## Original Article shRNA-mediated silencing of *PKHD1* gene promotes proliferation, migration and invasion of human intrahepatic cholangiocarcinoma HuCCT-1 cells

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Abstract: Intrahepatic cholangiocarcinoma (ICC), a type of cholangiocarcinoma, is characterized by insidious onset and lack of typical clinical symptoms at early onset, and the lack of effective treatments results in a poor prognosis. Identification of novel biomarkers and treatment targets is therefore of great significance to improve the survival for ICC patients. Polycystic kidney and hepatic disease 1 (PKHD1), a gene responsible for autosomal recessive polycystic kidney disease (ARPKD), has been linked to cancers, and mutation of the PKHD1 gene may cause abnormal proliferation and differentiation of bile duct epithelial cells. However, the role of the PKHD1 gene in the biological behaviors of ICC cells remains unknown until now. The present study was therefore designed to examine the effects of PKHD1 knockdown on the proliferation, migration and invasion of human ICC HuCCT-1 cells and investigate the underlying mechanisms. We transfected a lentiviral vector LV3-PKHD1 that contained the short hairpin RNA (shRNA)mediated silencing of the PKHD1 gene into HuCCT-1 cells, while GFP lentiviral vector LV3NC-transfected cells served as negative controls. The cell proliferation, migration and invasion were measured, and the ultrastructure of primary cilium was observed using scanning electron microscopy (SEM). The expression of PI3K/Akt signaling proteins was determined with Western blotting. gRT-PCR assay determined down-regulation of PKHD1 mRNA expression and Western blotting analysis revealed reduced FPC expression in HuCCT-1 cells post-transfection with LV3-PKHD1, which validated the effective silencing of the PKHD1 gene in HuCCT-1 cells. Wound scratch assay showed that the LV3-PKHD1 transfected HuCCT-1 cells had a greater healing ability of the scratch than the LV3NC-transfected and nontransfected cells at 24 and 48 h, and CCK-8 assay revealed that the LV3-PKHD1 transfected HuCCT-1 cells exhibited a greater proliferative ability than the LV3NC-transfected and nontransfected cells (P < 0.01). In addition, Transwell migration assay showed significantly more LV3-PKHD1 transfected HuCCT-1 cells penetrating through the Transwell chamber than the LV3NC-transfected and nontransfected cells (P < 0.01), and Transwell invasion assay revealed more LV3-PKHD1 transfected HuCCT-1 cells crossing the Matrigel than the LV3NC-transfected and nontransfected cells (P < 0.01). Moreover, Western blotting assay detected significant up-regulation of PI3K, Akt, p-Akt, and NF-kB expression in LV3-PKHD1 transfected HuCCT-1 cells as compared to that in the LV3NC-transfected and nontransfected cells (P < 0.05), and SEM displayed shorter length, less number and lower distribution density of primary cilium on the surface of LV3-PKHD1 transfected HuCCT-1 cells relative to LV3NC-transfected cells. The results of this study demonstrate that the silencing of the PKHD1 gene promotes the proliferation, migration and invasion of human ICC HuCCT-1 cells via the PI3K/Akt signaling pathway.

Keywords: Intrahepatic cholangiocarcinoma, PKHD1, proliferation, migration, invasion, PI3K/Akt signaling, gene silencing

#### Introduction

Cholangiocarcinoma is a primary bile tract malignant tumor that originates from the bile duct epithelial cells [1]. The incidence of cholangiocarcinoma is estimated to rank second only to hepatocellular carcinoma (HCC) in all hepatobiliary malignancies and comprises approximately 3% of all gastrointestinal tumors [2]. This malignancy is found to be highly prevalent in people at ages of approximately 70 years, with a bit higher incidence in men than in women [2]. Epidemiological data show an ongoing rise in both the incidence and mortality of cholangiocarcinoma worldwide [3].

Cholangiocarcinoma is anatomically classified into three groups, intrahepatic, perihilar, and distal [4]. Intrahepatic cholangiocarcinoma (ICC) originates from the intrahepatic bile duct and its branch to the interlobular ductile, which is characterized by insidious onset and lack of typical clinical symptoms at early onset [5-7]. Since the currently available diagnostic tools show a low sensitivity and there is a lack of specific biomarkers, most cases with ICC are identified as a late stage or having severe complications upon definite diagnosis [8]. Surgical resection and liver transplantation remain the most effective approaches for the treatment of ICC; however, surgical therapy has a low complete resection rate, which achieves 10% to 30% 5-year survival rate post-surgery [9], and there is a huge lack of matched liver donors for liver transplantation which suffers from a great deal of money [10-12]. Screening and identification of novel biomarkers for early diagnosis and prognosis prediction is therefore of great significance to improve the survival in ICC patients, which is urgently needed.

Polycystic kidney and hepatic disease 1 (PK-HD1), a gene responsible for autosomal recessive polycystic kidney disease (ARPKD), is encoded by the membrane-associated receptor-like protein fibrocystin/polyductin (FPC), which is composed of 4074 amino acids [13-15]. PKHD1 gene is expressed at a high level in the kidney and at lower levels in the liver and pancreas [16-18], while the PFC protein is mainly distributed on the primary cilia and apical membrane in the bile duct, which is involved in the maintenance of the normal tubular structure of the intrahepatic bile duct epithelial cells [19]. Mutation of the PKHD1 gene has been shown to cause abnormal proliferation and differentiation of bile duct epithelial cells [20], and the morphological defect of primary cilium and defective cilium-mediated intracellular signal transduction may lead to cell cycle abnormality and benign cell over-proliferation, resulting in cyst formation and enlargement [21, 22]. However, the role of the PKHD1 gene in the biological behaviors of ICC cells remains unknown until now. The present study was therefore designed to examine the effects of PKHD1 knockdown on the proliferation, migration and

invasion of human ICC HuCCT-1 cells and investigate the underlying mechanisms, so as to provide new insights into the screening of novel targets for the treatment of ICC.

### Materials and methods

## Cell lines and culture

Human ICC HuCCT-1 cell lines were purchased from Riken (Kyoto, Japan), and GFP lentiviral vector LV3NC (target sequence: TTCTCCGAAC-GTGTCACGT) transfected HuCCT-1 cells were provided by the Shanghai GenePharma Biotech Company (Shanghai, China). The lentiviral vector LV3-PKHD1 (target sequence: AAGCAGT-CCAAATCCAGGACC) that contained the short hairpin RNA (shRNA)-mediated silencing of the PKHD1 gene was constructed in vitro by the Shanghai GenePharma Biotech Company (Shanghai, China), and the LV3-PKHD1 was transfected into HuCCT-1 cells to construct stably transfected HuCCT-1 cells with low PKHD1 expression. Both vectors contained GFP. All cell lines were maintained in the Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 1.5 mM L-glutamine (Hyclone; Logan, UT, USA), 100 U/ ml penicillin(Hyclone; Logan, UT, USA) and 100 µg/ml streptomycin (Hyclone; Logan, UT, USA) at 37°C containing 5% CO<sub>2</sub>.

## Wound scratch assay

Six culture inserts (Ibidi GmbH; Munich, Germany) were placed in the center of a 24-well plate (Corning Inc.; Corning, NY, USA), and were divided into 3 groups, of two inserts for each group. Log-phase, LV3NC-transfecteds and LV3-PKHD1-transfected HuCCT-1 cells were seeded onto culture inserts at a density of  $3.5 \times 10^4$ cells in each insert, respectively. Cells were cultured overnight at 37°C in a saturated humidity incubator containing 5% CO<sub>2</sub> (Thermo Fisher Scientific; Cleveland, OH, USA). Then, the culture inserts were gently removed with a sterile forceps, and a scratch with 500 µm in width was produced. Each well that contained an insert was slowly transferred with approximately 500 µl fresh medium through the plate wall. and the changes of scratches in the culture plate was observed once every 4 to 6 h under an inverted microscope (Zeiss; Oberkochen, German), and photographed.

## CCK-8 assay

Log-phase, LV3NC-transfecte and LV3-PKHD1transfected HuCCT-1 cells were harvested, digested with 0.25% trypsin (Hyclone; Logan, UT, USA) and prepared into single-cell suspensions. Cells were seeded onto 96-well plates at a density of 2000 cells in each well and incubated overnight. Then, each cell-contained well was transferred quickly with 10  $\mu$ I CCK-8 solution along the plate wall, and gently shook for completely even mixture. Following 2 h incubation, the absorbance was measured at 260 nm with a NanoDrop2000 UV-Vis spectrophotometer (Thermo Fisher Scientific; Cleveland, OH, USA).

## Transwell migration and invasion assay

Log-phase, LV3NC-transfected and LV3-PKHD1transfected HuCCT-1 cells were incubated in the OPTI-MEM medium at 37°C containing 5% CO<sub>2</sub> for 24 h, harvested, and digested with 0.25% trypsin (Hyclone; Logan, UT, USA) and resuspended in the OPTI-MEM medium. Cells were then seeded onto the upper Transwell chamber (BD: San Jose, CA, USA) at a density of  $2 \times 10^4$  cells in each chamber, while the lower chamber was transferred with 500 µl complete medium. After 24 h, the chamber was washed with PBS, and fixed in methanol for 30 min. The cells remaining in the upper chamber were removed by gently swabbing a cotton bud against the membrane. The chamber was then stained with the crystal violet solution (Beijing Solarbio Life Sciences Co., Ltd.; Beijing, China) for 20 min, washed with clean water and photographed.

Cells were incubated in the OPTI-MEM medium at 37°C containing 5% CO<sub>2</sub> for 24 h, and harvested. Matrigel (BD; San Jose, CA, USA) was diluted with the OPTI-MEM medium at a ratio of 1:8, and then transferred to the upper Transwell chamber for incubation at 37°C overnight. Following OPTI-MEM hydration for 30 min, cells were seeded onto the upper Transwell chamber at a density of  $2 \times 10^4$  cells per chamber, while the lower chamber was transferred with 500 µl complete medium. After 24 h, the chamber was washed with PBS, and fixed in methanol for 30 min. The cells and Matrigel remaining in the upper chamber were removed by gently swabbing a cotton bud against the membrane. The chamber was then stained with the crystal violet solution for 20 min, washed with clean water and photographed.

### Scanning electron microscopy (SEM)

Sterile coverslips measuring 0.5 cm × 0.5 cm were transferred to 24-well plates. Log-phase, LV3NC-transfected and LV3-PKHD1 transfected-HuCCT-1 cells were digested with 0.25% trypsin and prepared into single-cell suspensions. Cells were then seeded onto 24-well plates at 2  $\times$  10<sup>4</sup> cells per well and incubated overnight at 37°C containing 5% CO<sub>2</sub>. When cells were adherent to the plate wall, they were rinsed gently with PBS to remove the dead cells, fixed in 2.5% glutaraldehyde, rinsed twice with PBS, dehydrated twice in an alcohol series (30%, 50%, 70%, 90% and 95%), of 5 min each time, and dehydrated twice in 100% alcohol, of 10 min each time. Then, the coverslips were collected, stuck to the conductive adhesive and dried in an EMITECH K850X critical point dryer (Quorum Technologies; Ashford, UK). Cells were plated with gold for 1 h in a Q150R rotarypumped sputter coater (Quorum Technologies; Ashford, UK), and the morphology of the primary cilium on cell surface was observed using SEM.

## qRT-PCR assay

The PKHD1 gene expression was determined using a qRT-PCR assay. Briefly, total RNA was extracted from cells using the TransZol Up Plus RNA Kit (Beijing TransGen Biotech Co., Ltd.; Beijing, China), and transcribed into cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific; Cleveland, OH, USA). gRT-PCR assay was performed on an ABI7500 Real-Time PCR System (Applied Biosystems; Foster City, CA, USA) in a 20 µl system containing 10 µl Bestar® SybrGreen gPCR Mastermix (DBI BioScience; Ludwigshafen, Germany), 0.5 µl of 10 µM each primer (forward: 5'-TCCAAA-CGCCGAGAATCACA-3' and reverse: 5'-TTCCTCT-CGGACAATGTGGC-3'), 1 µl cDNA template, and 8 µl ddH<sub>2</sub>O (Beijing Solarbio Life Sciences Co., Ltd.; Beijing, China) under the following conditions: at 95°C for 2 min; followed by 40 cycles of at 95°C for 10 s, at 60°C for 30 s, and at 72°C for 30 s; finally at 95°C for 1 min and at 60°C for 1 min, while GAPDH (forward: 5'-TCC-AAACGCCGAGAATCACA-3' and reverse: 5'-TTC-CTCTCGGACAATGTGGC-3') served as an internal control. Three PCR wells were assigned for



**Figure 1.** Transfection efficiency of the lentiviral vectors LV3NC and LV3-PKHD1 in HuCCT-1 cells under a microscope (× 100). A. LV3-PKHD1-transfected HuCCT-1 cells under an optical microscope; B. LV3-PKHD1-transfected HuCCT-1 cells under a fluorescence and phase-contrast microscope; C. LV3NC-transfected HuCCT-1 cells under an optical microscope; D. LV3NC-transfected HuCCT-1 cells under a fluorescence.

each cell sample, and the relative mRNA expression was quantified using the  $2^{-\Delta\Delta CT}$  method.

#### Western blotting analysis

The PKHD1 protein expression was determined with a Western blotting assay. Briefly, total protein was extracted from cells, and the protein concentration was quantified using the BCA assay (Beijing Zoman Biotechnology Co., Ltd.; Beijing, China). Total protein was separated by 10% SDS-PAGE, and the blots were transferred to the nitrocellulose (NC) membrane (Biosharp; Hefei, China). The blots were blocked in 5% BSA (for FPC; Biosharp; Hefei, China) or 5% TBST (for PI3K, Akt, p-Akt and NF-ĸB1) at room temperature for 2 h, and incubated in the primary anti-FPC, anti-PI3K, anti-Akt, and anti-p-Akt monoclonal antibodies (Abcam; Cambridge, CA, USA), and NF-kB1 polyclonal antibody (ABclonal Biotechnology Co., Ltd; Woburn, MA, USA) overnight, while  $\beta$ -actin (Elabscience Biotechnology Co., Ltd.; Wuhan, China) served as a loading control. Then, the blots were washed three times in TBST, of 10 min each time, and incubated in HRP conjugated-AffiniPure Goat Antirabbit/mouse IgG secondary antibody (1:10000 dilution; EarthOx Life Sciences; Millbrae, CA, China) at room temperature for 1 h. Subsequently, the blots were washed three times in TBST, of 10 min each time. The protein expression was visualized with enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific; Cleveland, OH, USA) and analyzed with the software Image Lab version 3.0 (BIO-RAD; Foster City, CA, USA).

#### Statistics

All measurement were described as mean  $\pm$  standard deviation (SD), and all statistical analyses were performed using the statistical soft-



**Figure 2.** *PKHD1 mRNA* expression and FPC protein expression in HuCCT-1 cells. A. Comparison of relative *PKHD1 mRNA* expression in the LV3-PKHD1, LV3NC transfected, and nontransfected HuCCT-1 cells, \**P* < 0.01; B. Comparison of PKHD1 protein expression in the LV3-PKHD1, LV3NC transfected, and nontransfected HuCCT-1 cells, \*\**P* < 0.05.

ware SPSS version 16.0 (SPSS, Inc.; Chicago, IL, USA). Differences of means were tested for statistical significance with Student t test, and a P value < 0.05 was considered statistical significance.

#### Results

#### Construction and validation of a stably transfected human ICC HuCCT-1 cell line with low PKHD1 expression

The GFP lentiviral vector LV3NC and the lentiviral vector LV3-PKHD1 containing shRNA-mediated silencing of the PKHD1 gene were transfected into HuCCT-1 cells, and the GFP expression was observed in cells under an inverted fluorescence microscope 72 h post-transfection. Microscopy showed intensely green fluorescent granules in both the LV3NC and LV3-PKHD1 transfected HuCCT-1 cells, with a transfection efficiency of approximately 70%. In addition, cells appeared well growth, and the green fluorescence was stably present in cell passages, indicating the construction of a stably transfected human ICC HuCCT-1 cell line with low PKHD1 expression (Figure 1). gRT-PCR assay detected significantly lower PKHD1 mRNA expression in LV3-PKHD1-transfected HuCCT-1



cells than in the LV3NC-transfected and nontransfected cells (P < 0.01), and no significant difference was seen in the PKHD1 mRNA expression between the LV3NC-transfected and nontransfected cells (P > 0.05). In addition, Western blotting assays revealed significantly lower FPC expression in LV3-PKHD1-transfected HuCCT-1 cells than in the LV3NC-transfected and nontransfected cells (P < 0.01), and no significant difference was seen in the FPC expression between the LV3NC-transfected and nontransfected cells (P > 0.05) (**Figure 2**). Our data suggest that the transfection of the lentiviral vector LV3-PKHD1 is effective for the silencing of the PKHD1 gene in HuCCT-1 cells.

## Effect of PKHD1 gene silencing on PI3K/Akt signaling protein expression

Western blotting analysis revealed significantly higher PI3K, Akt, p-Akt, and NF- $\kappa$ B expression in the LV3-PKHD1-transfected HuCCT-1 cells than in the LV3NC-transfected and nontransfected cells (P < 0.05), and no significant difference was seen in the PI3K, Akt, p-Akt, or NF- $\kappa$ B expression between the LV3NC-transfected and nontransfected cells (P > 0.05) (**Figure 3**). Our data suggested that silencing of the PKHD1 gene activated the PI3K/Akt signaling and up-



**Figure 3.** Western blotting assay determines PI3K, p-Akt/total Akt, and NF- $\kappa$ B protein expression in HuCCT-1 cells. A. Comparison of PI3K protein expression in the LV3-PKHD1, LV3NC transfected and nontransfected HuCCT-1 cells; B. Comparison of p-Akt and total Akt protein expression in the LV3-PKHD1, LV3NC transfected, and nontransfected HuCCT-1 cells; C. Comparison of NF- $\kappa$ B protein expression in the LV3-PKHD1, LV3NC transfected, and nontransfected HuCCT-1 cells; D. Western blotting assay determines PI3K, p-Akt/total Akt, and NF- $\kappa$ B protein expression in HuCCT-1 cells: \*P < 0.01, \*\*P < 0.05.



Figure 4. Growth curve of the LV3-PKHD1, LV3NC transfected, and nontransfected HuCCT-1 cells at various time points post-transfection. \*P < 0.01.

regulated PI3K, Akt, and NF-κB expression in the HuCCT-1 cells.

Effect of PKHD1 gene silencing on HuCCT-1 cell proliferation

The cell growth curve showed a significant faster growth of LV3-PKHD1-transfected HuCCT-1

cells than that of LV3NC-transfected and nontransfected cells (P < 0.01) (**Figure 4**), indicating that silencing of the PKHD1 gene promoted the proliferation of the HuCCT-1 cells.

Effect of PKHD1 gene silencing on HuCCT-1 cell migration

Wound scratch assay showed a greater healing ability of the scratch in the LV3-PKHD1transfected HuCCT-1 cells than in the LV3NCtransfected and nontransfected cells at 24 and 48 h (**Figure 5**).

## Effect of PKHD1 gene silencing on HuCCT-1 cell migration and invasion

Transwell migration assay showed that 557  $\pm$  34 LV3-PKHD1-transfected HuCCT-1 cells penetrated through the Transwell chamber, which was significantly greater than the LV3NC-transfected (431  $\pm$  31) and nontransfected cells



Figure 5. Wound scratch assay detects the healing ability of the LV3NC- and LV3-PKHD1-transfected HuCCT-1 cells.

(427  $\pm$  24) (P < 0.01) (**Figure 6**). In addition, Transwell invasion assay revealed that 255  $\pm$  22 LV3-PKHD1-transfected HuCCT-1 cells crossed the Matrigel, which was significantly greater than the LV3NC-transfected (73  $\pm$  5) and nontransfected cells (66  $\pm$  4) (P < 0.01) (**Figure 7**).

# Ultrastructure of primary cilium on HuCCT-1 cell surface

SEM displayed shorter and fewer primary cilium on the surface of the LV3-PKHD1-transfected HuCCT-1 cells relative to the LV3NC-transfected cells (**Figure 8**).

#### Discussion

*PKHD1*, which has been identified as the cause of ARPKD, is located on human chromosome

6p12.2, and its genome covers over 469 bp, which contains at least 86 exons [13]. FPC is the protein coding this gene, which plays a critical role in the proliferation, differentiation and apoptosis of multiple ductal epithelial cells via the primary cilium [20]. To date, more than 300 types of PKHD1 mutations have been identified [15, 23]. Pkhd1 exon 2-deficient mice exhibited hepatic, pancreatic, and renal abnormalities, grossly cystic and fibrotic livers, and progressive bile duct dilatation, as well as structural abnormalities and shortening of primary cilia in the bile ducts relative to the wild-type animals [24], and deletion of exon 40 on the Pkhd1 gene resulted in bile duct abnormality in mice [25]. In addition, mutation of the Pkhd1 gene by disrupting exon 4 downregulated FPC expression, resulting in intrahepatic bile duct proliferation with progressive cyst formation and associated periportal fibrosis in mice [26]. These findings demonstrate that mutation of

exon in the *Pkhd1* gene may cause cystic degeneration of liver, ciliary abnormalities in bile duct and abnormalities of bile duct itself.

Bile duct hamartoma, also termed von-Meyenburg's complex, is a rare, asymptomatic benign intrahepatic bile duct abnormality, which is mainly detected in laparotomy and autopsies [27]. This disorder usually occurs simultaneously with ARPKD and congenital hepatic fibrosis, suggesting these diseases may share the same genetic background [28]. Recently, there have been several cases of ICC arising from multiple bile duct hamartomas [29-31], demonstrating the increasing evidence proving the possible correlation between bile duct hamartoma, a previously identified benign disorder, and ICC. However, the molecular mechanisms of hamartoma-induced cholangiocarcinoma remain unclear until now.



**Figure 6.** Transwell migration assay detects the migration of HuCCT-1 cells. A. LV3-PKHD1-transfected HuCCT-1 cells (× 100); B. LV3NC-transfected HuCCT-1 cells (× 100); C. Nontransfected HuCCT-1 cells (× 100); D. Comparison of the number of LV3-PKHD1, LV3NC transfected, and nontransfected HuCCT-1 cells that penetrate through the Transwell chamber. \*P < 0.01.

The correlation between PKHD1 gene and tumors has been investigated [32]. Sequencing of well-annotated human protein-coding genes in colorectal cancer revealed that the PKHD1 gene may strongly correlate with the development of colorectal cancer [33]. Li and colleagues [34] observed a significantly rise in the number and size of intestinal tumors in the mouse hybrids after the crossing of ApcMin/+ mice with Pkhd1 knockout (Rkhd1-/-) mice, suggesting that the deletion of the PKHD1 gene may promote the intestinal tumorigenesis and malignant transformation of tumors in Apc<sup>Min/+</sup> mice. Among a series of the 1697 consecutive liver needle biopsies, a 0.35% incidence of multiple biliary hamartomas was diagnosed, most of whom were aged more than 40 years, and some cases showed atypical hyperplastic changes in the bile duct cells, suggesting the possible malignant transformation [35]. In addition, sequencing of a biliary hamartoma pedigree without concomitant renal disorders detected a c.4280delG deletion mutation on exon 32 of the *PKHD1* gene (unpublished data), which, for the first time, proved that the *PKHD1* gene alone may cause human bile duct hamartoma. However, the role of the *PKHD1* gene in the transformation from bile duct hamartoma to cholangiocarcinoma has not been demonstrated to date.

To examine the role of *PKHD1* in ICC, we designed a GFP-labeled LV3-PKHD1 vector, a shRNA lentiviral vector targeting the *PKHD1* gene, and transfected into human ICC HuCCT-1 cells. qRT-PCR assay determined down-regulation of *PKHD1 mRNA* expression and Western blotting analysis revealed reduced FPC expression in HuCCT-1 cells post-transfection with LV3-PKHD1, which validated the effective silencing of the *PKHD1* gene in HuCCT-1 cells, caused by the LV3-PKHD1 transfection. Wound



Figure 7. Transwell migration assay detects the invasion of HuCCT-1 cells. A. LV3-PKHD1-transfected HuCCT-1 cells ( $\times$  100); B. LV3NC-transfected HuCCT-1 cells ( $\times$  100); C. Nontransfected HuCCT-1 cells ( $\times$  100); D. Comparison of the number of LV3-PKHD1 and LV3NC transfected, and nontransfected HuCCT-1 cells that cross the Matrigel. \**P* < 0.01.



Figure 8. Ultrastructure of primary cilium in the LV3-PKHD1- and LV3NC-transfected cells (× 5000).

scratch assay showed that the LV3-PKHD1transfected HuCCT-1 cells had a greater healing ability of the scratch than the LV3NC-transfected and nontransfected cells at 24 and

48 h, and CCK-8 assay revealed that the LV3-PKHD1-transfected HuCCT-1 cells exhibited a greater proliferative ability than the LV3NCtransfected and nontransfected cells (P < 0.01). In addition, Transwell migration assay showed significantly more LV3-PKHD1-transfected HuCCT-1 cells penetrating through the Transwell chamber than the LV3NC-transfected and nontransfected cells (P < 0.01), and Transwell invasion assay revealed more LV3-PKHD1transfected HuCCT-1 cells crossing the Matrigel than the LV3NC-transfectedand nontransfected cells (P < 0.01). Our data suggest that the silencing of the PKHD1 gene promotes the proliferation, migration and invasion of HuCCT-1 cells.

It has been found that hepatocyte growth factor (HGF), stimulating factor (SF) and its c-Met receptor tyrosine-mediated signaling play an important role in angiogenesis, invasion and migration of cholangiocarcinoma cells [36, 37]. Following HGF stimulation, c-Met enters the cell nucleus in the presence of importin  $\beta$ 1 and scaffolding protein Gab (Grb-associated binding protein), to activate downstream signaling pathways [38]. Activated Gab1 may bind to downstream SH2-containing signaling molecules, such as p85 subunit, which participates in various signaling pathways, in which PI3K/ Akt signaling is one of the most important pathway [36]. It is therefore hypothesized that the silencing of the PKHD1 gene may promote the proliferation, migration and invasion of HuCCT-1 cells via the activation of the PI3K/Akt signaling.

PKHD1 gene has been shown to mediate cell proliferation and apoptosis via the PI3K/Akt pathway [39]. In polycystic kidney (PCK) rat models, the expression of PI3K, p-Akt and p-mTOR was found to increase in PCK cholangiocytes compared to normal cholangiocytes, and treatment with rapamycin and everolimus (inhibitors of mTOR complex 1), LY294002 (an inhibitor of PI3K) and NVP-BEZ235 (an inhibitor of PI3K and mTORC1/2), significantly increased the autophage and apoptosis of PCK cholangiocytes and inhibited the cell proliferative activity of PCK cholangiocytes [40], suggesting that the PKHD1 gene may be involved in cell proliferation and apoptosis via the PI3K/Akt pathway. As a signaling pathway closely associated with tumor development and progression, PI3K and Akt may phosphorylate and partici-

pate in the regulation of multiple proteins related to cell metabolism, proliferation, differentiation and apoptosis, which thus promotes cell growth and inhibits apoptosis, thereby participating in tumor cell adhesion, invasion and migration as well as tumor angiogenesis [41]. Following activation, PI3K produces PIP3 on cell membrane, and PIP3, as a second messenger, further activates downstream proteins like Akt [42]. As a ligand, PIP3 recruits PH domaincontaining proteins on cell membrane, and allows phosphorylation of these proteins by the kinases on the membrane; then, phosphorylated proteins trigger downstream signaling pathways [43]. NF-kB, a transcription factor of Akt, is involved in the cell proliferation and apoptosis induced by multiple growth factors and cytokines [40], and NF-kB expression has been found to promote cell growth and play a critical role in tumorigenesis [44]. It is therefore considered that PI3K/Akt signaling serves as a central control point in cell proliferation, growth, and differentiation [42]. Following transactivation, the ReIA/p65 subunit of NF-KB may activate intercellular cell adhesion molecule-1 (ICAM-1), vascular endothelial growth factor (VEGF) and multi-drug resistance gene (MDR), thereby regulating the biological behaviors of tumor cells, including proliferation, anti-apoptosis, invasion, migration and drug resistance [45]. In this study, Western blotting assay detected significant up-regulation of PI3K, Akt, p-Akt, and NF-kB expression in LV3-PKHD1transfected HuCCT-1 cells as compared to that in the LV3NC-transfected and nontransfected cells (P < 0.05). Our data suggest that PI3K, Akt, p-Akt, and NF-kB may be downstream target molecules of the PKHD1 gene, and the silencing of the *PKHD1* gene may activate the PI3K/Akt signaling pathway in HuCCT-1 cells. while PI3K/Akt pathway induces cancer cell division and proliferation and inhibits apoptosis [46].

Multiple hypotheses have been proposed to explain the cause of ICC; however, the exact pathogenesis of this malignancy remains unclear until now [47-49]. In addition, multiple signaling pathways and growth factors (EGFR, VEGF, HGF/MET) have been found to be involved in the ICC development and progression; however, the underlying mechanisms are not unclear to date [47]. FPC is reported to participate in the maintenance of the normal tubular structure of the renal collecting tubule and intrahepatic bile duct epithelial cells via the primary cilium [19]. As a mechanical, chemical and osmotic pressure sensor of intrahepatic bile duct epithelial cells, cilium, upon receiving physical and chemical stimuli in the tube cavity, transfers signals into intrahepatic bile duct epithelial cells [50]. In liver, primary cilium is present only in the bile duct epithelial cells, and cilia-related diseases are also called cholangiociliopathies [51], which include autosomal dominant polycystic kidney disease (ADPKD), ARP-KD, nephronophthisis-associated ciliopathies (NPHP), Bardet-Biedl syndrome (BBS) and Meckel-Gruber syndrome (MKS) [52]. shRNAmediated silencing of FPC was found to cause abnormalities in primary cilia [53], and siRNAinduced FPC knockdown was reported to result in cilial abnormalities in intrahepatic bile duct epithelial cells [54]. In the current study, SEM displayed shorter length, less number and lower distribution density of primary cilium on the surface of the LV3-PKHD1 transfected-HuCCT-1 cells relative to the LV3NC-transfected cells, which was similar to previous reports [50, 51]. Our data demonstrate that deficiency of FPC may cause changes in the size, number and distribution of primary cilium in the bile duct epithelial cells, and the length of primary cilium and interference of cilium-associated proteins may produce a cystic phenotype [55, 56]. It has been proved that the development of hepatic and renal cysts in polycystic kidney disease (PKD) correlates with ciliary length [57]. In mouse models of hepatic cyst and polycystic liver disease (PCK, pkd2<sup>ws25/-</sup> and Pkhd1<sup>del2/del2</sup> mice), the cystic cholangiocyte cilia were short and malformed [58]. These data suggest that deletion of the PKHD1 gene causes down-regulation of FPC expression, which may result in structural and functional alterations of the cilium, and finally induce cystic alteration of the bile duct epithelial cells. Taking these findings together, it is hypothesized that the structural and functional abnormalities of the cilium may be an indirect cause of cholangiocarcinoma; however, further studies are required to test our hypothesis.

In summary, the results of this study demonstrate that the silencing of the *PKHD1* gene results in the deletion of the FPC protein and induces structural and functional abnormalities of the cilium, which promotes the proliferation, migration and invasion of human ICC HuCCT-1 cells, and such a promotion is mediated via the PI3K/Akt signaling pathway. Our findings provide new insights into the elucidation of the ICC pathogenesis, and identification of novel targets for the treatment of ICC. Further *in-vivo* experiments to investigate the associations of PKHD1 with the clinico-pathologic characteristics of ICC patients seem justified.

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## Disclosure of conflict of interest

### None.

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