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ORIGINAL ARTICLE

UNC119 promoted cell growth and migration by Wnt/β-catenin signal and TGF-β/EMT signal pathway in hepatocellular carcinoma

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Summary

Purpose: *UNC119 was reported to be significantly up-regulated in hepatic cancer cells. However, the clinical significance of target UNC119 to reduce UNC119 expression and mechanisms in hepatocellular carcinoma (HCC) are not well understood. Our purpose was to study how UNC119 is expressed in HCC and its connection with HCC progression.*

Methods: *UNC119 expression was assessed with quantitative real-time PCR (qRT-PCR), western blot and immunohistochemical analyses in HCC cell lines and in tissues. The biological function of UNC119 for proliferation, growth and cell cycle of tumor cells were also analyzed both in vitro and in vivo.*

Results: *UNC119 expression was up-regulated both in HCC cell lines as well as in tissues through comparison with normal liver cells and tissues. Higher concentration level of UNC119 not only promoted proliferation, but also enhanced migration and invasion of HCC cells. UNC119 promoted the progression of cell cycle and significantly promoted HCC cells growth through Wnt/β-catenin signal pathway and enhanced tumor migration and invasion via TGF-β/epithelial-mesenchymal transition (EMT) pathway. Antibody for UNC119 (Anti-UNC119) efficiently inhibited HCC cells proliferation, migration and invasion by blocking Wnt/β-catenin and TGF-β/EMT signal pathway, respectively. Anti-UNC119 was not only beneficial for tumor remission, but also contributed to long-term survival of HCC-bearing mice.*

Conclusion: *UNC119 is significantly up-regulated and promoted cell growth and migration in hepatic cancer cells and tissues via Wnt/β-catenin signal pathway and TGF-β/EMT signal pathway, respectively. Anti-UNC119 treatment inhibited cell proliferation, growth, migration and invasion through inhibition of Wnt/β-catenin and GF-β/EMT signal pathway, respectively.*

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

Introduction

Hepatocellular carcinoma (HCC) is the major form of primary liver cancer that presents a high recurrence rate and ranks second in cancer death rates after treatment with radiotherapy, chemotherapy and surgery. HCC not only possesses high incidence among human cancers, but also the therapeutic schemes remain limited, especially for patients with advanced HCC in late stage. Currently, the common clinical therapies of surgery, chemotherapy and radiotherapy present only modest efficacy and considerable side effects during and the post-treatment period. Therefore, new clinical therapies are eagerly needed in order to enhance the curative effects, minimize the adverse events and even prolong survival of patients with HCC.

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Tumor immunotherapy is a novel cancer therapy strategy, which has potential curative effects without or few treatment-related adverse events and can lead to therapeutic improvements for HCC patients. Strategies of tumor immunotherapy have yielded a lot candidates by target antigen presenting molecules and bioinformatics studies, which showed many regulating immune responses for tumor cells. In this study, we demonstrated that mice with HCC showed beneficial effects after immunotherapy with rAd-IL-2, suggesting that rAd-IL-2 immunotherapy in mice may provide therapeutic advantages and improve the quality of life of patients with HCC.

Recently, genome-wide sequencing and RNA interference screening for identifying molecular alterations contributed significantly to our knowledge over cancer initiation, metastasis, target treatment and prognosis. Many reports have revealed many aberrant protein expression changes could accelerate tumorigenesis and cancer development. Among cancers, HCC has attracted considerable attention for its higher incidence and a high number of molecules were discovered for prevention, diagnosis and treatment for such patients. Investigating and understanding the molecular mechanisms of HCC metastasis as well as invasion have attracted researchers to deploy targeted therapies to suppress metastasis and invasion of tumor cells. Considering the significance of urgently unveiling the mechanisms of tumor cells growth and metastasis for HCC patients in future clinical options, molecular bioinformatics has made it possible to screen the targeted molecules for diagnostic and therapeutic protocols and offered chances of individual tailored medicine in patients who suffer of cancer or other diseases.

UNC119 was first identified as imperfect protein in *Caenorhabiditis elegans* mutants. This molecule revealed shortcomings in locomotion, feeding as well as chemosensation. Lei et al. argued that UNC119 kept a higher expression level and contributed to metastasis and invasion of HCC. Evidence has suggested that UNC119 may be included in the Wnt/β-catenin signal transduction pathway with a control over tumor growth, migration and invasion by modulating the G1/S stage transition to regulate cell proliferation. These data showed that UNC119 might be a possible target molecule to inhibit HCC cells growth.

UNC119, also known as HRG4, is specifically higher expressed in HCC cells needed for G protein trafficking in sensory neurons. In this study, we investigated the inhibitory efficacy of Anti-UNC119 in HCC cells in HepG2-bearing mice. We not only identified the clinical significance of Anti-UNC119 in HCC patients, but also studied the UNC119 biological functions in a HCC-bearing mice model. Furthermore, the biological functions of UNC119 mechanisms on migration were elaborated in HCC cells and tumors.

Methods

Ethics statement

This study was performed strictly in line with the suggestions in the Guide for the Care and Use of Laboratory Animals. All experimental protocols and animals were performed in accordance with National Institutes of Health guidelines with the approval of the Committee on the Ethics of Animal Experiments Defense Research. All operations and euthanasia were carried out to minimize animals' suffering.

Cells culture and reagents

Hep3B, HepG2, SMMC-7721, HUH7, SK-Hep1, MHCC-LM3, MHCC-97H and NCTC-1496 cell lines were purchased from Frederick Cancer Research Facility, the National Cancer Institute, Division of Cancer Treatment Tumor Repository (Frederick, USA), as well as American Type Cell Culture (Rockville, USA). All cells were cultured in DMEM medium with 10% heat-inactivated fetal bovine serum (FBS) (Biowhittaker, Walkersville, USA), 3 mM L-glutamine, 50μg/ml gentamicin (Biowhittaker) as well as 1% penicillin/streptomycin under standard culture conditions (5% CO_2, 37°C).

Comparative mRNA level by RT-PCR

RNeasy mini kit (Qiagen, Gaithersburg, USA) was used to extract total cell RNA and the 1 mu gRNA was synthesized into a cDNA synthesis kit (Bio-Rad, Hercules, USA). iQ SYBR Green Supermix (Bio-Rad) was used to make 1/10 of 25 mu LPCR in the iCycler heat circulator (Bio-Rad). The positive and reverse primers of CCND1, CCNE1, e-cadinin, waveform protein, ZEB1 and UNC119 were synthesized by Invitrogen Corp. (Thermo Fisher, Waltham, MA, USA). The PCR products were quantitatively amplified by measuring the computational cycle threshold (Ct) of the sample mRNA and GAPDH mRNA by 2-ΔΔCt calculation of relative changes of mRNA expression. The result is a difference of n times relative to the control (relative expression level).

MTT cytotoxicity assays

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

SDS-PAGE and western blot

The purified hTERTR-FAM96A fusion protein was homogenized, and SDS-PAGE analysis was performed under the condition of reduction. After non-reducing SDS- PAGE, the purified hTERTR-FAM96A fusion protein was transferred to the cellulose nitrate film. For western blot analysis, hTERT and Apoptin were prepared. Protein detection was performed by hTERT and apaf-1 with IFN-gamma as control. Details of the purchase are referenced in the previous description [16]. All experiments were performed in triplicate.

Animal study

Specific pathogen-free (SPF) female BALB/c (6-week old) nude mice were bought from Harbin Veterinary Research Institute (Harbin, China). Every animal was fed under pathogen-free conditions. 1×10^6 of HepG2 cells were injected subcutaneously to the mice in a total volume of 200 µl. Therapy for tumor-bearing mice by Anti-UNC119, UNC119 or PBS was initiated when tumor diameters reached 6 to 8 mm on day 7 after tumor inoculation. Mice with HCC were randomly separated into 3 groups (n=30 in each experimental group). The treatment was repeated 7 times at two day interval. Tumor diameters were assessed once every two days and tumor volume was counted with the formula:

$$0.52 \times \text{smallest diameter}^2 \times \text{biggest diameter}.$$

Immunohistochemical staining

Immunohistochemical staining was carried out by avidin-biotin-peroxidase technique. Paraffin-embedded tissue sections were prepared and antigen retrieval was performed for further analysis. The paraffin sections were exposed to hydrogen peroxide (3%) for 10-15 min, which subsequently was blocked by a regular blocking solution for 10-15 min at 37°C. Finally, the sections were incubated in anti-UNC119, anti-ZEB1, anti- EMT and anti-TGF-β antibodies at 4°C for 12 hrs after blocking. All sections were washed three times and incubated with secondary antibodies for 1 hr at 37°C and were counterstained with hematoxylin or DAPI.

Statistics

Data was analyzed using SPSS software (SPSS Inc., Chicago, Ill, USA). All data are shown as mean with standard error of the mean (SEM). Unpaired data was analyzed by Student's t-test. Comparisons of data between various groups were analyzed by one way analysis of variance (ANOVA). *p<0.05 and **p<0.01 were considered to be statistically significant.

Results

Expression level of UNC119 in HCC cell lines and in normal liver cells

In order to assess the expression level of UNC119 in HCC cell lines and normal liver cells, Hep3B, SK-Hep1, HUH7, SMMC-7721 (S-7721), MHCC-97H (M-97), MHCC-LM3 (M-LM3) and NCTC-1496 cells were analyzed by qRT-PCR and western blot. Figure 1A showed that UNC119 mRNA expression level was remarkably up-regulated in HCC cell lines compared with normal liver cells NCTC-1496 (**p=0.0032). Figure 1B and C also revealed that the protein level greatly increased in tumor cell lines compared with normal liver cells (**p=0.0072). Among the tumor cell lines, UNC119 expression was highest in HepG2 cells. Therefore, we chose HepG2 cells for further analyses. In addition, immunofluorescence for UNC119 in HepG2 and NCTC-1496 cells demonstrated that UNC119 was relatively higher in hepatic tumor cells compared to normal liver cells (Figure 1D). These observations indicated UNC119 might be a potential target in the HCC initiation or progression.

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

As UNC119 might be a possible molecular target for HCC therapy, a chimeric antibody of UNC119 (Anti-UNC119) was constructed to investigate its efficacy on hepatic tumor cells *in vitro*. The affinity of Anti-UNC119 was assessed by ELISA. Anti-UNC119 exhibited a higher affinity with UNC119 that presented neutralizing biological activity (Figure 2A). In addition, to investigate the biological functional roles of UNC119 on the growth in HCC cells *in vitro*, tumor growth was determined by MTT assay. Figure 2B showed that UNC119 significantly promoted tumor cell growth, but not in normal liver cells. Inversely, Anti-UNC119 showed great inhibitory effects on tumor cell growth. Furthermore, migration and invasion of tumor cells was evaluated after treatment with UNC119, Anti-UNC119 or vehicle. Tumor cell migration and invasion was inhibited in the Anti-UNC119-treated group (Figure 2C and D). However, UNC119 exhibited a promoting effect on tumor cells, while no such effect was observed in normal liver cells. These observations indicated that Anti-UNC119 efficiently inhibited tumor cell growth, migration and invasion but without influencing normal liver cells *in vitro*, which suggests that Anti-UNC119 may be not only an efficient but also safe anticancer agent for human cancer treatment.

Regulation mechanism of UNC119 for tumor cells through Wnt/β-catenin signal transduction pathway

In this study, we investigated whether UNC119 influenced CCND1 and CCNE1 expression in cyclins of hepatic tumor cells. The findings in Figure 3A-C revealed that mRNA as well as CCND1 and CCNE1 protein expression level was up-regulated

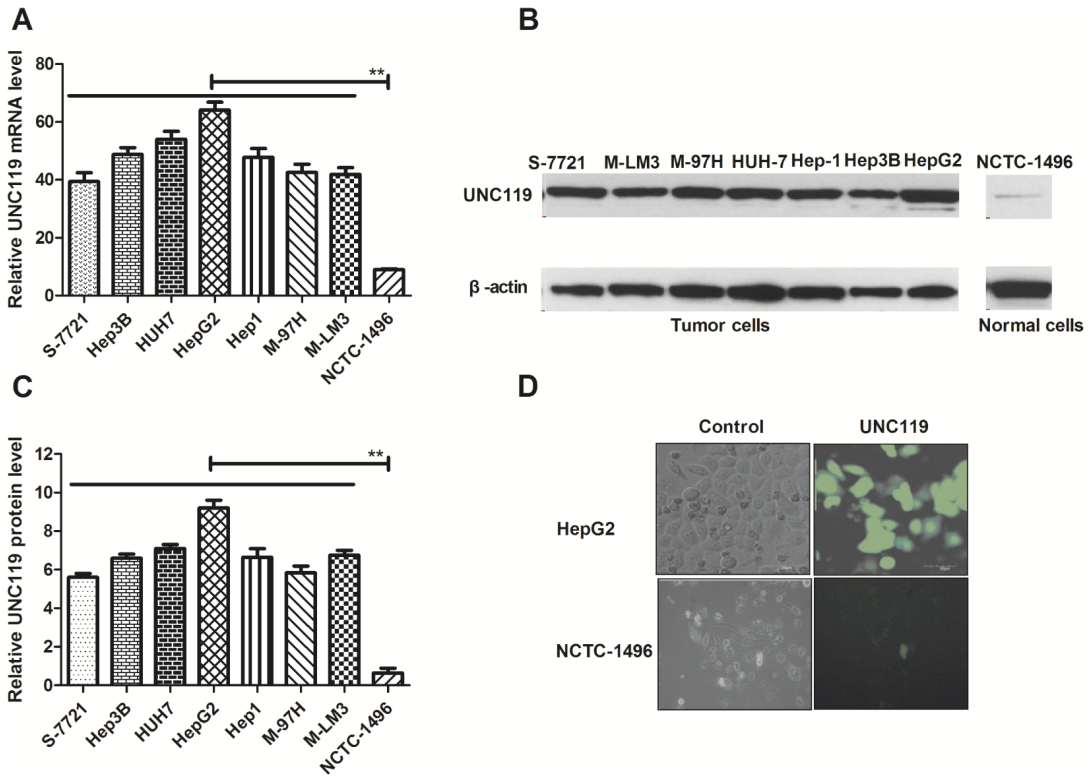

Figure 1. Expression of mRNA and protein levels of UNC119 in hepatocellular carcinoma cells. **A:** The changes of relative mRNA level of UNC119 in the hepatocellular carcinoma cells compared to normal liver cells. **B:** Protein expression level of UNC119 in the hepatocellular carcinoma cells compared to normal liver cells determined by SDS-PAGE, showing that protein level increased greatly in tumor cell lines compared to normal liver cells. **C:** The changes of relative protein level of UNC119 in the hepatocellular carcinoma cells compared to normal liver cells. **D:** Difference expression of UNC119 between HepG2 hepatic tumor cells and NCTC-1496 normal liver cells. Bars represent means and SEM. **p<0.01 versus normal liver cells (one-way ANOVA).

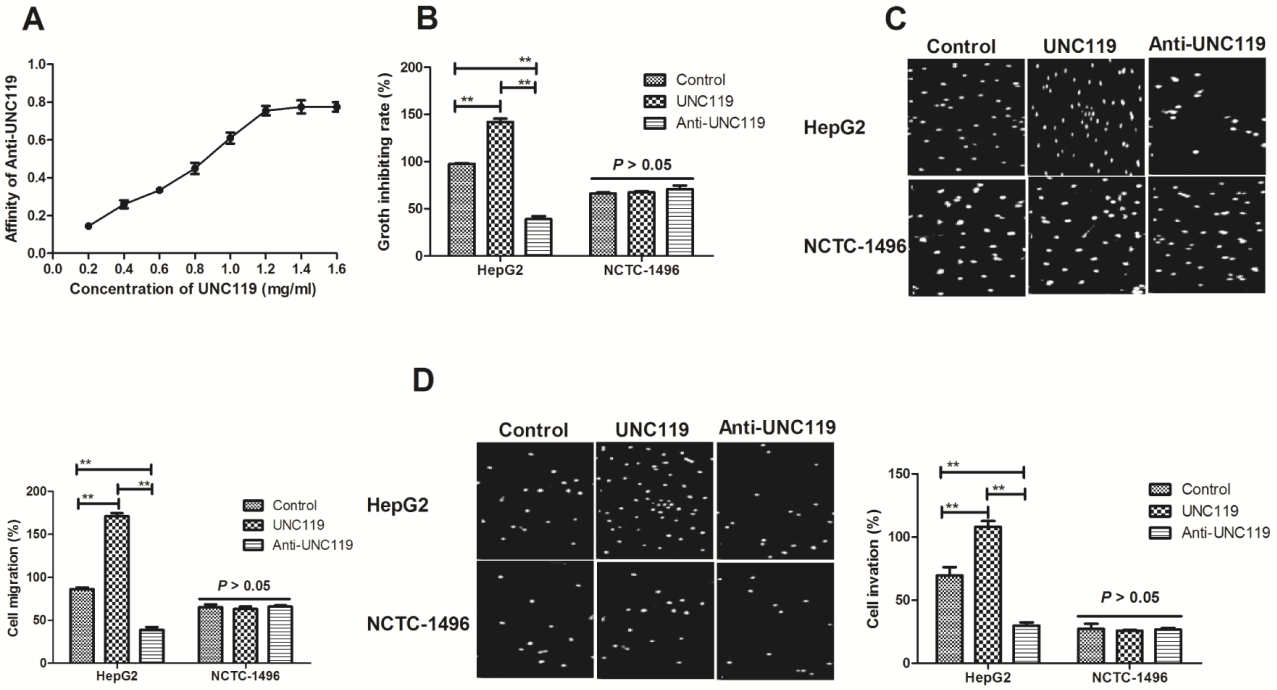

Figure 2. Inhibitory effects of Anti-UNC119 on hepatocellular carcinoma cells. **A:** High affinity of Anti-UNC119 in binding with UNC119 (p<0.05). **B:** Inhibition of tumor cells growth after treatment with Anti-UNC119. **C:** Inhibition of tumor cells migration after treatment with Anti-UNC119. **D:** Inhibition of tumor cells invasion after treatment with Anti-UNC119. Data are shown as means and SEM. **p<0.01 versus normal liver cells (one-way ANOVA).

after UNC119 treatment for 48 hrs in HepG2 cells. However, Anti-UNC119 canceled these effects in HepG2 cells. No great difference was found in NCTC-1496 normal liver cells. For more in-depth study into the fundamental mechanism of regulation in tumor cells cyclins, Wnt/β-catenin canonical signal pathway was analyzed in cancer cell growth. Luciferase reporter assay showed that UNC119 influenced the activity of Wnt/β-catenin signaling and Anti-UNC119 inhibited Wnt/β-catenin activity (Figure 3D). Also, these results indicated that Anti-UNC119 inhibited Wnt/β-catenin activity through regulating the expression level of CCND1 and CCNE1. In addition, it was found that UNC119 treatment up-regulated the activity of Wnt/β-catenin and Anti-UNC119 showed reverse effects in HepG2 cells. Furthermore, as TGF-β1 can facilitate the migration and invasion of tumor cells, we hypothesized Anti-UNC119 may inhibit TGF-β following suppressed EMT and the migration of cancer cells. Therefore, the expression of ZEB1 and EMT markers were analyzed in HepG2 tumor cells. According to the results, TGF-β1 and EMT markers expressions were greatly downregulated after treatment with Anti-UNC119 (Figures 3E and F). These results suggested that UNC119 promoted tumor cell growth through regulating Wnt/β-catenin signal transduction pathway and also promoted tumor cell migration by TGF-β/EMT pathway.

Therapeutic effects of Anti-UNC119 on HCC-bearing mice in vivo

We further examined its antitumor efficacy in HepG2-bearing mice *in vivo*. HepG2 cells were injected in nude BALB/c mice. The HepG2-bearing mice were subcutaneously injected with Anti-UNC119, UNC119 or PBS as control on day 7. The mice were treated for a total of 7 times. The xenograft

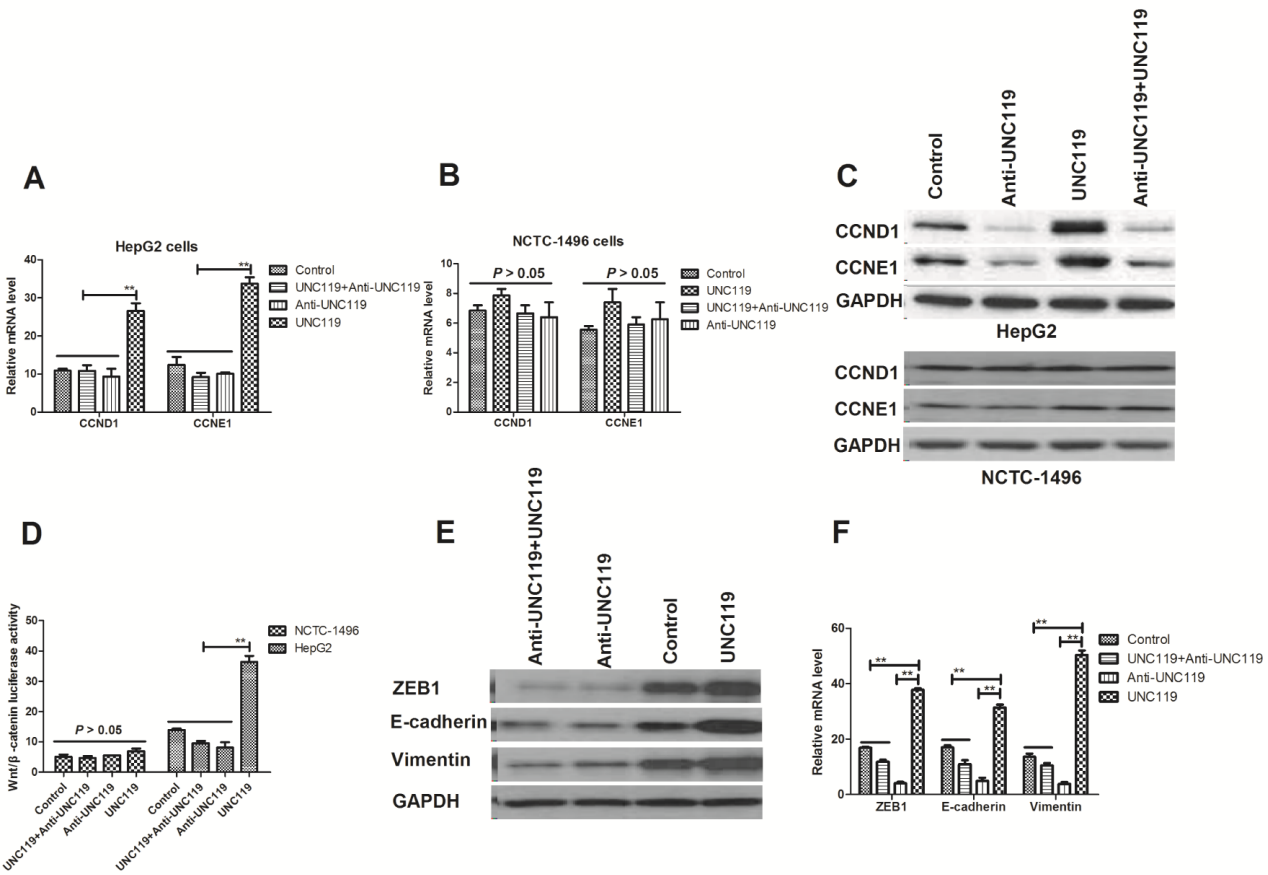

Figure 3. Mechanism of Anti-UNC119 in tumor cells growth and migration. **A:** mRNA expression changes of CCNE1 and CCND1 after treatment with UNC119, Anti-UNC119 or PBS in tumor cells. **B:** mRNA expression changes of CCNE1 and CCND1 after treatment with UNC119, Anti-UNC119 or PBS in tumor cells. **C:** Protein expression changes of CCNE1 and CCND1 after treatment with UNC119, Anti-UNC119 or PBS in tumor and normal cells. **D:** Inhibitory effects of Anti-UNC119 on Wnt/β-catenin signaling activity after treatment with UNC119, Anti-UNC119 or PBS in tumor and normal cells. **E:** Protein expression changes of ZEB1, Vimentin and E-cadherin after treatment with UNC119, Anti-UNC119 or PBS in tumor cells. **F:** Relative mRNA expression changes of ZEB1, Vimentin and E-cadherin after treatment with UNC119, Anti-UNC119 or PBS in tumor cells. Data represent means and SEM. **p<0.01 versus normal liver cells (data was analyzed by Student's t-test and one-way ANOVA).

tumor growth was monitored and recorded every two days. As illustrated in Figure 4A, the tumor growth was significantly suppressed in Anti-UNC119-treated group compared to UNC119 and PBS-treated groups. In addition, UNC119 expression was analyzed by immunohistochemistry and showed a significantly decrease in Anti-UNC119-treated tumors (Figure 4B). Also, β-catenin, TGF-β and EMT expressions were evaluated in the tumors of mice on day 25. The results in Figure 4C showed that β-catenin, TGF-β and EMT expression levels were decreased in Anti-UNC119-treated tumors and UNC119-treated tumors increased their expression compared to PBS-treated tumors. Furthermore, we observed that Anti-UNC119 treatment contributed to long-term survival in 120-day observation (Figure 4D). Overall, conclusively, Anti-UNC119 exhibited strong anticancer effect in the HCC mice model.

Discussion

The advent of target therapy raised expectations for improved hepatic tumors' treatments, and therapeutic benefits have been seen in preclinical and clinical trials. This research has shown that UNC119 mRNA and protein concentration were up-regulated in HCC cell lines, which promoted growth, migration as well as invasion of HCC cells (Figure 3). We elaborated that UNC119 promoted tumor cell growth through regulating Wnt/β-catenin signal transduction pathway and it also promoted tumor cell migration via TGF-β/EMT pathway. The mechanisms of UNC119 on promoting HepG2 cells growth, migration and invasion were illustrated in this study. Importantly, an efficient chimeric antibody targeting UNC119 was constructed and its biological activity was also studied *in vitro* and *in vivo*. Our data indicated that Anti-UNC119 could efficiently bind to UNC119 and decrease UNC119 expression both in mRNA as well as in protein levels (Figures 1 and 2). Our results have convincingly shown that Anti-UNC119 might be a possible anticancer agent for HCC treatment.

HCC incidence ranks 5th in men and 9th in women and accounts for more than 90% among the primary liver cancer cases. A previous study revealed that HCC was genetically complex, multifactorial and with great heterogeneity. Therefore, several opinions about anticancer protocols targeting different signal pathways have been proposed. These cellular signal pathways presented key signal transduction for various extracellular growth factors and receptors of HCC cells. To in-

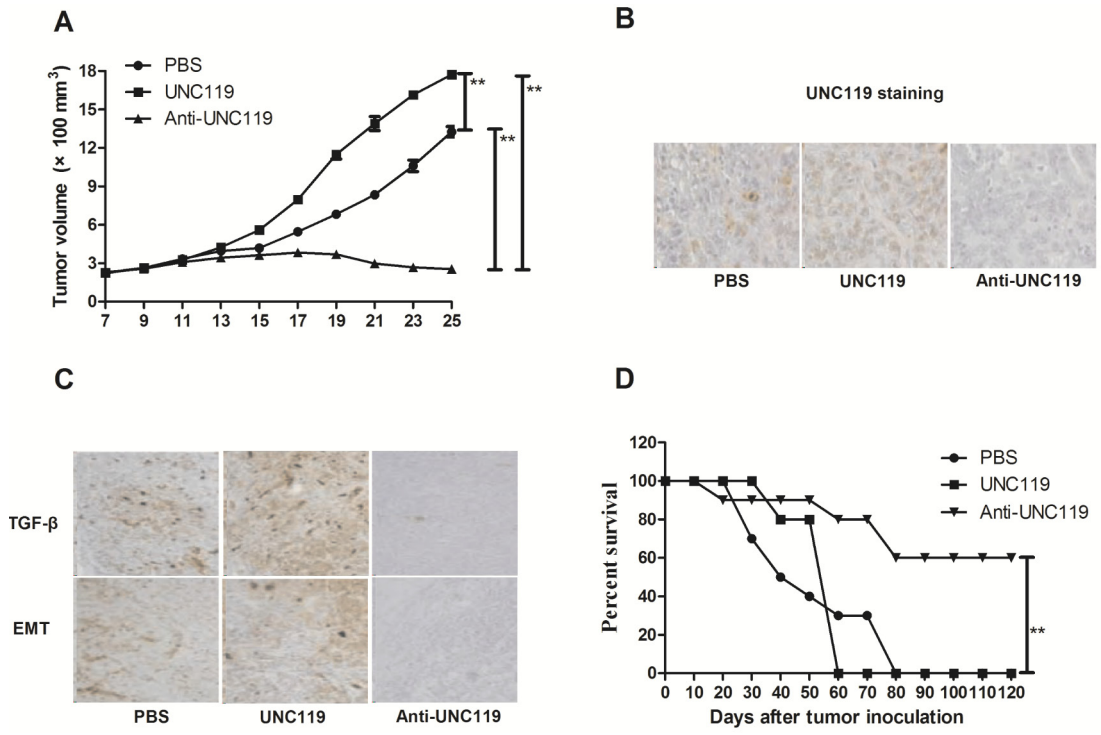

Figure 4. Therapeutic effects of Anti-UNC119 on HepG2-bearing mice. **A:** Inhibitory effects of Anti-UNC119 on tumor growth in HCC tumor-bearing mice. **B:** Down-regulation of UNC119 expression in tumors from Anti-UNC119-treated mice compared to UNC119 and PBS-treated groups. **C:** Down-regulation of ZEB1 and TGF-β expression in tumors from Anti-UNC119-treated mice compared to UNC119 and PBS-treated groups. **D:** Beneficial effect of Anti-UNC119 for long-term survival rate compared to UNC119 and PBS-treated groups. Data represent means and SEM. **p<0.01 versus normal liver cells (data was analyzed by Student's t-test and one-way ANOVA).

duce apoptosis and death, thus eliminating all tumor cells in patients, is the ultimate goal. However, the occurrence of tumor cells migration is a thorny problem in HCC therapy and tumor cells often invade surrounding tissues likely associated with acquired resistance to apoptosis induced by anticancer drugs. Tumors with acquired migration and invasion competences through different signal pathways and the consequent need for long-term medical therapy represents a significant clinical problem in HCC. Therefore, effective therapeutic agents inhibiting HCC cell growth are urgently needed due to the high rate of disease occurrence and easier metastasis in patients with HCC.

TGF-β/EMT pathway is an important mechanism regulating cancer cell invasion and metastasis. EMT has been characterized as the mechanism of migration and invasion of epithelial cells converted to mesenchymal cells (eg. fibroblast cells). Our data showed that UNC119 was greatly up-regulated in HCC cells as compared with normal liver cells. Our results also showed UNC119 up-regulated TGF-β/EMT expression levels in HCC cells and tissues to enhance tumor migration and invasion.

Currently, Wnt/β-catenin signaling pathway has been reported to be associated with tumor cells development in many kinds of cancers.

In the current study, the activity of Wnt/β-catenin signaling was positively correlated with the level of change of UNC119. In addition, the levels of target downstream genes CCND1 and CCED regulated by Wnt/β-catenin were increased after UNC119 treatment *in vitro* as well as *in vivo*. These observations indicated that down-regulation of UNC119 by Anti-UNC119 could suppress tumor cell growth and proliferation.

In conclusion, in the present study Anti-UNC119 was developed as an UNC119-targeting factor and impairing Wnt/β-catenin and TGF-β/EMT signal pathway. The antitumor character of Anti-UNC119 requires further investigation in preclinical studies.

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Conflict of interests

The authors declare no conflict of interests.

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