FULL LENGTH ARTICLE

Depletion of VPS35 attenuates metastasis of hepatocellular carcinoma by restraining the Wnt/PCP signaling pathway

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Abstract Vesicle Protein Sorting 35 (VPS35) is a novel oncogene that promotes tumor growth through the PI3K/AKT signaling in hepatocellular carcinoma (HCC). However, the role of VPS35 in HCC metastasis and the underlying mechanisms remain largely unclear. In this study, we observed that overexpression of VPS35 enhanced hepatoma cell invasion and metastasis by inducing epithelial—mesenchymal transition (EMT)-related gene expression. Conversely, knockout of VPS35 significantly inhibited hepatoma cell migration and invasion. Furthermore, depletion of VPS35 decreased the lung metastasis of HCC in nude mice. By transcriptome analysis, we determined that VPS35 promoted HCC metastasis by activating the Wnt/non-canonical planar cell polarity (PCP) pathway. Mechanistically, VPS35 activated the PCP pathway by regulating membrane sorting and trafficking of Frizzled-2 (FZD2) and ROR1 in hepatoma cells. Collectively, our results indicate that VPS35 promotes HCC metastasis via enhancing the Wnt/PCP signaling, thus providing a potential prognostic marker and therapeutic target for HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary malignant liver cancer, and constitutes a serious medical problem worldwide. With 841,000 new cases diagnosed and almost 782,000 deaths caused in 2018, HCC is estimated to be the sixth most frequently diagnosed cancer and is the fourth leading cause of cancer-related death globally. Despite the advances in diagnostic methods and surgical treatment, the overall 5-year survival rate remains poor. Metastasis and recurrence are the major causes of mortality in patients with HCC. Therefore, novel tools and approaches are needed to uncover new molecular mechanisms underlying HCC metastasis, and to identify novel therapeutic and diagnostic strategies to treat and prevent metastases.

The planar cell polarity (PCP) pathway is a non-canonical Wnt signaling pathway triggered by binding of Wnt ligand to its Frizzled (Fzd) receptor. The conserved PCP core components include specific Wnt ligands (usually Wnt5A, 5B, 7, and 11 in mammals), members of the Fzd receptor family, and co-receptors (such as Ror1/2, Ptk7, Ryk, Dachsous, and Fat). Binding of Wnt ligands leads to recruitment of Disheveled to Wnt/PCP Fzd receptors and activation of small GTPases of the Rho family (including Rac1, RhoA, and Cdc42), which are implicated in cytoskeleton remodeling and cell contractility, and the p62/JNK pathway. PCP signaling pathway plays a pivotal role in maintaining polarized epithelial tissue structure and promoting migration, convergence, and extension during development. Although previously overlooked, several studies have recently uncovered important contributions of the PCP pathway to tumor malignancy. Wnt/PCP components are upregulated in melanoma and cancer of the breast, ovary, and pancreas, and their upregulation is associated with poor prognosis. A recent study showed that FRIZZLED-7 and other Wnt/PCP components are upregulated in ovarian cancer cells, promoting cell proliferation, cell migration, and stemness in part via RhoA activity. Vesicle Protein Sorting 35 (VPS35) protein – a core component of the retromer complex – is involved in retrograde transport from the endosome to the plasma membrane or the trans-Golgi network (TGN). The retromer complex is essential for the maintenance of neurons. Impaired retromer function has been implicated in human neurodegenerative diseases, including Parkinson’s disease and Alzheimer’s disease. A recent study suggested that VPS35 silencing promoted association of N-Ras with cytoplasmic vesicles, attenuated GTP loading of Ras, and inhibited mitogen-activated protein kinase (MAPK) signaling and growth of N-Ras–dependent melanoma cells. Although the role of VPS35 in neurodegenerative diseases has been well documented, much less is known about its role in cancer.

In our previous study, we performed whole-exome and RNA sequencing in a small liver hepatocellular carcinoma (LHHC) cohort and characterized the transcriptional pattern of tumor-mutated alleles. We identified VPS35 as a novel oncogene that promotes tumor growth through the PI3K/AKT signaling. In this study, we further explored the function of VPS35 in the metastasis of HCC and elucidated the underlying mechanisms.

Material and methods

Tumor tissue specimens

A total of 40 HCC tumor samples and paired adjacent noncancerous liver tissues were obtained from curative resections at the Second Affiliated Hospital of Chongqing Medical University (Chongqing, PR China) between 2014 and 2017. The study was approved by the Institutional Review Board of Chongqing Medical University.

Antibodies

Antibodies were used as follows: anti-VPS35 (1:10,000, ab157220, Abcam, USA), anti-E-cadherin (1:10,000, ab40772, Abcam, USA), anti-N-cadherin (1:1000, 13116, Cell Signaling Technology, USA), anti-β-actin (1:2000, TA-09, ZSGB-BIO, China), anti-GAPDH (1:2000, AG019, Beyotime, China), anti-ROCK1 (1:500, WL01761, Wanleibio, China), anti-JNK (1:1000, GTX133806, Gene Tex, USA), anti-ROCK1 (1:1000, AJ518, Beyotime, China), anti-Na/K ATPase (1:2000, 619736, ZEN BIO, China), anti-Frizzled 2 (1:1000, 24272-1-AP, Proteintech, USA), anti-ROR1 (1 µg/ml, AF2000, R&D system, USA), anti-MMP9 (1:1000, S1241, Bioworld, USA), p62 (1:1000, 5114, Cell Signaling Technology, USA), anti-Rac (1:500, WL02851, Wanleibio, China), anti-RhoA (1:500, WL02853, Wanleibio, China), anti-Rac (1:1000, ab76535, Abcam, USA), anti-RhoA (1:1000, bs-5330R, Bioss, USA), anti-p-p62 (1:1000, GTX128171, Gene Tex, USA), anti-FZD1 (1:1000, GTX108181, Gene Tex, USA).

Cell lines and culture

Human HCC cell lines MHCC-97H, SK-Hep1, and Huh7 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). VPS35-knockout (KO) SK-Hep1 cells were generated by lentiviral CRISPR system as described previously. Adenoviral recombinant AdVPS35 was generated using the AdEasy system. Green fluorescent protein-expressing analogous adenovirus (AdGFP) was applied as a control. Cells were maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 µg/ml of streptomycin, and 100 unit/ml of penicillin in a humidified incubator at 37 °C with 5% CO2 and 95% air.

Immunofluorescence

E-cadherin, N-cadherin, and ROR1 were detected with the relative rabbit polyclonal antibodies. The specific signals were visualized with Alexa Fluor 488 or 594 secondary antibody (Invitrogen). Nuclei were stained with 1 µg/ml DAPI (10236276001, Roche Diagnostics GmbH, Mannheim, Germany) for 3 min. Stained sections were analyzed by a laser scanning confocal microscope (Leica TCS SP8, Solms, Germany).
Wound scratch assay

Cells were grown in 96-well plates until 90% confluent, then the cell surface was scraped with WoundMaker™ to create wounds. Before incubation in a serum-free medium, cells in suspension and debris were removed. After 18–36 h, cells migrating at the front of the wound were photographed by the IncuCyte ZOOM Live-Cell Imaging system (Essen Bioscience, Ann Arbor, MI, USA).

Transwell invasion assay

Cell invasion was evaluated using Cell Culture Insert (Transparent PET Membrane 8 μm pore size, 6.5 mm diameter, FALCON, USA). In brief, cells (6 × 10^4) were washed with PBS, then resuspended in 200 μl of serum-free medium and added to the upper chamber that was coated with Matrigel (BD Biosciences, San Jose, California, USA). At the same time, medium containing 10% FBS was added to the lower chamber. The chambers were incubated in 5% CO₂ at 37°C for 24 h. The invading cells were stained and quantified (ten random 200× fields per well) under Axio Imager A2 (ZEISS, Germany).

RNA extraction, reverse transcription, and quantitative real-time-PCR

Total RNA was extracted from HCC cells using TRIzol reagent (Invitrogen, Rockville, MD, USA) according to the manufacturer’s instructions. Purified RNA samples were reverse-transcribed into cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (RR047A, Takara, Tokyo, Japan). Quantitative Real-Time PCR analysis of target genes was performed using the SYBR Green qPCR Master Mix (Bio-Rad, Hercules, CA, USA) with specific primers (Table S1). Each sample was analyzed in triplicate. Relative mRNA levels were calculated using the 2^(-ΔΔCt) method using β-actin as the reference gene for normalization.

Western blotting

Cells or liver samples were lysed in cell lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with 1 mM of phenylmethylsulfonyl fluoride (Beyotime). Cell-surface and cytoplasmic proteins were extracted using the Membrane and Cytosol Protein Extraction Kit (Cat# P0033, Beyotime) according to the manufacturer’s instructions. Total proteins (30 μg) were separated by 10% SDS/PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The immunoblots were probed with the indicated antibodies. Protein bands were visualized with Ultra-sensitive chemiluminescence substrate kits (10300, New Cell &Molecular Biotech Co, Ltd, Su Zhou, China). GAPDH or β-actin were used as internal controls. All experiments were independently repeated three times.

RNA-sequencing (RNA-seq) analysis

Total RNA was extracted from parental or VPS35-KO SK-Hep1 cells using TRIzol reagent (Invitrogen, Rockville, MD, USA) according to the manufacturer’s instructions. RNA-seq was performed by the Beijing Genomics Institute (BGI, Shenzhen, China). Briefly, strand-specific RNA-seq libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Beverly, MA, USA). Six samples were sequenced on a HiSeq 4000 sequencing platform (Illumina, San Diego, CA, USA). We used the bioinformatics pipeline used in our previous study to analyze differential gene expression between VPS35-KO and parental cells. The RNA-seq data were submitted to the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) under the number SUB6834808.

Immunohistochemistry

Liver tissue samples were fixed in fresh 4% PFA and embedded in paraffin according to standard procedures. Sections were incubated with the indicated primary antibodies overnight at 4°C. Subsequently, the slides were incubated with a secondary anti-rabbit or anti-mouse IgG (ZSGB-BIO, Beijing, China) and visualized using 3,3-diaminobenzidine (ZSGB-BIO). Stained slides were scanned with the MoticEasyScanner (Motic, Hong Kong, China) and images were acquired using Motic DSAssistant Lite (Motic VM V1 Viewer 2.0).

Mouse studies

All animal experiments were performed using 6-week-old athymic nude mice. MHCC-97H cells were harvested using trypsin, washed twice with PBS, and re-suspended in PBS. The suspended cells (2 × 10⁶/200 μl) were injected into the lateral tail vein of male BALB/c nude mice. After 60 days, mice were sacrificed. Lung tissues were fixed in 4% PFA, embedded in paraffin, sectioned at 5 μm thickness, and then stained with hematoxylin and eosin (H&E). A minimum of 15 sections per mouse was examined under a light microscope. All animal experiments were carried out according to the guidelines of the Institutional Animal Care and Use Committee at Chongqing Medical University (project license number: 20170712). Animal care and use protocols adhered to national regulations for the administration of laboratory animals.

Statistical analysis

All data are presented as mean ± standard deviation (SD). GraphPad Prism 7.0 software (La Jolla, CA, USA) was used to perform all statistical analyses and prepare graphs. For statistical analysis, an unpaired, two-tailed t-test was applied, unless indicated differently in figure legends. Statistical significance was determined using one-way ANOVA for multiple comparisons. P < 0.05 was considered statistically significant.

Results

VPS35 depletion inhibits HCC cell migration and invasion

Cancer cell migration and invasion are crucial events for liver cancer metastasis. Therefore, we first explored the
role of VPS35 on HCC cell migration and invasion. Compared with L02 cell line, an increased endogenous expression of VPS35 in the majority of hepatoma cell lines rather than in Huh7 and SNU-449 cell lines (Fig. S1A). Wound scratch assays revealed that loss of VPS35 inhibited migration of MHCC-97H and SK-Hep1 cells (Fig. 1A). Instead, VPS35-overexpressing Huh7 cells showed enhanced wound closure (Fig. 1B). Next, invasive capacities were assessed using a transwell assay. As shown in Fig. 1C and D, respectively, VPS35 knock out in SK-Hep1 cells inhibited cell migration and invasiveness, while VPS35 overexpression in Huh7 cells had the opposite effect. To assess the role of VPS35 in the colonization potential of hepatoma cells in vivo, we injected VPS35-KO and parental MHCC-97H cells into the tail vein of nude mice and analyzed lung metastases after 8 weeks. Fewer lung metastases were found in VPS35-KO-injected mice than in parental MHCC-97H-injected mice (2/9 vs 5/9, respectively; Fig. 1E). These observations were confirmed by HaE staining of lung tissues (Fig. 1F). Taken together, our results suggest that VPS35 depletion decreased the migratory and invasive abilities of hepatoma cells in vivo and in vitro.

**VPS35 knockout reduces the expression of EMT-related genes in hepatoma cells**

Given that the epithelial–mesenchymal transition (EMT) program is critical for migration and invasiveness of most tumor, we next examined whether VPS35 regulates EMT-related gene expression in HCC cell lines. Quantitative RT-PCR showed that VPS35 overexpression upregulated the mesenchymal markers N-cadherin, Vitronectin, Matrix Metalloproteinase 14 (MMP14), Matrix Metalloproteinase 9 (MMP9) and Collagen type I alpha 2 (COL1A2) at the mRNA level. Conversely, VPS35-depleted MHCC-97H cells exhibited increased expression of E-cadherin, but decreased expression of N-cadherin, Fibronectin, MMP14, and MMP9 (Fig. 2A). Further, Western blot analysis revealed that VPS35 overexpression upregulated MMP9 and N-cadherin, but downregulated E-cadherin. Conversely, E-cadherin was upregulated, while MMP9 and N-cadherin were downregulated in VPS35-depleted MHCC-97H cells (Fig. 2B). Immunofluorescence analysis showed similar results (Fig. 2C). Consistent with the in vitro results, downregulation of E-cadherin and upregulation of N-cadherin and...
MMP9 were also observed in HCC clinical samples (Fig. 2D). These results suggest that VPS35 silencing inhibited tumor invasion and metastasis by downregulating EMT markers.

**Overexpression of VPS35 activates Wnt/PCP signaling pathway in hepatoma cells**

To investigate the molecular mechanism underlying VPS35-promoted hepatoma cell invasion and metastasis, we performed RNA-Seq analysis in VPS35-KO and parental SK-Hep1 cells. As shown in Fig. 3A and Fig. S1B, the differentially expressed genes (DEGs), which were significantly enriched in the Wnt/PCP signaling (e.g., Wnt7B, AXIN2, Rac3), were notably downregulated in VPS35-KO cells. These results were confirmed by qRT-PCR analysis of selected DEGs in VPS35-KO or VPS35-OE and parental MHCC-97H or Huh7 cells, respectively (Fig. 3B). Wnt/PCP signaling plays important roles in metastasis; therefore, we investigated if VPS35 promoted migration, invasion, and EMT via the activation of Wnt/PCP pathway in HCC cells. Overexpression of VPS35 in Huh7 cells reduced phosphorylation of RhoA at Ser188, Rac at Ser71 and p62 at Serine 403, while simultaneously increasing the expression of p62 and Rock1 and phosphorylation of JNK (Fig. 3C). Knockout of VPS35 in MHCC-97H cells showed the opposite effect (Fig. 3C). Wound scratch assays (Fig. 3D) and transwell assays (Fig. 3E) confirmed that the ROCK inhibitor GSK269962A can suppress VPS35-induced HCC cell migration and invasion. These results indicate that VPS35 might induce cell migration, invasion, and EMT through activation of Wnt/PCP signaling pathway.

VPS35 activates Wnt/PCP signaling pathway by regulating the sorting and trafficking of FZD2 and ROR1 to the plasma membrane

VPS35 directly associates with sorting signal cargo proteins. We next investigated the potential cytosolic retrieval

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**Figure 2** VPS35 knockout reduces the expression of EMT-related genes in hepatoma cells. (A) The mRNA expression levels of EMT-related genes in VPS35-OE and VPS35-KO cells were detected by quantitative real-time PCR. Data are means ± SD. (n = 3, performed in triplicate). *P < 0.05, **P < 0.01; ***P < 0.001. The protein expression of E-cadherin, N-cadherin, and MMP9 in hepatoma cells were examined by (B) western blotting and (C) immunofluorescence. Nuclei were counterstained with DAPI. (D) Immunohistochemical staining of normal (N) and tumor (T) tissues with anti-VPS35, anti-E-cadherin, anti-MMP9, and anti-N-cadherin antibodies.
proteins related to VPS35. Through global proteomic analysis in vitro and in non-polarized cultured cells, FRIZZLED-2 (FZD2) and ROR1 were regulated by VPS35, respectively. Therefore, we assumed that VPS35 activated the Wnt/PCP signaling by regulating the intracellular localization of FZD2 and ROR1. Consistently, immunoblots revealed a marked decrease in cell-surface FZD2 and ROR1 in VPS35-KO MHCC-97H cells, whereas overexpression of VPS35 increased the levels of cell-surface FZD2 and ROR1 (Fig. 4A). Immunofluorescence confirmed a substantial reduction in membrane localization of ROR1 in VPS35-KO cells, indicating a decreased recycling of ROR1. Conversely, VPS35-OE cells showed the opposite effect (Fig. 4B). Next, we analyzed VPS35 expression in clinical HCC samples and explored the potential association between expression of VPS35, FZD2, and ROR1 in HCC tissues. In an independent cohort of 371 HCC samples from the Cancer Genome Atlas (TCGA) database, VPS35 expression was positively correlated with ROR1 expression (r = 0.4702, P < 0.0001; Fig. 4C). To confirm these data, we analyzed the expression of these proteins in 16 HCC specimens and adjacent non-cancerous specimens. Immunoblot analysis showed that VPS35 expression was dramatically upregulated in tumor samples (Fig. 4D). Notably, in most cases, a higher expression of VPS35 was associated with higher expression of Wnt receptors FZD2 and ROR1, which indicated the potential important roles of VPS35 played in HCC metastasis (Fig. 4D). Taken together, these results demonstrated that VPS35 activated the Wnt/PCP signaling through sorting and trafficking of FZD2 and ROR1 to the plasma membrane in hepatoma cells.

Discussion

In our previous study, we identified VPS35 as a novel potential oncogene in HCC. We showed that VPS35 is upregulated in HCC tissues and indicates a poor prognosis for patients with HCC. Furthermore, the overexpression of VPS35 promotes the proliferation of HCC cells by activating the PI3K/AKT signaling pathway. However, its potential function in the metastasis of HCC was thus far poorly understood. In this study, we found that VPS35 overexpression promotes HCC cell migration and invasion in vivo and in vitro and accelerates EMT progression by activating the...
Wnt/PCP signaling pathway. Mechanistically, VPS35 activates the Wnt/PCP pathway by regulating the sorting and trafficking of FZD2 and ROR1 to the plasma membrane. The EMT of neoplastic hepatocytes is a key event in metastasis. Several studies have reported the role of the retromer complex in regulation of membrane proteins involved in EMT. VPS35 is the central cargo-binding subunit of the retromer complex. Crumbs (Crb), a key polarity protein in EMT, is a retromer cargo that interacts with VPS35. Moreover, several members of the SNX family in the retromer pathway can regulate intracellular trafficking of E-cadherin. Here, we found that VPS35 upregulated MMP9 and N-cadherin, but downregulated E-cadherin. Conversely, VPS35 depletion upregulated E-cadherin, but downregulated MMP9 and N-cadherin. Therefore, we proposed that VPS35 promotes HCC cell migration and invasion by inducing EMT. However, the detailed transport mechanism of the three key EMT markers mediated by VPS35 needs to be further studied.

Our transcriptomic studies indicate that VPS35 deficiency inhibits Wnt/PCP signaling pathway in HCC cells. The
VPS35 promotes HCC cell metastasis and mediates EMT by upregulating p62 and p-JNK. Importantly, VPS35-promoted HCC invasion and migration were partially suppressed by the ROCK inhibitor GSK269962A, indicating that VPS35 exerts its oncogenic role via activation of Wnt/PCP pathway.

VPS35 interacts directly with the conserved transmembrane protein MiG-14/WLs, it retrieves endocytosed WLs from the endosome to the TGN, after which WLs is reused for further Wnt secretion. In this study, we demonstrated that VPS35 participates in non-canonical Wnt signaling. The membrane distribution of FZD2 and ROR1 was increased in VPS35-OE Huh7 cells, while it was markedly reduced in VPS35-KO MHCC-97H cells. These findings indicate that VPS35 might facilitate the recycling of membrane-localized FZD2 and ROR1, thus promoting the activation of downstream Wnt/PCP signaling. We assumed that the increased membrane recycling and/or decreased lysosomal degradation of FZD2 and ROR1 could contribute to the increased levels of these proteins in VPS35-OE cells, which in turn might boost the activation of the Wnt/PCP signaling. However, the detailed molecular mechanism by which VPS35 mediates FZD2 and ROR1 recycling remains to be further investigated.

In summary, the present work is the first to reveal that VPS35 promotes HCC cell metastasis and mediates EMT by activating the Wnt/PCP signaling pathway. These results further broaden our understanding of the role of VPS35 in EMT process and HCC metastasis. Our study may facilitate the evaluation of VPS35 as a potential prognostic biomarker of HCC, as well as offer a new therapeutic target in HCC metastasis therapy.

Conflict of Interests

The authors have no financial conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2020.07.009.

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