Original Article

PORCN promotes hepatocellular carcinogenesis via Wnt/β-catenin-dependent manner

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Background: Porcupine O-acyltransferase (PORCN), a membrane-bound O-acyltransferase, is crucial in Wnt ligand palmitoylation. However, the roles of PORCN in the development of hepatocellular carcinoma (HCC) remain unknown.

Methods: Western blot, real-time quantitative polymerase chain reaction (RT-qPCR) assays, and The Cancer Genome Atlas (TCGA) database were used to study the expression and prognostic values of PORCN in patients with HCC. Following this, Cell Counting Kit-8 (CCK-8), wound-healing tests, Transwell assay, and a xenograft mouse model were employed to examine the effect of PORCN on HCC cells. Finally, the underlying molecular mechanisms involved in cell proliferation and migration caused by PORCN were identified.

Results: The protein and messenger RNA (mRNA) levels of PORCN in HCC tissues were higher than those of adjacent normal tissues. The analysis of TCGA database indicated that patients with higher PORCN expression had a lower overall survival (OS) rate. Overexpression of PORCN could promote the proliferation and migration abilities of HCC cells both *in vitro* and *in vivo*. Gene set enrichment analysis (GSEA) showed that the effect of PORCN on the biological characteristics of HCC cells mainly centered on the Wnt-β-catenin signaling pathway. Mechanically, immunofluorescence staining and subcellular protein fraction assays showed that PORCN could induce epithelial-mesenchymal transition (EMT) by promoting the translocation of β-catenin from the cytoplasm to nucleus, ultimately promoting the progression of HCC.

Conclusions: The findings of this study suggest that PORCN can promote HCC cell proliferation and migration by stimulating the Wnt-β-catenin signaling pathway. Therefore, PORCN may be a promising therapeutic target for HCC.

Keywords: Porcupine O-acyltransferase (PORCN); membrane-bound O-acyltransferase (MBOAT); hepatocellular carcinoma (HCC); Wnt-β-catenin signaling

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Introduction

Hepatocellular carcinoma (HCC) is one of the most serious malignant cancers, accounting for 90% of primary hepatic carcinoma, and has become the leading cause of death from cancer worldwide (1-3). Due to the heterogeneity of HCC, its pathogenesis is complex and involves multiple genes and signaling pathways. Recently, considerable progress has been made in the clinical diagnosis and treatment of HCC,

but the curative effect is not ideal (4). Only 10–13% of patients with HCC are cured via liver transplantation and surgical treatment (5). Therefore, HCC poses a huge threat to global health.

Porcupine O-acyltransferase (PORCN), a member of the membrane-bound O-acyltransferase (MBOAT) family, is a multichannel integral membrane enzyme expressed on the endoplasmic reticulum (6,7). PORCN palmitoylates Wnts and secretes them out of the cell membrane (8). In all vertebrates, the acylation of PORCN is necessary for normal secretion and the activity of all Wnts, and thus the loss of PORCN is embryo-lethal (9-11). When Wnt is not activated, intracellular Axin, APC, and GSK3β proteins form complexes that bind and phosphorylate β-catenin for degradation by ubiquitination modification (12,13). Activated Wnt binds to the frizzled receptors (FZDs) on the surface of the cell membrane, activates the dishevelled (Dvl) protein in the cell, and inhibits the degradation of β-catenin by the complex formed by GSK3β and other proteins so that the non-phosphorylated β-catenin protein is free in the cytoplasm. Non-phosphorylated β-catenin directly enters into the nucleus, then binds to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of the transcription factors, and initiates Wnts to regulate the expression of target genes (14-16). According to The Cancer Genome Atlas (TCGA) database, about 18% of stomach cancers have mutations in the *RNF43* gene. The proliferation, migration and invasion of cancer cells with higher PORCN expression are enhanced in gastric cancer while apoptosis is weakened (17). In addition, the loss of function due to *RNF43* mutations is observed in about 5–10% of patients with pancreatic cancer cases, rendering them sensitive to PORCN inhibition (18). Before they were applied clinically, PORCN inhibitors were demonstrated to inhibit Wnt ligand biosynthesis and thus inhibit pancreatic cancer progression with *RNF43* mutations. This suggests that targeting PORCN has a promising effect in treating pancreatic cancer with *RNF43* mutations (19).

HCC is a seriously life-threatening, and PORCN inhibitors have been proven to be crucial in many other cancers. However, relevant studies on PORCN in HCC are lacking. We aimed to understand the mechanism of PORCN expression in patients with HCC, with the hope of improving patient prognosis and lengthening survival time. We investigated the effects of PORCN overexpression on the proliferation and migration of HCC cells *in vitro* and *in vivo* by establishing stably expressed HCC cell lines. We found that increased PORCN expression can alter the location of β-catenin in a Wnt/β-catenin-dependent manner, promote the transcription of downstream genes, and thus promote the invasion of HCC cells. Therefore, intervention of PORCN expression in patients with HCC may improve prognosis and lengthen survival time. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-191/rc).

Highlight box

Key findings

- The expression of porcupine (PORCN) in HCC tissues was elevated, which was negatively correlated with the poor prognosis of patients HCC and promoted HCC progression in a Wnt-β-catenin-dependent manner.

What is known and what is new?

- PORCN has been reported to palmitoylate Wnt and is involved in maintaining its activity and normal secretion.
- High expression of PORCN promotes the proliferation and migration of HCC cells. High PORCN expression can predict poor prognosis in patients with HCC.

What is the implication, and what should change now?

- The Wnt-β-catenin signaling pathway is crucial in the development of HCC. PORCN is closely related to the modification and secretion of Wnt, and its inhibition may be a promising avenue in HCC therapy.

Methods

Patients and animals

The samples were collected from patients undergoing surgery who had not received other treatment before surgery in the Affiliated Hospital of Nantong University. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the ethics committee of the Affiliated Hospital of Nantong University (No. 2020-L093). Informed consent was obtained from all the patients. Animal experiments were performed under a project license (No. S20200315-009) granted by the ethics committee of the Affiliated Hospital of Nantong University and in compliance with institutional guidelines for the care and use of animals.

Cell and transfection

SMMC-7721 was transfected with overexpressed plasmid (SMMC-7721-PORCN) and a negative control (SMMC-

7721-vector) targeting PORCN. The projection and synthesis of overexpressed and negative control plasmids were carried out by Miaolingbio (Wuhan, China). After 6 h, the medium was changed to one containing serum. After 48 h, cells were acquired for RNA and protein extraction, and real-time quantitative polymerase chain reaction (RT-qPCR) and Western blotting were used to test the transfection level.

Western blotting

Radioimmunoprecipitation assay buffer (Beyotime, Nantong, China) containing protease and phosphatase inhibitors was used to cleave tissues and cells and extract proteins, with electrophoresis transfer membrane sealing, incubation, washing, and other processes also being conducted. PORCN (ab201793, 1:1,000, Abcam, Cambridge, UK), β-catenin (GB12015, 1:1,000, Servicebio, Wuhan, China), E-cadherin [3195T, 1:1,000, Cell Signaling Technology (CST), Danvers, MA, USA], N-cadherin (13116T, 1:1,000, CST), and α-smooth muscle actin (SMA) (ab5694, 1:10,000, Abcam) antibodies were used in this study. The trend of the protein band was quantitatively analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

RNA extraction and RT-qPCR

TRIzol reagent (Ambion, Austin, TX, USA) was used to extract total RNA from HCC cells and various tissues, which was then reverse-transcribed into complementary DNA (cDNA) using a reverse transcription kit (Vazyme, Nanjing, China). A SYBR Green PCR Kit (Qiagen, Hilden, Germany) in the Poche Light Cycler 480 system (Roche Holding AG, Basel, Switzerland) was used to detect the differential expression of various molecules in tissues. The primer sequences (Sangon Biotech, Shanghai, China) involved in this study were as follows: PORCN, forward 5'-CCCCTCCATCCTTGACC-3' and reverse 5'-CTCCCCTTCTCTGTTTCCC-3'; and 18s, forward 5'-CGCCGCTAGAGGTGAAATTC-3' and reverse 5'-CCAGTCGGCATCGTTTATGG-3'.

Cell proliferation assays

In 96-well plates, 2×10^3 cells were added into 100 μL of the medium, and 3 replicates were performed. After 72 h, each well was added with Cell Counting Kit-8 (CCK-8) reagent and incubated for 2 h. Absorbance at 450 nm was measured using an enzyme label.

Wound-healing assay

Cells were replicated 3 times on a 6-well plate. When the cells reached 90% confluence, a 100-μL pipette head of was used to scratch a wound. The medium was changed to a medium without fetal bovine serum (FBS), and then the wound healing was observed under a microscope and analyzed using ImageJ software.

Transwell migration assay

Cells (3×10^4) were suspended in FBS-free medium and then inoculated into the superior cavity, while Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS was inoculated into the inferior cavity. After a 24-h or 48-h culture, the cells were passed through micropores, fixed with formaldehyde, and stained with 0.1% crystal violet. Five regions were randomly selected, and ImageJ software was used to calculate and count cells.

Immunofluorescence analysis

In the circular slides of 24-well plates, 3×10^3 cells were placed. Cells were fixed with 4% paraformaldehyde for half an hour and washed 3 times in a shaker with phosphate-buffered saline with Tween 20 (PBST). After this, 0.2% Triton X-100 was infiltrated for 10 minutes, and the slides were washed 3 times. Next, 10% bovine serum albumin was added, the slides were sealed at 4 ℃ overnight, and the primary antibodies were incubated at 4 ℃ overnight, washed 3 times, incubated with species-specific fluorophore conjugate secondary antibodies, and then washed 3 times again. After 4',6-diamidino-2-phenylindole (DAPI) incubation, the circular slides were placed upside down on the slides and photographed under microscopy.

Subcellular protein fractionation

Cytoplasm and nuclear protein extractions were performed with a nuclear and cytoplasm protein extraction kit (Beyotime Biotechnology, Shanghai, China) as per the manufacturer's protocol. Western blotting was performed on the extracted proteins for analysis, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and lamin A/C were used as internal parameters of cytoplasm and nuclear protein.

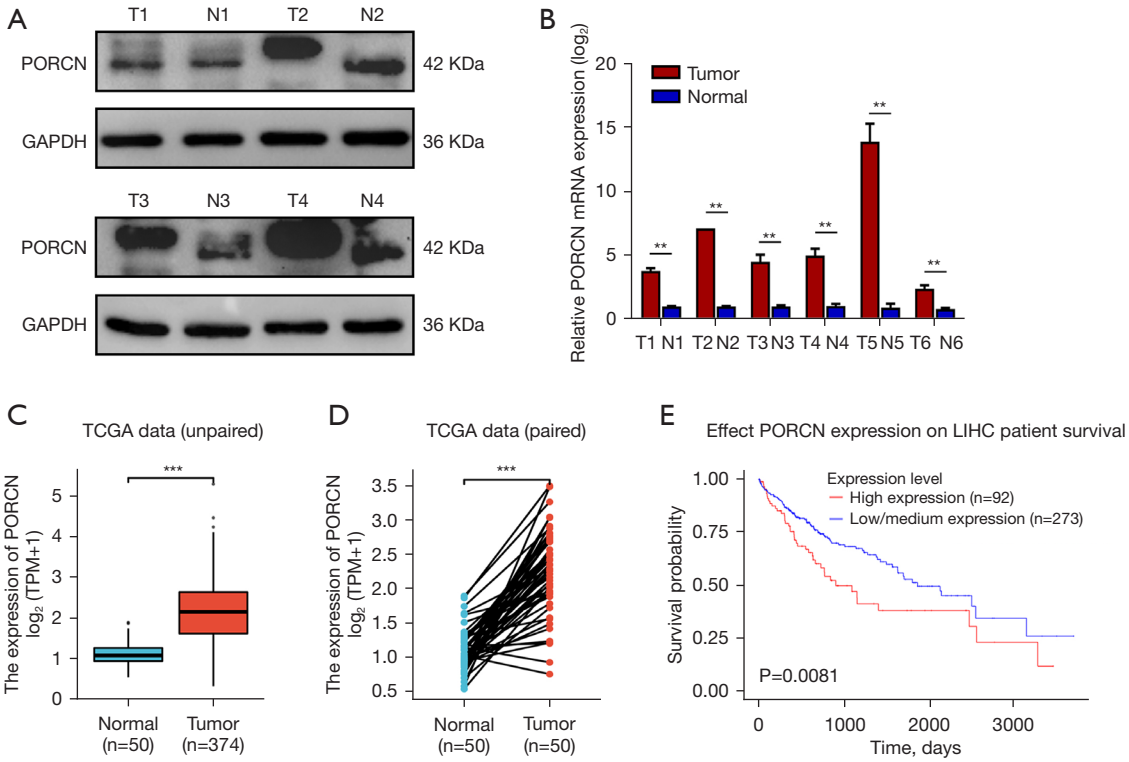

Figure 1 PORCN was elevated in HCC tissues and had value as a prognostic indicator. (A) Representative images of PORCN expression obtained in HCC tissues and nearby normal tissues in Western blotting analysis. GAPDH was used as the internal parameter. (B) Histogram of PORCN mRNA expression obtained using RT-qPCR analysis in HCC tumor tissues and matched nontumor tissues. Loading control was assessed using 18S. (C,D) Relative expression of PORCN mRNA in tumor tissues and normal tissues. (E) Kaplan-Meier survival curves revealed a correlation between PORCN expression and the overall survival rate of patients with HCC patients in TCGA database. **, P<0.01; ***, P<0.001. T, HCC tumor tissue; N, paired nontumor tissue; PORCN, porcupine O-acyltransferase; HCC, hepatocellular carcinoma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-qPCR, real-time quantitative polymerase chain reaction; mRNA, messenger RNA; TCGA, The Cancer Genome Atlas; TPM, transcripts per million; LIHC, liver hepatocellular carcinoma.

Statistical analysis

The results are expressed as mean ± standard error. All statistical analyses were performed using GraphPad Prism 8.0.2 (GraphPad Software, San Diego, CA, USA). The data obtained from the paired liver cancer and its adjacent tissue were analyzed using the paired rank-sum test. Differences between 2 groups were compared using a two-tailed Student's *t*-test. Statistical difference was set at P<0.05.

Results

The PORCN expression was elevated in HCC tissues and was correlated with a worse prognosis

PORCN function was investigated in HCC by assessing its levels in 4 HCC pairs and adjacent normal tissues. Western blotting results indicated that the PORCN protein expression level in HCC tissues was upregulated in comparison to that of adjacent normal tissues (*Figure 1A*). PCR results likewise showed the increased messenger RNA (mRNA) level of PORCN in HCC tissues in comparison to adjacent normal tissues (*Figure 1B*). Subsequently, TCGA database was used to analyze the PORCN expression level. In both paired and unpaired samples, the PORCN mRNA level in tumors was significantly higher than that in adjacent normal tissues (*Figure 1C,1D*). Kaplan-Meier survival curves of patients in TCGA database showed that the overall survival (OS) rate of patients with HCC in the group with high PORCN expression was significantly worse than in those in with low PORCN expression (P=0.0081) (*Figure 1E*).

These results indicate that PORCN expression was elevated in HCC tissues and negatively correlated with

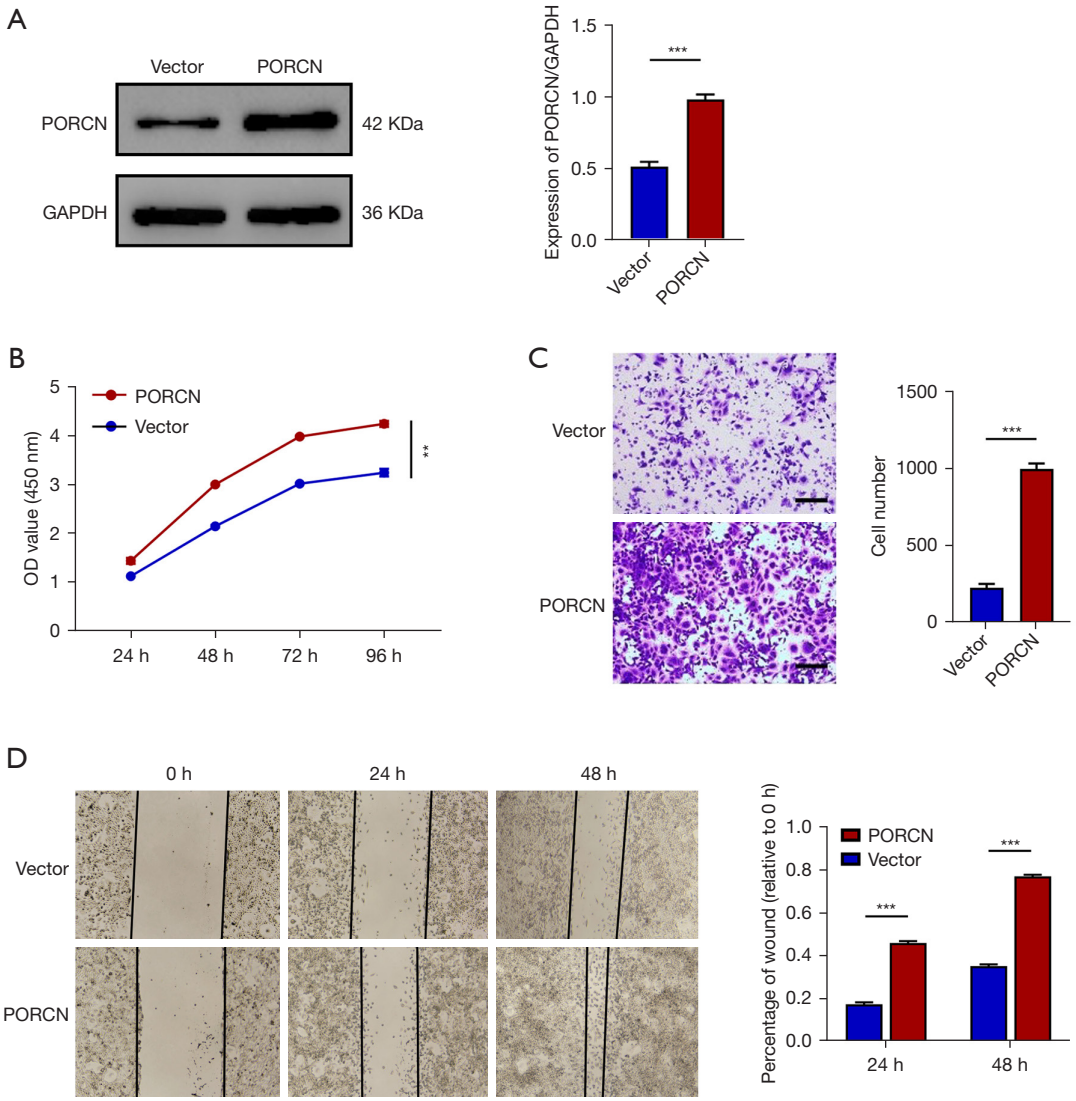

Figure 2 PORCN promoted HCC cell growth and metastasis *in vitro*. (A) Western blotting analysis verified the efficiency of PORCN overexpression in SMCC-7721 cells. GAPDH was used as the internal parameter. (B) CCK8 assays showed that PORCN overexpression promoted the growth of SMMC-7721. (C) Transwell assay was used to determine the migration capacity of HCC cells. Crystal violet staining; scale bars: 100 μm. (D) Wound healing assays revealed the metastasis capacity of HCC cells (magnification, ×4). **, P<0.01; ***, P<0.001. PORCN, porcupine O-acyltransferase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OD, optical density; HCC, hepatocellular carcinoma; CCK8, Cell Counting Kit-8.

prognosis of patients. In the future, PORCN can possibly be a prognostic indicator for HCC patients.

Upregulation of PORCN promoted the proliferation and migration HCC cells

To clarify the role of PORCN in HCC, we transfected SMMC-7721 cells with the plasmid and established a stable overexpression cell line (*Figure 2A*). With the CCK8 assay, overexpression of PORCN promoted the proliferation of HCC cells (*Figure 2B*). Transwell migration and wound-healing experiments revealed that the motility of HCC cells was significantly enhanced after PORCN overexpression (*Figure 2C,2D*).

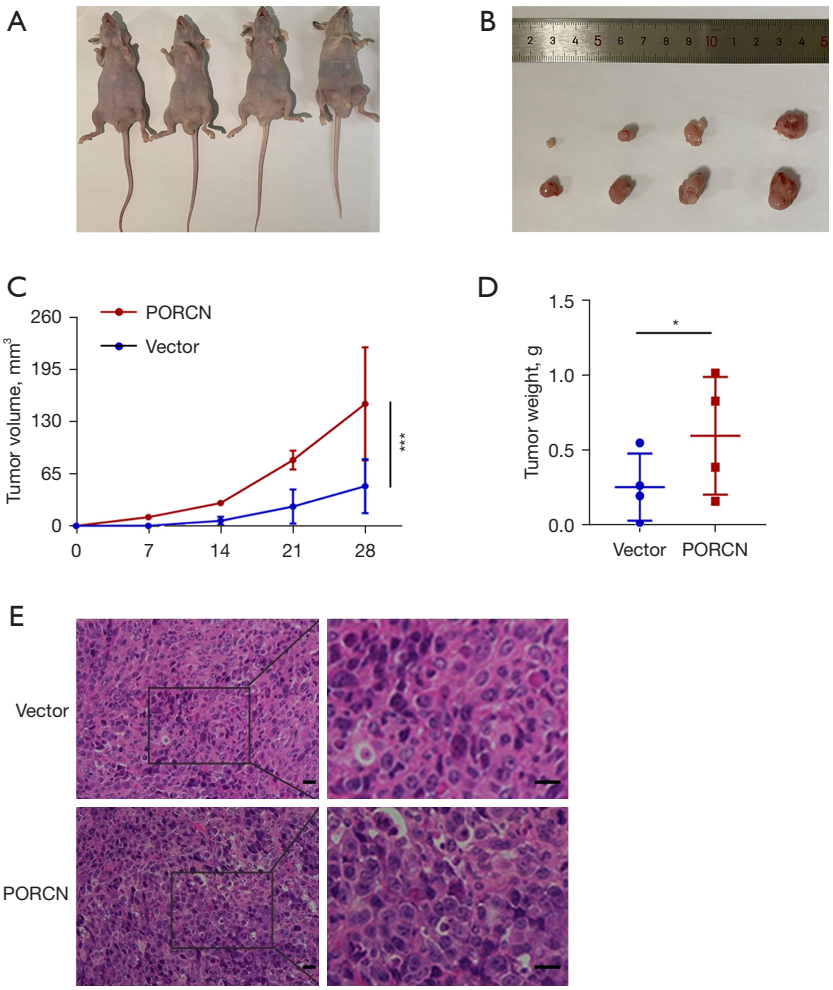

Figure 3 PORCN accelerated tumor growth and metastasis *in vivo*. (A-D) Size and weight of subcutaneous transplanted tumor in nude mice injected with SMCC-7721 cells. (E) Representative images of hematoxylin and eosin staining for subcutaneous transplanted tumor in nude mice. Scale bars: 100 μm. *, P<0.05; ***, P<0.001. PORCN, porcupine O-acyltransferase.

Subsequently, we studied the impact of PORCN on HCC cell growth and progression *in vivo*. SMMC-7721 cells overexpressing PORCN and empty controls were implanted into the armpit skin of both sides of the nude mice. The volume and weight of the subcutaneous tumor in the SMMC-7721-PORCN group were significantly higher than those in the SMMC-7221-vector group (*Figure 3A-3D*), suggesting that overexpression of PORCN promotes tumor growth *in vivo*. Hematoxylin and eosin (HE) immunohistochemical staining demonstrated that the pathological features of the tumors (*Figure 3E*).

In conclusion, PORCN can accelerate HCC cell proliferation and migration both *in vivo* and *in vitro*.

Upregulation of PORCN promoted the epithelial-mesenchymal transition (EMT) phenotype of HCC cells

According to the HCC tissue information derived from TCGA database, high- and low-PORCN expression groups were formed, and the signaling pathways associated with tumor function potentially involved in PORCN were identified using gene set enrichment analysis (GSEA). *Figure 4* shows that the impacts of PORCN on the biological characteristics of HCC cells mainly center around the Wnt-β-catenin signaling pathway. The Wnt-β-catenin pathway, also called the classical Wnt signaling pathway, influences cell differentiation, proliferation, and apoptosis, which in turn affects the regulation of intercellular

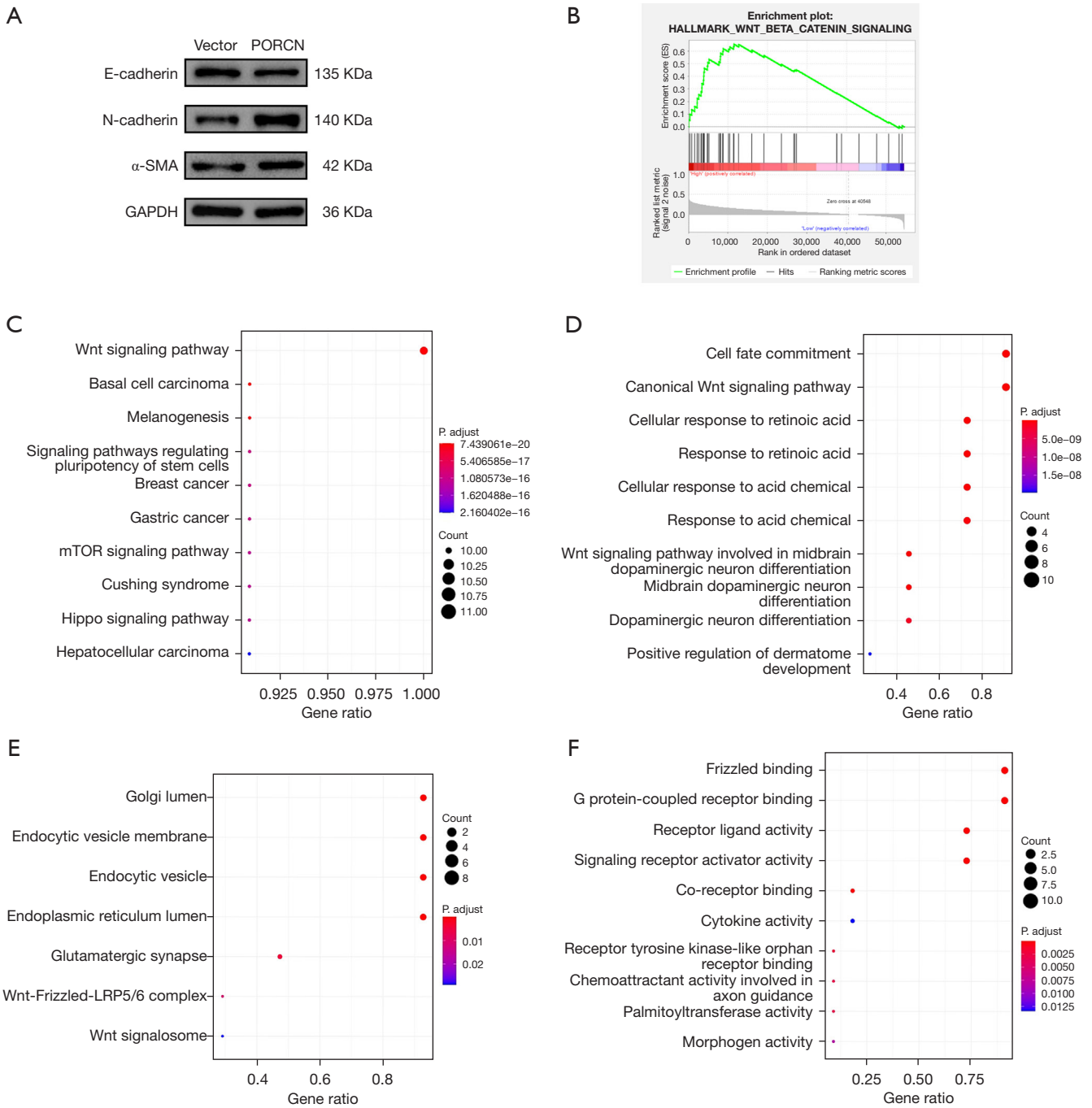

Figure 4 PORCN promoted EMT in HCC cells. (A) EMT-related markers E-cadherin, N-cadherin, and α-SMA expression levels in related cells. GAPDH was used as the internal parameter. (B-F) GSEA was used to determine the impact of PORCN on the biological characteristics of HCC cells. PORCN, porcupine O-acyltransferase; α-SMA, α-smooth muscle actin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mTOR, mammalian target of rapamycin; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; GSEA, gene set enrichment analysis.

connectivity and the maintenance of epithelial phenotype/tissue homeostasis (20). Abnormal activation of this pathway may lead to EMT (21,22).

In order to further clarify the possible molecular mechanisms of PORCN promoting the development of HCC, we first determined whether PORCN regulated the expression of EMT marker. Analysis of the epithelial-labeled E-cadherin, mesenchymal-labeled N-cadherin, and α-SMA protein levels showed that overexpression of PORCN could induce EMT. PORCN overexpression increased the expression of N-cadherin and α-SMA but decreased the expression of E-cadherin (*Figure 4A*). The result suggests that PORCN is crucial in the tumorigenesis of HCC by influencing the EMT process.

Upregulation of PORCN facilitated the EMT process through regulating the entry of β-catenin into the nucleus

GSEA showed that the function of PORCN was mainly concentrated in the Wnt-β-catenin signaling pathway (*Figure 4B-4F*). The Wnt-β-catenin signaling pathway is often activated during HCC progression and participates in the EMT process (23-25). Therefore, we further investigated the role of the β-catenin signaling pathway in the regulation of the EMT process by PORCN. First, we detected the protein level of total β-catenin in SMCC-7721-PORCN and SMCC-7721-vector cells, and found that the expression level of β-catenin in cells was upregulated when PORCN was overexpressed (*Figure 5A*). The subcellular localization of β-catenin was detected using immunofluorescence assay. In the SMCC-7721-PORCN cells, the excess expression of PORCN facilitated β-catenin entry into the nucleus (*Figure 5B*). β-catenin was obviously transferred into the nucleus in PORCN-overexpressed cells as demonstrated by further subcellular isolation experiments, whereas the cytoplasmic β-catenin levels remained unchanged (*Figure 5C*). These results suggest that PORCN promotes HCC cell EMT by upregulating β-catenin entry into the nucleus.

Discussion

Primary liver cancer is characterized by a high morbidity and fatality rate, seriously threatening people's health and safety. High metastasis and invasiveness are important factors contributing to death in patients with liver cancer. Therefore, elucidating the mechanism of HCC progression is still an important topic of HCC research. Over the past few years, PORCN has been found to figure prominently in the occurrence and progression of many types of malignant tumors (17). Studies have found that PORCN can palmitoacylate Ku70, an important protein in DNA damage repair, and DNA damage repair is the target of many chemotherapeutic drugs (18). In breast cancer cells, knockdown or inhibition of PORCN can delay and degrade the DNA damage repair process of cancer cells, increase the sensitivity of cancer cells to chemotherapeutic drugs, and thus improve the prognosis of patients with breast cancer. A study using TCGA database also demonstrated PORCN to be negatively correlated with the prognosis of patients with breast cancer (19). PORCN is the only acyltransferase responsible for the acidification of all Wnts palmitoacylate. PORCN inhibitors reduce cells' capacity to generate Wnt ligands, whereas the potential to produce some ligands is unaffected (26). Clinical data of PORCN inhibitors have also shown good therapeutic effects on patients with tumor (27). Thus, PORCN is considered to be a promising drug target for treating Wnt-dependent cancers (28,29). Several PORCN inhibitors, including LGK974 or WNT974 (NCT01351103), ET-159 or ET-1922159 (NCT02521844), CGX1321 (NCT02675946), and RXC004 (NCT034474), have entered phase 1 clinical trials for the therapy of pancreatic, melanoma, lung, cervical, breast, respiratory, head and neck, and biliary tract cancers (30-32). For example, a phase 1 trial of WNT974 enrolled 94 patients with advanced solid tumors being administered (10 mg) of oral WNT974 once a day plus an additional dosing regimen. The results showed that WNT974 alone was well tolerated, which may affect the recruitment of immune cells to tumors and enhance the activity of WNT974 at checkpoint (33).

Despite HCC being a potentially lethal disease and PORCN inhibitors being critical in the treatment of many other cancers, there are no relevant studies on PORCN in HCC. Therefore, we actively explored the role of PORCN in HCC. We determined that PORCN, a unique acyltransferase responsible for palmitoylation, has a tremendous impact on HCC progression. Our study found that PORCN was highly expressed in liver cancer tissues, and overexpression of PORCN promoted the proliferation and migration of liver cancer cells. In the cause of tumor development, EMT promotes tumor cells to acquire invasive and motility properties. The regulation of intercellular adhesion components such as E-cadherin and N-cadherin is the primary cause of HCC metastasis (34-36). In the present study, we found that PORCN overexpression

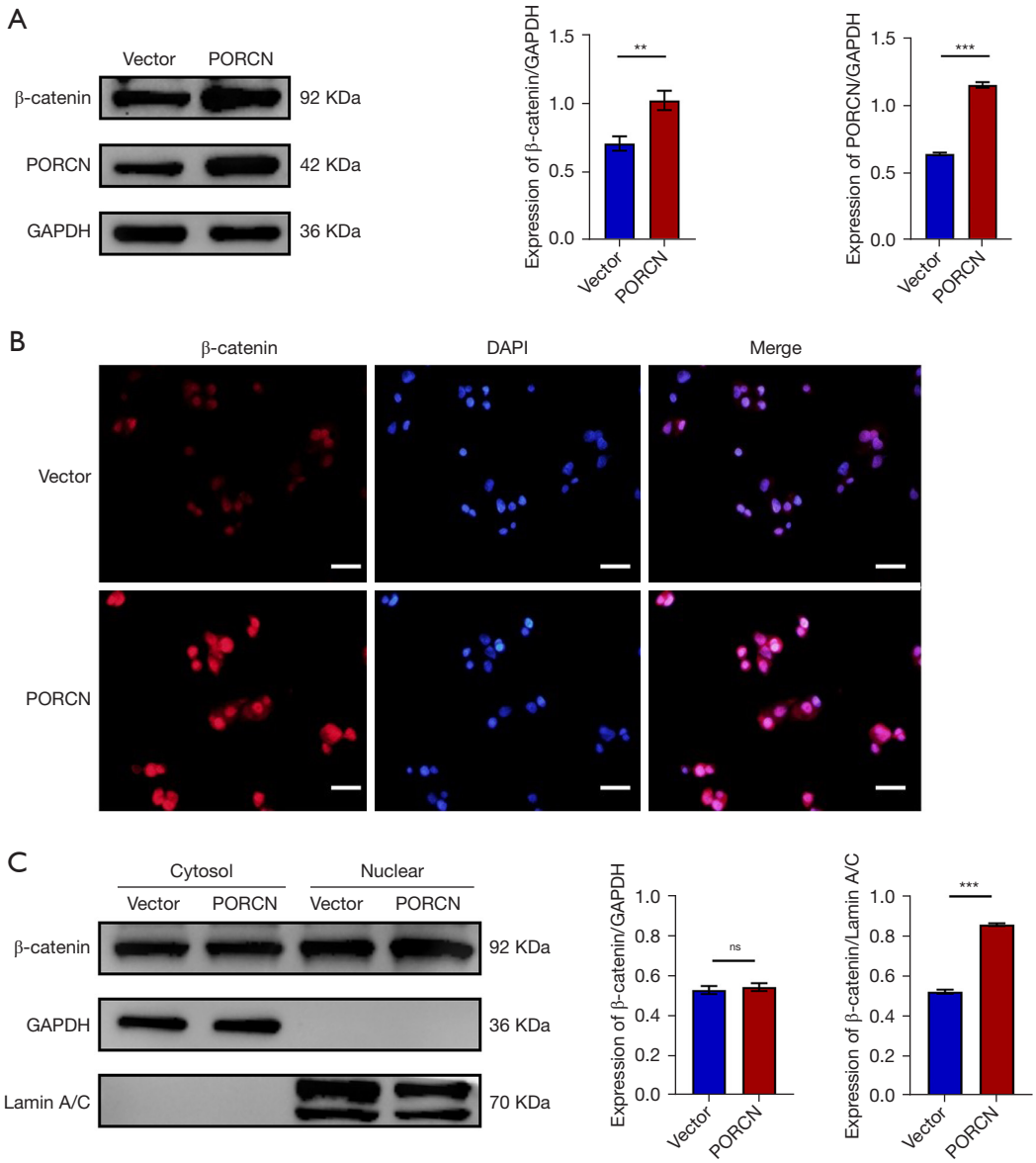

Figure 5 PORCN facilitated the EMT process by promoting β-catenin entry into the nucleus. (A) PORCN and β-catenin expression levels in related cells. GAPDH was used as the internal parameter. (B) Representative immunofluorescence images showed PORCN increased the entry of β-catenin into the nucleus. Scale bars: 100 μm. (C) Protein separation indicated that β-catenin was expressed in the cytoplasm and nucleus of cells. The loading control was assessed using GAPDH and lamin A/C. **, P<0.01; ***, P<0.001; ns, not significant. PORCN, porcupine O-acyltransferase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EMT, epithelial-mesenchymal transformation; DAPI, 4',6-diamidino-2-phenylindole.

increased the expression of N-cadherin and α-SMA but decreased the expression of E-cadherin. The results suggest that PORCN affects tumorigenesis of HCC by influencing the EMT process.

Wnts are secreted fatty-acid modified glycoproteins (37), and PORCN is required for the lipid modification of Wnt proteins. Wnt ligands bind to receptors and co-receptors on the surface of target cells, triggering a series of downstream signal transduction events, especially β-catenin-mediated transcription, in which β-catenin stabilization and nuclear translocation are important links. Studies have shown that abnormal activation of Wnt/β-catenin signaling contributes

to the initiation of EMT and the formation of invasive carcinoma (38-40). The data from GSEA database proved that PORCN participates in the binding process of Wnt ligands and frizzled-LRP5/6 co-receptors on the membrane surface, regulating the classical Wnt signaling pathway. Our study demonstrated that PORCN regulates β-catenin transfer from the cytoplasm to the nucleus, promoting downstream gene transcription and thus mediating the EMT process.

Conclusions

PORCN plays a key role in HCC progression and is dependent on the entry of β-catenin into the nucleus. Therefore, PORCN may be promising therapeutic target for HCC.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-191/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-191/dss

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-191/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-191/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the ethics committee of the Affiliated Hospital of Nantong University (No. 2020-L093). Informed consent was taken from all the patients. Animal experiments were performed under a project license (No. S20200315-009) granted by the ethics committee of the Affiliated Hospital of Nantong University and in compliance with institutional guidelines for the care and use of animals.

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