Catalytically defective receptor protein tyrosine kinase PTK7 enhances invasive phenotype by inducing MMP-9 through activation of AP-1 and NF-κB in esophageal squamous cell carcinoma cells

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ABSTRACT

Protein tyrosine kinase 7 (PTK7), a member of the catalytically defective receptor protein tyrosine kinase family, is upregulated in various cancers including esophageal squamous cell carcinoma (ESCC). Here, we have explored the molecular mechanism of PTK7-dependent invasiveness in ESCC cells. PTK7 knockdown reduced gelatin degradation and MMP-9 secretion in cultures of ESCC TE-10 cells, and showed reduced levels of MMP9 mRNA using real-time RT-PCR and luciferase reporter assays. PTK7 knockdown decreased not only phosphorylation of NF-κB, IκB, ERK, and JNK, but also nuclear localization of NF-κB and AP-1 consisting of c-Fos and c-Jun. Activation of AP-1 and NF-κB requires PTK7-mediated activation of tyrosine kinases, including Src. In addition, NF-κB activation by PTK7 involves the PI3K/Akt signaling pathway. PTK7-mediated upregulation of MMP9 was also observed in other ESCC cell lines and in three-dimensional cultures of TE-10 cells. Moreover, MMP-9 expression positively correlated with PTK7 expression in ESCC tumor tissue. These findings demonstrate that PTK7 upregulates MMP9 through activation of AP-1 and NF-κB and, thus increases invasive properties of ESCC cells.

INTRODUCTION

Protein tyrosine kinase 7 (PTK7) consists of an extracellular domain with seven immunoglobulin-like loops, a transmembrane domain, and a tyrosine kinase domain that lacks detectable kinase activity; therefore, PTK7 is a member of the family of catalytically defective receptor protein tyrosine kinases (RPTKs) [1–4]. Homozygosity for a truncated PTK7 gene was perinatally lethal in mice and associated with severe developmental defects, including defective neural tube closure [5]. PTK7 mutant mice phenotypically overlap with knockdown mice of Vangl2, a core PCP gene. PTK7 also interacts with Wnt/PCP ligand, and induces JNK activation during morphogenetic movements in Xenopus [7]. These findings suggest that PTK7 regulates PCP, canonical and non-canonical Wnt signaling pathways during development.

PTK7 is upregulated in esophageal squamous cell carcinoma (ESCC) [8], colorectal cancer [9, 10], and other cancers [11–15]. PTK7 enhances proliferation, survival, and migration of various cancer cells [8, 11, 13, 16]. PTK7 increases activation of ERKs, JNK, and p38 in ESCC and vascular endothelial cells [8, 17], and decreases expression of BAX and cleavage of caspase-3, -8, and -9 in cholangiocarcinoma [15]. In colon cancer and ovarian cancer, PTK7 sensitizes canonical Wnt and non-canonical Wnt/PCP pathways, respectively [6, 18]. However, PTK7 also has a tumor-suppressive role in some cancer types [19–22]. The mechanism(s) underlying the contradictory roles played by PTK7 in different cancer types is unclear. Recently, we demonstrated that PTK7 displays phenotypes
ranging from oncogenic to tumor-suppressive depending on its concentration relative to those of its binding partners, such as kinase insert domain receptor (KDR) [17]. Our finding of a biphasic function of PTK7 explains in part the discrepancy in the expression-level-dependent oncogenic functions of PTK7.

In a previous report, we described increased PTK7 expression in tumor tissue of ESCC patients and its correlation with poor prognosis [8]. Moreover, PTK7 knockdown inhibited invasiveness and other oncogenic phenotypes of ESCC cells. In an attempt to identify a proteolytic enzyme responsible for the PTK7-mediated invasiveness, we performed fluorescent gelatin degradation assay and gelatin zymography. We identified matrix metalloproteinase (MMP)-9 as an enzyme responsible for the invasiveness, analyzed signaling pathways involved in induction of MMP-9, and described the molecular mechanism underlying PTK7-mediated invasiveness in ESCC TE-10 cells. We also demonstrate the correlation of PTK7 expression and MMP-9 induction in multiple ESCC cell lines and patients.

RESULTS

PTK7 knockdown inhibits gelatin degradation by reducing MMP-9 secretion in ESCC TE-10 cells

We analyzed whether PTK7 stimulates focal proteolytic degradation of extracellular matrix (ECM) components in ESCC TE-10 cell cultures using a fluorescent gelatin degradation assay. Two lines of PTK7 knockdown cells, PTK7-KD-6433 and PTK7-KD-6434, showed significantly decreased degradation of FITC-labeled gelatin compared to control vector-transfected cells (Figure 1). To examine whether the gelatinases MMP-2 and MMP-9 are involved in PTK7-mediated gelatin degradation, extent of gelatin degradation was analyzed in TE-10 cells overexpressing tissue inhibitor of metalloproteases (TIMP)-1 and TIMP-2 (Figure 2A). TIMP-1 expression significantly reduced gelatin degradation to the similar extent as PTK7 knockdown in TE-10 cells. However, TIMP-2 expression inhibited gelatin degradation poorly in TE-10 cells. It is known that TIMP-1 inhibits both MMP-2 and MMP-9 and that TIMP-2 inhibits MMP-2, but not MMP-9 [23]. Thus, this observation suggests that PTK7-induced gelatin degradation is mediated by increased MMP-9 secretion in TE-10 cells.

To further confirm this finding, conditioned medium of control vector-transfected and PTK7-knockdown cells was analyzed by gelatin zymography and western blotting (Figure 2B). MMP-9 secretion was significantly decreased in PTK7 knockdown cells compared to control cells. Expression of exogenous PTK7-FLAG in the PTK7 knockdown cells restored MMP-9 secretion (Figure 2B, left panel). Moreover, gradual decrease of PTK7 expression using single and double transfection of two knockdown vectors is in parallel with decrease of MMP-9 secretion in TE-10 cells. PTK7 knockout almost completely abolished MMP-9 secretion (Figure 2B, right panel). MMP-2 secretion was not detected in ESCC TE-10 cells, regardless of PTK7 expression status. These results demonstrate that PTK7 is indispensable for MMP-9 expression in TE-10 cells.

PTK7 shedding is not involved in PTK7-induced MMP-9 secretion in TE-10 cells

We previously reported that a cytosolic domain (CTF2) of PTK7 generated by sequential cleavage by ADAM17 and γ-secretase translocates into nucleus and enhances oncogenic phenotype of colon cancer cells [24]. However, the extracellular domain of PTK7, which can be produced by its shedding, was not detected in the conditioned medium of TE-10 cells (Supplementary Figure S1A). Incubation with GW280264X (ADAM 17/10 inhibitor) and/or DAPT (gamma-secretase inhibitor) to inhibit generation of PTK7-CTF2, did not change the secreted MMP-9 level in TE-10 cells (Supplementary Figure S1A). Therefore, we assume that PTK7-CTF2 is not produced in TE-10 cells. In addition, ectopic expression of PTK7-CTF2 did not change the secreted MMP-9 level although some of the expressed PTK7-CTF2 was detected in nucleus (Supplementary Figure S1B). Therefore, we conclude that PTK7-CTF2 does not affect induction of MMP-9 expression in TE-10 cells.

PTK7 knockdown decreases transcription of MMP9 mRNA in TE-10 cells

To examine whether decreased MMP-9 secretion level is the result of decreased MMP9 mRNA level, control vector-transfected cells and PTK7-knockdown cells were analyzed by reverse transcription (RT)-PCR and reporter assays. In PTK7-KD-6433 and PTK7-KD-6434 cells, MMP9 mRNA levels were reduced to 20% and 15% and PTK7 mRNA levels were reduced to 26% and 12%, respectively, compared to control cells (Figure 3B). Consistent with the absence of secreted MMP-2 (Figure 2B), MMP2 mRNA was not detected in TE-10 cells (Figure 3A). Luciferase activity driven by the MMP9 promoter was decreased to 45% and 39% of control values in PTK7-KD-6433 and 6434 cells, respectively (Figure 3C). These findings show that PTK7 upregulates MMP9 at the transcriptional level.

PTK7 knockdown inhibits phosphorylation of IκB, NF-κB, and mitogen-activated protein kinases (MAPKs) in TE-10 cells

MMP9 gene expression is increased primarily by transcription factors AP-1 and NF-κB [25]. Therefore, we
analyzed phosphorylation of IκB and NF-κB, as indicators of NF-κB activation status, and of ERK and JNK, as indicators of AP-1 activation status, in PTK7 knockdown cells (Figure 4A). Phosphorylation of IκB, NF-κB, ERK, and JNK was reduced in the PTK7 knockdown cells, and nuclear levels of AP-1 consisting of c-Fos and c-Jun, and NF-κB were decreased in the PTK7 knockdown cells compared to control cells (Figure 4B). These observations show that PTK7 upregulates MMP-9 by activating the NF-κB and AP-1 signaling pathways.

**RPTKs and Src are involved in PTK7-mediated activation of NF-κB and AP-1 in TE-10 cells**

To elucidate the signal transduction pathway involved in PTK7-mediated NF-κB activation, phosphorylation of various signaling proteins in the presence of signaling inhibitors was analyzed in control and PTK7 knockdown cells. Total tyrosine phosphorylation was decreased in PTK7-KD-6433 and -6434 cells compared to control vector cells (Figure 5A). Because a dramatic decrease in tyrosine phosphorylation was detected in proteins of approximately 60 kDa and 125–130 kDa, phosphorylation levels of p60<sup>Src</sup>, p125<sup>FAK</sup>, and p130<sup>CAS</sup> were analyzed. Phosphorylation of all three proteins was decreased in PTK7 knockdown cells (Figure 5B). Incubation of control vector-transfected TE-10 cells with a pan-PTK inhibitor (genistein), a multitarget RPTK inhibitor (TKI-258), and Src family kinase inhibitors (PP1 and PP2) decreased not only tyrosine phosphorylation of total cellular proteins and Src, but also phosphorylation of IκB, NF-κB, ERK, and JNK, to the same extent as seen in PTK7 knockdown cells treated with vehicle control dimethyl sulfoxide (DMSO) (Figure 5C). Moreover, expression of dominant-negative mouse Src (mSrc-DN) decreased phosphorylation of Src, IκB, NF-κB, ERK, and JNK, as much as the levels in PP1-treated or PTK7-knockdown TE-10 cells (Figure 5D). These results suggest that PTK7 activates AP-1 and NF-κB signaling pathways through RPTKs and Src in TE-10 cells.

**PI3K and Akt are required for PTK7-mediated NF-κB activation in TE-10 cells**

The PI3K/Akt pathway activates NF-κB through phosphorylation of the IκB kinase (IKK) complex in colorectal cancer cell [26]. In TE-10 cells, PTK7 knockdown decreased phosphorylation of Akt, IκB, and NF-κB (Figure 6A). As expected, PI3K inhibitor LY294002 decreased phosphorylation of IκB and NF-κB in control vector-transfected cells, whereas MEK inhibitor PD98059 and JNK inhibitor SP600125 did not inhibit phosphorylation of either protein. In PTK7 knockdown cells, phosphorylation of Akt, IKKa (Thr23), IKKα/β (Ser176/180), and IκB was significantly decreased, to the same extent as observed in LY294002-treated and dominant-negative Akt-overexpressing TE-10 cells (Figure 6B). These findings indicate that PTK7 enhances NF-κB phosphorylation through the PI3K-Akt signaling pathway in ESCC TE-10 cells.

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**Figure 1: Effect of PTK7 knockdown on gelatin degradation by TE-10 cells.** Control vector-transfected and PTK7 knockdown (PTK7-KD-6433 and -6334) TE-10 cells were plated at 4 × 10<sup>4</sup> cells/well of 24-well plate on FITC-gelatin-coated cover glasses and incubated for 48 h at 37°C. The cells were stained with rhodamine-phalloidin and DAPI, and analyzed by fluorescence microscopy (×100). Western blot on right shows PTK7 levels in control and PTK7 knockdown cells. GAPDH served as loading control. Relative gelatin degradation was shown as FITC-gelatin degraded area normalized to DAPI intensity of the sample referred to that of the control vector-transfected cells. ***P < 0.001 vs. control vector-transfected cells.
Blockade of PTK7-induced signaling pathways attenuates \textit{MMP9} upregulation in 2D and 3D cultures of TE-10 cells

To confirm that PTK7-induced ERK, JNK, PI3K/Akt, and NF-κB signaling pathways are responsible for PTK7-mediated \textit{MMP9} upregulation, MMP-9 expression and gelatin degradation were analyzed in 2D and 3D cultures of TE-10 cells in the presence of inhibitors of MEK, JNK, PI3K, and NF-κB. In 2D culture, treatment of cells with all tested inhibitors, as well as PTK7 knockdown, significantly decreased the level of secreted MMP-9 (Figure 7A). In 3D culture, intensity of fluorescence generated by degradation of fluorogenic DQ-gelatin was reduced by treatment with MEK, JNK, PI3K, and NF-κB inhibitors to 31\%, 35\%, 21\%, and 10\%, respectively, of control values (Figure 7B and 7C). In PTK7-KD-6434 cells, fluorescence intensity was reduced to 17\% of control. These results demonstrate that PTK7-mediated transactivation of the \textit{MMP9} gene occurs by AP-1 activation, which is associated with activation of ERK and JNK, and by NF-κB activation, which requires activation of PI3K.

\begin{figure}
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\includegraphics[width=\textwidth]{figure2}
\caption{Identification of a gelatinase induced by PTK7 in TE-10 cells. (A) TE-10 cells overexpressing TIMP-1 or TIMP-2 were grown on FITC–gelatin-coated coverslips, stained with rhodamine-phalloidin and DAPI, and analyzed by fluorescence microscopy (×100). Western blot on right shows TIMP-1 and TIMP-2 levels in conditioned medium and PTK7 level in cell lysates. Relative gelatin degradation was shown as FITC-gelatin degraded area normalized to DAPI intensity of the sample referred to that of the control vector-transfected cells. ** \(P < 0.01\), *** \(P < 0.001\) vs. control vector-transfected cells. (B) Levels of secreted MMP-2 and MMP-9 and PTK7 were analyzed by gelatin zymography and western blotting in conditioned medium and cell lysates. PTK7 knockdown (PTK7-KD-6433 and 6434) TE-10 cells transfected with empty vector (Vector) or PTK7 overexpression vector (PTK7-FLAG) (left panel) and PTK7 knockdown (PTK7-KD-6433, 6434, and 6433/6434) or PTK7 knockout (2 cell lines of PTK7-KO-F369 and F849) TE-10 cells (right panel) were used. HT-1080 cell conditioned medium was used to show positions of MMP-9 and MMP-2.}
\end{figure}
PTK7 expression is correlated with MMP-9 expression in other cells and tumor tissue of ESCC

We analyzed levels of PTK7 and secreted MMP-9 in non-neoplastic esophageal epithelial cell lines and ESCC cell lines (Figure 8A). Both PTK7 and MMP-9 were expressed at a low level in the non-neoplastic esophageal epithelial cell lines NE1 and NE2. In the ESCC cell lines TE-5, TE-6, TE-9, TE-10, TE-11, and TE-14, levels of MMP-9 secretion varied. Interestingly, cells showing elevated PTK7 expression also showed elevated MMP-9 secretion (Figure 8A). PTK7 knockdown in TE-6, TE-9, and TE-10 cells reduced levels of secreted MMP-9 (Figure 8B). In PTK7 knockdown TE-6, TE-9, and TE-10 cells, PTK7 mRNA levels were decreased to 34%, 27%, and 12% respectively, of control values, and in these cells MMP9 mRNA levels were decreased to 20%, 30%, and 14%, respectively, of control (Figure 8C and 8D). Thus, we are confident that PTK7 enhances MMP9 expression at the transcriptional level in other ESCC cells as well as in TE-10 cells.

To investigate whether PTK7 expression was correlated with that of MMP-9 in vivo, tumor tissue from 155 ESCC patients was immunohistochemically stained with anti-PTK7 and anti-MMP-9 antibodies and staining intensity was analyzed. Representative images of PTK7 and MMP-9 staining in each tissue block are shown in Figure 9A. PTK7 and MMP-9 are enriched at pericellular region of cancer cells but not around normal cells. Spearman’s rank correlation analysis showed that PTK7 expression was significantly correlated with MMP-9 expression (Figure 9B; Spearman’s rank: $r = 0.4$ and $P = 4.5 \times 10^{-7}$). These findings support the idea that PTK7 expression is responsible for the induction of MMP-9 in ESCC tumor tissue.

![Figure 3: Effect of PTK7 knockdown on MMP9 transcription in TE-10 cells.](image-url)

- **A** and **B**: MMP9 mRNA levels in control vector-transfected and PTK7 knockdown (PTK7-KD-6433 and -6434) TE-10 cells were analyzed by conventional RT-PCR (A) and real-time RT-PCR (B). (A) PCR products were separated by PAGE in 5% gels and visualized by ethidium bromide staining. (B) Relative expression levels of MMP9 and PTK7 mRNA determined by real-time PCR. **$P < 0.01$, ***$P < 0.001$ vs. those in control vector-transfected cells.
- **C**: Control vector-transfected and PTK7 knockdown TE-10 cells were cotransfected with pRL-TK and pGL3-M9P-wt (MMP9 promoter) or pGL3-Basic (promoterless). Luciferase activity is shown as ratio of firefly/Renilla luciferase activity. **$P < 0.01$ vs. luciferase activity in control vector-transfected TE-10 cells transfected with pGL3-M9P-wt.
DISCUSSION

In this study, we found that PTK7 knockdown reduced focal degradation of underlying gelatin in TE-10 cell cultures. MMP-9 is the major enzyme responsible for PTK7-dependent gelatin degradation, and PTK7 induces MMP9 at the transcriptional level. Transactivation of the MMP9 gene is stimulated primarily by transcription factors AP-1 and NF-κB [27]. We found that PTK7 increased nuclear levels of c-Fos and c-Jun through activation of ERK and JNK and increased the level of activated NF-κB by phosphorylation and degradation of IκB and phosphorylation of RelA/p65.

It is interesting to understand how PTK7 activates MAPKs and IKK to activate AP-1 and NF-κB. Knockdown of PTK7 decreased tyrosine phosphorylation of cellular proteins in TE-10 cells. Although PTK7 is a catalytically defective RPTK, it might recruit protein tyrosine kinases and enhances downstream signaling pathways. It was well known that HER3, a catalytically defective RPTK, can heterodimerize with other EGFR family members upon ligand binding such as neuregulin and activates downstream signaling proteins such as Akt and Erk [28]. We have previously demonstrated that PTK7 binds to and activates KDR, one of VEGF receptor [17]. However, KDR expression was not detectable in TE-10 cells. Nevertheless, pan-PTK inhibitor genistein and multitarget RPTK inhibitor TKI-258 inhibited phosphorylation of ERK, JNK, IκB, and NF-κB. Thus, RPTKs other than KDR might work with PTK7 for downstream activation. We also found that PTK7 knockdown decreases phosphorylation of Src and Src substrate CAS [29]. Indeed, treatment of Src family kinase inhibitors PP1 and PP2 and expression of dominant-negative Src decreased activation of ERK, JNK, IκB, and NF-κB to the same extent as PTK7 knockdown. It was reported that PTK7 coprecipitates with Src and enhances Src activity [30]. In addition, Src is known to activate NF-κB, as well as ERKs and JNK [31, 32]. These reports support the idea that Src may be a PTK involved in PTK7-mediated activation of AP-1 and NF-κB.

Interestingly, we found that PTK7-mediated phosphorylation of IκB and NF-κB was inhibited by a PI3K inhibitor, LY294002. IKKa is phosphorylated at Thr23 by Akt, and IKKa/β is phosphorylated at Ser176/180 by NIK and TAK1 or autophosphorylated by the IKK complex [33]. In our study, phosphorylation of IKKa at Thr23 and of IKKa/β at Ser176/180 was decreased by LY294002 treatment and overexpression of dominant-negative Akt. Thus, the PTK7-activated PI3K/Akt pathway appears to activate IKK in ESCC TE-10 cells. The signaling pathways involved in PTK7-induced MMP-9 expression are summarized in Figure 10.

We have shown that the pro-invasive effect of PTK7 occurs through enhanced MMP-9 secretion in 2D culture conditions. In vivo, cells are surrounded by ECM and grow

Figure 4: Effect of PTK7 knockdown on NF-κB and AP-1 activation in TE-10 cells. Activation of NF-κB and AP-1 was analyzed in control vector-transfected and PTK7 knockdown (PTK7-KD-6433 and 6434) TE-10 cells. (A) Phosphorylation of NF-κB and IκB as indicators of NF-κB activation, and phosphorylation of ERK and JNK as indicators of activation of AP-1 were analyzed by western blotting. (B) Nuclear and cytosolic fractions were prepared from control and PTK7 knockdown cells. Levels of AP-1 complex components, c-Fos and c-Jun, and NF-κB were analyzed by western blotting. GAPDH and lamin A/C served as cytosolic and nuclear markers, respectively.
or migrate through ECM proteolysis [34]. Use of a 3D culture system that incorporates Matrigel and DQ-gelatin to mimic in vivo conditions, we demonstrated that PTK7 knockdown, as well as inhibition of MEK, JNK, PI3K, and NF-κB activation, significantly inhibited degradation of ECM components.

Gelatinase activity is frequently increased in ESCC and is correlated with tumor invasion and metastasis [35]. We previously demonstrated that PTK7 expression is related to poor prognosis of ESCC patients [8]. Here, we showed that PTK7 expression is required for transcription and secretion of MMP-9 in multiple ESCC cell lines that express PTK7. Although MMP9 induction is controlled by various stimuli and signaling pathways, we have shown that PTK7 expression is positively correlated with MMP-9 expression in samples of ESCC tumor tissue from 155 patients.

We showed that PTK7 enhances invasiveness through MMP-9 induction by AP-1 and NF-κB activation. Chemoresistant cancer cells often show NF-κB activation, and NF-κB knockdown strengthens the effect of chemotherapeutic agent 5-FU on cell death in cancer cells [36–38]. Consistently, we have shown that PTK7 knockdown sensitizes cells to 5-FU–induced death [8]. These data suggest the possibility that inhibition of PTK7 function reduces NF-κB activation and enhances the effect

![Figure 5: Involvement of PTK in PTK7-mediated activation of NF-κB and AP-1 in TE-10 cells.](image)

(A and B) Tyrosine phosphorylation of cellular proteins (A) and p60Src, p125FAK, and p130CAS (B) was analyzed by western blotting in control vector-transfected and PTK7 knockdown (PTK7-KD-6433 and 6434) TE-10 cells. Tyrosine phosphorylation of cellular proteins was detected by anti-phosphotyrosine (4G10) and anti–phospho-Src family (Tyr416) antibodies, respectively. Tyrosine phosphorylation of p125FAK and p130CAS was monitored by immunoprecipitation with anti-FAK and anti-CAS antibodies, respectively, and western blotting with anti-phosphotyrosine (4G10) antibody. (C) Subconfluent control vector-transfected and PTK7 knockdown (PTK7-KD-6434) TE-10 cells were incubated with genistein (100 μM, pan-PTK inhibitor), TKI-258 (200 nM, multi-targeted RTK inhibitor), PP1 or PP2 (10 μM, Src family kinase inhibitors), or DMSO (vehicle) for 30 min. (D) Control vector-transfected cells were transfected with dominant-negative mouse Src expression construct (mSrc-DN). Levels of total and phosphorylated forms of the indicated signaling molecules were analyzed by western blotting.
of 5-FU in chemoresistant cancer cells. Taken together, the data show that PTK7 plays a role in tumorigenesis, invasiveness, and chemoresistance. Thus, attenuation of PTK7 function would be a valuable therapeutic means to control ESCC and other cancers that express PTK7.

MATERIALS AND METHODS

Reagents and antibodies

Quenched fluorogenic DQ Gelatin and rhodamine-conjugated phalloidin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fluorescein isothiocyanate (FITC)-conjugated gelatin, anti-FLAG antibody, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Genistein, PP1, PP2, and LY294002 were purchased from AG Scientific (San Diego, CA, USA). TKI-258 was purchased from ApexBio (Houston, TX, USA). PD98059 and SP600125 were purchased from Tocris Bioscience (Bristol, UK). QNZ and antibodies against phospho-ERK, ERK2, c-Fos, lamin A/C, phospho-IKKα (Thr23), IKKα, NF-κB p65, CAS, FAK, Src, and HA tag were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-JNK, JNK, c-Jun, phospho-Src family (Tyr416), phospho-Akt (Ser473), Akt, phospho-IKKα/β (Ser176/180), IKKβ, phospho-IκBα (Ser32), IκBα, phospho-NF-κB p65 (Ser536), and MMP-9 were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-phosphotyrosine antibody (clone 4G10) and anti–MMP-2 antibody were purchased from Millipore (Billerica, MA, USA). Anti-GAPDH antibody was purchased from AbClone (Seoul, Korea). Horseradish peroxidase-conjugated goat anti-mouse IgG, rabbit IgG, and rabbit anti-goat IgG were purchased from KOMA Biotech (Seoul, Korea). Anti-PTK7 antibody was described previously [39].

Cell culture

Human ESCC TE-5, TE-6, TE-9, TE-10, TE-11, and TE-14 cells were obtained from the RIKEN BioResource
Center (Tsukuba, Japan) and human fibrosarcoma HT-1080 cells was obtained from Korean Cell Line Bank (Seoul, Korea). These cells were maintained in Dulbecco’s modified Eagle medium (DMEM; Gibco/Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS). Non-neoplastic esophageal epithelial cell lines NE1 [40] and NE2 [41] were provided by Professor G.S.W. Tsao (University of Hong Kong, Hong Kong SAR, China). These cells were maintained in a 1:1 mixture of Epilife medium (Gibco/Thermo Fisher Scientific) and Defined Keratinocyte-SFM (Gibco/Thermo Fisher Scientific) containing 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Expression vectors, knockdown vectors, and knockout vectors

Expression vectors pcDNA3.1-TIMP-1 [42] and pcDNA3.1-TIMP-2 [43] encoding human TIMP-1 and TIMP-2, respectively, and pcDNA3-hPTK7-FLAG [17] encoding human PTK7 with a C-terminal Flag tag were described previously. Expression vector encoding K297R/Y529F dominant-negative mouse Src (pLNCX-mSrc [K297R/Y529F]) was generated by site-directed mutagenesis using pLNCX-mSrc, which was a generous gift from Professor E.-S. Oh (Ewha Womans University, Korea), as a template and primer pairs listed...
Expression vector encoding HA-tagged K179M kinase-deficient AKT (pCMV6-HA-Akt-KD [K179M]) was a kind gift of Professor K.-Y. Choi (Yonsei University, Korea). Constructs pLKO.1-shRNA-PTK7–6433 and –6434 for human PTK7 knockdown vectors and pLKO.1-control (Sigma-Aldrich) were described previously [8]. Constructs LentiCRISPRv2-sgRNA-PTK7-F369 and –F849 for human PTK7 knockout vectors were generated by cloning of oligonucleotide sequences listed in Supplementary Table S2 into LentiCRISPRv2 vector (Addgene, Cambridge, MA, USA).

**Generation of PTK7 knockdown and PTK7 knockout ESCC cells**

Production of PTK7 knockdown and PTK7 knockout lentiviruses and infection into ESCC cells were performed as described previously [8]. Puromycin-resistant cells were enriched by incubation of the cultures with 2.5 μg/ml puromycin for 14 d. A mixed culture of PTK7 knockdown cells and individual clones of PTK7 knockout cells were maintained in the presence of 1 μg/ml puromycin.

**Fluorescent gelatin degradation assay**

Cross-linked FITC–gelatin-coated coverslips were prepared as described previously [44]. Briefly, acid-washed glass coverslips were coated with 20 μg/ml FITC-gelatin for 2 h at 37°C. The coverslips were washed with phosphate-buffered saline (PBS), cross-linked with 0.5% glutaraldehyde for 15 min, and then quenched with 5 mg/ml sodium borohydride for 3 min at 25°C. The FITC-

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**Figure 8: Analysis of PTK7-dependent MMP-9 expression in esophageal epithelial and ESCC cell lines.** In non-neoplastic esophageal epithelial cell lines (NE1 and NE2) and ESCC cell lines (TE-5, 6, 9, 10, 11, and 14) (A) and control vector-transfected and PTK7 knockdown (PTK7-KD-6434) TE-6, -9, and -10 cells (B), levels of secreted MMP-9 levels in the medium and levels of PTK7 and GAPDH in cell lysates were analyzed by western blotting. HT-1080 cell lysates served as an MMP-9-positive control. MMP9 and PTK7 mRNA levels were analyzed by conventional (C) and real-time (D) RT-PCR in control and PTK7 knockdown TE-6, -9, and -10 cells. **p < 0.01, ***p < 0.001 vs. level in control vector cells.
Figure 9: Relationship between PTK7 and MMP-9 expression based on immunohistochemical analysis of ESCC tissue. (A) Representative images of immunohistochemical staining of PTK7 and MMP-9 in serial sections of each ESCC tissue block (×200). Areas inside blue boxes were shown at a higher resolution. Scale bar indicates 50 μm. (B) Distribution (top) and linear relationship determined by Spearman’s rank correlation test (bottom) of PTK7 and MMP-9 staining intensity in 155 ESCC tissue samples. r and P values of Spearman’s rank correlation are shown.

Figure 10: Proposed model for PTK7-mediated MMP-9 induction and invasiveness in TE-10 cells. PTK7 activates the PI3K/AKT and Ras/MAPK pathways via activation of unidentified RPTK and Src. The PI3K–Akt–IKK signal cascade activates NF-κF. ERK and JNK activate AP-1 complex components c-Fos and c-Jun. NF-κF and AP-1 transactivate MMP9 and enhance invasive phenotype of ESCC cells. Solid and dashed arrows indicate activation at the protein and transcriptional levels, respectively.
gelatin-coated coverslips were washed with PBS again and incubated with 5% FBS in DMEM for 1 h at 37°C. Cells (4 × 10^4/well of 24-well plate) were seeded on the coverslips and incubated for 48 h at 37°C to allow gelatin degradation. The cells were fixed with 3.7% paraformaldehyde and treated with 1% Triton X-100. Actin filaments were stained with rhodamine-phalloidin (500 ng/ml). Nuclei were counterstained with DAPI (250 ng/ml). Immunofluorescence staining was observed with an Axio fluorescence microscope (Zeiss, Jena, Germany). FITC-gelatin degraded area was measured by ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to DAPI intensity for quantitation of gelatin degradation.

**Preparation of conditioned medium and gelatin zymography**

Subconfluent cells were incubated in serum-free medium for 24 h. The conditioned medium was collected by centrifugation at 2000 g for 5 min and secreted proteins in the supernatant were precipitated with cold trichloroacetic acid (TCA; Sigma-Aldrich). The precipitated proteins were analyzed using gelatin zymography as described previously [45].

**RNA isolation and reverse transcription (RT)-PCR analysis**

Total RNA was isolated from each cell line using TRIZol reagent (Invitrogen). First-strand cDNA was synthesized from total RNA using oligo (dT)\textsubscript{15} primers and the AMV RT system (Promega, Madison, WI, USA) according to the manufacturer’s instructions. PCR was carried out under conditions of 27 cycles of denaturation at 94°C for 30 s, annealing (see Supplementary Table S3 for annealing temperatures) for 60 s, and extension at 72°C for 30 s. PCR products were analyzed by polyacrylamide gel electrophoresis (PAGE) through 5% gels. Real-time PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) and the CFX Real-Time system (Bio-Rad, Hercules, CA, USA.).

**Dual-luciferase reporter assay**

Cells (8 × 10\textsuperscript{3}/well) were seeded in 96-well plates 16 h prior to transfection. Luciferase reporter construct pGL3-M9P-wt containing the human wild-type MMP9 promoter or the promoterless vector pGL3-basic was cotransfected with reporter vector pRL-TK encoding Renilla luciferase using Lipofectamine 2000 [46]. After 24 h, luciferase activity was measured using a dual-luciferase reporter assay system (Promega) and normalized to Renilla luciferase activity.

**Fractionation of cytosolic and nuclear proteins**

Subconfluent cells were separated into cytoplasmic and nuclear fractions as described previously [24], and proteins were analyzed by western blotting.

**Preparation of cell lysates, immunoprecipitation, and western blotting**

Subconfluent cells were lysed with RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing 1 mM NaF, 1 mM Na\textsubscript{3}VO\textsubscript{4}, and Sigmafast protease inhibitor tablets (2 mM AEBSF, 300 nM aprotinin, 130 μM bestatin, 1 mM EDTA, 14 μM E-64, and 1 μM leupeptin; Sigma-Aldrich). Immunoprecipitation and western blotting were performed as described previously [17].

**Gelatinolytic activity in 3D culture**

3D cell culture in Matrigel (Phenol Red-Free; Corning Inc., Corning, NY, USA) containing fluorogenic DQ Gelatin was performed as previously described [47, 48]. Briefly, cells (5 × 10\textsuperscript{4}/100 μl) were incubated in phenol red-free DMEM containing signaling inhibitors for 30 min, mixed with 100 μl of 6 mg/ml Matrigel containing 50 μg/ml DQ Gelatin and signaling inhibitors, and seeded in 13-mm glass-bottomed dishes (SPL Life Sciences, Pocheon-si, Gyeonggi-do, Korea) coated with 50 μl Matrigel. After polymerization of Matrigel, 2 ml culture medium containing signaling inhibitors were added, and the dishes were incubated at 37°C for 72 h. Cells were incubated with Hoechst 33258 (2 μg/ml) for 30 min to counterstain nuclei. Intensity of fluorescence generated by DQ Gelatin cleavage was determined and normalized to that of Hoechst 33258 staining using a confocal microscope (LSM700; Zeiss) and ImageJ software.

**Immunohistochemical (IHC) staining of ESCC tissue and statistical analysis**

Preparation and IHC staining of ESCC tissue were performed as described previously [8]. Micrographs were taken of stained tissue and evaluated for staining intensity by a pathologist blinded to the identity of the samples. Intensity of PTK7 or MMP-9 staining was scored on a scale of 0 (low) to 4 (high).

Statistical analysis of association of PTK7 and MMP-9 expression was carried out using Spearman’s rank correlation. Correlation and statistical comparisons were performed using the R package ‘pspearman’ (version 0.3–0).
Statistical analysis

All data subjected to statistical analysis were obtained from at least three independent experiments and are expressed as mean ± standard deviation. Statistical significance was analyzed by Student’s t-test unless specified otherwise. A p-value < 0.05 was considered significant.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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