UNC119 promotes the growth and migration of hepatocellular carcinoma via Wnt/β-catenin and TGF-β/EMT signaling pathways

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Summary
Purpose: It has been reported that UNC119 is significantly up-regulated in liver cancer cells. However, the role of UNC119 in liver cancer and the clinical significance of reducing its expression in hepatocellular carcinoma (HCC) are not well understood. The aim of this study was to investigate the expression profile of UNC119 in HCC and its connection with the progression of HCC.

Methods: UNC119 expression in HCC cell lines and tissues was assessed using quantitative real-time PCR, western blot and immunohistochemical analyses. The biological functions of UNC119 during the proliferation, growth and other different life cycles of tumor cells were also analyzed both in vitro and in vivo.

Results: UNC119 expression was up-regulated in both HCC cell lines and tissues. A higher level of UNC119 not only promoted HCC cell proliferation, but also enhanced its ability of migration and invasion. UNC119 promoted the progression of cell cycles and significantly induced HCC cell growth through the Wnt/β-catenin signaling pathway. In addition, UNC119 enhanced tumor migration and invasion through the TGF-β/epithelial-mesenchymal transition (EMT) pathway. The antibody against UNC119 (Anti-UNC119) efficiently inhibited the proliferation, migration and invasion of HCC cells by blocking the Wnt/β-catenin and TGF-β/EMT signaling pathways, respectively. Anti-UNC119 not only facilitated tumor remission, but also extended long-term survival of HCC-bearing mice.

Conclusion: UNC119 was significantly up-regulated in liver cancer cells and tissues. It promoted cell growth and migration through the Wnt/β-catenin and TGF-β/EMT signaling pathways, respectively. The anti-UNC119 treatment inhibited tumor cell proliferation, growth, migration and invasion by inhibiting the Wnt/β-catenin and GF-β/EMT signaling pathways, respectively.

Key words: HCC, UNC119, Anti-UNC119, Wnt/β-catenin, TGF-β/EMT

Introduction
Hepatocellular carcinoma (HCC) is a major type of primary liver cancer with a high recurrence rate, and ranks second in cancer-related mortality after radiotherapy, chemotherapy and surgery [1]. HCC is associated with a high incidence in humans, but its treatment is hindered by limited therapeutic regimens, especially for patients with advanced HCC [2]. Currently, the common choices of surgery, chemotherapy and radiotherapy can only achieve a modest result, yet generate many side effects during and after treatment. Therefore, new clinical therapies are urgently needed to enhance treatment efficacy, to minimize adverse responses and to prolong the survival of HCC patients [3,4]. Tumor immunotherapy is a novel strategy to treat cancer and shows a strong efficacy without causing too many treatment-related adverse events, thus leading to
Overall improvement in HCC patients [5,6]. So far, bioinformatics studies on tumor immunotherapy have found many candidates which target antigen presenting molecules to regulate immune responses in tumor cells [7,8]. It was demonstrated that a Rad-IL-2 immunotherapy elicited positive responses in mice harboring HCC, suggesting that such an immunotherapy may improve the conditions of HCC patients.

Recently, genome-wide sequencing and RNA screening have helped to identify molecular alterations involved in the initiation, metastasis, targeted treatment and prognosis of cancer [9,10]. In addition, many reports have revealed that aberrant changes in protein expression could accelerate the tumorigenesis and development of cancer [11]. Due to the higher incidence of HCC, many candidate molecules were proposed to aid the prevention, diagnosis and treatment of HCC patients. Furthermore, investigations on the molecular mechanisms underlying HCC invasion and metastasis have promoted the development of therapies aiming to suppress these two characteristics of tumor cells [12]. In the future clinical treatment of HCC, it is expected that the regulation of tumor cell growth and metastasis will play a critical role, whereas molecular bioinformatics has made it possible to screen targeting molecules of diagnostic and therapeutic potentials while offering the option of personalized medicine for the patients.

UNC119 was first identified as an imperfect protein in Caenorhabditis elegans mutants with defects in locomotion, feeding and chemosensation capabilities [13]. Lei et al. argued that a higher level of UNC119 expression contributed to invasion and metastasis of HCC [14]. Evidence has suggested that UNC119, which modulates the G1/S transition in cells, may be involved in the regulation of tumor proliferation, migration and invasion via the Wnt/β-catenin signal transduction pathway [14]. These data showed that UNC119 might become a possible target in inhibiting the growth of HCC cells.

UNC119 is well known as HRG4, whose expression in HCC cells is specifically high, and is needed for the trafficking of G proteins in sensory neurons [15]. In this study, the inhibitory effect of anti-UNC119 on HCC cells was investigated in HepG2-bearing mice. We not only investigated the clinical significance of anti-UNC119 in HCC patients, but also studied the biological functions of UNC119 in a HCC mice model. Furthermore, the mechanisms underlying the biological functions of UNC119 during cancer migration were studied in HCC cells and tissues.

Methods

Ethics statement

This study was performed strictly in line with the Guide for the Care and Use of Laboratory Animals. All experimental protocols were in accordance with the guideline issued by the National Institutes of Health and were approved by the Committee on the Ethics of Animal Experiments Defense Research. All surgeries and euthanasia were carried out in a way to minimize suffering.

Cells culture and reagents

Hep3B, HepG2, SMMC-7721, RUH7, SK-Hep1, MHCC-LM3, MHCC-97H and NCTC-1494 cells were obtained from Frederick Cancer Research Facility, the National Cancer Institute, Division of Cancer Treatment Tumor Repository (Frederick, MD) as well as American Type Cell Culture (Rockville, MD), respectively. All cells were cultured under standard tissue culture conditions (5% CO₂, 37°C) in a DMEM medium containing 10% heat-inactivated FBS (Biowhittaker, Walkersville, MD), 3 mM L-glutamine, 50 μg/ml gentamycin (Bio-whittaker) as well as 1% penicillin/streptomycin.

Determination of mRNA levels by real time RT-PCR

A RNeasy mini kit (Qiagen, Gaithersburg, MD) was used to extract total RNA from cells and 1 μg RNA was converted to cDNA using a cDNA synthesis kit (Bio-Rad, Hercules, CA). iQ SYBR Green Supermix (Bio-rad) was used to prepare 1/10 of 25 μl PCR in an iCycler heat circulator (Bio-Rad). The forward and reverse primers of CCND1, CCNE1, e-cadinin, waveform protein, ZEB1 and UNC119 were synthesized by Invitrogen. The PCR products were quantitatively amplified by measuring the computational cycle threshold (Ct) of the sample and GAPDH mRNA. Using 2-ΔΔCt method, the relative change in mRNA expression was calculated. The results were presented as fold differences relative to the control (relative expression levels).

MTT cytotoxicity assay

For each testing condition, ACHN, 786-0 and RuCa cells were incubated with Lenvatinib or/and sensitized lymphocytes in 96-well plates for 48, 72 and 96 hrs in triplicates, respectively, and PBS was used as the control. At each time point, 20 μl of PBS, which contained 5 mg/ml MTT, were added into each well and the plate was further incubated for 4 hrs. Most of the medium was removed and 100 μl of DMSO (dimethylsulfoxide) was added into each well to solubilize the crystals. The optical density (OD) value was measured by a Bio-Rad (ELISA) plate reader at a wavelength of 450 nm.

SDS-PAGE and western blot

The purified hTERTR-FAM96A fusion protein was homogenized to perform SDS-PAGE and SDS-PAGE analyses under reductive conditions. The purified hTERTR-FAM96A fusion protein was transferred onto a cellulose nitrate film. For western blot analysis, hTERTR-FAM96A fusion protein was transferred onto a cellulose nitrate film. For western blot analysis, hTERTR-FAM96A fusion protein was transferred onto a cellulose nitrate film. For western blot analysis, hTERTR-FAM96A fusion protein was transferred onto a cellulose nitrate film.
and Apoptin were prepared. Protein detection was performed with hTERT and apafl, and IFN- gamma was used as the control. Details of the assay have been described elsewhere [16]. All experiments were performed in triplicates and repeated at least three times.

**Animal study**

Specific pathogen-free (SPF) female BALB/c (six-week old) nude mice were purchased from Harbin Veterinary Research Institute (Harbin, China). All animals were housed under pathogen-free conditions. 1×10^6 HepG2 cells were injected into the right side of each mouse in a total of 200 μl liquid. Anti-UNC119, UNC119 and PBS treatments in tumor-bearing mice were initiated when the tumor diameter reached 6 to 8 mm on day 7 after tumor inoculation. HCC-bearing mice were randomly separated into 3 groups (n=30 in each group). The treatments were repeated 7 times with a 2-day interval. Tumor diameters were measured once every 2 days to calculate the tumor volume using the following formula: 0.52 × smallest diameter^2 × biggest diameter.

**Immunohistochemical staining**

Immunohistochemical staining was carried out using the avidin-biotin-peroxidase technique. Paraffin-embedded tissue sections were prepared and underwent epitope retrieval for subsequent analysis. The paraffin sections were incubated with hydrogen peroxide (3%) for 10-15 min and blocked with a regular blocking solution for 10-15 min at 37°C. Finally, the sections were incubated with anti-UNC119, anti-ZEB1, anti-EMT and anti-TGF-β antibodies, respectively, at 4°C for 12 hrs. All sections were then rinsed twice and incubated with secondary antibodies for 1 hr at 37°C. In the last step, the sections were counterstained with hematoxylin or DAPI.

**Statistics**

All data were shown as mean ± standard error of the mean (SEM). Unpaired data were analyzed by the Student’s t test using SPSS 20.0 software (IBM, Armonk, NY, USA). Comparisons of data among various groups were done by one-way ANOVA. *p<0.05 and **p<0.01 were deemed statistically significant.

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**Figure 1.** Expression of UNC119 mRNA and protein in hepatocellular carcinoma cell lines and normal liver cell line. **A:** Relative mRNA levels of UNC119 in hepatocellular carcinoma cell lines compared to normal liver cell line NCTC-1496. **B:** Western blot determined the protein level of UNC119 in hepatocellular carcinoma cell lines compared to normal liver cell line NCTC-1496. **C:** Relative protein level of UNC119 in hepatocellular carcinoma cell lines compared to normal liver cell line NCTC-1496. **D:** Different expression of UNC119 between HepG2 cells and NCTC-1496 cells by immunofluorescence assays. Data represent means and SEM, **p<0.01 versus normal liver cells (one-way analysis of variance).
Results

Expression of UNC119 in HCC and normal liver cells

In order to measure the expression of UNC119 in HCC and normal liver cell lines, Hep3B, SK-Hep1, HUH7, SMMC-7721(S-7721), MHCC-97H (M-97), MHCC-LM5 (M-LM5) and NCTC-1496 cell line were analyzed by qRT-PCR and western blot. In Figure 1A, it is shown that the mRNA expression of UNC119 was remarkably up-regulated in HCC cell lines as compared to regular NCTC-1496 liver cell line (**p=0.0032). The protein level of UNC119 (Figure 1B and C) was also greatly increased in tumor cell lines compared to regular NCTC-1496 cell line (**p=0.0072). Among these tumor cell lines, UNC119 expression was the highest in HepG2 cells. Therefore, HepG2 cells were chosen to carry out further analyses. In addition, immunofluorescence assays of HepG2 and NCTC-1496 cell line demonstrated that UNC119 expression was higher in hepatic tumor cells as compared to normal liver cells (Figure 1D). These observations indicated that UNC119 might act as a potential target in the initiation or progression of HCC.

Inhibitory effects of Anti-UNC119 on the proliferation, development, migration and invasion of HCC cells in vitro

Since UNC119 might act as a possible target in HCC therapies, a chimeric antibody targeting UNC119 (anti-UNC119) was constructed to investigate its therapeutic effect on hepatic tumor cells in vitro. The affinity of anti-UNC119 was measured by ELISA. Anti-UNC119 exhibited a high affinity to UNC119 (Figure 2A). In addition, MTT assay was used to evaluate the growth of HCC cells in order to investigate the biological roles of UNC119. The results in Figure 2B showed that UNC119 significantly promoted the growth of tumor cells but not the growth of normal liver cells. In contrast, anti-UNC119 greatly inhibited tumor cell growth. Furthermore, migration and invasion of tumor cells were evaluated by treating the cells with UNC119, anti-UNC119 or an empty vehicle. The migration and invasion of tumor cells were inhibited in the anti-UNC119-treated group (Figure 2C and D). However, UNC119 promoted the migration and invasion of tumor cell line Hep G2 but showed no effects on regular NCTC-1496 liver cell line. These observations indicated that anti-UNC119 effectively inhibited the growth, migration and invasion of tumor cells but did not affect normal liver cells, suggesting that anti-UNC119 may become an efficient and safe anticancer agent.

UNC119 regulates tumor cells through the Wnt/β-catenin signal transduction pathway

A previous study indicated that CCND1 and CCNE1 proteins were essential for G1/S transition, which was elaborated in various types of tumor

Figure 2. Inhibitory effects of anti-UNC119 on the proliferation, migration and invasion of HCC cells. A: High affinity of anti-UNC119 in binding with UNC119. B: Inhibition of tumor cell growth by anti-UNC119 on HepG2, compared to NCTC-1496. C: Inhibition of tumor cell migration by anti-UNC119 on HepG2, compared to NCTC-1496. D: Inhibition of tumor cell invasion by anti-UNC119 on HepG2, compared to NCTC-1496. Data represent means and SEM, **p<0.01 versus normal liver cells (one-way analysis of variance).
cells [17]. In this study, whether UNC119 affected CCND1 and CCNE1 expression in the cyclins of hepatic tumor cells was investigated. The results in Figure 3A-C showed that the mRNA and protein level of CCND1 and CCNE1 was up-regulated in HepG2 cells at 48 hrs after the UNC119 treatment. However, anti-UNC119 abolished the effects of UNC119 on HepG2 cells and no significant difference was found between anti-UNC119-treated HepG2 cells and normal NCTC-1496 liver cells. In order to gain a more in-depth understanding on the fundamental mechanism underlying the above observations, the effect of Wnt/β-catenin canonical signal pathway on cancer cell growth was analyzed. Luciferase reporter assays showed that UNC119 promoted the activity of Wnt/β-catenin signaling, whereas anti-UNC119 inhibited the Wnt/β-catenin activity (Figure 3D). In addition, anti-UNC119 inhibited Wnt/β-catenin activity by regulating the expression of CCND1 and CCNE1 in the tumor cells. It was also found that the UNC119 treatment up-regulated the activity of Wnt/β-catenin, whereas the treatment by anti-UNC119 exerted opposite effects on HepG2 cells. Furthermore, since TGF-β1 is involved in the migration and invasion of tumor cells, it was hypothesized in this study that anti-UNC119 may inhibit the activity of TGF-β by suppressing EMT and migration of cancer cells [18]. The expression of ZEB1 and EMT markers, E-cadherin and vimentin, in HepG2 cells was analyzed, and the results showed that the expression of ZEB1 and EMT markers (E-cadherin and vimentin) was greatly inhibited after the treatment with anti-UNC119 (Figure 3E and F). These results suggested that UNC119 promoted tumor cell growth and migration by regulating the Wnt/β-catenin and TGF-β/EMT signaling pathways, respectively.

Figure 3. Underlying mechanism of UNC119 regulating tumor cells. A: mRNA expression of CCNE1 and CCND1 in HepG2 cells after treatment with UNC119, anti-UNC119, UNC119+anti-UNC119 or PBS. B: mRNA expression of CCNE1 and CCND1 in NCTC-1496 cells after treatment with UNC119, anti-UNC119, UNC119+anti-UNC119 or PBS. C: Protein expression of CCNE1 and CCND1 in HepG2 and NCTC-1496 cells after treatment with UNC119, anti-UNC119, UNC119+anti-UNC119 or PBS. D: Wnt/β-catenin signaling activity in HepG2 and NCTC-1496 cells after treatment with UNC119, anti-UNC119, UNC119+anti-UNC119 or PBS. E: Protein expression of ZEB1, Vimentin and E-cadherin in tumor cells after treatment with UNC119, anti-UNC119, UNC119+anti-UNC119 or PBS. F: Relative mRNA expression of ZEB1, Vimentin and E-cadherin in tumor cells after treatment with UNC119, anti-UNC119, UNC119+anti-UNC119 or PBS. Data represent means and SEM, **p<0.01 versus normal liver cells NCTC-1496 (data were analyzed by Student’s t tests and one-way analysis of variance).
Therapeutic effects of anti-UNC119 on HCC-bearing mice in vivo

Despite the finding that anti-UNC119 exerted inhibitory effects on tumor cells in vitro, the antitumor efficacy of anti-UNC119 was further examined in HepG2-bearing mice in vivo. HepG2 cells were injected in nude BALB/c mice to obtain HepG2-bearing mice, which were then subcutaneously injected with anti-UNC119, UNC119 or PBS on day 7. These mice were treated for a total of 7 times. The xenograft tumor growth was monitored and recorded every two days. As illustrated in Figure 4A, the tumor growth was significantly suppressed in the anti-UNC119 group compared to the UNC119 and PBS groups. In addition, UNC119 expression was analyzed by immunohistochemical assays and the results showed a significantly decreased UNC119 expression in anti-UNC119-treated tumor (Figure 4B). Furthermore, the expression of β-catenin, TGF-β and EMT in the tumor was measured on day 25. The results in Figure 4C showed that the expression of β-catenin, TGF-β and EMT was decreased in anti-UNC119-treated tumor, whereas the expression of these markers increased in the UNC119-treated tumor compared to the PBS-treated tumor. Furthermore, the anti-UNC119 treatment improved the long-term survival of HCC mice during the 120-day observation period (Figure 4D). Overall, anti-UNC119 exhibited a strong anticancer efficacy in the treatment of HCC mice.

Discussion

The progress of target therapies has raised expectations for improved treatments of hepatic tumors, whereas the therapeutic benefits of such treatments have been observed in preclinical and clinical trials [19]. It has been shown that the mRNA and protein expression of UNC119, which promotes the growth, migration and invasion of HCC cells, is up-regulated in HCC cell lines (Figure 3). It is proposed in this study that UNC119 may promote tumor cell growth and migration by regulating the Wnt/β-catenin and TGF-β/EMT pathways, respectively. The mechanisms underlying
the functions of UNC119 have been illustrated in this study. More importantly, an effective chimeric antibody targeting UNC119 was constructed to study its biological activities both in vitro and in vivo. The data indicated that anti-UNC119 could efficiently bind to UNC119 and decrease the mRNA and protein expression of UNC119 (Figures 1 and 2). These results showed that anti-UNC119 might act as a possible anticancer agent for HCC treatment.

HCC is one of the most extensively studied cancers (the incidence of HCC ranks 5th in men and 9th in women) and accounts for more than 90% of primary liver cancers [20,21]. Previous studies have revealed that HCC is a genetically complex, multifactorial and heterogeneous disease [22,23]. Therefore, several oncolytic approaches targeting different signal pathways have been characterized [24,25]. These cellular signaling pathways provide key signals for various extracellular growth factors and receptors in HCC cells. The ultimate goal in cancer treatments is to induce apoptosis of all tumor cells. However, tumor cell migration is a thorny problem in clinical HCC treatments, whereas many anticancer drugs can induce acquired resistance of tumor cells against apoptosis, which often leads to invasion of tumor cells into the surrounding cells and tissues [26,27]. Tumor can acquire the ability of migration and invasion through different signaling pathways, and the subsequent demand for medical therapies with a long-term efficacy represents a significant clinical problem faced by HCC treatments [28]. Therefore, effective agents that can inhibit the growth of hepatic tumor cells are urgently needed to prevent the high rate of metastasis in HCC patients.

The TGF-β/EMT pathway is an important pathway involved in cancer cell invasion and metastasis [29]. EMT has been identified as the mechanism underlying the migration and invasion of epithelial cells, which can be converted into fibroblasts and lose their characteristics of epithelial cells [30]. The results from this study showed that UNC119 was significantly up-regulated in HCC cells, thus increasing the expression of TGF-β/EMT to enhance the ability of tumor migration and invasion.

Currently, it has been known that the Wnt/β-catenin signaling pathway is associated with the development of many types of tumor cells [31,32]. The results from this study also showed that the activity of Wnt/β-catenin signaling was positively correlated with the level of UNC119. In addition, it was found both in vitro and in vivo that the UNC119 treatment increased the expression of CCND1 and CCED, downstream genes in the Wnt/β-catenin pathway, and further promoted the development of HCC cells. These observations indicated that the down-regulation of UNC119 by anti-UNC119 could suppress tumor cell growth and proliferation.

In conclusion, anti-UNC119 was developed to target UNC119 and impair the Wnt/β-catenin and TGF-β/EMT signaling pathways. The results showed that anti-UNC119 had the potential to inhibit the activity of hepatic carcinoma cells. The anti-tumor characteristics of anti-UNC119 should be further studied to evaluate its potential clinical value in HCC treatments.

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Conflict of interests

The authors declare no conflict of interests.

References

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