Supporting Information

The catalytically defective receptor protein tyrosine kinase EphA10 promotes tumorigenesis in pancreatic cancer cells.

Won-Sik Shin[1], Mi Kyung Park[2], Young Hun Lee[1], Kyung Woo Kim[1], Ho Lee[2], and Seung-Taek Lee[1]*

[1]Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul, Republic of Korea, [2]Department of Cancer Biomedical Science, Graduate School of Cancer Science and Policy, National Cancer Center, Gyeonggi, Republic of Korea

***Corresponding author:**

Seung-Taek Lee, Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul 03722, Republic of Korea. Phone: +82-2-2123-2703, FAX: +82-2-362-9897, E-mail: stlee@yonsei.ac.kr

1 Supporting Materials and Methods

1.1 Cell culture

Human pancreatic adenocarcinoma PANC-1, MIA PaCa-2, and AsPC-1 cells; breast cancer MDA-MB-436 cells; and melanoma MDA-MB-435 cells were purchased from the Korea Cell Line Bank (Seoul, Korea). HEK 293 cells expressing SV40 T antigen (HEK293T) were kindly provided by Dr. Jong Bae Park (National Cancer Center, Goyang, Korea). All cells were grown in Dulbecco's Modified Eagle Medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% bovine serum (BS) for HEK293T cells or 10% fetal bovine serum (FBS) for other cells, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37°C in 5% CO_2.

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

Total RNA was isolated from cultured cells using TRIzol reagent (Ambion, Carlsbad, CA, USA). First-strand cDNA was synthesized from total RNA using oligo $(dT)_{15}$ primers and the AMV RT system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The primer pair sequences for RT-PCR were as follows: 5'-GAGTGAGGTCCCAGAATCCGTTGG-3' (nt. 5251–5275 of GenBank NM_001099439.1) and 5'-GGCTTGCAGCCCACCTCTGTC-3' (nt. 5389-5369 of GenBank NM_001099439.1) for human EphA10 cDNA, 5'-TGGGCTACGTGACCTATGAC-3' (nt. 2100–2119 of GenBank NM_004994) and 5'-CAAAGGTGAGAAGAGAGGGC-3' (nt. 2290–2271 of GenBank NM_004994) for human MMP-9 cDNA, and 5'-ACTGCTTAGCACCCCTGGCCA-3' (nt. 488–508 of GenBank BC023632) and 5'-TTGGCAGTGGGGACACGGAAG-3' (nt. 740–720 of GenBank BC023632) for human GAPDH cDNA. PCR amplification was carried out under the following conditions: initial denaturation at 94°C for 30 s and 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for EphA10 or 62°C for GAPDH for 60 s, and extension at 72°C for 30 s. PCR products were analyzed by polyacrylamide gel electrophoresis (PAGE). Quantitative RT-PCR was performed under conditions of initial heat activation at 95°C for 15 s, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for EphA10, 59°C for MMP-9, or 62°C for GAPDH for 30 s, and extension at 72°C for 20 s using the QuantiTect SYBR Green PCR Kit (Qiagen, Venlo, Netherlands) and the QuantStudio3 Real-Time system (Applied Biosystems, Waltham, MA, USA).

The EphA10 or MMP-9 mRNA level was normalized by the GAPDH mRNA level.

1.3 Construction of EphA10 expression vector

To construct an expression vector for the human EphA10 polypeptide, an *EphA10* cDNA encoding the EphA10 polypeptide and a C-terminal FLAG-tag was amplified by PCR. An oligo $(dT)_{15}$-primed cDNA mixture of breast cancer MDA-MB-436 cells was used as a template, and PrimeSTAR GXL DNA polymerase (TaKaRa, Kusatsu, Japan) was used to synthesize DNA products. The upstream primer (5'-CCCAAGCTTGCCACC**ATG**GAGACCTGCGCCGG-3') contained a *Hin*dIII site (underlined) and 20 nucleotides of *EphA10* cDNA (nt. 84-103 of NM_001099439.1) around the start codon (bold). The downstream primer (5'-CCGCTCGAG**TCA***CTTATCGTCGTCATCCTTGTAATC*CACCTGCACCCCCTGGCCC-3') contained 19 nucleotides of *EphA10* cDNA (nt. 3110-3092 of NM_001099439.1) in front of the stop codon, a FLAG-tag coding sequence (italicized), a termination codon (bold), and a *Xho*I site (underlined). The PCR product was digested with *Hin*dIII and *Xho*I restriction enzymes and inserted between the *Hind*III-*Xho*I sites of the mammalian expression vector, pcDNA3.1 (Thermo Fisher Scientific, Waltham, MA, USA). The resulting construct, pcDNA 3.1-EphA10-FLAG, was bi-directionally sequenced to avoid PCR errors.

To construct a lentiviral transfer vector for EphA10 expression, a DNA fragment including the EphA10-FLAG-coding sequence was amplified by PCR, using the pcDNA 3.1-EphA10-FLAG vector as a template and PrimeSTAR GXL DNA polymerase. The upstream primer (5'-GATCTCGACGCGGCCGC*TACGACTCACTATAGGGAGACCCAAGCT*-3') contained 17 nucleotides of pHRST-IRES-eGFP (nt. 2839-2855), including a *Not*I site (underlined) and 28 nucleotides of pcDNA3.1 (nt. 866-893; italicized). The downstream primer (5'-GGGCGGAATTGGATCC*GAAGGCACAGTCGAGGCTGATCAG*-3') contained 16 nucleotides of pHRST-IRES-eGFP (nt. 2871-2856), including a *Bam*HI site (underlined) and 24 nucleotides of pcDNA3.1 (nt. 1037-1014; italicized). The PCR product was ligated into the *Not*I and *Bam*HI sites of the pHRST-IRES-eGFP vector using In-fusion HD cloning kit (Takara). The resulting construct, pHRST-EphA10-FLAG-IRES-eGFP, was bi-directionally sequenced to avoid PCR errors.

1.4 Generation of *EphA10* knockdown and overexpression lentivirus and infection of pancreatic cancer cells

EphA10 knockdown constructs (pLKO.1-shRNA-EphA10-341 and 387) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lentiviruses were produced in HEK293T cells by co-transfection of 10 μg of the *EphA10* knockdown vector (pLKO.1-shRNA-EphA10-341 or 387), 10 μg of the knockdown control vector (pLKO.1-Control), 13 μg of the *EphA10* overexpression vector (pHRST-EphA10-FLAG-IRES-eGFP), or 10 μg of the overexpression control vector (pHRST-IRES-eGFP) as well as 7.4 μg of packaging vector (psPAX2) (Addgene, Cambridge, MA, USA) and 2.6 μg of envelope vector (pMD2.G) (Addgene). Pancreatic cancer cells infected with *EphA10* knockdown lentiviruses were incubated with 1 μg/ml puromycin for 14 days, and puromycin-resistant *EphA10*-knockdown colonies were pooled in a mixed culture. Cells infected with *EphA10*-overexpression lentiviruses were used without further selection.

1.5 Transfection of siRNA

The siRNA mixture against human MMP-9 or EphA7 (Dharmacone, Lafayette, CO, USA) was transfected into Mia PaCa-2 cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s procedure.

1.6 Antibodies

The following antibodies were used: anti-EphA10 from GeneTex (Hsinchu City, Taiwan); anti-Ki-67 and anti-CD31 from Abcam (Cambridge, UK); anti-phospho-ERK, anti-NF-κB p65, anti-FAK, and anti-β-actin from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-phospho-AKT (Ser473), anti-AKT, anti-phospho-JNK, anti-JNK, anti-phospho-NF-κB p65 (Ser536), anti-MMP-9, and anti-EphA7 from Cell Signaling Technology (Danvers, MA, USA); anti-phospho-FAK (Tyr397) from Merck Millipore (Burlington, MA, USA); anti-ERK2 from Bioss (Boston, MA, USA); anti-phospho-EphA7 (Tyr791) from Thermo Fisher Scientific; anti-FLAG from Sigma-Aldrich; anti-GAPDH from AbClone (Seoul, Korea); and horseradish peroxidase-conjugated goat anti-mouse IgG and rabbit IgG from KOMA Biotech

(Seoul, Korea).

1.7 Immunoprecipitation and western blot analysis

For immunoprecipitation assay, sub-confluent cultured cells were lysed with NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40) containing 1 mM NaF, 1 mM Na_3VO_4, and Sigmafast protease inhibitor tablet (2 mM AEBSF, 300 nM aprotinin, 130 μM bestatin, 1 mM EDTA, 14 μM E-64, and 1 μM leupeptin; Sigma-Aldrich). The lysates were incubated with the indicated antibodies and protein-A/G agarose (Amicogen, Jinju, Korea). The protein-bound resins were washed with NP-40 lysis buffer. For western blot analysis, cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) for 10 min at 4°C. Immunoprecipitated proteins or lysates were subjected to SDS-PAGE and transferred onto a PVDF membrane. Blots were incubated with the indicated antibodies, and the immune-reactive bands were visualized using West-Q PICO Dura ECL solution (GenDepot, Barker, TX, USA), Immobilon Western Chemiluminescent HRP Substrate (Millipore), and a LAS-3000 imaging system (Fujifilm, Tokyo, Japan). The band intensities were quantified with the MultiGauge software (Fujifilm, Tokyo, Japan).

1.8 Cell proliferation assay

The cell proliferation assay was performed as previously described.[1] Briefly, cells (0.1 ml, 3×10^4 cells/ml) were plated in a 96-well flat-bottom cell culture plate (SPL, Pocheon, Korea) in DMEM supplemented with 10% FBS and incubated for 1 to 3 days. To measure the number of live cells, cells were washed with phosphate-buffered saline (PBS; 8.06 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.6 mM KCl; pH 7.4) and incubated with 0.1 ml DMEM containing 0.5 mg/ml MTT for 4 h. After removal of the medium, cells were washed with PBS and solubilized with 0.1 ml DMSO. Absorbance was measured at 565 nm using a SpectraMax M3 microplate reader (Molecular Devices, San Jose, CA, USA).

1.9 Cell adhesion assay

The cell adhesion assay was analyzed as previously described.[2] Briefly, detached cell

suspensions (1×10^4 cells/0.1 ml) were loaded onto 96-well plates coated with rat tail type I collagen (1 μg/well) and were incubated for 90 min. Then, the cells were fixed with 3.7% paraformaldehyde in PBS and stained with 0.005% crystal violet. The stained cells were lysed with 1% SDS, and the absorbance was measured at 600 nm.

1.10 Chemotactic migration and invasion assays

Chemotactic migration assays were performed as previously described.[3] Briefly, detached cell suspensions (1×10^5 cells/0.2 ml) were loaded into the upper compartment of transwells (Corning, Tewksbury, MA, USA). The bottom surface of each transwell was coated with 10 μl of 0.1% gelatin. The lower compartment of each well was filled with 0.6 ml DMEM with 10% FBS as a chemoattractant. The chamber was incubated at 37°C for 24 h. Invasion assays were identical to chemotactic migration assays, except that the top surface of the filter was coated with 25 μg of growth factor-reduced Matrigel (Corning) and the incubation lasted for 48 h. Cells that migrated or invaded to the bottom surface of the filter were fixed with 3.7% paraformaldehyde in PBS for 15 min and stained with 0.005% crystal violet for 5 min. After removal of non-invasive cells with a cotton swab, the stained cells were solubilized with 1% SDS, and absorbance was measured at 600 nm.

1.11 Wound healing assay

A wound was introduced by scraping the subconfluent monolayer with a 1000-μl micropipette tip. The cells were then incubated for 48 h in DMEM supplemented with 10% FBS, fixed with 3.7% paraformaldehyde in PBS, and assessed by light microscopy.

1.12 Fluorescent gelatin degradation assay

Cross-linked FITC-gelatin-coated coverslips were prepared as previously described.[4] Briefly, acid-washed glass coverslips were coated with 20 μg/ml FITC-gelatin at 37°C for 2 h. The coverslips were washed with PBS, cross-linked with 0.5% glutaraldehyde for 15 min, and quenched with 5 mg/ml sodium borohydride for 3 min at 25°C. The coverslips were placed on 24-well plates, washed with PBS, and incubated with 5% FBS in DMEM for 1 h at 37°C. Detached cells (4×10^4 cells/0.5 ml) were loaded into the 24-well plate and incubated for 48 h at 37°C. The cells on the coverslips were

fixed with 3.7% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100. Actin filaments and nuclei were stained with rhodamine-phalloidin (500 ng/ml) and DAPI (250 ng/ml), respectively. Immunofluorescence staining was observed with an Axio fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany). FITC-gelatin degraded areas were measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to DAPI intensity for quantitation of gelatin degradation.

1.13 Gelatin zymography

Gelatin zymography was analyzed as previously described.[5] Sub-confluent cells were incubated for 24 h in serum-free medium. The conditioned medium was collected by centrifugation at 2,000 *g* for 5 min, and secreted proteins in the supernatant were precipitated with cold trichloroacetic acid (TCA; Sigma-Aldrich). The precipitated proteins were loaded in 7.5% SDS gels containing 0.1% gelatin. After electrophoresis, the gels were incubated for 1 h at room temperature in refolding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5% Triton X-100) and for 16 h at 37°C in reaction buffer (50 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$). Then, the gels were stained with Coomassie brilliant blue R-250.

1.14 Preparation of paraffin blocks

The tumor tissues were fixed in 10% neutral-buffered formalin (Sigma-Aldrich) for 15 h and processed with 70% ethanol, 95% ethanol, 100% ethanol, xylenes, and paraffin in a HistoCore Pearl tissue processor (Leica Biosystems, Wetzlar, Germany). The tumor tissues were embedded in paraffin, cut into 4-μm sections by Rotary Microtome Microm HM355S (Thermo Fisher Scientific), and mounted onto silane-coated glass slides (Muto Pure Chemicals, Tokyo, Japan).

1.15 Immunohistochemical (IHC) staining

After paraffin removal and dehydration, tissue sections were quenched with 3% H_2O_2 in methanol for 20 min and incubated in 10 mM citrate buffer solution (pH 6) at 90 °C for 10 min for antigen retrieval. After washing with PBS, 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, stained with DAB Chromogen (ScyTek Laboratory, West Logan, UT, USA) for 5 min at room temperature, washed with PBS and deionized water, and counterstained with hematoxylin. To detect cell morphology in the xenograft tumor tissues, deparaffinized sections were stained with hematoxylin and eosin (H&E). For immunohistochemical quantification analysis, randomly selected images (n = 10) were analyzed in each animal per group using the NIH ImageJ software version 1.8.0_112 (National Institutes of Health, Bethesda, MD).[6]

1.16 Ethics statement

All experimental animal procedures were reviewed by the Institutional Animal Care and Use Committee of the National Cancer Center of Korea. Animal studies were performed in accordance with the guidelines of the Committee.

1.17 Statistical analysis

Statistical analyses were performed using Graphpad Prism 5 (GraphPad Software, La Jolla, CA, USA) for public database analysis and Excel (Microsoft, Redmond, WA, USA) for all other analyses. Statistical significance was analyzed using Student's t-tests. For all tests, *P* values less than 0.05 were considered statistically significant.

2 Supporting References

1. Lee YH, Seo EK, Lee ST. Skullcapflavone II Inhibits Degradation of Type I Collagen by Suppressing MMP-1 Transcription in Human Skin Fibroblasts. *Int J Mol Sci*. 2019;20:2734.
2. Shin WS, Shim HJ, Lee YH, et al. PTK6 Localized at the Plasma Membrane Promotes Cell Proliferation and MigratiOn Through Phosphorylation of Eps8. *J Cell Biochem*. 2017;118:2887-2895.
3. Shin WS, Gim J, Won S, Lee ST. Biphasic regulation of tumorigenesis by PTK7 expression level in esophageal squamous cell carcinoma. *Sci Rep*. 2018;8:8519.
4. Shin WS, Hong Y, Lee HW, Lee ST. Catalytically defective receptor protein tyrosine kinase PTK7

enhances invasive phenotype by inducing MMP-9 through activation of AP-1 and NF-kappaB in esophageal squamous cell carcinoma cells. *Oncotarget*. 2016;7:73242-73256.

5. Choi YJ, Lee YH, Lee ST. Galangin and kaempferol suppress phorbol-12-myristate-13-acetate-induced matrix metalloproteinase-9 expression in human fibrosarcoma HT-1080 cells. *Mol Cells*. 2015;38:151-155.
6. Vrekoussis T, Chaniotis V, Navrozoglou I, et al. Image analysis of breast cancer immunohistochemistry-stained sections using ImageJ: an RGB-based model. *Anticancer Res*. 2009;29:4995-4998.