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Supplemental Information

**hsa_circ_001653 Implicates in the Development
of Pancreatic Ductal Adenocarcinoma
by Regulating MicroRNA-377-Mediated HOXC6 Axis**

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Supplemental Methods

Cell Transfection

The Capan-2 cells in logarithmic growth phase were selected and transfected with the plasmids as follows: hsa_circ_001653 empty vector, si-hsa_circ_001653-1, si-hsa_circ_001653-2, miR-377 mimic, miR-377 inhibitor as well as its NC, HOXC6 overexpression empty vector and HOXC6 overexpression alone or in combination. The BxPC3 cells in logarithmic growth phase were also selected and transfected with hsa_circ_001653 overexpression empty vector plasmids and hsa_circ_001653 overexpression plasmids. All target plasmids were purchased from Dharmacon Company (Lafayette, Co., Wisconsin, USA). Then, the collected cells were inoculated into a 6-well plate at a density of 5×10^5 cells/well. When cell confluence approached over 80%, transfection was conducted using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Subsequently, 4 μ g target plasmids and 10 μ L lipofectamine 2000 were diluted by a total of 250 μ L serum-free Opti-MEN9 (Gibco, Carlsbad, California, USA) medium, which was then allowed to stand for 5 min at room temperature. The two were mixed together and allowed to stand for 20 min. Then the mixture was added to the cells and incubated in 5% CO₂ at 37°C. After 6 h, the medium was renewed, and the cells were harvested after 24 - 48 h of transfection.

Western Blot Analysis

After 48 h of transfection, Capan-2 and BxPC3 cells were collected and lysed with cold radio-immunoprecipitation assay (RIPA) lysis buffer and centrifuged (14000 rpm, 4°C) for 30 min. The supernatant proteins were determined using the bicinchoninic acid assay (BCA) method and stored at -20°C. Next, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a nitrocellulose membrane, which was then

blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. Subsequently, the membrane was incubated overnight at 4°C with the following diluted primary antibodies: rabbit antibody to HOXC6 (ab151575, 1 : 1000), PCNA (ab92552, 1 : 1000), matrix metalloproteinase-2 (ab92536, 1 : 1000), MMP-9 (ab76003, 1 : 1000), VEGFR1 (ab32152, 1 : 1000) and cleaved Caspase-3 (ab32042, 1 : 500). The next day, the membrane was rinsed with phosphate buffer saline-Tween 20 (PBST), and incubated with horseradish peroxidase (HRP)-labeled secondary goat anti-rabbit immunoglobulin G (IgG; 1 : 5000, Arctic Zhongshan Biotechnology Co., Ltd., Guangzhou, China). All antibodies were purchased from Abcam Company (Cambridge, MA, USA). The protein bands were developed using enhanced chemiluminescence (ECL) solution (ECL808-25, Biomiga Inc., San Diego, California, USA). Images were photographed by X-ray (36209ES01, Qcbio Science & Technologies Co., Ltd., Shanghai, China). With GAPDH as the internal reference, the ratio of the gray values between the target band and internal reference band was taken as the relative expression of protein.

Transwell Assay

The Transwell chambers were placed in a 24-well plate, and the diluted Matrigel (1 : 8; Sigma-Aldrich, SF, CA, USA) was added to cover the membrane of apical chamber, following by drying at room temperature. After 48 h of transfection, Capan-2 cells and BxPC3 cells were starved for 24 h in serum-free medium. After conventional detachment, cells were rinsed with PBS and resuspended in RPMI-1640 medium. A total of 200 μ L cell suspension at a concentration of 1×10^5 cells/mL was added into the apical chamber, and 600 μ L of RPMI-1640 medium containing 20% FBS was added into the basolateral chamber. After 24 h of conventional culture, the cells on the interior epidermis of the apical chamber were wiped with a cotton swab. The migrated cells were

then fixed with 4% paraformaldehyde for 15 min and stained with 0.5% crystal violet solution (prepared by methyl alcohol) for 15 min. Afterwards, the invasive cells were analyzed in 5 randomly selected visual fields under an inverted microscope ($\times 200$) (Shanghai Caikon Optical Instrument Co. Ltd., Shanghai, China). A total of 3 duplicated wells were set for each group, and the invasive cells were counted with the mean values calculated.

FISH Assay

FISH assay was conducted to identify the subcellular localization of hsa_circ_001653 based on the instructions of RiboTM lncRNA FISH Probe Mix (Red) (Guangzhou RiboBio Co., Ltd., Guangzhou, China). The cells in logarithmic growth phase were seeded on a cover slip in a 6-well plate (3×10^4 cells/well), followed by 2-day culture to make the cell confluence reach about 80%. Then, the cells were fixed with 1 mL 4% paraformaldehyde for 10 min at room temperature, and then treated with protease K (2 $\mu\text{g}/\text{mL}$), glycine and ethylphthalate reagent successively. Then, cells were incubated with 250 μL prehybridization solution for 1 h at 42°C, and hybridized with 250 μL hybridization solution containing 300 ng/mL probe at 42°C overnight, followed by washing with PBST. Subsequently, the nucleus was stained with PBST-diluted 4',6-diamidino-2-phenylindole (DAPI) (1 : 800) dye liquor for 5 min. The slide was then washed by PBST and sealed with anti-fluorescence quencher. Lastly, cells were observed and photographed in 5 different visual fields under a fluorescence microscope (Olympus Optical Co., Ltd, Tokyo, Japan).

Dual-Luciferase Reporter Assay

The web-available database Starbase was used to predict the potential relationship among hsa_circ_001653, HOXC6 and miR-377. The full-length sequence of hsa_circ_001653 and 3' UTR

of HOXC6 were amplified and cloned into the luciferase vector of pmirGLO (E1330, Promega Corporation, Madison, WI, USA), then named phsa_circ_001653-Wt and pHOXC6-Wt. Then, the bioinformatics software was applied to predict the binding sites between miR-377 and hsa_circ_001653 as well as between miR-377 and HOXC6 in addition to their site-directed mutation. The phsa_circ_001653-Mut and pHOXC6-Mut vectors were constructed respectively, and the pRL-TK vector (E2241, Promega, Madison, Wisconsin, USA) expressing renilla luciferase was taken as the internal reference. Afterwards, miR-377 mimic or miR-377 NC was co-transfected with luciferase reporter vectors into the Capan-2 cells. After 48 h, the cells were collected and lysed. At last, the fluorescence intensity was detected by Dual Luciferase Reporter Assay Kit (GM-040502A, Qcbio Science & Technologies Co., Ltd., Shanghai, China) (detailed procedures in Supplementary methods) at 560 nm (the relative luminous unit [RLU] of firefly) and at 465 nm (the RLU of renilla), and the binding intensity was quantified according to the ratio of firefly RLU to renilla RLU.