colon cancer cells inhibits proliferation and induces caspase-10-dependent apoptosis through the mitochondrial pathway.

Consistent with those studies, we showed here that PTK7 knockdown decreases proliferation of TE-10 and TE-11 ESCC cells and survival of TE-10 cells, and inhibits wound healing and invasion of TE-10 cells. A previous study showed that soluble PTK7 treatment, which has an effect comparable to that of PTK7 knockdown, inhibited vascular endothelial growth factor-induced activation of FAK and paxillin in HUVECs.[24] Focal adhesion kinase is involved in the adhesion and migration of cells[35] and, more specifically, in tumor invasion and lymph node metastasis in ESCC.[36] Activation of Akt and MAPKs promotes cell survival, proliferation, and migration.[37,38] We found that knockdown of PTK7 decreased phosphorylation of Akt, Erk, JNK, p38 MAPK, and FAK in ESCC cells. Therefore, given our *in vitro* findings regarding PTK7-dependent proliferation, survival, wound healing, and invasion, we propose that PTK7 may be involved in tumorigenesis and metastasis of ESCC cells, specifically through the activation of factors that promote proliferation, cell survival, migration, and invasion.

In summary, we showed upregulation of PTK7 in clinical samples of ESCC and highlighted the oncogenic role of PTK7 in ESCC cells *in vitro*. Increased expression of PTK7 was inversely correlated with overall survival of ESCC patients, and grading of IHC samples based on PTK7 expression was able to predict ESCC prognosis in a multivariate analysis. Our findings consistently suggest that PTK7 is a potentially valuable prognostic marker for ESCC and might be applied as a therapeutic target molecule in the management of ESCC.

Acknowledgments

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. χ^2-test for the cross-tabulations of immunohistochemical staining grade and other categorical clinical variables ($n = 157$).

Fig. S1. Protein tyrosine kinase 7 (PTK7) expression and knockdown in esophageal squamous cell carcinoma cell lines.