miR-577 suppressed the metastasis, EMT and viability via NF-κB pathway by targeting CXCL5 through in hepatocellular carcinoma

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Research

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Abstract

Background

Hepatocellular carcinoma (HCC) is a common malignant cancer worldwide. miR-577 have a role in inhibiting cell viability, metastasis in many tumors. This research was to explore the great role of miR-577 in hepatocellular carcinoma.

Methods

RT-qPCR and western blot were performed to evaluate the the miR-577 and genes mRNA and protein levels. Transwell assay and CCK-8 were applied to measure the viable and invasive abilities. Meanwhile, Kaplan-Meier method was used to assess the survival of HCC patients.

Results

miR-577 was downregulated in HCC tissues, which predicted a worse overall survival in HCC. miR-577 targeted to CXCL5 and mediated its expression in HCC. miR-577 suppressed cell invasion and EMT in HuH-7 cells. miR-577 inhibited cell viability via NF-κB pathway. In addition, miR-577 overxpression impaired the xenograft growth of HuH-7 cells.

Conclusion

miR-577 inhibited cell invasion, EMT and viability via NF-κB pathway by targeting to CXCL5 in HCC. The newly identified miR-577/CXCL5 axis provides novel insight into the pathogenesis of hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is a major cause of cancer death especially in Africa and Asia [1, 2]. Due to the hepatitis C virus epidemic, the incidence of HCC is increasing in western countries [3]. The current treatment for hepatocellular carcinoma is limited to surgical resection, but resection results in a recurrence rate of more than 70% within 5 years, while 80% of present are not suitable to surgery [4]. Therefore, it is urgent to explore biomarkers for the treatment.

The discovery of MicroRNAs (miRNAs) opened up a new generation of cognition of in hepatocellular carcinoma [5, 6]. miRNAs negatively mediated gene expression through translational repression or mRNA degradation to be involved in the development of tumors [7]. Several miRNAs, including miR-122, miR-325, miR-206, miR-122 and miR-224 were played great roles in hepatocellular carcinoma [8–11]. miR-577 acted tumor suppressor to suppress tumor growth and enhances chemosensitivity in colorectal cancer

[12]. miR-577 regulated cell proliferation and promoted G1-S phase transition in esophageal squamous cell carcinoma [13]. Similarly, miR-577 inhibited pancreatic β-cell function and survival in pediatric diabetes [14]. In non-small cell lung cancer, miR-577 suppressed cell growth and EMT in regulating WNT2B via Wnt/β-catenin pathway [15]. However, there was little studies elucidated the roles of miR-577 in HCC, thus, the experiments were performed to explore the vital functions of miR-577 in HCC.

C-X-C motif chemokine ligand 5 (CXCL5), known as ENA-78 or SCYB5 was a member of CXC subfamily of chemokines, binds the G-protein coupled receptor chemokine (C-X-C motif) receptor 2 to recruit neutrophils, to promote angiogenesis and to remodel connective tissues [16]. CXCL5 was thought to play roles in cell proliferation, migration, and invasion of cancer [17, 18]. CXCL5 citrullination may exert inflammatory properties by recruiting monocytes to inflamed joint tissue in a mouse model of inflammatory arthritis [19]. CXCL5 acted as an important angiogenic factor in idiopathic pulmonary fibrosis and non-small cell lung cancer [20, 21]. CXCL5 was involved in the interaction between cholangiocarcinoma cells and cancer-associated fibroblasts and inhibition of tumor-stromal interactions [18]. In our study, we discovered that miR-577 enhanced cell viability and invasion through binding to CXCL5 in HCC. miR-577 promoted invasion EMT and viability through PI3K/AKT pathway in HCC.

Material And Methods

Clinical specimens

Pairs of HCC tissues and peritumoral normal tissues were gathered from 48 hepatocellular carcinoma patients in Shandong Provincial Hospital affiliated to Shandong University during January 2016 to December 2018. Specimens was immediately frozen in liquid nitrogen and then stored at -80℃ after surgery. We obtained the written informed consent and the Ethics Committees of Shandong Provincial Hospital affiliated to Shandong University approved for this study.

Cell culture

We purchased HCC cells HuH-7 and a normal hepatocyte cell L-02 from American Type Culture Collection (ATCC; Rockville, MD, USA). All the cells were incubated in DMEM medium (Invitrogen, Carlsbad, CA, USA) with 10% FBS (Sigma-Aldrich, Louis, MO, USA) at 37℃ in a humidified chamber with 5 % CO_2.

Transfection

The specific plasmids of miR-577 mimic or miR-577 inhibitor as well as their negative control were designed and synthetized from Gene-Pharma (Shanghai, China). The transfection was carried out using HuH-7 cells that were incubated in 6-well plate. The Lipofectamine 2000 Reagent (Invitrogen, USA) diluted using Opti-MEM/Reduced serum medium (Thermo Scientific, Shanghai, China) was used to perform the transfection. Geneticin (G418; Thermo Scientific, Shanghai, China) was used to select the stable transfection cells, while we harvest the transient transfection cells after transfected 48 h.

Quantitative real-time PCR

TRIzol Reagent (Invitrogen) and miRNeasy Mini Kit (Qiagen, Hilden, Germany) were employed to extract total mRNAs and miRNAs from tissues or cells. Omniscript Reverse Transcription Kit (Qiagen) and TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) were used to synthesize the first cDNA chain; followed QuantiTect SYBR Green PCR Kit (Qiagen) and miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems) were conducted to carry out the qPCR in a Quantitect SYBR green PCR system (Qiagen). The relative levels of mRNA and miRNA were derived using $2^{-\Delta\Delta Ct}$ method, the GAPDH and U6 small nuclear RNA utilized as normalization. The primers used for RT-qPCR were as follows: miR-577 forward 5'-TGCGGTAGATAAAATATTGG-3', reverse 5'-GTGCAGGGTCCGAGGT-3'; U6 forward 5'-GCTTCGGCAGCACATATACTAAAAT-3', reverse 5'-CGCTTCACGAATTTGCGTGTCAT-3'; CXCL5 forward 5'-AGCTGCGTTGCGTTTGTTTAC-3', reverse 5'-TGGCGAACACTTGCAGATTAC; GAPDH forward 5'-AAGGTGAAGGTCGGAGTCAA-3', reverse 5'-AATGAAGGGGTCATTGATGG-3'.

Western blot analysis

The total proteins were lysed by RIPA Lysis Buffer (Sigma, USA) containing 10% PMSF (Sigma). The SDS-PAGE was applied to separate the protein and then the blots were electro-transferred to PVDF membranes (Millipore, USA). After being blocked by 5% fat-free milk at ream temperature for 1 h, the membranes were incubated with primary antibodies. The primary antibodies were against CXCL5 (1:1000; Abcam, Cambridge, USA), E-cadherin (1:1000; Abcam), N-cadherin (1:1000; Abcam), Vimentin (1:1000; Abcam), c-Myc (1:1000, Abcam), TRAF6 (1:1000, Abcam). Next, the blots were incubated by secondary anti-rabbit HRP-conjugated antibody (Cell Signaling). The protein signals were captured using Enhanced Chemiluminescence Detection Kit (ECL, Pharmacia Biotech, Arlington, USA).

MTT assay

The HuH-7 cells were plated into 96-well plates and cultivated for 24h, 48h, 72h and 96h. We added 20 μl of MTT (5 mg/ml, Sigma) into each well and followed cultured for 6 h. Next, we discarded the supernatant and added 100 μl of DMSO (Sigma) to each well. After agitating for 10 min, the absorbance at a wavelength of 570 nm was evaluated using an ELISA reader (Bio-Rad, Hercules, CA, USA).

Transwell assay

The transwell insert (8 μm membrane, Corning, Cambridge, MA) were placed in 24-well plate to evaluate the cell invasive ability. The HuH-7 cells were suspended by FBS free RPMI-1640 medium and we added 200 μl in the upper chamber, whereas the lower chamber was filled with 500 μl medium containing 15% FBS, which acted as inducer. After the cells were incubated for 24 h at 37°C, the non-invasive cells, which still on the upper surface, were removed by cotton swabs. We fixed and then stained the invasive cells using 4% paraformaldehyde and 10% crystal violet respectively; and followed counted the cells under a microscope (Olympus Corporation, Tokyo, Japan).

miRNA targets prediction and dual-luciferase reporter assay

TargetScan was conducted to peform the prediction of target genes of miR-577 and we discovered that CXCL5 was one of potential target gene. We mutated the binding sequences from UUUAUCU to AAAUAGA to confirm miR-577 binding to CXCL5 in HCC cells. Followed, we inserted the wild type and the mutational 3'-UTR of CXCL5 into the dual luciferase reporter vectors, which were designated as WT or MUT. We utilized Lipofectamine 2000 Reagent (Invitrogen, USA) to co-transfect miR-577 mimic and WT or MUT vector into HuH-7 cells. Finally, the luciferase activity was measured using dual luciferase reporter assay system (Promega, USA).

Statistical analysis

All the statistical analysis was performed to use SPSS 16.0 software (IBM, Armonk, NY, USA) and the data were presented as mean ± SD. Student's t test was performed to compare the differences between two groups, besides, one-way ANOVA was utilized to compare the differences between three or more groups. The association between miR-577 expression and the overall survival for HCC patients were assessed by Kaplan-Meier curve and log-rank test. P< 0.05 was considered to be statistical significant.

Results

miR-577 downregulation predicted poor prognosis of HCC

The miR-577 level was assessed in 48 pairs of HCC and peritumoral normal tissues and we found that miR-577 expression was overexpression in HCC tissues versus corresponding peritumoral normal tissues (P<0.05) (**Figure 1A**). Kaplan-Meier method elucidated the miR-577 expression has association with poor overall survival of HCC patients (P<0.05) (**Figure 1B**).

miR-577 inhibited cell viability and invasion in HuH-7 cells

miR-577 expression were evaluated in HCC cells HuH-7 and a hepatocyte cell L-02. miR-577 expression was higher in L-02 cells than HuH-7 (P< 0.01) cells (**Figure 2A**). To assess the roles of miR-577, miR-577 mimic and miR-577 inhibitor were employed to up- (P< 0.01) or down-regulate (P< 0.05) miR-577 in HuH-7 cells calculated by RT-qPCR (**Figure 2B**).

MTT assay illuminated that miR-577 mimic suppressed (P< 0.05) cell viability, while miR-577 inhibitor promoted (P< 0.05) the proliferative ability in HuH-7 cells (**Figure 2C**). Transwell assay indicated that miR-577 mimic inhibitor (P< 0.05) cell invasive ability whereas miR-577 enhanced (P< 0.05) (**Figure 2D**). All the results revealed miR-577 inhibited the viability and invasion in HCC cell HuH-7.

miR-577 regulated CXCL5 expression through binding to CXCL5 mRNA 3'-UTR

CXCL5 was predicted as a target gene of miR-577 using TargetScan, and the binding site was located at 249 to 255 on CXCL5 mRNA 3'-UTR. To validate miR-577 binding to the potential binding site of CXCL5,

we mutated the potential binding sites, and then calculated the luciferase activity (**Figure 3A**). The luciferase reporter assay proved that miR-577 reduced ($P< 0.05$) the luciferased activity of HuH-7 cells that transfected wild type CXCL5 3'-UTR, however, it make no difference ($P> 0.05$) on the luciferase activity of cells transfected mutated CXCL5 3'-UTR (**Figure 3B**). Moreover, we evaluated CXCL5 mRNA levels after transfected miR-577 mimic or miR-577 inhibitor in HuH-7 cells, and miR-577 overexpression inhibited CXCL5 mRNA level ($P< 0.05$), while knockdown miR-577 promoted CXCL5 expression in HuH-7 cells ($P< 0.05$) (**Figure 3B**). All the results indicated that miR-577 mediated CXCL5 expression by targeting to its mRNA 3'-UTR in HCC cells HuH-7.

miR-577 suppressed cell invasion, EMT and viability through PI3K/AKT signal pathway

RT-qPCR was employed to assess the CXCL5 expression in tissues and cells. The CXCL5 expression in HCC tissues was higher than that in peritumoral normal tissues (P< 0.05) (**Figure 4A**). As expected, the CXCL5 expression was lower in hepatocyte cell L-02 than HCC cells HuH-7 (P< 0.05) (**Figure 4B**). Moreover, the proteins levels of EMT and pathway associated were assessed by western blot in HuH-7 cells. Research found that miR-577 mimic suppressed CXCL5 and E-cadherin expression, while improved N-cadherin and Vimentin expression in HuH-7 cells (**Figure 4C**), which suggested that miR-577 suppressed cell EMT through CXCL5. In addition, miR-577 inhibited CXCL5, c-Myc and TRAF6 expression in HuH-7 cells (**Figure 4D**), which proved that miR-577 inhibited cell proliferation through NF-κB pathway. All the results revealed that miR-577 inhibited cell invasion, EMT and viability through NF-κB signal pathway.

miR-577 impaired the xenograft growth in vivo

The nude mice were inject the HuH-7 cells stably transfected miR-577 mimic or control plasmid at subcutaneous. The volumes of xenograft tumors were measured every 3 days and the group of transfecting miR-577 mimic had a slower growth rate than control group, which indicated that miR-577 inhibited the HCC growth in vivo (**Figure 5A**). After 26 days of training, the nude mice were sacrificed. The volumes were calculated and the tumor volume of cells overexpressed miR-577 was smaller than the control group (P<0.05) (**Figure 5B**).

Discussion

Hepatocellular carcinoma is one of the most common causes of cancer-related death worldwide with a lower 5-year survival rate [22, 23]. However, it is remain incompletely understood of the molecular mechanisms of hepatocellular carcinoma.

miRNAs were associated with translational repression and mRNA degradation at post transcriptional level [24, 25]. miR-577 acted as tumor suppressor to inhibit cell proliferation, migration and invasion in papillary thyroid carcinoma [26]. miR-577 suppressed metastasis and EMT of breast cancer [27]. Consistent with all the findings, we proposed that miR-577 was downregulated and miR-577 inhibited cell viability and invasion in HCC. We also revealed that miR-577 low expression predicted worse outcome of

HCC patients. miR-577 suppressed tumor growth of hepatocellular carcinoma, which was consistent with the findings in glioblastoma [28].

CXCL5 acted as oncogene and enhanced cell growth and metastasis in several tumors, including bladder cancer, pancreatic cancer, cervical cancer and cutaneous melanoma [29–32]. CXCL5 was overexpressed in intestinal epithelium in inflammatory bowel disease and also in malignant pancreatic diseases [33, 34]. CXCL5 directly enhance tumor cell survival and proliferation in gastric cancer [35]. Consistent with all the findings, we discovered CXCL5 was upregulated in HCC tissues and cells. CXCL5 overexpression was associated with poor prognosis of HCC patients. In colorectal cancer, CXCL5 promoted tumor angiogenesis via AKT/NF-κB pathway [36]. It's the first time to propose that CXCL5 was a target gene of miR-577 in HCC. miR-577 regulated cell invasion, EMT and viability through NF-κB signaling pathway by regulating CXCL5 in HuH-7 cells.

Conclusion

miR-577 was low expressed in HCC tissues and miR-577 downregulation predicted poor prognosis of HCC patients. CXCL5 was a target gene of miR-577 and its expression was regulated by miR-577 in HCC. miR-577 impaired cell invasion, EMT and through NF-κB pathway in HuH-7 cells by targeting to CXCL5. miR-577 overexpression inhibited xenograft growth of HuH-7 cells.

Declarations

Acknowledgements

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Not applicable.

Author contribution

LT and JL are responsible for the conception or design of the work. WL contributes the acquisition, analysis, or interpretation of data for the work. XS provides the tissue samples. HX helps in the follow-up of the patients. JL helps in reviewing the histopathology slides. All authors finally approved the manuscript version to be published.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval and consent to participate

The study was approved by Ethical Committee of Jinan Infectious Diseases Hospital affiliated to Shandong University and conducted in accordance with the ethical standards. Signed written informed consents were obtained from the patients and/or guardians.

Consent for publication

Not applicable.

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Figures

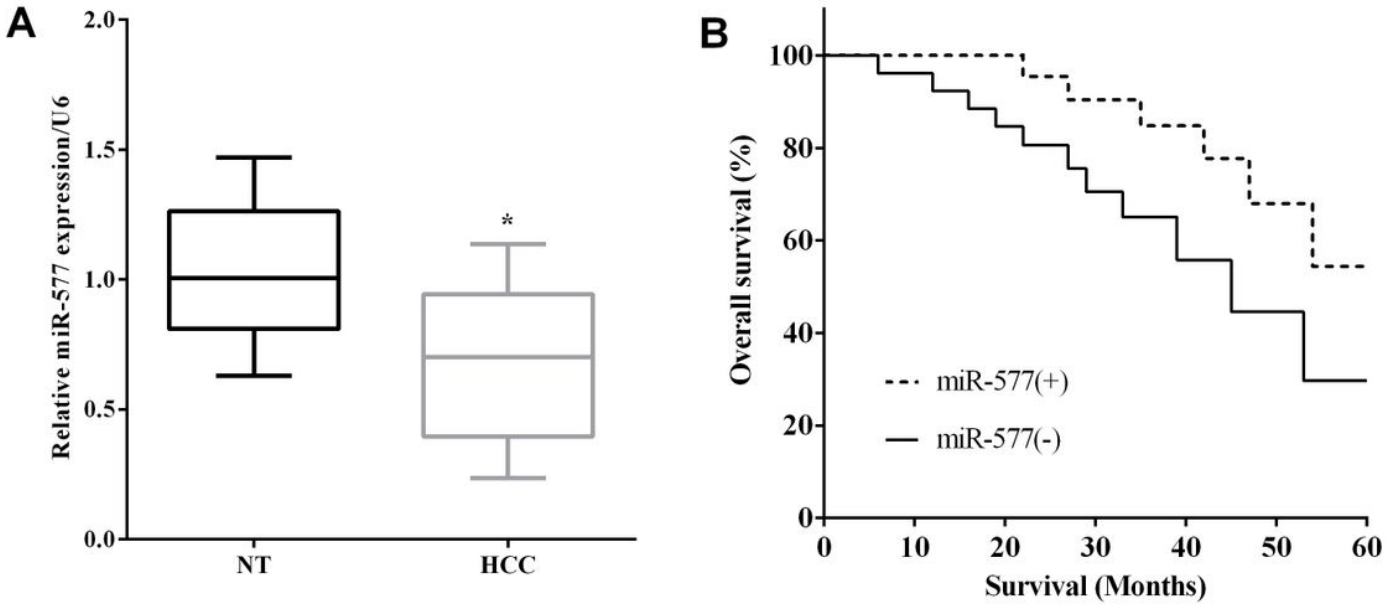

Figure 1

Downregulation of miR-577 predicted poor prognosis of HCC (A) miR-577 expression was downregulated in HCC tissues versus corresponding peritumoral normal tissues. (B) miR-577 downregulation predicted poor 5-year survival in HCC.

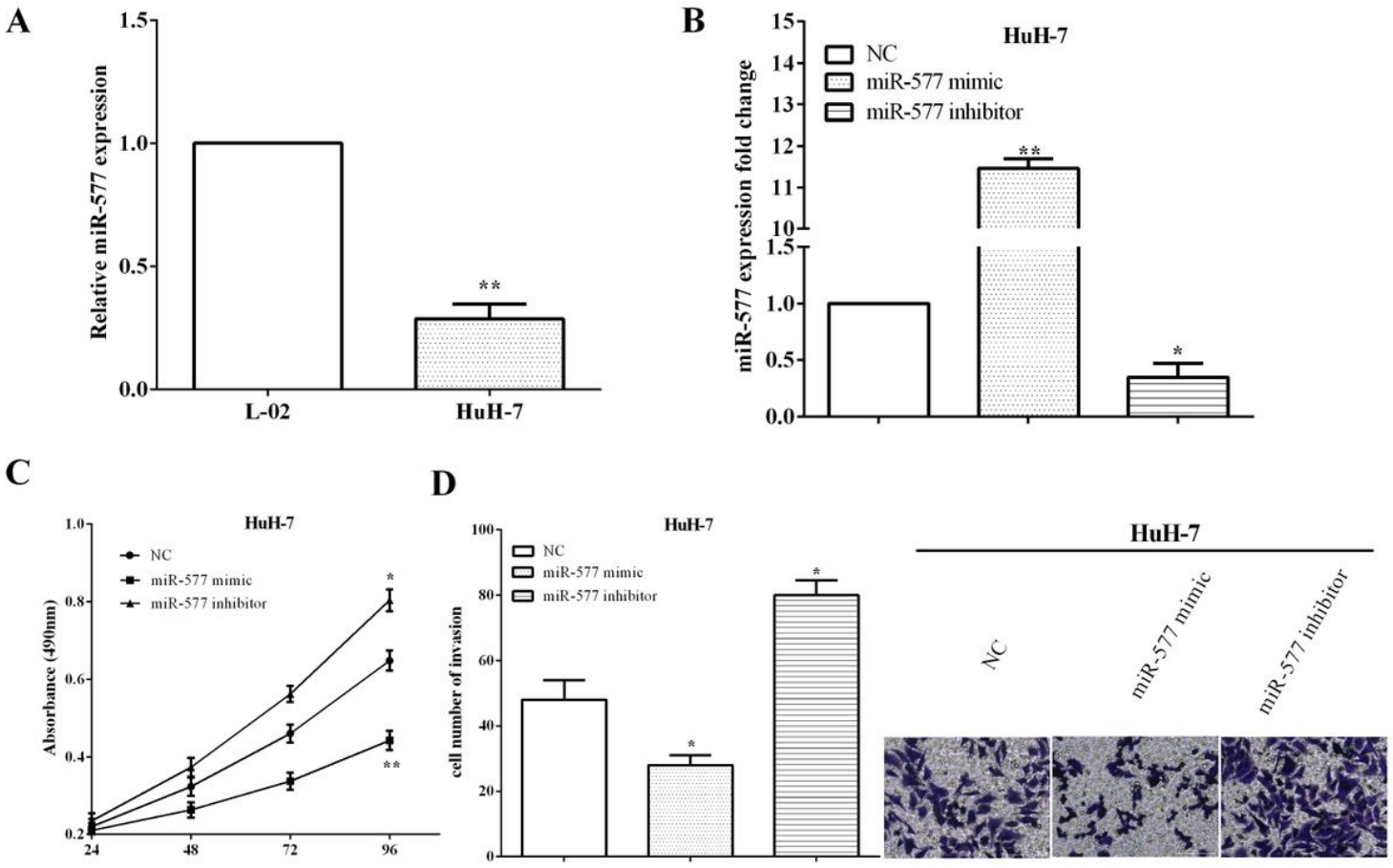

Figure 2

miR-577 suppressed cell viability and invasion in HuH-7 cells (A) miR-577 expression was higher in hepatocyte cell L-02 than HuH-7 cells. (B) RT-qPCR was utilized to measure the efficiency of transfecting miR-577 mimic and miR-577 inhibitor in HuH-7 cells. (C) MTT assay revealed cell viability was inhibited by miR-577 mimic, whereas enhanced by miR-577 inhibitor in HuH-7 cells. (D) Transwell assay elucidated miR-577 mimic impaired cell invasive ability while it was promoted by miR-577 inhibitor in HuH-7 cells.

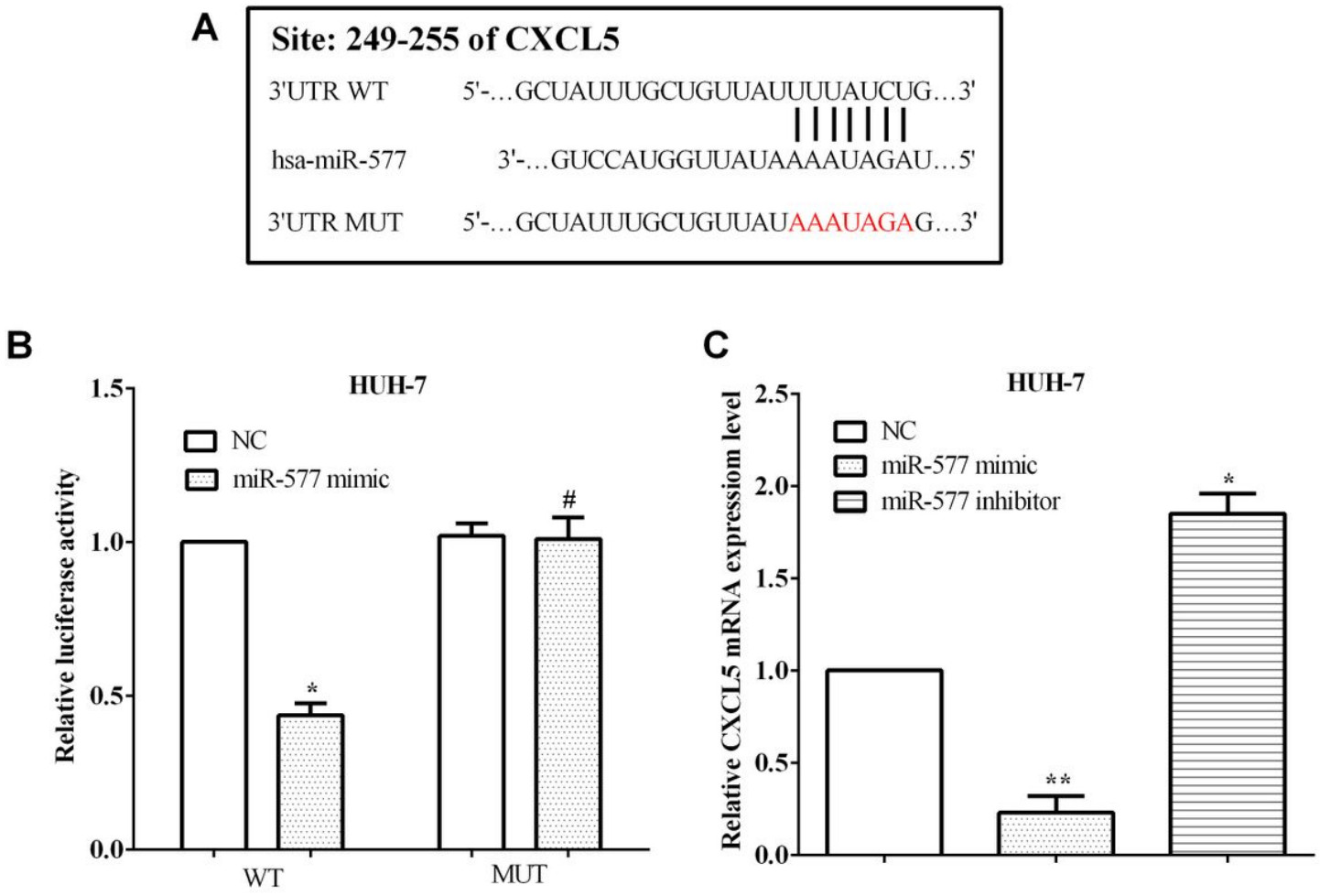

Figure 3

miR-577 regulated CXCL5 expression through binding to its mRNA 3'-UTR (A) TargetScan predicted CXCL5 was a target gene of miR-577. (B) miR-577 increased the luciferased activity of HuH-7 cells that transfected wild type CXCL5 3'-UTR. (C) miR-577 reduced CXCL5 mRNA level, whereas knockdown miR-577 improved CXCL5 expression in HuH-7 cells.

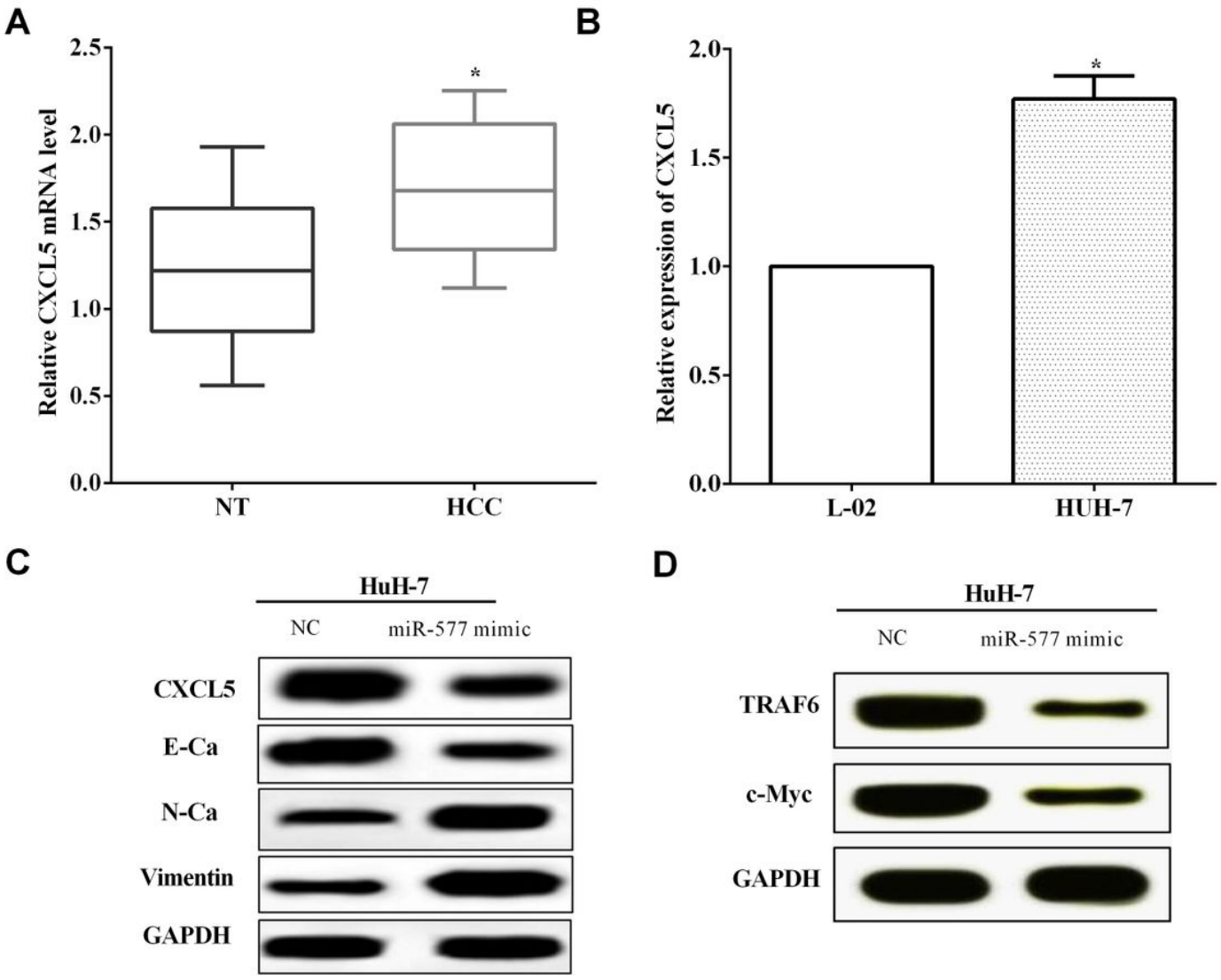

Figure 4

miR-577 inhibited cell invasion, EMT and viability through NF-κB signal pathway (A) The relative expression of CXCL5 in HCC tissues and peritumoral normal tissues. (B) CXCL5 expression in HCC cells and normal cells. (C) CXCL5 expression was higher in HuH-7 cells than hepatocyte cell L-02. (D) miR-577 suppressed cell invasion, EMT and suppressed viability through NF-κB signal pathway.

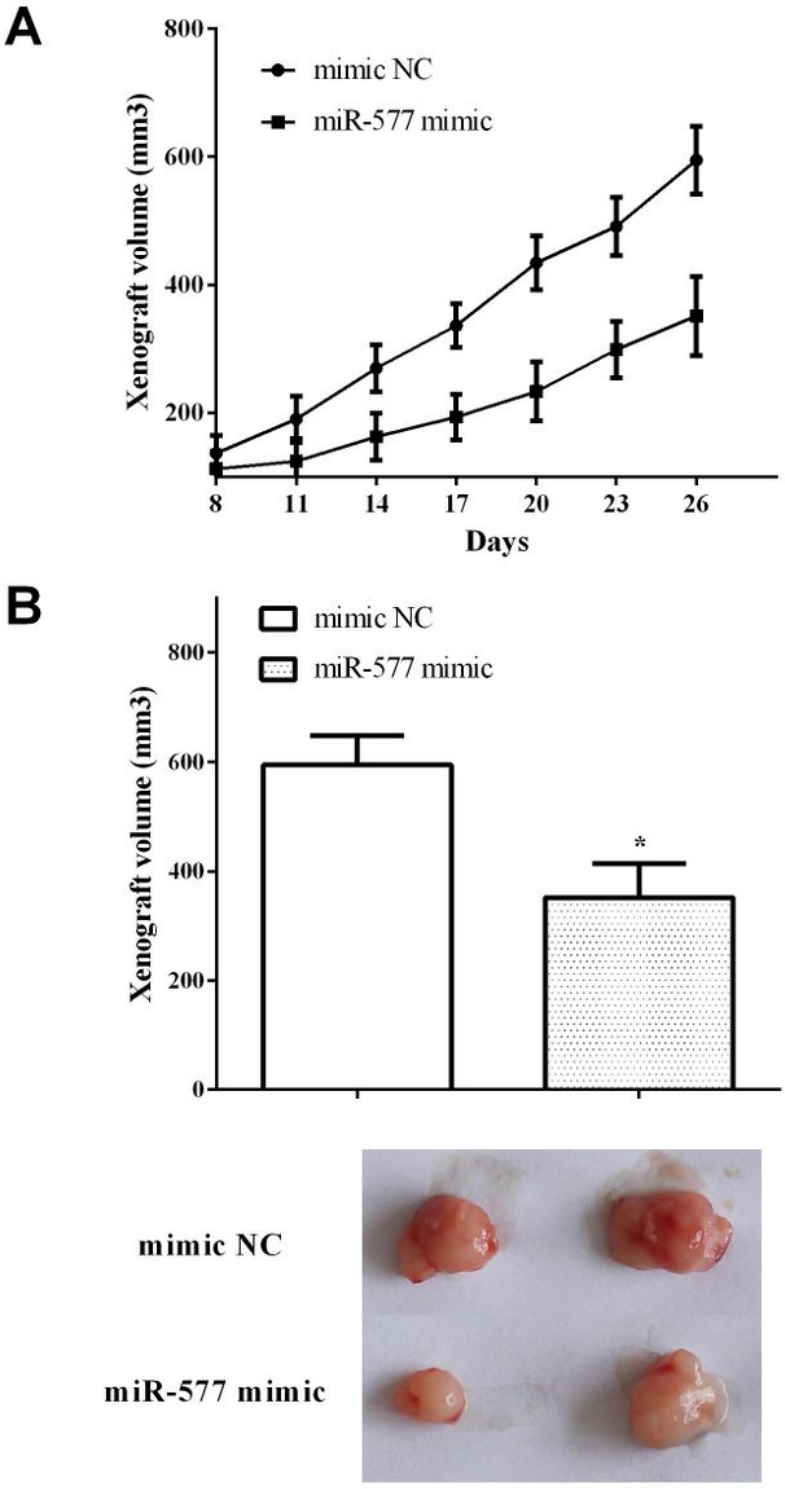

Figure 5

miR-577 suppressed the xenograft growth in vivo (A) The group of transfecting miR-577 mimic had a slower growth rate than control group. (B) The tumor volume of cells overexpressed miR-577 was smaller than the control group.