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Original Article

MiR-1179 represses cell proliferation, migration and invasion of hepatocellular carcinoma through suppression of NUAK2

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Abstract: Objective: To investigate the function and mechanism of miR-1179 in the tumorigenesis of hepatocellular carcinoma (HCC). Methods: The levels of miR-1179 and NUAK2 in clinical tissues or cell lines were examined using quantitative Real-time PCR (qRT-PCR). Cell Counting Kit-8 (CCK-8), EdU assay, colony formation, wound healing, Transwell assays and flow cytometry assays were conducted to examine the impact of miR-1179 on HCC cells. The protein expression of NUAK2 was detected using Western blotting assay. Bioinformatics analysis and luciferase reporter assays were conducted to reveal the association of miR-1179 with NUAK2. Results: MiR-1179 expression was significantly downregulated in HCC specimens and cell lines compared to normal samples and cells. The miR-1179 overexpression inhibited HCC cell migration, invasion and proliferation through targeting NUAK2. Overexpression of NUAK2 can reverse the effect of miR-1179 on hepatocellular carcinoma cells. Conclusion: miR-1179 suppresses HCC developmen through targeting NUAK2. Which can be used as a potent HCC diagnostic marker.

Keywords: microRNAs, hepatocellular carcinoma, miR-1179, NUAK2

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death worldwide [1]. The pathogenesis of HCC is complex, which is involved in a large number of genes and proteins [2, 3]. Aflatoxin, hepatitis B and C and alcohol are all risk factors for HCC [4]. Currently, HCC is mainly detected by blood indicators, imaging techniques and histopathology, but it's screening is far from satisfactory [5-7]. Though great advance has been achieved in the treatment of HCC, the recurrence rate of HCC patients within 5 years after operation remains high [8].

The aberrantly expressed microRNAs (miRNAs) have been a hot topic in a wide range of diseases in recent years. They are a type of small conserved noncoding RNA molecules with approximately 18-25 nucleotides [9]. MiRNAs have been validated to function as regulators by interacting with 3'-untranslated region (3'-UTR) of targeted mRNAs in different disorders, thereby altering the expression levels of targeted genes [10, 11]. Many researches have shown that miRNA is associated with a wide range of biological processes, including cell multiplication, apoptosis, separation, and metastasis [12-15]. Lots of investigations have affirmed that miRNAs work as oncogenic genes in diverse malignant tumors, including HCC [16, 17]. For instance, miR-301a-3p inhibits HCC progression through targeting VGLL4 [18]. MiR-766-3p inhibits the progression of HCC by suppressing Wnt3a [19]. MiR-218 inhibits HCC cell growth by suppressing the expression of the proto-oncogene Bmi-1 [20]. MiR-1179 has been reported to suppress the development of many cancers, such as pancreatic cancer, lung adenocarcinoma, gastric cancer, and papillary thyroid carcinoma [21-24]. MiR-1179 suppresses gastric cancer cell proliferation by targeting HMGB1 [25]. MiR-1179 inhibits cell growth of glioblastoma through directly targeting E2F transcription factor 5 [26]. Therefore, miR-1179 plays a major role in the development of can-

cers, but the details of its effects and mechanisms are still deficient.

NUAK2, as a member of the SNF1/AMPK kinase family, could promote cell division via regulating cell cycle [27, 28]. At present, a number of studies have demonstrated that the deregulation of NUAK2 leads to the occurrence and progression of different tumors [28-30]. In this study, bioinformatics analysis has confirmed that NUAK2 is a potential downstream target of miR-1179 in HCC, but the correlation between miR-1179 and NUAK2 remains unclear. Hence, this study investigate the relationship between miR-1179 and NUAK2 in HCC and the molecular mechanism.

Materials and methods

Sample collection

A total of 30 pairs of HCC tissues and adjacent normal tissues (> 5 cm from HCC tissues) were acquired from patients undergoing surgery who had been admitted to Jiangsu Cancer Hospital from March 2017 to December 2018.

Inclusion criteria: Patients were diagnosed as HCC for the first time; patients received no antitumor therapy, such as radiotherapy or chemotherapy; patients with complete comprehensive clinicopathological data.

Exclusion criteria: Patients with other malignant tumors or hematological diseases; HCC patients with distant tumor metastasis; Patients with severe mental illness; patients who had received preoperative radiotherapy or chemotherapy; patients with poor treatment compliance.

Patientsaged from 42 to 65 years old were included in the study. The Ethics Committee of Jiangsu Cancer Hospital approved this study (2017[025]). Writen informed consents were obtained from all patients. Tissues harvested from those patients were stored at -80°C before being used.

Cell culture

HepG2 (Human liver cancer cell line), Huh-7, MHCC97-H and SNU-475 (human hepatocellular carcinoma cell lines) and THLE-2 (human normal hepatocytes) were were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). MHCC97-H, a metastatic HCC cell line, was generated from the MHCC97 parental cell line. HCC cell line, SNU-475, was obtained from the tumor tissues of HCC patients. All of these cells were cultured at 37°C in the presence of 5% CO_2 in RPMI 1640 medium (Invitrogen, Carlsbad, USA) containing 1% penicillin/streptomycin and 10% FBS.

Cell transfection

For overexpression of miR-1179 mimic: 5'-AAGCAUUCUUUCAUUGGUUGG-3' and NC mimic: 5'-AAUUCGUAGCUUGCAUGCAAGC-3' were procured from Invitrogen (Carlsbad, USA). To knock down the expression of NUAK2, shRNA against NUAK2 (sh-NUAK2: 5'-GCAAGATCTGATGCACATACG-3') was utilized and non-specific RNA served as negative control (sh-NC: 5'-CACGTAGAGGCATGATGCCGT-3'). The NUAK2 (full-length coding domain) was inserted into pcDNA3.1 vectorto to construct pcDNA3/NUAK2 (named as NUAK2). Empty vector was utilized as negative control. Lipofectamine 2000 (ThermoFisher Scientific, Waltham, USA) was used for the transfection of all these vectors and reagents into cells. Forty-eight hours later, transfected cells were collected for subsequent use.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from HCC cells using the TRIzol® reagent (Invitrogen), according to the manufacturer's instructions. The QuantiTect Reverse Transcription Kit was used to convert RNA into cDNA (QIAGEN, Valencia, USA). NUAK2 expression was measured in an Applied Biosystems PCR 7900 (Thermo Fisher Scientific, Waltham, USA) using SYBR Premix ExTaqTM (Takara, Dalian, China) and β-actin as an internal control. Detection of miR-1179 level was performed with the TaqMan MicroRNA assays (Thermofisher Scientific) and U6 was taken as an endogenous control. $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels. The following primers were used in PCR assay: miR-1179: 5'-AGCATTCTTTCATTGGTTG-3' (F) and 5'-GAACATGTCTGCGTATCTC-3' (R); NUAK2: 5'-CTACCATCAAGGCAAGTTCCTGC-3' (F) and 5'-CCAGGATGTAGAGGAGAACACC-3' (R); β-actin: 5'-CCTCGCCTTTGCCGATCC-3' (F) and 5'-GGATCTTCATGAGGTAGTCAGTC-3' (R);

U6: 5'-CGCTTCGGCAGCACATATACTA-3' (F) and 5'-CGCTTCACGAATTTGCGTGTCA-3' (R).

Cell counting kit-8 (CCK-8) assay

Transfected cells were seeded at a density of 2×10^3 cells/well in 96-well plates and incubated for 0, 24, 48, or 72 hours. The CCK-8 solution (10 μL, Dojindo, Kumamoto, Japan) was then added to each well. After another 4-hour incubation at 37°C, the absorbance at 450 nm was measured.

Colony formation assay

The cells were digested by trypsin and seeded on a 6-well plate containing RPMI 1640 and 10% FBS at 1×10^3 cells/well. The media was replaced every two days. After two weeks, the cells were stained using crystal violet solution (1% in cold PBS). Seventy-five μm cell lines or 50 cells were considered that the cells were colonized.

5-Ethynyl-20-deoxyuridine (EdU) assay

To assess cell proliferation, an EdU assay kit (Sigma-Aldrich, St Louis, USA) was used. Transfected HCC cells were seeded and incubated in 96-well plates at a density of 5×10^3 cells per well for 2 hours before being treated with EdU solution (50 nmol/l). Images were captured using a fluorescence microscope after the nuclei were counterstained with DAPI.

Cell apoptosis assay

Following trypsinization and centrifugation, 1×10^6 transfected HCC cells were collected and treated with 500 μl buffering agent containing 5 μl Annexin V-FITC and 5 μl PI for 20 minutes at room temperature in a dark environment. Then, the rate of cell apoptosis was obtained from flow cytometry (Beckman Coulter, Inc., Brea, USA).

Wound healing assay

HCC cells were seeded into 6-well plates and grown to 90% confluence in 10% FBS medium. Then, the wound was scratched using a 10 μl micropipette tip. After cellular debris was washed with PBS, cells were incubated at 37°C. The wound size was monitored every day and images were captured at 0 and 48 h after scratching. Wound healing rate = [(wound area at 0 h-wound area at 48 h)/wound area at 0 h] ×100%.

Cell migration and invasion assays

Transwell chamber assay was used to detect cell migration and invasion. Cells were seeded into the upper chamber of transwell inserts with 8 μm pore size (Corning Costar, Cambridge, USA). The 1×10^5 cells in 200 μl medium without serum were added to the upper chamber, while the lower compartment was filled with 600 μl complete medium containing 10% FBS. The cells were then incubated for 24 hours. After that, the cells on the lower surface of the membrane were fixed with 4% polyformaldehyde for 10 min and stained with Giemsa. For cell invasion assay, the lower transwell chambers were covered with matrigel (BD Biosciences, San Jose, USA). HCC cells were treated in the same manner as cell migration assay. Cells were imaged and counted in at least five random fields under a digital microscope.

Western blot analysis

Proteins were isolated from HCC cells with the use of M-Per Mammalian Protein Extraction Reagent (Pierce, Rockford, USA), which were then loaded onto a 10% SDS-PAGE and electrophoretically transferred to a PVDF membrane. After that, the membranes were sealed in 5% fat-free milk for an hour, then probed by primary antibodies overnight at 4°C, hatched with the HPR-conjugated secondary antibodies (1:1000) for an hour and visualized by an ECL detection kit (Pierce Chemical, Rockford, USA). The primary antibodies including anti-Bcl-2, anti-Bax, anti-Cleaved-caspase 3, anti-Cleaved-caspase 9 and anti-β-actin (all these antibodies were bought form Abcam and diluted at 1:1000); anti-Cox-2, anti-MMP2, anti-MMP9 and anti-NUAK2 (all these antibodies were bought form Cell Signaling Technology and diluted at 1:2000) were used. β-actin was taken as an internal reference.

Luciferase reporter assay

NUAK2-WT or NUAK2-Mut was created by synthesizing wild-type or mutant NUAK2 fragments containing miR-1179 binding sites and inserting them into the pGL3-basic plasmid (Promega, Madison, USA). HCC cells were co-

transfected with the aforementioned reporter vectors and either a negative control NC mimic or a miR-1179 mimic. At 48 hours after transfection, the luciferase activity was detected by Dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical analysis

Each experiment was repeated at least three times, and all data were expressed as mean ± standard deviation (SD). For the comparison between two groups, the Student's t-test was used, while one-way ANOVA followed by Tukey test was employed to analyze differences among multiple groups. Survival analysis was performed using univariate and multivariate Cox regression hazard analysis and survival curves derived from Kaplan-Meier survival analysis. The statistical analysis described above was carried out using GraphPad Prism 6.0. (GraphPad, San Diego, USA). The difference was significant at $P<0.05$ level.

Results

The miR-1179 expression was reduced in HCC

The miR-1179 expression level in HCC was determined using RT-qPCR. The results showed that miR-1179 was reduced in HCC samples (**Figure 1A**). Similarly, we found that miR-1179 expression was clearly downregulated in Huh-7, MHCC97-H, SNU-475 and HepG2 compared to THLE-2 cells (**Figure 1B**). Thus, miR-1179 was overexpressed to conduct gain-of-function assays to explore the function of miR-1179 in HCC. Given that Huh-7 and HepG2 presented the lower expression level of miR-1179, these cells were adopted in subsequent experiments. Furthermore, survival analysis revealed that patients with low miR-1179 expression had significantly shorter survival times than those with high miR-1179 expression ($P=0.0210$). GFP and RT-qPCR assays delineated that transfection with miR-1179 mimic prominently elevated the level of miR-1179 (**Figure 1D**, **1E**). Collectively, these results revealed that miR-1179 level significantly descended in HCC tissues and cells.

MiR-1179 overexpression inhibited cell proliferation and induced apoptosis in HCC cells

The CCK-8 assay revealed that miR-1179 overexpression reduced the viability of HCC cells (**Figure 2A**). Likewise, colony formation assay showed that the overexpression of miR-1179 contributed to the decreased colony numbers (**Figure 2B**). Besides, the inhibitory role of miR-1179 in cell proliferation was further confirmed by EdU assay (**Figure 2C**). Flow cytometry analysis also indicated that miR-1179 mimic induced the apoptosis of HCC cells (**Figure 2D**). Moreover, the results revealed that the upregulation of miR-1179 led to reduction of Bcl-2 proteins, whereas the Bax, Cleaved-caspase 3 and Cleaved-caspase 9 expression levels increased (**Figure 2E**). Therefore, we drew a conclusion that miR-1179 retarded the development of HCC by inhibiting cell proliferation and inducing cell apoptosis.

HCC cell migration and invasion were hampered by increased miR-1179 expression

Based on the results above, we investigated the role of miR-1179 in cell migration and invasion. It indicated that the healing rate of wounds was reduced due to overexpression of miR-1179 (**Figure 3A**). the results of transwell chamber assays demonstrated that the migration and invasion abilities of HCC cells were restricted by a miR-1179 mimic, which was in line with the results mentioned above (**Figure 3B**). Furthermore, enhanced expression of miR-1179 decreased the levels of proteins including Cox-2, MMP2 and MMP9 (**Figure 3C**). Based on these results, we concluded that miR-1179 suppressed the migration and invasion of HCC cells.

NUAK2 was proved to be a downstream target of miR-1179

Bioinformatics analysis was used to elucidate the molecular mechanism underlying miR-1179. By using the Targerscan, we discovered that NUAK2 harbored the potential miR-1179 bind sites (**Figure 4A**). It is worth noting that we chose NUAK2 for in-depth study because of its essential role in the development of liver cancer [27]. NUAK2 was found to be expressed at various levels in HCC tissues and cells, according to RT-qPCR results. Data in **Figure 4B** and **4C** showed that NUAK2 was involved in HCC cell proliferation. This result was supported by the findings in Figure S1. Besides, the relationship between NUAK2 and miR-1179 was evaluated through luciferase reporter assay. The results revealed that miR-1179

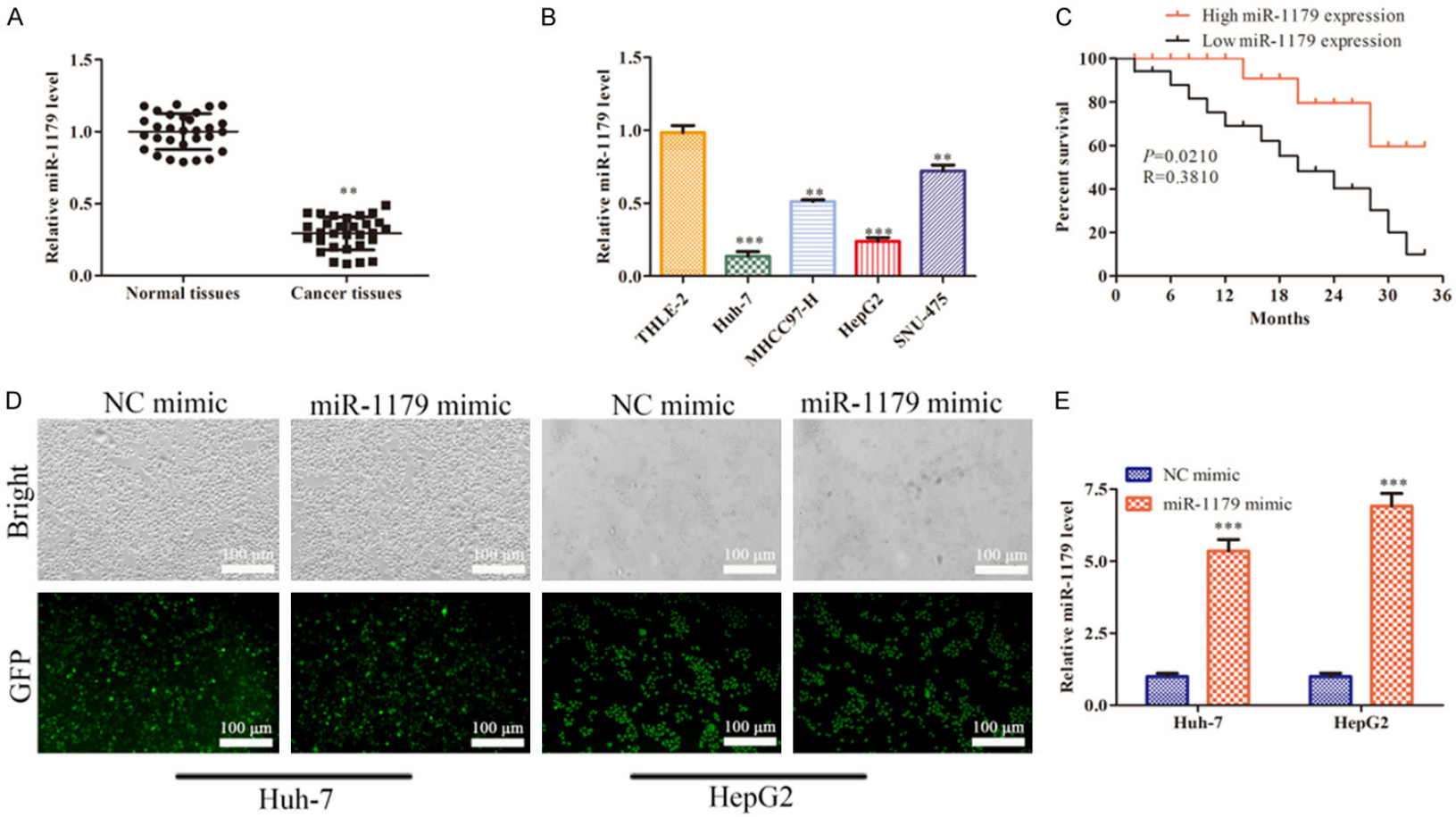
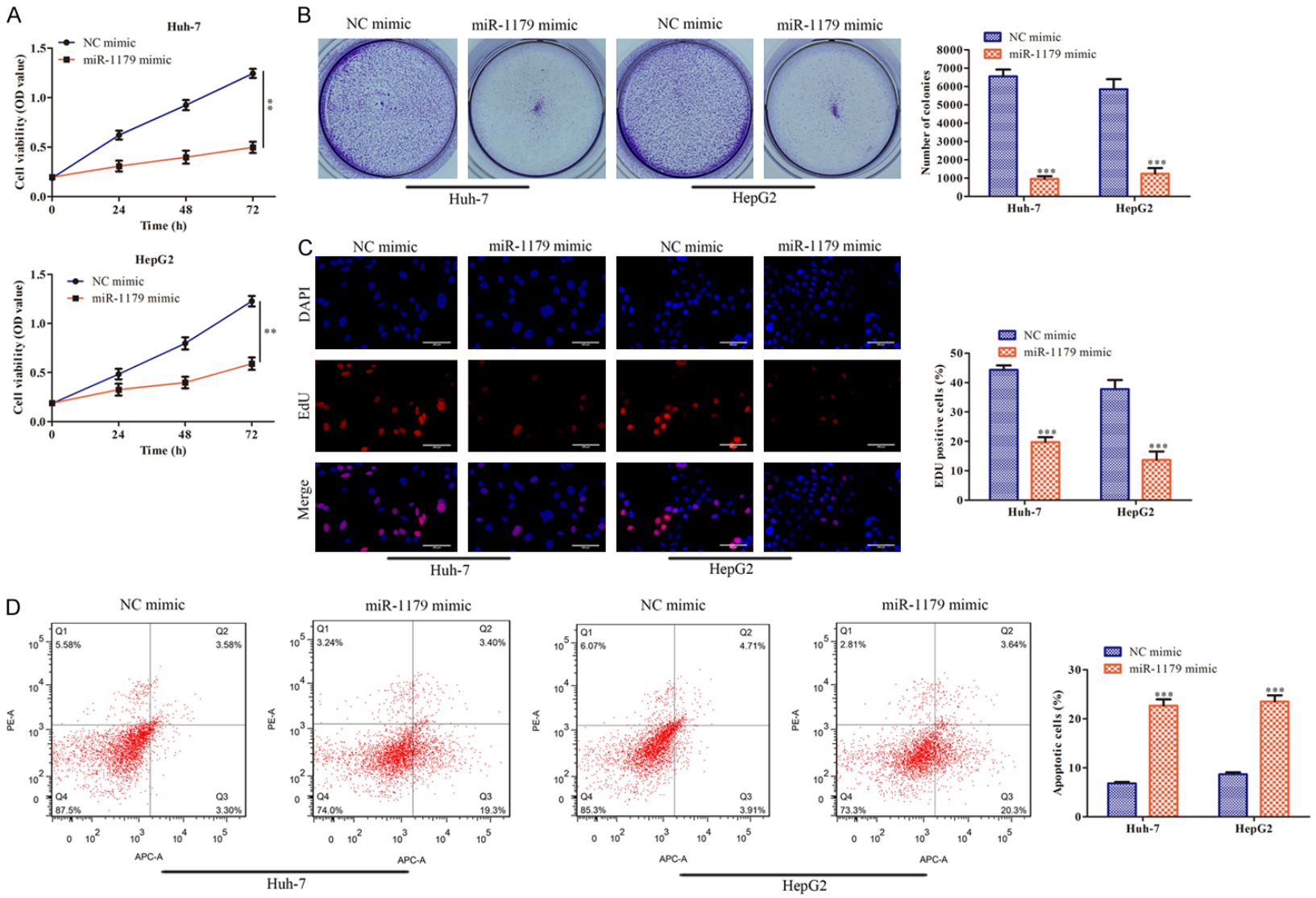


Figure 1. MiR-1179 expression was decreased in HCC. A: RT-qPCR analysis of miR-1179 expression level in 30 pairs of tumor tissues and matched non-cancer samples of HCC patients; B: RT-qPCR was carried out to determine the expression level of miR-1179 in HCC cells (Huh-7, MHCC97-H and SNU-475) and human liver cancer cell line (HepG2), as well as in human normal hepatocytes THLE-2; data were normalized to THLE-2; C: Kaplan-Meier curves for survival time in patients with HCC patients divided according to miR-1179 expression: a significantly shorter survival times for those with low miR-1179 expression than for those with high miR-1179 expression (P=0.0210). D: GFP assay was used to detect the transfection efficiency of miR-1179 overexpression, scale bars, 100×; E: RT-qPCR analysis was utilized to certify the transfection efficiency of miR-1179 overexpression; data were normalized to mimics NC. All results were expressed as mean ± SD, and each experiment was performed three times. Compared with control group, **P<0.01, ***P<0.001. HCC: Hepatocellular carcinoma; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.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Huh-7
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miR-1179 mimic
Cell viability (OD value)
Time (h)
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B
NC mimic
miR-1179 mimic
Huh-7
HepG2
Number of colonies
C
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Merge
EDU positive cells (%)
D
Q1
Q2
Q3
Q4
PE-A
APC-A
Apoptotic cells (%)

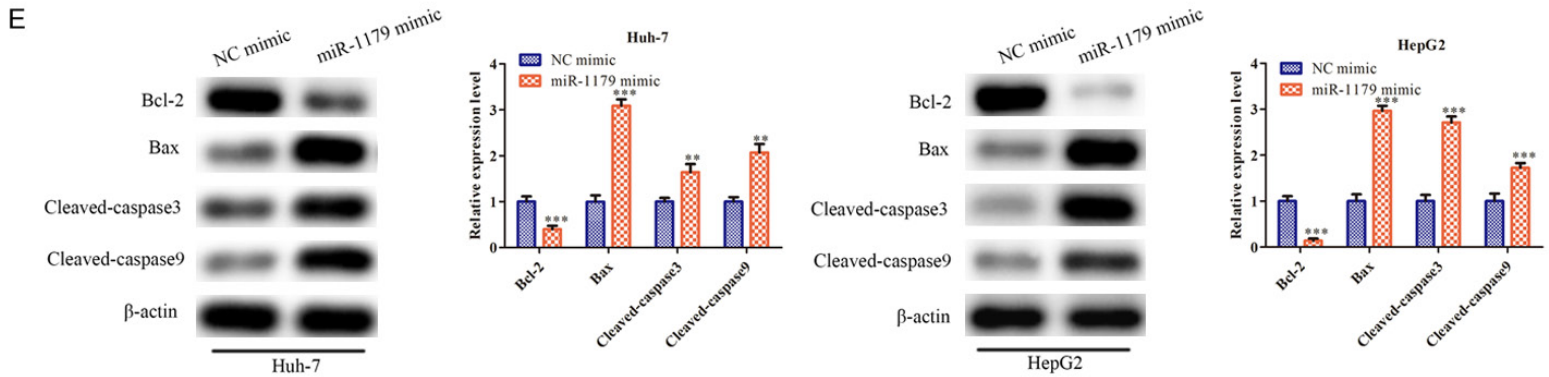


Figure 2. Overexpression of miR-1179 suppressed cell proliferation and induced the apoptosis of HCC cells. A: Detection results of CCK-8 assay at 0, 24, 48 and 72 h after incubation; B, C: Cell proliferation determined by Colony formation and EdU assays, scale bars, 100×; D: Cell apoptosis determined by Flow cytometry analysis; E: Western blot analysis of the expression levels of Bcl-2, Bax, Cleaved-caspase 3 and Cleaved-caspase 9. All results were expressed as mean ± SD and each experiment was performed three times. Compared with NC mimic group, $^{**}P<0.01$, $^{***}P<0.001$. EdU: 5-Ethynyl-20-deoxyuridine; HCC: Hepatocellular carcinoma.

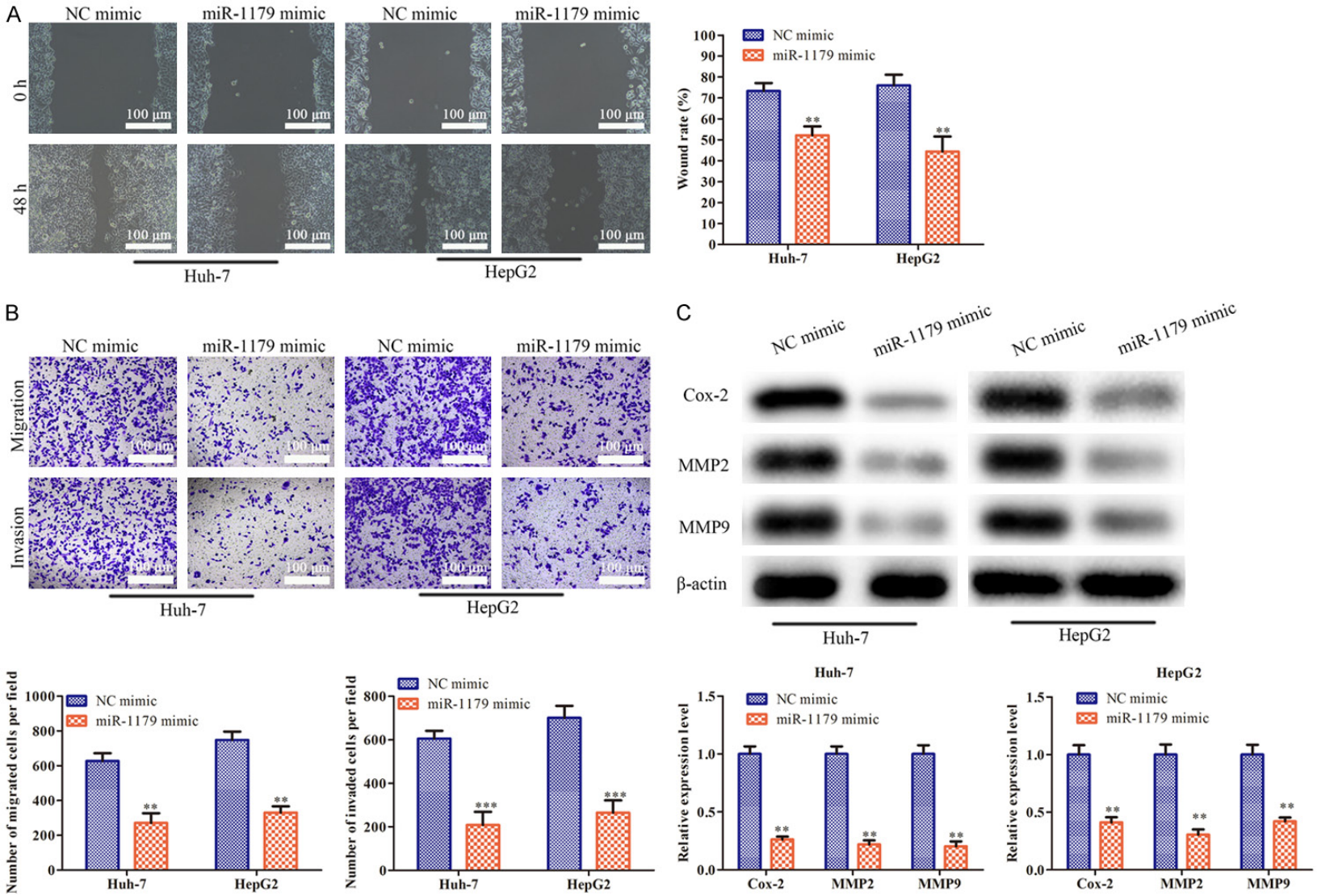


Figure 3. Overexpression of miR-1179 impeded the migration and invasion of HCC cells. A: Wound healing assay was adopted for evaluation of cell migration capacity, scale bars, 100×; B: Cell migration and invasion assessed by transwell chamber assays, scale bars, 100×; C: Western blot analysis of Cox-2, MMP2 and MMP9 levels; data are normalized to mimics NC. All results were expressed as mean ± SD and each experiment was performed three times. Compared with NC mimic group, $^{**}P<0.01$, $^{***}P<0.001$. HCC: Hepatocellular carcinoma.

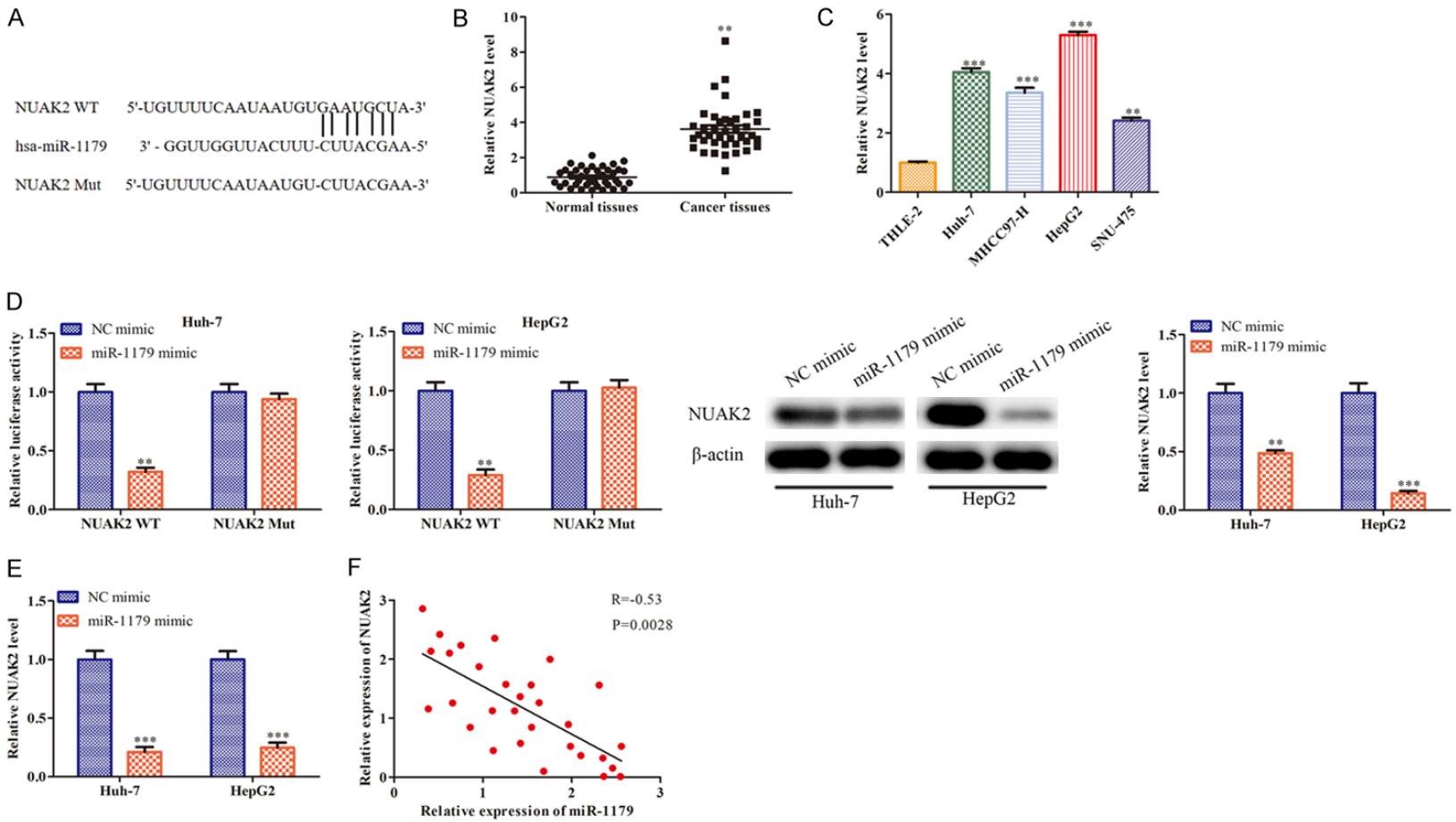


Figure 4. NUAK2 was proved to be a downstream target of miR-1179. A: The putative miR-1179 binding sites for NUAK2; B: RT-qPCR analysis of NUAK2 expression level in 30 pairs of tumor tissues and matched non-cancer samples of HCC patients; C: RT-qPCR was carried out to determine the expression level of NUAK2 in HCC cells (Huh-7, MHCC97-H and SNU-475) and human liver cancer cell line (HepG2), as well as in human normal hepatocytes THLE-2; D: Luciferase reporter assay was applied to assess the relationship between miR-1179 and NUAK2; E: RT-qPCR and western blot analyses of the mRNA and protein levels of NUAK2; F: Person correlation analysis was conducted to assess the association between miR-1179 and NUAK2 expression in tumor samples (n=30). All results were expressed as mean ± SD and each experiment was performed three times. Compared with control group, $^{**}P<0.01$, $^{***}P<0.001$. RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.

mimic indeed impaired the luciferase activity of NUAK2 3'-UTR, whereas the mutant form of NUAK2 3'-UTR promoter had no response to miR-1179 mimic, which further validated that miR-1179 directly bound to NUAK2 (**Figure 4D**). RT-qPCR and western blot analyses revealed that miR-1179 overexpression reduced NUAK2 expression at both the transcriptional and translational levels (**Figure 4E**). Furthermore, we discovered an inverse relationship between NUAK2 expression and miR-1179 expression in clinical tumor tissues (**Figure 4F**). In summary, our study showed that miR-1179 negatively modulated NUAK2 expression via directly binding to NUAK2 3'UTR.

Silencing of NUAK2 restrained the malignant behaviors of HCC cells

We used loss-of-function assays to further validate the role of NUAK2 in HCC. When Huh-7 and HepG2 cells were transfected with sh-NUAK2, RT-qPCR analysis confirmed that NUAK2 expression was reduced (**Figure 5A**). CCK-8 and EdU assays suggested that knockdown of NUAK2 suppressed the proliferative capacity of Huh-7 and HepG2 cells (**Figure 5B** and **5C**). We also found out that inhibition of NUAK2 markedly induced cell apoptosis (**Figure 5D**), which was consistent with the results above. Furthermore, transwell assays indicated that the knockdown of NUAK2 overtly repressed cell migration and invasion in HCC (**Figure 5E**). In a word, NUAK2 played an oncogenic role in HCC development.

MiR-1179 repressed the progression of HCC by targeting NUAK2

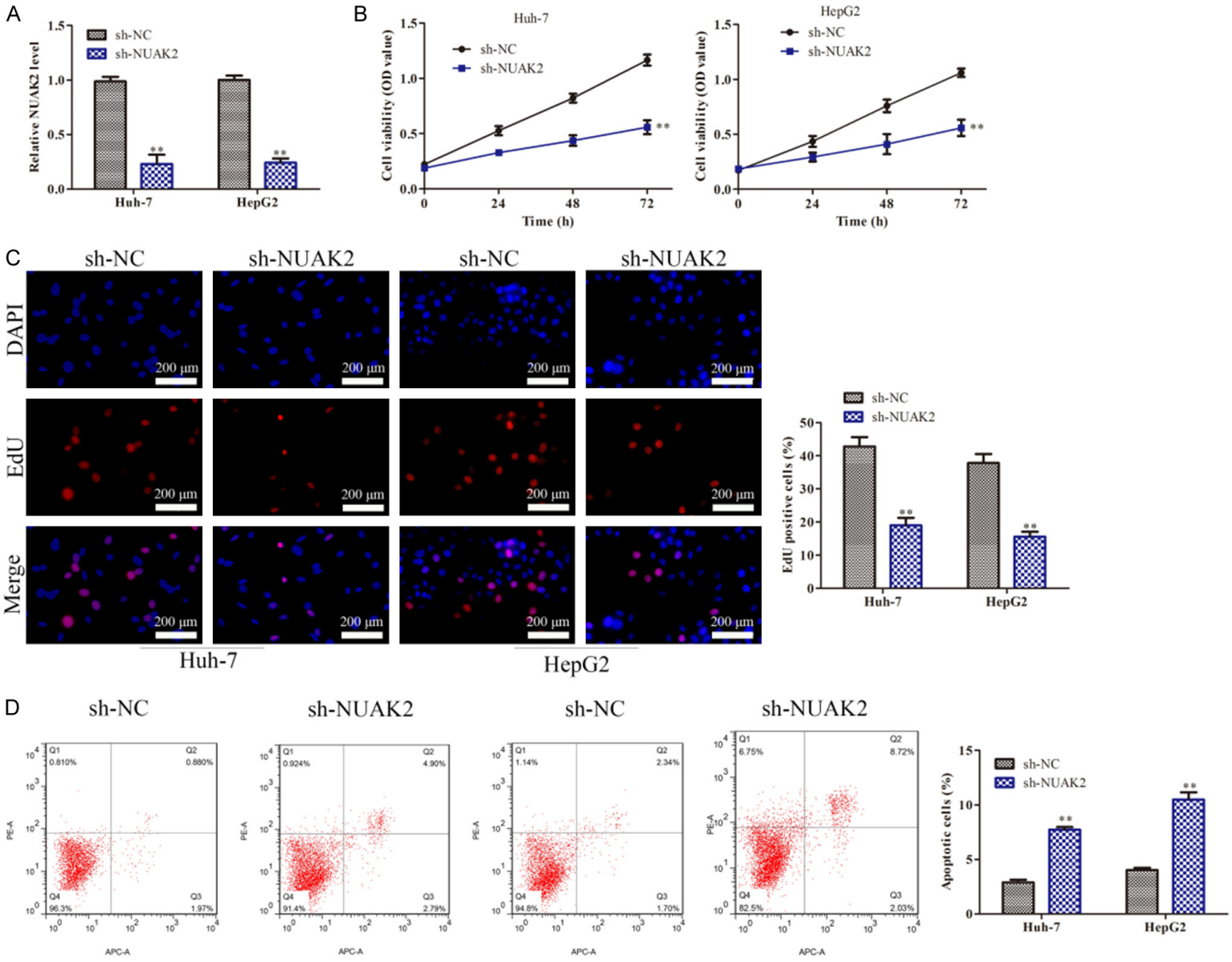
We overexpressed the NUAK2 in presence of miR-1179 mimics in HCC cells, as to confirm that the tumor silencer function of miR-1179 was achieved by regulating NUAK2. The efficiency of NUAK2 overexpression was verified by RT-qPCR (**Figure 6A**). The CCK-8 and EdU assays revealed that overexpression of NUAK2 promoted the proliferation of HCC cells that suppressed by miR-1179 mimic (**Figure 6B** and **6C**). Moreover, Flow cytometry assay revealed that the increased apoptosis rate of HCC cells caused by miR-1179 mimics was reversed when NUAK2 was overexpressed (**Figure 6D**). Transwell chamber assays showed that NUAK2 overexpression counteracted the inhibitory impacts of miR-1179 overexpression on cell migration and invasion in HCC (**Figure 6E**), which conformed to the results mentioned above. Therefore, it was concluded that overexpression of NUAK2 promoted proliferation, metastasis and inhibited HCC cell apoptosis, and MiR-1179, as a tumor silencer, played an important role in HCC via targeting NUAK2.

Discussion

HCC remains one of the most prevalent malignancies with severe mortality worldwide [31]. Elucidating the pathogenesis of HCC is indispensable to develop the treatment strategy for patients with HCC. In this study, we discovered that miR-1179 expression was significantly downregulated in clinical HCC samples and cell lines. Furthermore, the inhibitory function of miR-1179 in the malignant behaviors of HCC cells was confirmed. miR-1179 acted as a tumor suppressor in HCC progression by targeting NUAK2.

A number of researches have confirmed that miRNAs play a role in the onset and development of various cancers, including HCC [32-34]. Explorations of miRNAs have recently revealed promising potential clinical biomarkers and therapeutic targets for the treatment of HCC [35, 36]. Multiple evidences have shown that miR-1179 negatively regulates the development of various malignant tumora. For example, miR-1179 targets HMGB1 to repress gastric cancer cell proliferation [25]. The overexpression of MiR-1179 restrains cell metastasis in breast cancer via modulating Notch signaling pathway. Meanwhile, it is associated with favorable prognosis [37]. MiR-1179 inhibits NSCLC progression by regulating sperm-associated antigen 5-mediated Akt signaling [38]. However, the potential of miR-1179 in the occurrence and development of HCC is still unclear. In the current study, we aimed to further characterize its function in the progression of HCC. We discovered that miR-1179 expression was reduced in HCC specimens and cells. Furthermore, gain-of-function tests indicated that miR-1179 overexpression repressed HCC cell proliferation, migration and invasion, while promoted cell apoptosis.

NUAK2, as a member of the SNF1/AMPK kinase family, is located at 1q32, and is controlled by tumor suppressor liver kinase B1 (LKB1) and death receptor signaling via nuclear factor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Huh-7
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Cell viability (OD value)
Time (h)
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sh-NC
sh-NUAK2
DAPI
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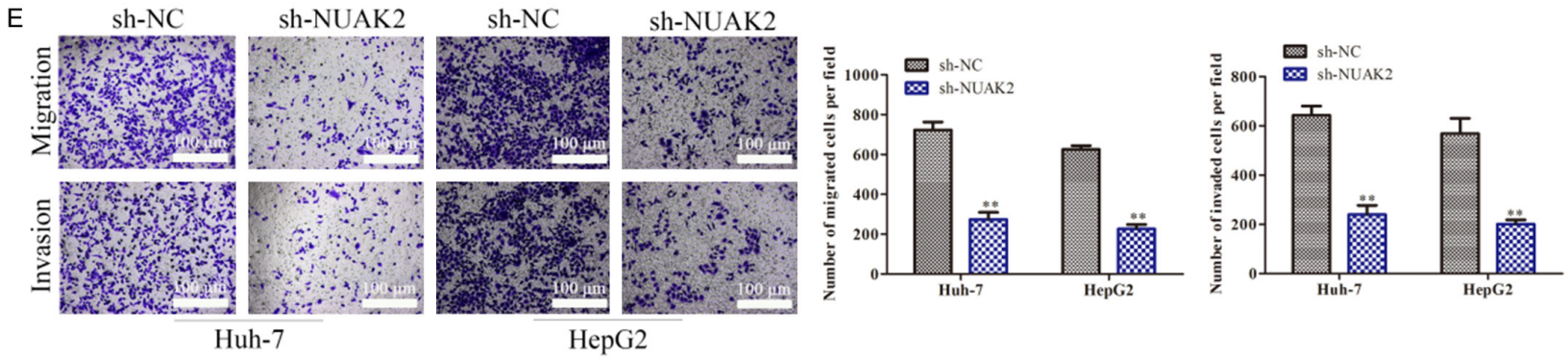
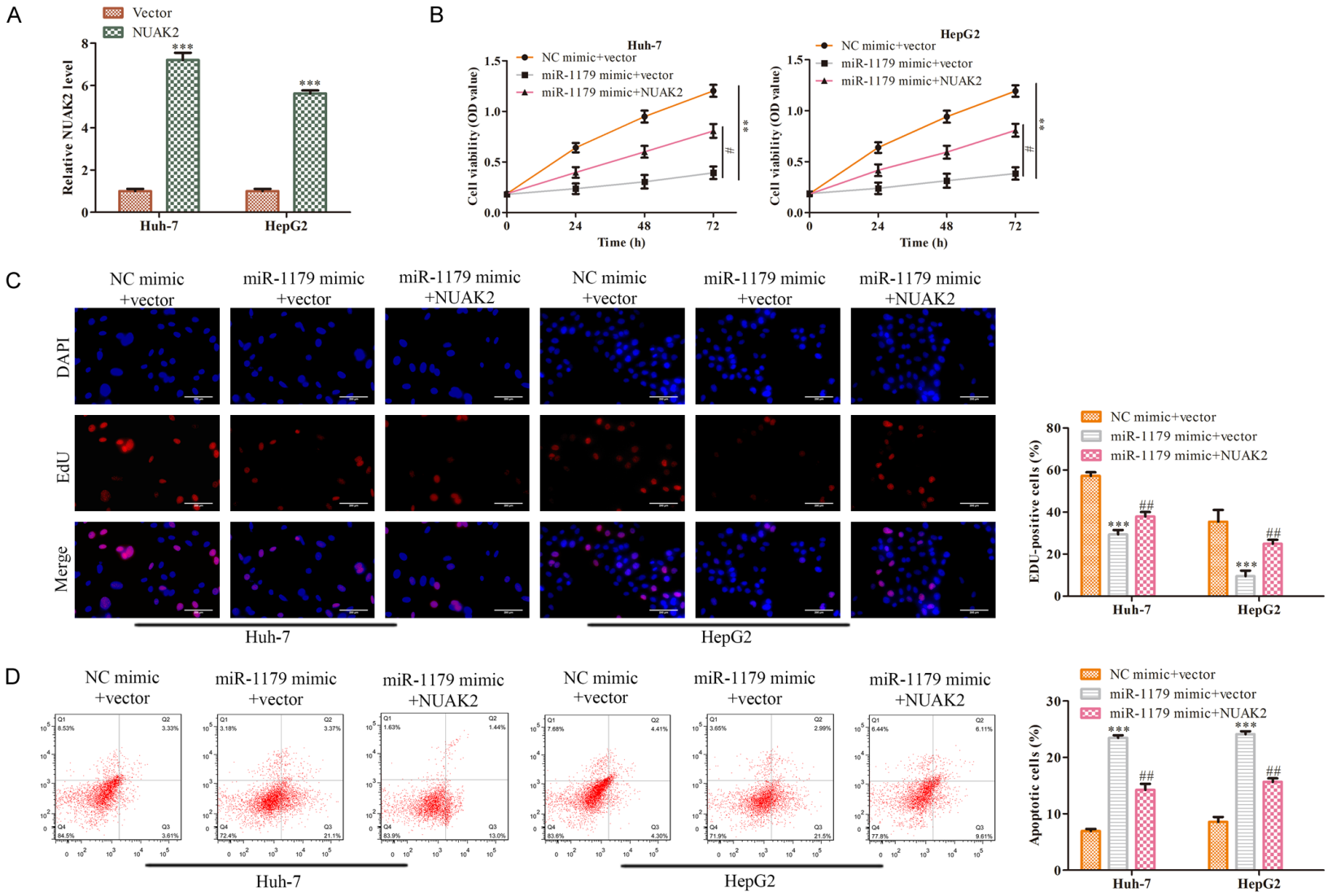


Figure 5. Silencing of NUAK2 restrained the malignant behaviors of HCC cells. A: The efficiency of NUAK2 knockdown was verified by RT-qPCR assay; B: Detection results of CCK-8 assay at 0, 24, 48 and 72 h after incubation; C: EdU assays, scale bars, 400×; D: Flow cytometry analysis; E: Transwell assays were implemented to determine the effects of NUAK2 knockdown on cell migration and invasion, scale bars, 100×. All results were expressed as mean ± SD and each experiment was performed three times. Compared with sh-NC group, $^{**}P<0.01$. HCC: Hepatocellular carcinoma; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.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Relative NUAK2 level
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miR-1179 mimic+vector
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Cell viability (OD value)
Time (h)
HepG2
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Merge
EDU-positive cells (%)
D
Apoptotic cells (%)

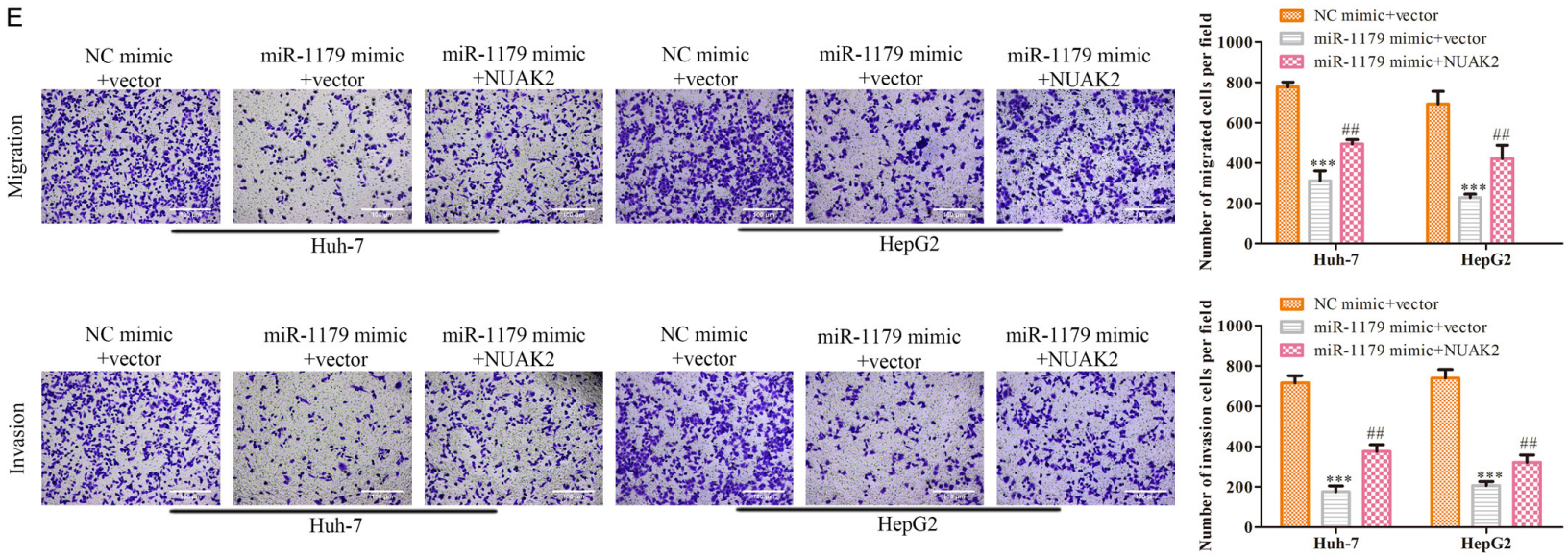

Figure 6. MiR-1179 repressed the progression of HCC by targeting NUAK2. A: NUAK2 expression level detected by RT-qPCR; B: CCK-8 results assay at 0, 24, 48 and 72 h after incubation; C: EdU assay, scale bars, 400×; D: Flow cytometry analysis; E: Transwell chamber assays, scale bars, 100×. All results were expressed as mean ± SD and each experiment was performed three times. Compared with vector group, $^{***}P<0.001$. Compared with NC mimic + vector group, $^{**}P<0.01$. Compared with miR-1285-3p mimic + vector group, $^{\#}P<0.05$, $^{\#\#}P<0.01$. HCC: Hepatocellular carcinoma; EdU: 5-Ethynyl-20-deoxyuridine; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.

(NF)-κB [39-41]. NUAK2 has been proven to play an oncogenic role in gastric cancer, melanoma, glioblastoma and liver cancer [27, 42-44]. We discovered the miR-1179 binding sites in 3'UTR of NUAK2 via bioinformatics analysis. Hence, we speculated that NUAK2 was a target of miR-1179. Our findings demonstrated that NUAK2 directly bound to miR-1179 and was negatively regulated by miR-1179. Subsequently, we confirmed that NUAK2 contributed to the malignant phenotypes of HCC cells. Moreover, enhanced expression of NUAK2 abrogated the influences of miR-1179 on HCC progression. We also found that although NUAK2 overexpression alone modestly affected the progression of HCC cells, such an overexpression specifically recuperated the miR-1179 activating or inhibitory effects on the HCC cells progression, indicating NUAK2 was a bone fide downstream target of miR-1179. In addition, previous study indicated that NUAK2 modulated matrix gene responses to TGF-β signaling [45].

COX-2, MMP2 and MMP9 are widely used as biomarkers of EMT. MMP2 and MMP9 are two major members of MMPs, which play an essential role in cancer metastasis and are related to epithelial-mesenchymal transformation (EMT) and angiogenesis [46]. COX-2 can activate the expression of MMP protein, thus promoting the progress of EMT [47]. By detecting these proteins, we found that miR-1179 can inhibit the EMT process of HCC through targeting NUAK2, thus inhibiting the malignant degree of the tumor.

In this report, we aimed to clarify the function of miR-1179 and its regulatory mechanism in HCC. Our findings demonstrated that that miR-1179, acting as a tumor suppressor, played a key role in the deterioration of HCC by inhibiting NUAK2 expression and could serve as a useful target for the treatment of HCC.

Disclosure of conflict of interest

None.

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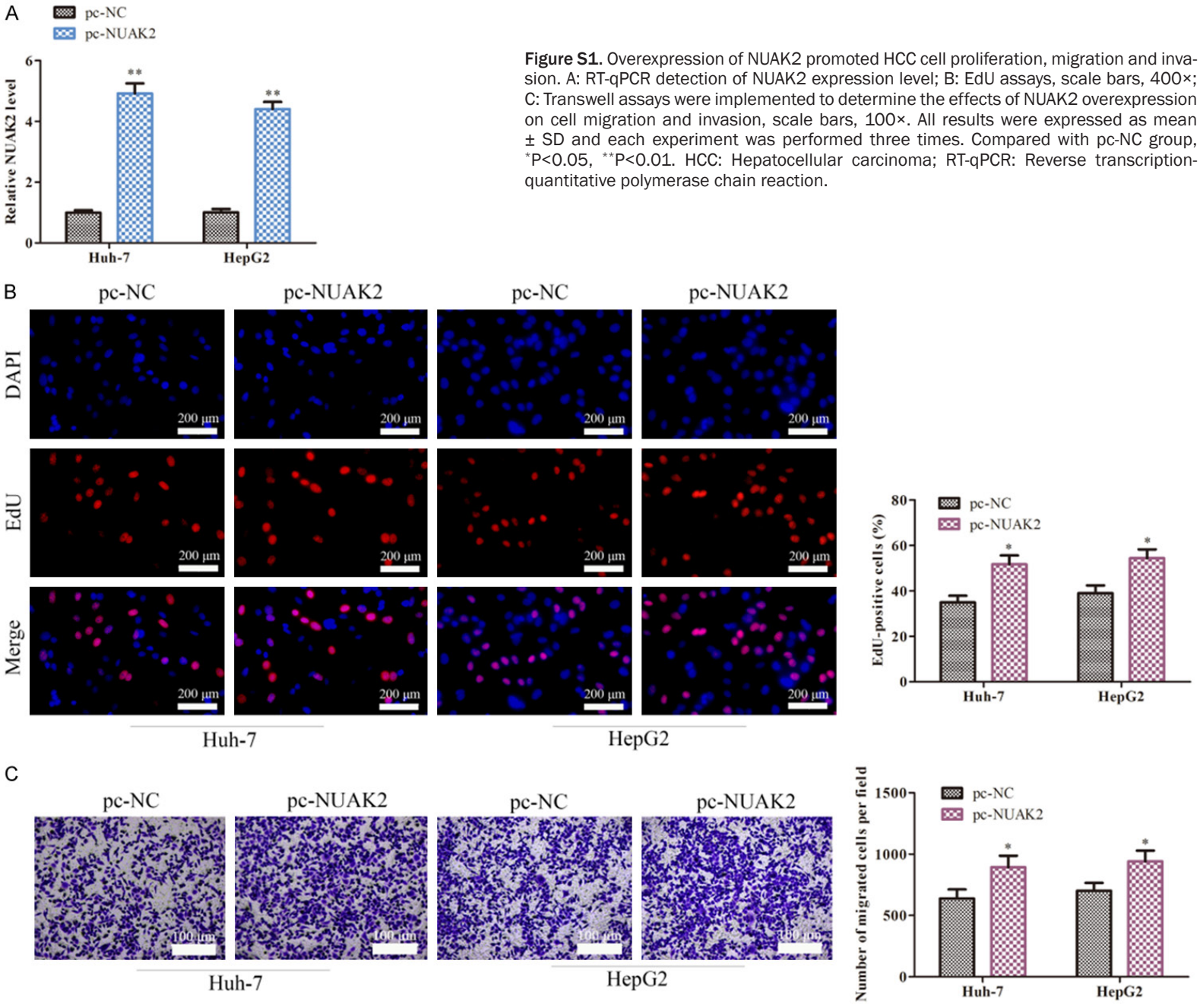

Figure S1. Overexpression of NUAK2 promoted HCC cell proliferation, migration and invasion. A: RT-qPCR detection of NUAK2 expression level; B: EdU assays, scale bars, 400×; C: Transwell assays were implemented to determine the effects of NUAK2 overexpression on cell migration and invasion, scale bars, 100×. All results were expressed as mean ± SD and each experiment was performed three times. Compared with pc-NC group, $^{*}P<0.05$, $^{**}P<0.01$. HCC: Hepatocellular carcinoma; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.