Cell-based assays for potential prognostic biomarkers in PDAC

Do Young Hyeon
  Seoul National University  https://orcid.org/0000-0002-1825-0097

Dowoon Nam
  Korea University

Youngmin Han
  Seoul National University College of Medicine

Duk Ki Kim
  Seoul National University College of Medicine

Gibeom Kim
  Seoul National University

Daeun Kim
  Ajou University

Jingi Bae
  Korea University

Seunghoon Back
  Korea University

Dong-Gi Mun
  Korea University

Inamul Hasan Madar
  Korea University

Hangyeore Lee
  Korea University

Su-Jin Kim
  Korea University

Hokeun Kim
  Korea University

Sangyeop Hyun
  Ajou University

Chang Rok Kim
  Seoul National University

Seon Ah Choi
  Seoul National University

Yong Ryoul Kim
Abstract

The associated publication reports proteogenomic analysis of human pancreatic ductal adenocarcinoma (PDAC), where we provided significantly mutated genes (SMGs)/biomarkers, cellular pathways, and cell types as potential therapeutic targets to improve stratification of patients with PDAC. This protocol describes the detailed methods for cell-based assays for potential prognostic biomarkers in PDAC, including cell culture, viral transduction, and cell-based assays.

Procedure

Cell culture

AsPC1 (KCLB No. 21682) and PANC1 (No. 21469) cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI and DMEM media, respectively, containing 10 % foetal bovine serum (FBS) and ZellShield (Minerva Biolabs) in a humidified incubator at 37 °C and 5 % CO₂. HEK 293T (CRL-11268) cell was obtained from American Type Culture Collection (ATCC). Patient-derived cell lines, including SNU3608, 2571, 3615, and 3573 cells, were cultured in RPMI media containing 10 % FBS and ZellShield. All cells used in this study were tested for mycoplasma contamination.

Viral transduction

For knockdown with shRNAs, virus particles were produced in HEK293T cells by co-transfection of the pLKO construct expressing the indicated shRNAs with pMD2.G and psPAX2 in ratios of 5:1.25:3.75 by mass. The viral supernatant was collected 48 h after transfection. The target cells were transduced with 0.45 μm filtered viral supernatant and polybrene (8 μg/mL). The transduced cells were selected with puromycin treatment (2.5 μg/mL) and maintained in a medium containing puromycin. The following sequences of prognostic biomarkers were targeted by shRNAs:

shDCBLD2-1: CGGCCAAATCAGTGTTGTAAT;
shDCBLD2-2: CGCTTCAGAAAGAGAACTGTT;
shPPL-1: CGCCAAGTTCACTGAAGTTTA;
shPPL-2: CCGCTATGTCAACAAGGATAT;
shPTGES-1: CTACTCCTTTCTGGGTCCTAA;
shPTGES-2: GAACGACATGGAGACCATCTA;
shTPI1-1: CTCAGAGAAGGCATGTCTT;
shTPI1-2: GCAGAAGCTAGATCCCAAGAT.

We used empty pLKO.1 vector that contains no shRNA sequences as a shCtrl.

**Validation of shRNA-mediated knockdown by Immunoblot analysis**

Knockdown efficiency of each shRNA was validated by immunoblot analysis. Cell lysates were separated by electrophoresis and transferred to PVDF membrane. Anti-DCBLD2 antibody from Sigma (HPA0169090, 1:1,000 dilution), anti-PPL antibody from Bethyl lab (A301-005A, 1:2,000), anti-PTGES antibody from Cayman chemical (#160140, 1:50 dilution), anti-TPI1 antibody from Abcam (ab196618, 1:5,000 dilution), and anti-Tubulin antibody from Abfrontier (LF-PA0146A, 1:2,500 dilution) were used to detect endogenous protein.

**Proliferation curve**

Transduced AsPC1 and PANC1 cells were seeded at the density of $1 \times 10^3$ cells/100 mL in 96-well plates in triplicate. 10 mL of D-Plus CCK (Donginbio) was treated to cells in each well on the indicated days and plates were incubated for 2 hours in the incubator. After incubation, absorbance under light of wavelength 450 nm was measured using Multiskan Go spectrophotometer (Thermo Scientific).

**Proliferation assay for patient-derived cells**

SNU3608, 2571, 3615, and 3573 cells were seeded at $1 \times 10^5$ cells/mL in 12-well plates in duplicate. The cell numbers were counted using a Countess II FL Automated Cell Counter (Thermo Fisher) on the indicated days. Trypan blue staining was used to stain the dead cells.

**Spheroid formation assay**

Spheroid formation assay was performed with transduced AsPC1 and PANC1 cells. 500 cells were seeded in the 96-well Clear Round Bottom Ultra-Low Attachment Microplate (Corning). After seeding, plates were centrifuged by 1000g for 10 minutes in room temperature. Images of spheroids were taken using a JuLI Stage in 4× magnification 7 days after seeding. Spheroid volumes were measured using the JuLI Spheroid STAT software. 5 technical replicates of every transduced cell lines were used to generate data.
Wound healing assay

AsPC1 cells transduced with shRNAs were seeded in 24-well plates and cultured until they covered the well surface. The cells were scratched using a 200 L micro-pipette tip. After scratching, the cells were incubated in media containing 1 % FBS at 37 °C for 24 h. Photomicrographs were taken by JuLI Stage (NanoEnTek) at 4× magnification. Wound closure (%) was measured using JuLI STAT software, which calculates the wound area by excluding the cell-covered area. Cell covered area is coloured in yellow.

Transwell migration and Matrigel invasion assays

Transwell migration assay was performed using Transwell permeable supports (8.0 mm pore size, 24-well, Corning). In the case of Matrigel invasion assay, we coated the permeable supports with 50 ml of Growth factor reduced Matrigel (Corning) in 0.125 ml/mg concentration and dried the coating overnight. AsPC1 and PANC1 cells transduced with shRNAs were seeded in the upper chamber at a density of 5 × 10⁴ cells in 200 mL of serum-free medium in duplicate. The lower chamber was filled with 750 mL of 20 % FBS-containing medium. The chamber was incubated at 37 °C for 48 h and fixed with 3.7 % formaldehyde for 30 min. The cells were permeabilized with methanol for 20 min and stained with 0.05 % crystal violet for 30 min at room temperature. After each step, the chamber was washed twice with water. The cells on the upper surface of the membrane were removed using a cotton swab. Images were obtained using an EVOS FL Auto 2 at 20 × magnification. Four images were taken from every section of the well and quantified using the ImageJ software.