Highly expressed UNC119 promotes hepatocellular carcinoma cell proliferation through Wnt/β-catenin signaling and predicts a poor prognosis

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Abstract: UNC119, also known as Retinal Protein 4 (HRG4) is significantly up-regulated in hepatocellular carcinoma (HCC) tissues. However, the clinical significance of UNC119 and its biological roles and associated mechanisms in HCC tumorigenesis remain unknown. In current study, quantitative real-time PCR, western bolt and immunohistochemical analyses were applied to evaluate the expression of UNC119 in HCC tissues and cell lines. The roles of UNC119 in cell proliferation, cell growth and cell cycle were analyzed by both loss- and gain-function assays in vitro and in vivo. Luciferase reporter assays and western blot were employed to investigate the mechanisms. We found that compared with normal liver, UNC119 is highly expressed in HCC tissues both in published GSE datasets and in our fresh or TMA tissues. We further found that highly expressed UNC119 is not only closely correlated with liver cirrhosis, tumor size and TNM stage but also predicts a poor prognosis of patients. Cellular function assays reveal that UNC119 promotes cell proliferation. In addition, it might through enhancing the activity of Wnt/β-catenin signaling to promote the progression of cell cycle and ultimate promotes cell proliferation and growth. Taken together, we found that UNC119 is commonly highly expressed HCC tissues and the expression pattern makes a difference in the prognosis of patients. Furthermore, it promotes cell proliferation and growth through Wnt/β-catenin signaling.

Keywords: Hepatocellular carcinoma, UNC119, proliferation, Wnt/β-catenin signaling

Introduction

Hepatocellular carcinoma (HCC) is the third cause of cancer-related mortality and it ranks sixth in terms of global incidence [1]. Currently, only 30%-40% of HCC patients are diagnosed in the early stage and are eligible for potential curative treatment [2]; furthermore, patients in the intermediate stages and advanced stages also could be manipulated in certain therapies such as chemoembolization and sorafenib treatment [3]. However, despite this promising breakthrough has gained, HCC patients still have dismal prognosis. Therefore, the pathological molecular mechanisms remain to be elucidated.

Recent years, Genome-wide exome sequencing and Genome-wide RNAi screening for identifying molecular alterations which contributes to HCC initiation and progression have revealed many aberrant molecular changes might accelerate HCC tumorigenesis. Schulze K et al. recently reported that they identified 161 putative driver genes which associated with 11 recurrently altered pathways and their findings not only identified risk factors specific mutational signatures but also defined related aberrant pathways, which will be useful to design target therapy for HCC patients [4]. Previously, Lars Z et al. performed an oncogenomics-based in vivo RNAi screen and identified 12 tumor suppressors in liver cancer and those genes including XPO4, DDX20, GJD4, NRSN2 and so on [5] and subsequently, Jianchuan X et al. validated that NRSN2 is down-regulated in HCC tissues and might be a tumor suppressor [6].

Dafang W et al. performed a large-scale cDNA transfection screening for genes related to cancer development and progression in 2004 [7]. They found that 3806 cDNA species possess the ability could affects cancer cell growth. However, large amount of those genes were still
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Among those genes we found that UNC119 could promote cell growth and the fold change was 19 compared with control. While the detailed biological functions, mechanisms and clinical significance in HCC still unknown.

UNC119, also known as HRG4, is specifically expressed in the photoreceptors in the retina and is required for G protein trafficking in sensory neurons [8-10]. It has been reported that as an activator of SRC-type tyrosine kinases is essential for T cell activation and could regulate myofibroblast differentiation [11-13]. In current study, we not only identified the clinical significance of UNC119 but also investigated its biological functions and associated mechanisms in HCC.

Materials and methods

Ethic statement

18 fresh tumor tissues and matched adjacent tissues were collected from patients with pathologically and clinically confirmed HCC. All human tumor tissues were obtained with written informed consent from patients. The Institutional Review Board of Cangzhou Central Hospital approved the use of the tumor sample and animal in this study. Commercial HCC tissue microarray (TMA, HLiv-HCC180Sur-04), which containing 90 malignant tissues and paired adjacent normal tissues was purchased from Shanghai Outdo Biotech CO., LTD. All procedures of animal experiments were performed in accordance with The Animal Care and Use Committee of Cangzhou Central Hospital.

Cell culture

Hep3B, SK-Hep1 and HuH7 cells were purchased from ATCC, SMMC-7721, MHCC-97H and MHCC-LM3 were obtained from Cell Bank of the Chinese Academy of Sciences. All of those cells were maintained under standard culture conditions (37°C, 5% CO2) in culture medium recommended by ATCC or Cell Bank of the Chinese Academy of Sciences. Wnt/β-catenin signaling inhibitor IWR-1-endo was purchased from selleck (Selleck Chemicals, USA) and dissolved with sterile water to indicated concentration.

RNA isolation and quantitative real-time PCR

Total RNA was purified from HCC and adjacent tissues or cells using TRIzol (Invitrogen) following the manufacturer’s protocol. RNA (1 μg) was reverse transcribed using SuperScript Reverse Transcriptase III (Invitrogen). Quantitative real time PCR was performed using SYBR green Supermix (ABI) in ABI 7500 PCR system. Housekeeping gene GAPDH was used as an internal standard. Primers using in this study were described in Table 1.

Western blots

Cells were lysed in WB/IP lysis buffer (P0013, Beyotime) and nuclear proteins were extracted using lysis buffer (P0028, Beyotime), all the procedures were following the manufacturer’s protocol. Subsequently the cell lysates were boiled in 5X SDS-PAGE loading buffer for 10 min and then resolved by 8% SDS-PAGE and transferred to nitrocellulose membrane. The following antibodies were used in this study: UNC119 (GeneTex), GAPDH (Proteintech), β-catenin (Proteintech). Bound antibodies were visualized with the ECL kit (Thermo Scientific).

Construct stable cell lines

To generate stable silenced UNC119 cell lines, Vectors containing shRNAs were purchased.
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**Table 2. Correlations between UNC119 and key clinicopathological parameters**

<table>
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<td>Low</td>
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<tr>
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<td>( T_1 )</td>
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<td>( T_2 )</td>
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<td></td>
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<tr>
<td>( T_3, T_4 )</td>
<td>9</td>
<td>43</td>
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*P<0.05.

from Sigma-Aldrich. To over-express UNC119, vectors containing the ORF of UNC119 were obtained from Genecopoeia. We transfected those vectors to 293T cells with lipofectamine 2000 (Invitrogen) and all the procedures were following the manufacture’s protocol. The supernatant media containing virus was collected by centrifugation to remove cellular contaminant. The resulting viruses were used to infect indicated cells, and then integrated cells were selected by 2 μg/ml puromycin for 2 weeks. The alterations of UNC119 in those cells were confirmed by western blots before further analysis. The sequence of sh RNAs were described in Table 2.

**CCK8 cell viability assays**

Cells were seeded into a 96-well plate at 3×10^3 cells per well with 100 ul cultured medium and cultured at 37°C, 5% CO₂. The cell viability was quantified by addition 10 μl of cell counting kit (CCK8, Dojindo). After 1.5 hours incubation, the plates were monitored by Power Wave XS microplate reader (BIO-TEK) at an absorbance 450 nm.

**Colony formation assays**

Colony formation in soft agar was tested to assay anchorage-independent growth. Stable transfected cell lines were suspended in the upper layer which consisting of culture medium with 1% FBS and 0.35% agar, which is above a basal layer of 0.6% agar in 6-well plates in a triplicate manner. The cell density was 2,500 cells per well. Colonies were stained with 0.05% crystal violet, and all the visible colonies were counted by microscopy after 21-28 days.

**Flow cytometry cell cycle assays**

The indicated cells (20×10^4/well) were seeded in the 6-well plates. Then these cells were collected at 12 h, 24 h and 36 h. Cells were washed twice with 1× PBS, then re-suspended and fixed in 2 ml 70% ethanol at -20°C. Cells were then stained with PI (BD) and followed as the manufacture’s protocol.

**In vivo tumor formation assay**

100×10^4 stable SMMC-7721 and Hep3B cells and control were subcutaneously injected into right flank of 5 BALB/c (nu/nu) mice in each group. Tumor sizes were measured once a week and mice were sacrificed for the analysis of tumor burden after 4 weeks.

**Luciferase reporter assays**

Indicated cells were seeded in 96-well plates and transfected with TCF/β-catenin reporter plasmid (Wnt/β-catenin signaling) and 10 ng Renilla following the recommended protocol for the Lipofectamine 2000 transfection system. After 48 hours incubation, firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega, Madison, WI) from the cell lysates.

**Statistics analysis**

Data are expressed as the mean ± standard deviation. The correlation between UNC119 expression and the clinicopathological parameters was evaluated using the \( \chi^2 \) test by SPSS software. Two tail student’s t-test was used for comparisons between control and test groups and P<0.05 was considered statistically significant difference.

**Results**

The level of UNC119 was significantly upregulated in HCC

We retrieved the expression of UNC119 Oncomine Database. Surprisingly, we found that
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UNC119 expression was significantly upregulated in HCC tissues compared with normal liver in different datasets. As illustrated in Figure 1, in Chen liver dataset, the mRNA level was remarkably elevated in 103 HCC tissues compared with 75 normal liver, P=0.03. Convincingly, the similarity results were found in two Roessler liver datasets and Wurmbach liver dataset, and p values were 0.001, 2.20E-8 and 0.013, respectively. This observation highlighted a potential role of UNC119 in the initiation or progression of HCC.

To confirm the above findings, we performed quantitative real-time PCR assays to analyze the expression of UNC119 in 18 matched HCC tissues and adjacent tissues. As shown in Figure 2A, the mRNA levels of UNC119 were significantly increased in malignant tissues compared with the paired normal tissues.

To indentify the protein level of UNC119 changed in HCC tissues, we performed IHC assays to analyze the expression of UNC119 in a commercial TMA which containing 91 cases of HCC and paired adjacent tissues. The analysis from two independently experienced pathologists revealed that the protein level of UNC119 was more commonly upregulated in HCC tissues compared with counterparts, as shown in Figure 2B and 2C. UNC119 was elevated in 58 (64.53%) cases. We then analyzed the correlation between the levels of UNC119 and the clinicopathological parameters and found that highly expressed UNC119 was significantly associated with liver cirrhosis (P=0.035), tumor size (P=0.028) and TNM stage (P=0.042) as shown in Table 2. Furthermore, Kaplan-Meier survival analysis revealed that the positive expression of UNC119 indicates a poor prognosis of HCC patients (P=0.036), as show in Figure 2D.
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These results suggested that both mRNA and protein levels of UNC119 were elevated in HCC patients and might play a role in HCC initiation or tumor growth. In addition, the highly expressed UNC119 predicts a worse prognosis of patients. We then performed cell function assays to investigate the biological functions of UNC119 in vitro and in vivo.

**UNC119 contributes to accelerate proliferation and growth of HCC cells in vitro**

To get insight into the biological function roles of UNC119 in tumorigenesis, firstly, we performed in vitro proliferation assays using HCC cell lines. As presented in Figure 2E and 2F, both quantitative real-time PCR and western
UNC119 promotes HCC cell proliferation

Figure 3. Knockdown of UNC119 in HCC cells inhibits cell proliferation. (A, B) The protein level of UNC119 was reduced remarkably in Hep3B (A) and MHCC-LM3 (B) after silencing. (C, D) Silencing UNC119 inhibits cell viability significantly both in Hep3B (C) and MHCC-LM3 (D) cells. (E, F) Knockdown of UNC119 inhibits the formation of cellular clones on the soft agar both in Hep3B and MHCC-LM3 cells (E), and statistics were illustrated in (F). (G, H) Flow cytometry illustrates that silencing UNC119 the cell cycle was arrested in G0/G1 stage both in Hep3B and MHCC-LM3 cells. *P<0.05, **P<0.01. Data presented here are representative of three to five independent experiments.
UNC119 promotes HCC cell proliferation

A

 UNC119

 GAPPDH

 SMMC-7721

 Vector UNC119

 B

 UNC119

 GAPPDH

 SK-Hep1

 Vector UNC119

 C

 OD 450

 Days

 SMMC-7721-Vector SMMC-7721-UNC119

 **

 **

 D

 OD 450

 Days

 SK-Hep1-Vector SK-Hep1-UNC119

 **

 **

 E

 Vector UNC119

 SMMC-7721

 SK-Hep1

 F

 Number of cell clones/n

 SMMC-7721 SK-Hep1

 **

 **

 G

 Percentage of distribution

 SMMC-7721

 Vector UNC119

 *

 G0/G1 S G2/M

 H

 Percentage of distribution

 SK-Hep1

 Vector UNC119

 **

 **

 G0/G1 S G2/M
blot assays revealed that UNC119 was relatively abundant in Hep3B and MHCC-LM3 cell lines, while lowly in SMMC-7721 and SK-Hep1 cells. We then stably silenced UNC119 in Hep3B and MHCC-LM3 cell lines, overexpressed the full-length of UNC119 in SMMC-7721 and SK-Hep1 cells. As illustrated in Figures 3A, 3B and 4A and 4B, the expression of UNC119 was significantly altered when transfected with indicated factors.

As shown in Figure 3C, 3D, when silencing UNC119, the cell viability was remarkably decreased in Hep3B and MHCC-LM3 cells. Convincingly, when heightening the level of UNC119 in SMMC-7721 and SK-Hep1 cells, the viability of cells was significantly elevated in these two cell lines, as shown in Figure 4C and 4D.

Furthermore, we also performed Anchorage-independent growth assay in these transfected cell lines, as presented in Figure 3E and 3F, we found that when silenced UNC119 the ability of clone formation in Hep3B and MHCC-LM3 cell lines was significantly inhibited. Moreover, the
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Figure 6. UNC119 influences the activity of Wnt/β-catenin signaling. (A, B) The expression of CCND1 and CCNE1 were remarkably reduced (A) or elevated (B), when UNC119 silencing in Hep3B or over-expressing in SMMC-7721 cells. Normalized to GAPDH. (C, D) Luciferase reporter assays reveal that Wnt/β-catenin signaling was inhibited when UNC119 were silenced in Hep3B (C), while it was elevated when UNC119 was heighted in SK-Hep1 (D). (E) The level of nuclear β-catenin was reduced when UNC119 silenced in Hep3B and while it was increased in SK-Hep1 when UNC119 over-expressed, lamin A/C and GAPDH were used as loading control. *P<0.05 **P<0.01. Data are representative of three to five independent experiments. (F) β-catenin inhibitor IWR-1-endo significantly inhibits SK-Hep1 proliferation.

effect was totally reversed in SMMC-7721 and SK-Hep1 cell lines which were over-expressed UNC119, as shown in Figure 4E and 4F.

To extend these findings, we next examined whether UNC119 through modulating the process of cell cycle to regulate cell proliferation. We performed cell cycle assays by PI staining and detected by flow cytometry. Interestingly, we found that when knockdown UNC119, the cell cycle was arrested in the G0/G1 stage (Figure 3G, 3H), and the arrestation was removed and cells were mainly distributed in S and G2/M stage when heightening the level of UNC119 in SMMC-7721 and SK-Hep1 (Figure 4G, 4H). These results suggested that UNC119
UNC119 promotes HCC cell proliferation through modulates the G1/S stage transition to regulate cell proliferation.

Stably silencing UNC119 inhibits tumor growth in vivo

Next, we examined the in vivo roles of UNC119 in HCC development. We selected stable transfected SMMC-7721 and Hep3B cells to perform in vivo tumor formation assay. Cells were injected subcutaneously in nude mice and tumor growth was monitored. As illustrated in Figure 5A, tumors formed from SMMC-7721 cells which were over-expressed UNC119, the tumor size was significantly larger than control group. And tumors from Hep3B which were knockdown UNC119, the tumor size was significantly smaller than negative control group. Furthermore, both the tumor growth speed and tumor weight were also positively correlated with the level of UNC119 (Figure 5B, 5C). Convincingly, we found that Proliferating Cell Nuclear Antigen (PCNA), which is a marker of active proliferate cells, was significantly lower expressed in silenced group compared with negative control group in Hep3B (Figure 5D).

UNC119 influences the distribution of β-catenin

As we known, cyclins, which are dispensable during cell cycle transition [14]. Especially, CCND1 and CCNE1 which are required in G1/S transition were observed highly expressed in many tumors [15-19]. In current study, we examined whether UNC119 affects the expression of these two cyclins. And we found that inhibiting the expression of UNC119, concomitantly, the mRNA level of CCND1 and CCNE1 was attenuated in stable knockdown Hep3B cells (Figure 6A). Confidently, the effects of UNC119 in these two cyclins were totally reversed in SMMC-7721 overexpressed cell (Figure 6B). These results confirmed that UNC119 through modulating the level of CCND1 and CCNE1 to regulate cell proliferation.

To investigate the mechanism underlying the effects that UNC119 affects the expression of these cyclins. As we known, Wnt canonical signaling plays an essential role in cancer cell growth; in addition, CCND1 is a confirmed target gene of Wnt/β-catenin [20]. Therefore, we next performed Luciferase reporter assays to examine whether UNC119 influence the activity of Wnt/β-catenin signaling. Encouragingly, the results from Hep3B stable cell lines indicate that silencing UNC119 results in reducing the activity of Wnt/β-catenin signaling (Figure 6C). In agreement with results from Hep3B, the reversed effects were observed from stable SK-Hep1 cells, when elevated the expression of UNC119, the activity of Wnt/β-catenin were increased (Figure 6D). Next, we further detected the β-catenin in Hep3B and SK-Hep1 stable cells, and we found that the level of nucleus β-catenin was decreased when UNC119 silenced in Hep3B, while, the level of nucleus β-catenin was increased in SK-Hep1 which UNC119 elevated.

We further used IWR-1-endo, which is an inhibitor of Wnt/β-catenin signaling by stabilizing the Axin destruction complex [21] to promote β-catenin degradation, performed cell viability assays in stable SK-Hep1 cells. As shown in Figure 6F, when treated with IWR-1-endo, the cell viability were significantly decreased in control group compared with over-expressing group.

These results reveal that UNC119 through regulating β-catenin in to the nucleus to regulate the expression of CCND1 to promotes cell proliferation and tumor growth.

Discussion

As we known, HCC is a prevalent and highly aggressive malignancy worldwide and its clinical outcome remains challenging [22], therefore a better understanding of underlying molecular mechanisms is highly desirable. Tumor initiation is resulted in uncontrolled cell proliferation [23], and it is widely accepted that the alterations, including genetic and epigenetic alterations, contribute to this malignant feature [24]. Previously, Dafang W. et al. reported that a large cohort genes could contribute to cell growth [7], among then we found that UNC119 is stand out for its robust effects in promoting cell growth. However, the clinical significance and detailed roles and mechanisms remain to be elucidated in HCC.

In current study, we firstly screened the published GSE datasets, and found that UNC119 is highly expressed in four cohorts of HCC patients’ malignant tissues compared with normal liver tissues. These results remind us th-
at UNC119 is commonly up-regulated in HCC tissues and must play a role in HCC tumorigenesis. Furthermore, we demonstrated that UNC119 is significantly up-regulated in HCC tissues and over-expression is closely correlated with liver Cirrhosis, tumor size and TNM stage. These clinical significances suggested that UNC119 involved in HCC initiation and tumor growth. In addition, Kaplan-Meier analysis reveals that the highly expression of UNC119 is closely associated with poor prognosis; these results indicate that UNC119 is implicated in the progression of HCC.

Both loss- and gain- function cellular function assays revealed that UNC119 could promotes HCC cell proliferation and growth, furthermore, in vivo tumor formation assays validated these findings. As we known, aberrant cell cycle progression contributes to uncontrolled cell proliferation [23]. Cancer cells employed many ways, such as mutation of tumor suppressor p53 and PTEN or aberrant overexpression of cyclins, to over cell cycle checkpoints to promote cell proliferation [25-27]. In current study, we found that the expression of CCND1 and CCNE1 is concomitantly altered with the alteration of UNC119; this finding suggested that UNC119 might partly through modulating cyclins to promote cell proliferation.

As we known, abnormal activation of Wnt/β-catenin signaling was observed in numerous solid tumors, including HCC [28, 29]. And CCND1 is a downstream target gene of Wnt/β-catenin signaling [20]. In current study, we found that the activity of Wnt/β-catenin signaling is positively correlated with the level change of UNC119. Additionally, we found that the level of nucleus β-catenin, which functions as a transcription factor to target downstream genes in nucleus [30], was subsequently changed when the level of UNC119 is changed. These findings suggested that elevated UNC119 could in some way to promoteβ-catenin enter nucleus, to regulate the expression of CCND1 and ultimate promotes cell proliferation and growth. While, Wnt/β-catenin signaling obtains a centre role in the Physiological process of cell and it also could target many other oncogenes, like c-myc, TCF-1 and MMP7 [31], all of these genes could contribute to tumor progression.

In conclusion, we first report the clinical significance of UNC119 in HCC patients, furthermore, we found that UNC119 through promoting β-catenin enter nucleus to promote cell proliferation and growth.

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Disclosure of conflict of interest

None.

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