PLVAP Regulates the Occurrence and Development of Hepatocellular Carcinoma via Wnt/B-Catenin Signaling Pathway

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Introduction. Recurrence and metastasis are one of the reasons for the poor prognosis and high fatality rate of hepatocellular carcinoma (HCC). However, the current treatment methods are limited, so finding effective treatment targets is an effective expansion of the treatment methods for patients with HCC. The research base on specific high expression of PLVAP in the RNA sequencing of HCC patients in the TCGA database, and its expression level has a significant impact on the proliferation, migration and invasion of the HCC highly metastatic cell line MHCC97H. At the same time, there is a significant correlation for the clinical prognosis of patients. The mechanism of regulating HCC is to affect the activation of Wnt/β-catenin signaling pathway. We believe that PLVAP will be one of the key targets of HCC treatment, providing scientific basis for clinical treatment.

Key word: PLVAP; hepatocellular carcinoma; Wnt/β-catenin signaling pathway;

INTRODUCTION

Hepatic malignancies is organized with primary hepatocellular carcinoma (HCC) and secondary hepatocellular carcinoma (HCC)[1]. Hepatocellular carcinoma is the most frequent and commonly primary hepatocellular carcinoma, accounting for more than 90% of primary hepatocellular carcinoma cases, and is an important cause of global tumor-related deaths [2], more than half a million patients die each year due to hepatocellular carcinoma [3], and the number of deaths due to HCC is increasing year by year. As we all know, it is a heterogeneous tumor, mainly derived from chronic liver inflammation, with a variety of etiologies, including chronic viral hepatitis

infection, alcoholism and fatty liver[4]. The etiology and exact molecular mechanism of primary liver cancer are not fully understood. At present, it is believed that its pathogenesis is a complex process with multiple factors and multiple steps, which is affected by the dual factors of environment and diet.

To explore the key pathogenic gens of HCC, we analyzed the comparison of RNA sequencing results of all liver cancer patients in the TCGA database with normal liver tissues, FC >2, P< 0.05. Among them, 3966 genes were up-regulated and 405 genes were down-regulated. Our analysis of the top 10 genes up-regulated that Plasmalemma vesicle associated protein (PLVAP) may be a key gene that causes hepatocellular malignancy into hepatocellular carcinoma, and the level of expression is significantly related to the malignancy of tumors. In Previous studies have reported roles for PLVAP in the regulation of basal permeability, leukocyte migration and angiogenesis[5-7], and PLVAP has been investigated as a novel target in cancer therapy, it can promotes angiogenesis of cholangiocarcinoma through the DKK1/CKAP4/PI3K signaling pathway[5, 8]. And now it has been found that PLVAP also has an immunosuppressive effect by regulating macrophages in hepatocellular carcinoma[9, 10]. PLVAP is also required for the formation of pits and stomata through the endothelial passage. The function in the permeability of capillaries and the endothelial window facilitates the passage of water and solutes and regulates the cross-cell and paracellular flow in different organs, and at the same time regulates embryonic development.

PLVAP is an endothelial-associated protein that is involved in endothelial diaphragm formation and maintenance of basal vascular permeability[11, 12]. In past studies, it has been related to the study of PLVAP genes in hepatocellular carcinoma and pancreatic cancer[13, 14], and significant efficacy, but the mechanism of action is not clear. Therefore, we explain the specific phenotypes and mechanisms affecting the occurrence and development of hepatocyte cancer from the perspective of PLVAP.

RESULTS

PLVAP plays an important role in the occurrence and development of hepatocellular carcinoma

We analyzed the RNA sequencing results of patients with hepatocellular carcinoma in TCGA data, RNA sequencing, volcanograph (Fig 1a) and thermostat (Fig 1b), with Fc> 2, P <0.05 as threshold, discovery of up-regulating genes 3966, down-regulation of 405. Extraction of RNA with 6 cancer tissue and cancer tissue of patients with hepatocellular carcinoma was selected, and the PLVAP was found to be a gene (Fig 1c), which was the most significant expression difference, thus selected PLVAP as a research objective. In GEPIA 2 data, it was found that PLVAP had a significant correlation with prognosis of patients with hepatocytes (Fig 1d). We selected LO2 as the normal hepatocyte control group, compared mRNA and protein expression levels in PLVAP in hepatocyte cancer cell line Bel-7402, Hep-G2, HCCLM3, MHCC97L, MHCC97H. The results showed that the level of expression of hepatocyte cancer cell line was significantly higher than that of normal control cell lines, and PLVAP was expressed in high metastatic cell line MHCC97H (Fig 1e and f).

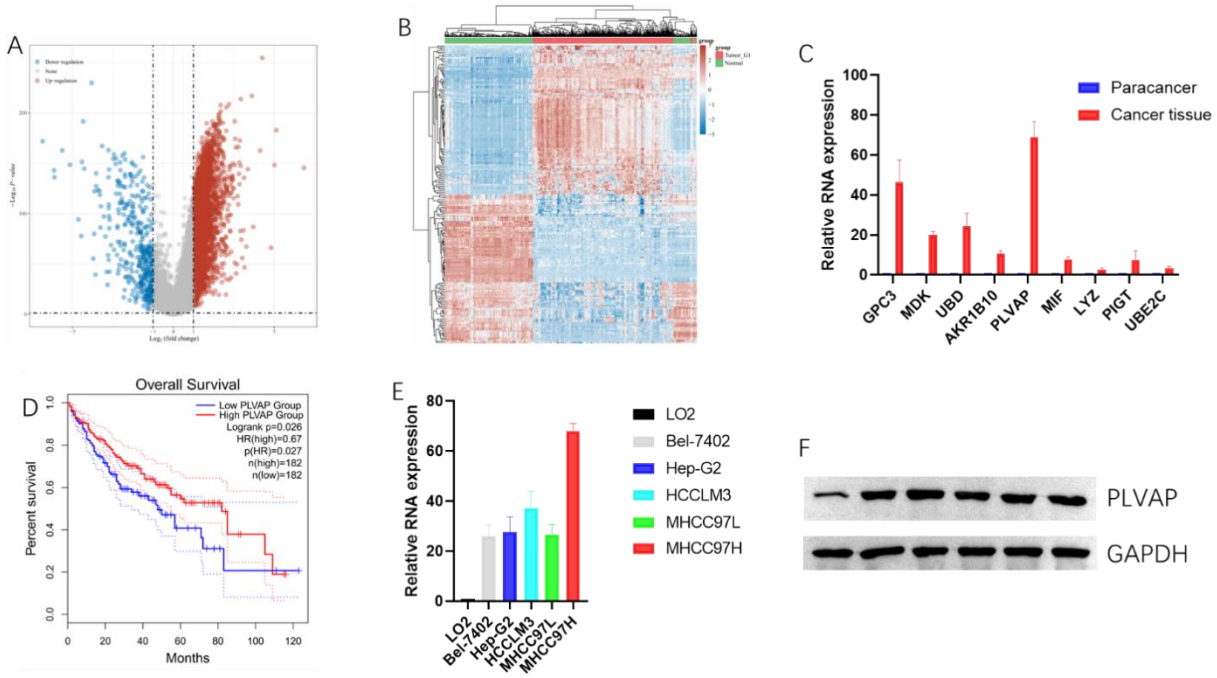

Fig 1 PLVAP is an important factor affecting hepatocyte cancer

PLVAP is a key factor in regulating proliferation transfer of hepatocyte cancer

In our clinical data analysis and large data sequencing, PLVAP has a significant effect on prognosis of patients with hepatocellular carcinoma while discovery that PLVAP has a difference in expression in MHCC97H (97H is hepatocyte cancer high transfer cell line). We have chosen at 97H hepatocyte cancer cell lines as a study, and siRNA silence PLVAP was transfected by LiP3000, and the siRNA concentration was 50 nmol. After the analysis of Western-blot and qRT-PCR analysis, siRNA can significantly inhibit PLVAP expression, the silencing efficiency is approximately 80% (Fig 2A and 2b). After 50 nmol siRNA was transferred by Lip3000 for 48h, the cells were digested into a single cell suspension and counted, using CCK8 to detect the proliferation speed of each group of 97H, and there is a significant difference in the proliferation speed of the 5day, and the 7day is further expanded (Fig 2c); The result of cloning formation capabilities show that the silencing PLVAP can significantly inhibit the formation of cloning of 97H, and its inhibitory effect is positively correlated with siRNA's silencing effect (Fig 2d); Migration and invasive data show that PLVAP expression has a significant impact on the migration and invasive ability of 97H cells. After the expression of silence 97h, the migration and invasive levels are significantly reduced, and there is significant difference than the Blank group and NC groups(Fig 2e and 2f).

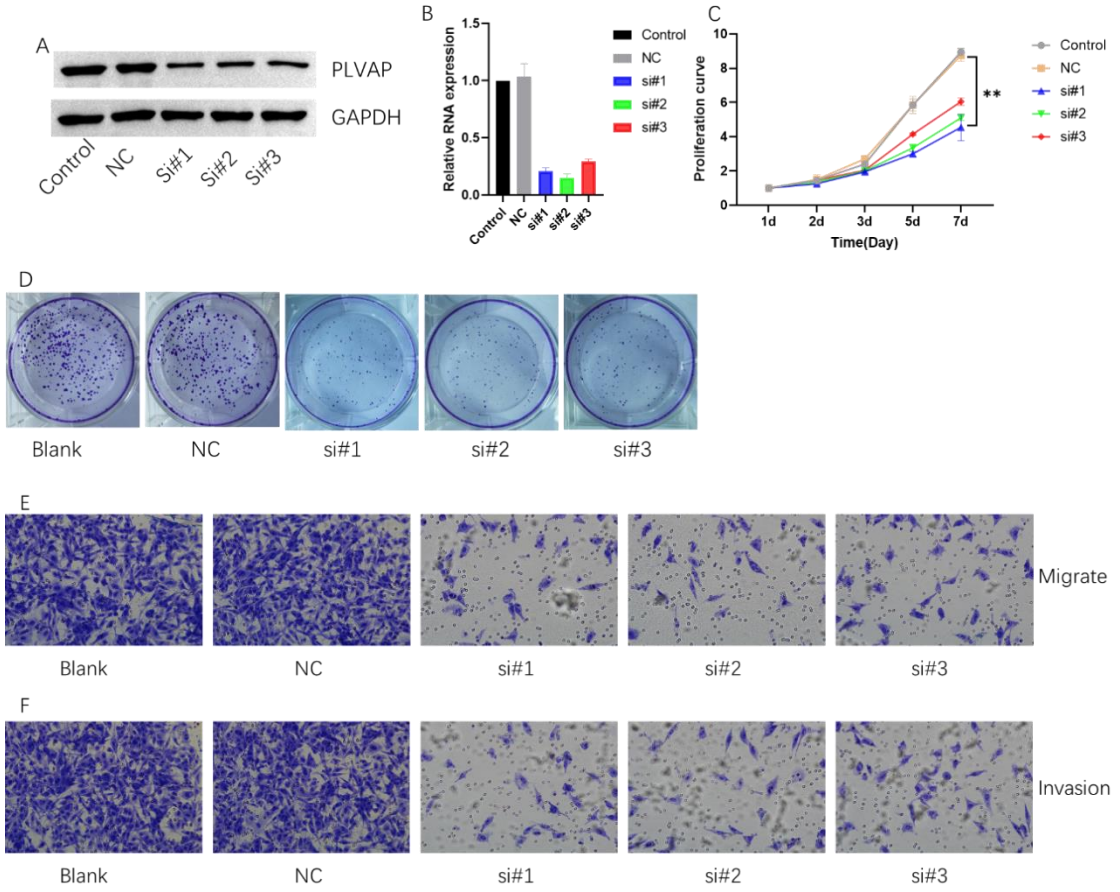

Fig 2 PLVAP regulates the proliferation and migration of MHCC97H

PLVAP regulates the development of liver cancer via Wnt / β-catenin signal

PLVAP is a key target of regulating hepatocyte cancer proliferation and migration invasion, but its regulation mechanism is currently not clear. In our study, it is found that it may be related to the regulation of Wnt / β-catenin signal pathway, which promotes the secretion of Wnt1, thereby enhancing β-catenin phosphorylation, activating Wnt / β-catenin signaling pathway. In our analysis, it was found that after the silencing PLVAP can significantly reduce the expression of Wnt1, further inhibit phosphorylation of β-catenin, reduce the expression of TCL, its effect is stronger than Wnt / β-catenin pathway inhibitor (ICG-001， a Wnt/β-catenin pathway antagonist) (Fig 3a). Further, the silencing PLVAP can significantly increase the ubiquitinization level of β-catenin, thereby reducing the stability of β-catenin (Fig 3b).

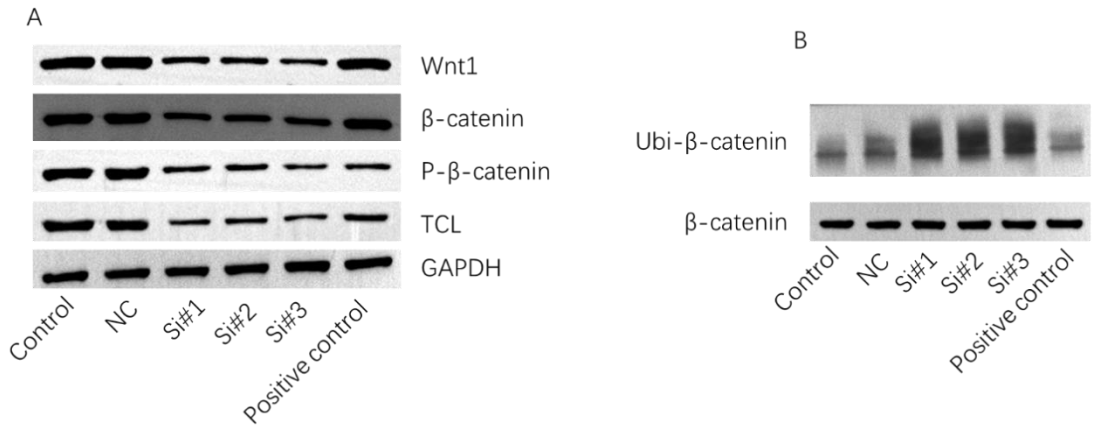

Fig 3 PLVAP regulates the expression of Wnt / β-catenin signaling pathway

DISCUSSION

The Wnt/β-catenin signaling pathway is consists of Wnt, the Wnt receptor, and the key signaling molecule, β-catenin[15]. β-catenin has a key role in the Wnt/β-catenin signaling, which harmonizes transcription of Wnt target genes[16]. Stabilized β-catenin activates gene expression of the Wnt/β-catenin signaling pathway and upregulates tumorigenesis, further promoting the occurrence and development of tumors[17, 18]. Specifically for HCC, hepatocytes transformed into cancer cells under regulation of promoted carcinogenic genes, the regulation of Wnt / β-catenin signals played important functions, but we found that the main cause may be specifically expressed in the carcinogenic PLVAP. The conserved Wnt / β-catenin pathway regulates the polyvilness of stem cells and determines the differentiation of the cells during the development process[19, 20]. This developmental level is combined with other pathways, including retinoic acid, fibroblast growth factor (FGF), transforming growth factor β (TGF-β) and bone morphological protein (BMP), existence in various different Cell type and tissue[21-23]. Studies have shown that Wnt is integrated with other signal paths to promote the development process of EMT[24]. The promoting carchatom is combined with the Wnt / β-Catenin signaling path to GSK-3β, up - regulate the level of SNAil, which in turn reduces the expression of E-Cadherin, promoting the occurrence of EMT and tumor metastasis[25-27].

PLVAP is classified to the caveolae and diaphragmed fenestrae structures of fenestrae endothelia, which minute adjustment endothelial permeability[28]. Therefore, PLVAP may plays a vital role in maintaining vascular integrity and homeostasis, not only normal but also pathological conditions[5]. The expression of PLVAP will affect the integrity of the blood vessels and the stability of the substrate, which has correlation with the occurrence and development of tumors. It affects the transformation of the EMT by regulating the Wnt / β-Catenin signal pathway, resulting in the occurrence and metastasis of hepatocytes. Its specific mechanism is PLVAP to promote the secretion of Wnt1, thereby activating Wnt / β-catenin signaling pathways related to WNT signals.

Clinically, PLVAP is an independent prognostic predictor of poor survival, and which related to occurrent and development of HCC. Our experimental results show that the PLVAP could directly activate Wnt/β-catenin signaling. Inhibition of PLVAP in the hepatocyte cancer cell line, it can significantly reduce the proliferation activity of cells and inhibit its migration and invasiveness. Tumor tissues in patients with hepatocellular carcinoma grow rapidly, the progress of the disease is difficult to control, and the prognosis is poor [29]. Therefore, looking for an effective treatment target is imminent, and we believe that PLVAP will be an effective target of hepatocellular carcinoma treatment.

METHODS

Patients and tissue samples.

HCC samples were obtained from ATCC Cell Bank. All patients gave consents to collect tissues. 6 pairs of HCC tissues after surgery. All human samples were collected with informed consents from the donors according to the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). The study was performed after approval by the institutional review board (IRB) of Chaohu Hospital of Anhui Medical University.

Premiers for relative mRNA in human breast cancer cell

PLVAP: 5`- CGGTGTCCATGCCTTCCAT -3`

5`- GGGAGGCTGTTACTGTCATGC-3`

Online dataset. The expression of PLVAP1 in HCC patient with clinicopathological features and survival outcome of TCGA database was analyzed on the GEPIA website (http://gepia.cancer-pku.cn/index.html).

Cell cultures and treatment. MHCC97H HCC cells was obtained from Zhong Qiao Xin Zhou Biotechnology Co.,Ltd.(Shanghai, China) and grown according to standard protocols. MHCC97H cells were cultured in DMED with 10% FBS, 1% Penicillin and Streptomycin.

Western-Blot Cells were lysed in RIPA lysis buffer (Beotime, China) supplemented with 1% protease and phosphatase inhibitors (Life Technologies, USA). Protein samples were subjected to 10% SDS-PAGE according to the mass of protein and transferred to PVDF membranes (Bio-Rad, USA). Membranes were then blocked with 5% non-fat milk in 0.1% TBST buffer for 2 h and incubated with primary antibody overnight at 4 °C. Primary antibodies against phospho-β-catenin(Cell Signaling, 1:1000), total β-catenin (Cell Signaling,1:1000), PLVAP(Cell Signaling, 1:500), UB (Cell Signaling,1:1000), TCL(Abcam,1:1000), GAPDH(Abcam,1:1000).

Colony formation assay. $1*10^4$ cells were plated in 6-well plates and cultured for 15 days. The colonies were stained with 1% purple crystal violet for after fixation with 4% formaldehyde for 15 min.

CCK8 assay. MHCC97H cells were seeded in 6-well plates (30,000 cells per well) and then treated with the siPLVAP for 24 h. At specific timepoints, cell viability was measured using 1% CCK8 assay. Read the absorbance at 450 absorbance and analyze the proliferation curve

Statistics. The in vitro data were presented as mean ± S.D. of three independent experiments. All statistical analyses were performed using SPSS 21.0 statistical software package and Graphpad Prism 8. Unpaired two-sided Student's t test and one-way ANOVA was used to compare cell viability, colony formation. In all cases, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

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