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Original Article

MiR-767-3p promotes the progression of hepatocellular carcinoma via targeting CASP-3/-9

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Abstract: Objective: The aim of this study was to investigate the relationship between miR-767-3p and hepatocellular carcinoma (HCC). Method: We examined the expression of miR-767-3p in both HCC tissues and HCC cell lines by qRT-PCR and western blot assay. We also investigated the influence of miR-767-3p on HCC by transfecting HCC cells with either miR-767-3p mimics or inhibitors. Result: MiR-767-3p expression was increased in HCCs and cell lines. Functional analyses demonstrated that miR-767-3p increased HCC cell proliferation and prevented apoptosis both in vitro and in vivo, whereas miR-767-3p inhibition had the opposite effect. Caspase-3 and caspase-9 were found to be direct targets of miR-767-3p in HCC cell lines, and overexpression of miR-767-3p suppressed caspase-3/-9 production. Caspase-3 and caspase-9 siRNA knockdown revealed similar promoting of cell proliferation and inhibiting of cell apoptosis produced by miR-767-3p overexpression, whereas caspase-3/-9 siRNAs inhibited miR-767-3p knockdown-induced inhibition of cell proliferation and promotion of cell apoptosis. Conclusion: MiR-767-3p promoted proliferation and prevented apoptosis in human hepatocellular carcinoma (HCC) through inhibiting the caspase-3/caspase-9 pathway.

Keywords: miR-767-3p, hepatocellular carcinoma, casepase

Introduction

Despite the use of a variety of therapeutic techniques such as surgical resection, chemotherapy, targeted therapy, and liver transplantation, hepatocellular carcinoma (HCC) remains one of the major causes of tumor-related fatalities. HCC is the most common primary liver cancer, accounting for approximately 75%-85% of all primary liver cancers. Its early symptoms are not noticeable, the diagnostic rate is low, and most patients missed the best time for surgical resection when they are diagnosed. As the importance of molecular targeted therapy in the non-surgical treatment of HCC is more recognized, the treatment effect has increased, but patient's survival remains dismal. Every year, more than one million people are affected by HCCs globally [1-3], leading to approximately 700,000 deaths annually [4-6]. The reasons are due to unresectable tumors because of advanced stages at diagnosis, chemotherapy resistance, frequent recurrence, and tumor metastasis [5, 6]. Recent studies have reported that multiple genes and RNAs are involved in HCC development, especially those involved in cell growth and apoptosis.

miRNA, a small non-coding RNA with a length of about 22nt, is commonly found in eukaryotes and has been called the "dark matter" in living organisms for a long time. Most miRNA genes are transcribed into primary transcripts, pre-miRNA, in the nucleus by the action of RNA polymerase II; the latter becomes precursor miRNA, pre-miRNA, by the action of Drosha enzyme, and is further transported to the cytoplasm by binding to the export protein; under the action of Dicer enzyme and The mature miRNA, with a light group at the 3' end and a peptide group at the 5' end, is highly conserved in terms of structure and function evolution, and is expressed only in specific tissue cells or at specific stages of cells, with tissue specificity and temporal sequence. Functionally, miRNAs, as regulatory molecules, can specifically inhibit

gene expression by base pairing with the 3' untranslated region of target genes, and achieve post-transcriptional silencing of genes by blocking or degrading their translation. Although miRNAs represent a small percentage of non-coding RNAs, more than 60% of transcripts are regulated by miRNAs. Numerous studies have shown that miRNAs are involved in a series of important life processes such as embryonic development, organ formation and differentiation, hematopoietic processes, cell proliferation and apoptosis. In tumors, most miRNAs are lowly expressed and act as oncogenes; some tumors have upregulated miRNA expression, which promote tumorigenesis and development by inducing oncogenes or downregulating the expression of oncogenes, [7, 8]. By silencing various target mRNAs, miRNAs are involved in almost all cellular activities, including developmental timing, proliferation, differentiation, apoptosis, organ development, tumorigenesis [9, 10]. In recent years, microRNAs have been shown to play a role in the development and progression of a variety of human cancers, including hepatocellular carcinoma (HCC). Accumulating data has suggested that microRNAs can be prognostic biomarkers or even therapeutic targets for HCC, and numerous miRNA-targeted innovative therapeutic methods have been reported to have achieved promising outcomes [8].

It has been demonstrated that the cancer-related microRNA known as miR-767-3p has either an oncogenic or a tumor suppressive role in a variety of human malignancies. miR-767-3p is involved in the progression and carcinogenesis of gastric cancer; miR-767-3p regulates growth and migration of lung adenocarcinoma cells by regulating CLDN18; miR-767-3p inhibited the expression of ASF1B, which had an effect on the progression of melanoma; and miR-767-3p inhibited the expression of ASF1B [11-14]. On the other hand, it is not yet known whether or not miR-767-3p plays a part in the progression of HCC. To investigate the potential role of miR-767-3p in HCC and to hypothesize about how it might work, we performed experiments on cell survival and proliferation, as well as colony formation, flow cytometry, and gene overexpression and knockdown assays. In addition, the results of an in vivo xenograft tumor growth model demonstrated that miR-767-3p inhibited the caspase-3/caspase-9 pathway, which resulted in increased HCC proliferation.

Materials and methods

Clinical samples and cell lines

A total of 60 patients with liver cancer had an average age of 45±7.55, including 32 males and 28 females. Patients who are getting their primary therapy at the hospital's hepatobiliary surgery department, where they are having radical hepatocellular carcinoma resection. Patients were subjected to stringent screening, and the criteria for inclusion were as follows: 1. Age between 25 and 70 years old; 2. Primary patients who had not received any prior treatment, such as radiotherapy, transarterial chemoembolization (TACE), radiofrequency or microwave ablation, immunotherapy, targeted therapy, etc.; 3. Specimens were pathologically confirmed as having hepatocellular carcinoma. Patients with other systemic malignancies, autoimmune diseases, hematological diseases, organ failure, etc. Patients treated with warfarin, vitamin K, or vitamin K antagonists during the study period; Incomplete data and withdrawal from the study. A backup collection of specimens has been kept in liquid nitrogen after they were gathered. Each patient signed a paper indicating their understanding and agreement to the specimen collection and usage for the purposes of the study. The Ethical Committees of the Hospital of Chengdu University of Traditional Chinese Medicine in Chengdu, China, gave their stamp of approval to each and every method (No: 2020DL-007). ATCC in Washington, District of Columbia, United States, supplied the human HCC cell lines PLC/PRF/5, Hep3B, and HepG2, as well as normal human liver cells. The Chinese Academy of Sciences supplied the human HCC cell lines SMMC7721, Bel7402, and Huh7, as well as normal human liver cells HL7702; both sets of cells were used in the study (Shanghai, China). Following the instructions provided by the manufacturer, the cells were grown in medium (RPMI-1640 or -MEM) from Gibco (USA), which contained 10% fetal bovine serum (FBS, Gibco) and 1% plating supplement. All of the cells were grown in an environment that was humidified at 37 degrees Celsius and contained 5% carbon dioxide.

RNA preparation and real-time quantitative PCR (RT-qPCR)

Following the instructions provided by the manufacturer of the mir-Vana miRNA Isolation Kit

Table 1. The sequences of the primers

Gene	Forward (5'-3')	Reverse (5'-3')
CASP3	ATGCTGTGTAACACAAAC	CTGCAACGTGCAACGTCA
miR-767-3p	ATCGTGGCCAGTGCTGAACGT	TGCCAACGTGCCAAACACGTGC
CASP9	TGCGTGCCATGCAACGTGGAA	TGCCACTGTGTGCAACACACA
U6	CATGTGCAACAGTAATATAT	TACACAGCAACGTGCAAA
GAPDH	TTGCAATGCAACTTCAGATG	TGCATGATATACGTGATTG

(Ambion, USA), RNAs were isolated from clinical tissue or cell lines by utilizing the kit. cDNA was produced using Primescript RT Reagent (TaKaRa, Shiga, Japan), and then real-time quantitative PCR (RT-qPCR) was performed using a SYBR Premix Kit (TaKaRa) on an iQ5 RT-PCR system (Bio-Rad) in accordance with the instructions provided by the manufacturer. The endogenous control for the calculation of relative miRNA expression was found to be U6 snRNA, whereas the relative mRNA expression was standardized against GAPDH. The relative fold-change of miRNAs and mRNAs was quantified using the $2^{-\Delta\Delta Ct}$ method. The primer sequence is shown in **Table 1**.

Plasmid transfection and generation of stable cell lines

We cloned the human pre-miR-767-3p gene into the retroviral transfer plasmid pMSCV-puro (RiboBio Corporation, Guangzhou, China), which resulted in the production of the plasmid pMSCV-miR-767-3p. This allowed us to produce miR-767-3p in a stable form. We also used a miR-767-3p-inhibitor that was commercially manufactured by RiboBio Corporation (Guangzhou, China) in order to suppress the function of miR-767-3p. miR-767-3p mimics (cells transfected with miR-767-3p mimic sequence, 5'-UGAUAAUGUGCUUACUUGCAGA-3'), miR-767-3p inhibitors (cells transfected with miR-767-3p inhibitor sequence, 5'-GCUUAUAUGCAGACUGAAUGUU-3'). This inhibitor was a 20-O-methyl-modified antisense oligonucleotide (ASOmiR-767-3p). RiboBio was also the vendor for the acquisition of small interfering RNAs (siRNAs) that targeted caspase 3 and caspase 9, as well as their respective control RNAs. Following the instructions provided by the manufacturer, we transfected plasmids, miRNAs, and siRNAs using Lipofectamine 3000 from Life Technologies in New York, United States.

Dual luciferase reporter assay

In order to determine the likely downstream target gene of miR-767-3p, we employed TargetScan, which is the prediction technique that is utilized the most commonly. In order to investigate the direct interaction between miR-767-3p and caspase 3/caspase 9 mRNA, HCC cells were transfected with miR-767-3p mimics (50 nmol/L), a normalization control (1 ng), firefly luciferase reporter vector (100 ng) with the sequence of caspase 3-3'UTR (named CASP3-3'UTR), caspase 9-3'UTR (named CASP Following a transfection time of 48 hours, the cells were harvested, and a Dual Luciferase Reporter Assay Kit (Promega, USA) was used to assess the luciferase activity in the samples.

Western blotting (WB) analysis

After transfection, HCC cells were lysed with RIPA buffer, and the protein was prepared. The protein was then isolated using 10% SDS-PAGE, and it was transferred to immobilon membranes and nitrocellulose membranes. This was done so that the protein levels of caspase 3 and caspase 9 could be determined (Amersham Biosciences, NJ, USA). Following an incubation of the membranes at room temperature for two hours with blocking buffer, the membranes were treated overnight at four degrees Celsius with specific antibodies targeting caspase 3, caspase 9, or GAPDH (all from Cell signaling, Shanghai, China). The membranes were then subjected to a two-hour incubation at room temperature with secondary antibodies, and the results were analyzed with a ChemiDoc XRS+ system (Bio-Rad, USA).

Cell viability and proliferative detection

24 hours were spent cultivating transfected HCC cells in 96-well plates at a density of 2×10^3 cells per well. At the fifth day after the transfection, the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was utilized to determine the level of vitality of the cells. In addition, the rate of cell proliferation was determined 12 hours, 24 hours, 48 hours, and 72 hours after the transfection by measuring the optical density at 450 nm with a CCK-8 assay in accordance

with the methods provided by the manufacturer. On order to conduct an assay measuring the formation of tumor colonies, 1 × 10^3 HCC cells were grown in 12-well plates for two weeks. After that, crystal violet was used to stain the cells, and the number of colonies was determined by counting them under a microscope (Nikon, Japan).

Cell apoptosis assay

Apoptosis in the cells was measured using a technique called flow cytometry. In six-well plates, 1 × 10^5 HCC cells were cultivated for a total of 48 hours. After that, the cells were incubated for 15 minutes using the conventional procedure with a FITC Annexin V apoptosis Detection Kit from BD Pharmingen in California, United States of America. Within one hour, apoptosis in cells was examined with the use of flow cytometry (BD Biosciences).

In vivo xenograft tumor growth model

The Medical Experimental Animal Care Commission of the Hospital of Chengdu University of Traditional Chinese Medicine gave their approval for every in-vivo animal study before it was carried out, and the research was carried out in accordance with the standards set forth by the international community. In order to evaluate the growth of in vivo xenograft tumors, 16 male nude mice that were 4 weeks old were randomly separated into four groups: a mimics NC group, a miR-767-3p mimics group, an inhibitors NC group, and an inhibitors miR-767-3p group (4 animals in each group). The right flank of each mouse received a subcutaneous injection of transfected HCC cells at a concentration of 1 × 10^7 cells, which were maintained in suspension in 150 l of FBS-free RPMI 1640 media. Using an external caliper, the volume of each group's tumor was determined, and then the volume was computed using the following formula: Volume = (Length (mm) With 2 (mm^2))/2. After 21 days of receiving the injection, the mice were humanely executed by cervical dislocation, and their tumors were collected, weighed, and then frozen in 4% paraformaldehyde. Tumor samples were then embedded in paraffin, sectioned, stained with immunohistochemistry (IHC) [15, 16] and TUNEL staining [17, 18]. The standard procedures of staining were carried out previously described [15-18].

Statistical analysis

Every experiment was carried out, conducted, and evaluated on an individual basis at least three times. The data were all presented with the mean and standard deviation. The software program SPSS 18.0 was utilized in order to carry out the statistical analyses (Chicago, USA). The two-tailed Student's t test was used to analyze the results of the comparisons between the groups. It was determined that the data were statistically significant if the *P* value was less than 0.05.

Results

miR-767-3p was upregulated in HCC tissues and HCC cell lines

The expression profile of miR-767-3p in HCC tissues and the matching nontumorous tissues is the first thing that we present here. The expression of miR-767-3p was found to be significantly higher in clinical HCC samples when compared with the matching non-tumor tissues (**Figure 1A**). In addition to this, the expression of miR-767-3p was analyzed in HCC cell lines as well as human liver cells. The expression of miR-767-3p was shown to be statistically substantially greater in HCC cell lines compared to that found in normal human liver cells (**Figure 1B**). This revealed that expression of miR-767-3p might possibly be related with the formation of HCC tumors.

miR-767-3p promotes HCC cell viability and proliferation in vitro

The expression level of miR-767-3p was higher in HepG2 and Hep3B cells when compared to all other HCC cell lines that we researched (**Figure 1B**). As a result, HepG2 and Hep3B cells were selected for use in the investigations that followed. We transfected Hep3B and HepG2 cells with either an inhibitor of miR-767-3p or a mimic of miR-767-3p, along with their respective control vectors. The findings of the RT-qPCR experiment demonstrated that the miR-767-3p inhibitor as well as the miR-767-3p mimics were successfully stably transfected into the Hep3B and HepG2 cells (**Figure 1C**). Overexpression of miR-767-3p led to an increase in the viability of the cells. Both cell lines had a significant drop in their levels of viable cells as a direct consequence of miR-

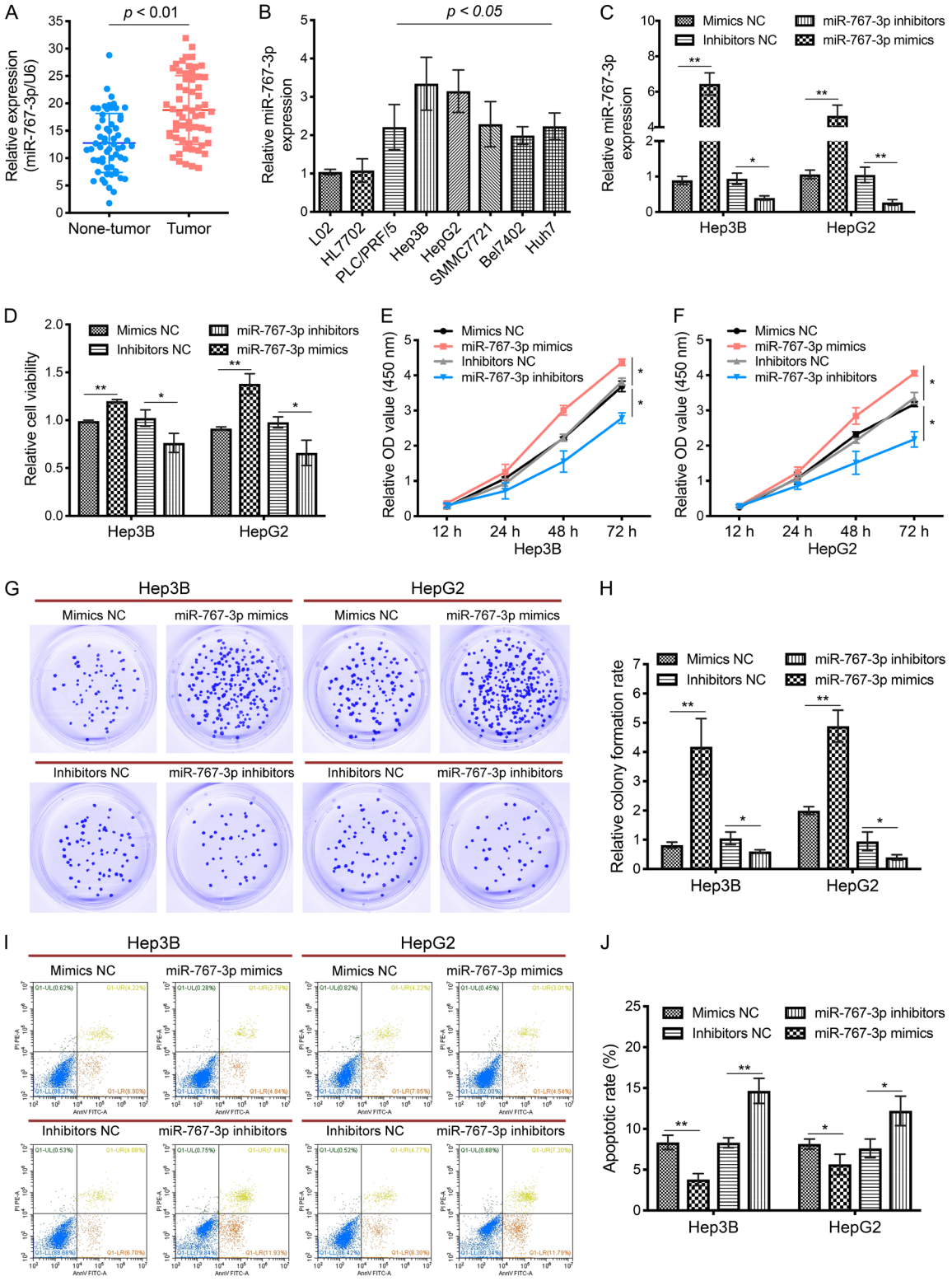

Figure 1. miR-767-3p promotes HCC cell viability and proliferation in vitro. A. miR-767-3p expression was increased in HCC tissues, compared with corresponding non-tumor specimens. B. PCR analysis showed that the expression of miR-767-3p was significantly higher in HCC cell lines compared with that in normal human liver cells. C. Confirmation of transinfection using PCR analysis. D. CCK-8 analysis for cell viabilities evaluation five days after transinfection. E, F. CCK-8 analysis for OD value five days after transinfection. G, H. Colony formation assay and its quantification. I, J. Flow cytometry of cell apoptosis and its quantification. *P < 0.05, **P < 0.05 compared with relative control groups. NC: Negative Control.

767-3p inhibition (**Figure 1D**). The results of this experiment were verified by a test of cell proliferation (**Figure 1E**, **1F**).

miR-767-3p promotes the colony formation and inhibits cell apoptosis in HCC cells in vitro

In accordance with the findings of the cell viability and proliferation assays, the colony formation assay further demonstrated the stimulatory effect of miR-767-3p on cell growth. On the other hand, inhibition of miR-767-3p resulted in a reduction in the capacity of both Hep3B and HepG2 cells to form colonies (**Figure 1G**, **1H**). The cell apoptosis assay was used to determine whether or not miR-767-3p had an effect on the level of cell death seen in HCC cell lines. Flow cytometry demonstrated that overintroducing miR-767-3p into Hep3B and HepG2 cells resulted in less cell death, whereas inhibiting miR-767-3p resulted in more cell apoptosis. This was the case regardless of whether or not the cells were treated with miR-767-3p (**Figure 1I**, **1J**).

miR-767-3p targets the 3'-UTR of CASP3 and CASP9 in HCC cell lines

Analyses in bioinformatics were carried out with the assistance of TargetScan and miRDB, which contain the prediction methods that are utilized the majority of the time. We discovered 6658 potential targets of miR-767-3p in TargetScan and 1128 potential targets of miR-767-3p in miRDB, respectively, when 1045 putative downstream mRNA targets were identified using both prediction algorithms (**Figure 2A**). A putative binding site for miR-767-3p was found in the 3'-UTR of CASP3 and CASP9, which was one of the 1045 possible targets (**Figure 2B**). After that, we utilized real-time quantitative PCR (RT-qPCR) in order to investigate the potential connection between miR-767-3p and CASP3/CASP9. When compared with the negative control groups, miR-767-3p overexpression treated HCC cells had significantly lower CASP3/CASP9 expression, whereas miR-767-3p inhibition resulted in enhanced expression of CASP3/CASP9 (**Figure 2C**). This was validated by Western blot analysis (**Figure 2D**, **2E**). Following this, we utilized reporter vectors that included either wild-type or mutant sequences inside the respective CASP3 or CASP9 3'-UTR segments. Accordingly, the findings of the dual luciferase reporter assay revealed that miR-767-3p transfection in Hep3B cells caused a significant reduction in luciferase activity in wild-type reporters that contained 3'-UTR of CASP3 or CASP9, whereas in mutant reporters this activity remained statistically unchanged (**Figure 2F**, **2G**).

Inhibition of CASP3/CASP9 exhibits similar promoting effects of miR-767-3p overexpression on HCC

The findings of the Western blot analysis showed that the use of siRNAs was successful in knocking down CASP3 and CASP9 (**Figure 3A-D**), and this was corroborated by the results of the RT-qPCR study (**Figure 3E**, **3F**). According to the findings of the cell proliferation assay, suppression of CASP3/CASP9 led to an increase in the number of Hep3B cells (**Figure 3G**, **3H**). The results of the tumor colony formation experiment demonstrated that knocking down CASP3/CASP9 had a stimulatory effect on the growth of Hep3B cells, which was in agreement with the findings of the cell proliferation assay (**Figure 3I**, **3J**). Flow cytometry, on the other hand, revealed that suppression of CASP3/CASP9 significantly decreased the amount of cell death seen in Hep3B cells (**Figure 3K**, **3L**).

In the following step of our study, we inhibited miR-767-3p while simultaneously knocking down CASP3/CASP9 in Hep3B cells and observed that knockdown of CASP3/CASP9 greatly decreased the suppression of miR-767-3p-induced cell growth inhibition in Hep3B cells (**Figure 4A**, **4B**). Additionally, the suppression of miR-767-3p was able to prevent the induction of cell apoptosis. This was achieved through the knockdown of CASP3/CASP9 (**Figure 4C**, **4D**). When taken together, our findings suggest that miR-767-3p stimulates the growth of HCC cells by inhibiting the pathway involving caspase-3 and caspase-9.

miR-767-3p promotes HCC tumor growth in vivo

In addition to this, we used a subcutaneous xenograft tumor development mice model to investigate the effect of miR-767-3p on HCC in vivo. When compared to the negative control groups, Hep3B cells with overexpression of miR-767-3p showed clearly evident effects that promoted the growth of tumors (**Figure 5A**).

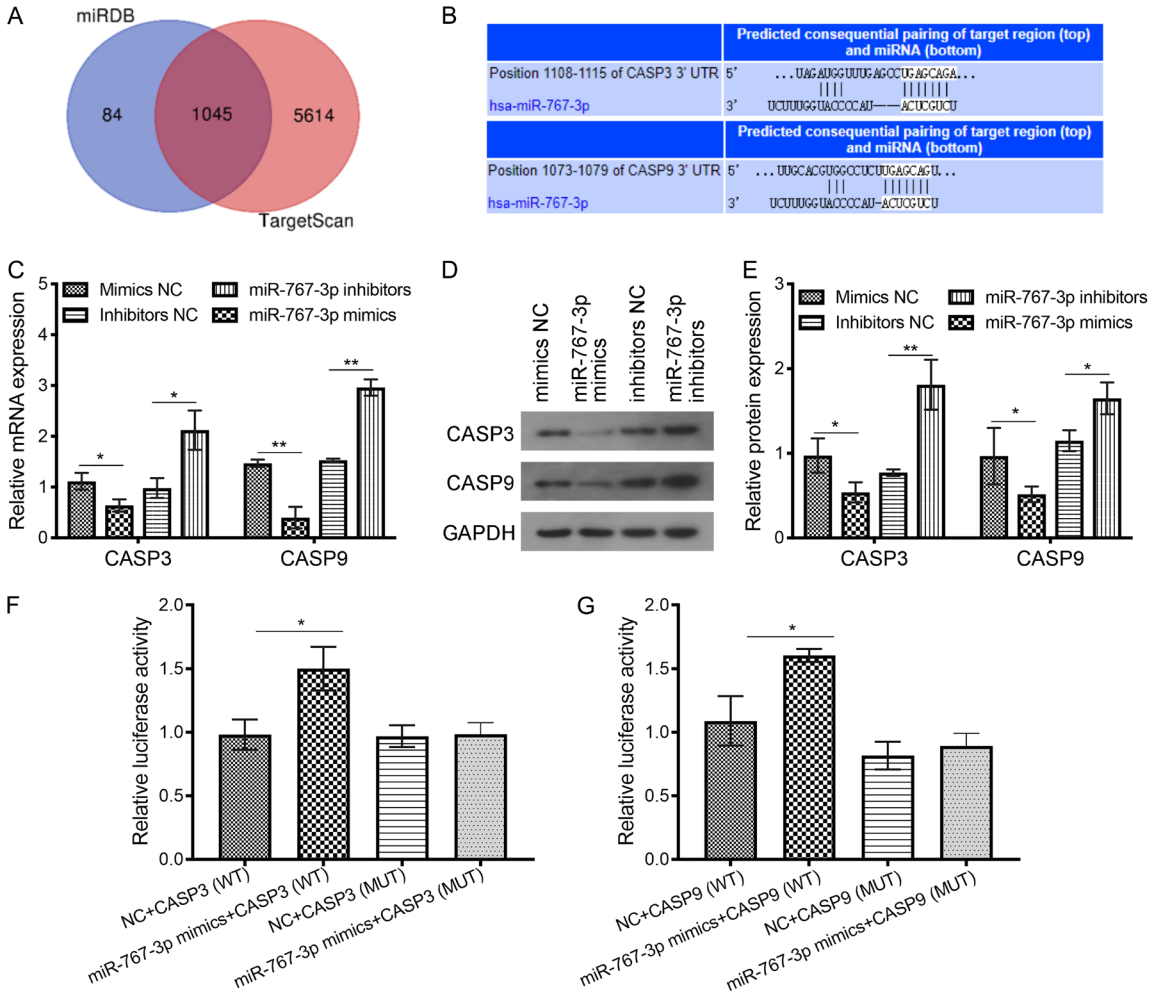

Figure 2. miR-767-3p targets the 3'-UTR of CASP3 and CASP9 in HCC cell lines. A. Bioinformatics analyses using TargetScan and miRDB. B. Putative binding sites for miR-767-3p was identified in the 3'-UTR of CASP3 and CASP9. C. RT-qPCR analysis to uncover the possible relationship between miR-767-3p and CASP3/CASP9. D, E. WB analysis and its quantification. F, G. Results of dual luciferase reporter assay. *P < 0.05, **P < 0.05 compared with relative control groups. NC: Negative Control; CASP3: caspase 3; CASP9: caspase 9.

These effects could also be seen by measuring the volume of the tumors (**Figure 5B**). Tumor weight in the miR-767-3p mimics group was greater than that in the mimics NC group, and tumor weight in the miR-767-3p inhibitors group was lower than that in the inhibitors NC group (**Figure 5C**). There was no significant difference in body weight among the groups (**Figure 5D**). Mice in the miR-767-3p mimics group have poor survival (**Figure 5E**). On the other hand, preventing miR-767-3p from functioning properly in Hep3B cells produced the opposite effect. In addition, immunohistochemical staining showed that overexpression of miR-767-3p led to an increase in the production of Ki-67 and Bcl-2 and it led to a decrease in the production of cleaved caspase-3/-9 and Bax. The expression of Ki-67 and Bcl-2 was inhibited by miR-767-3p suppression and the expression of cleaved caspase-3/-9 and Bax was increased by miR-767-3p suppression (**Figure 5F**). TUNEL staining performed on slides of tumor tissue revealed that overexpression of miR-767-3p in Hep3B cells led to a lower rate of cell apoptosis, whereas suppression of miR-767-3p induced a higher rate of cell death. These findings were in line with those obtained in vitro (**Figure 5G**). Our findings suggest that miR-767-3p may be responsible for the formation of tumors in HCC.

Discussion

microRNAs are known to play critical roles in the development of HCCs at early stages and

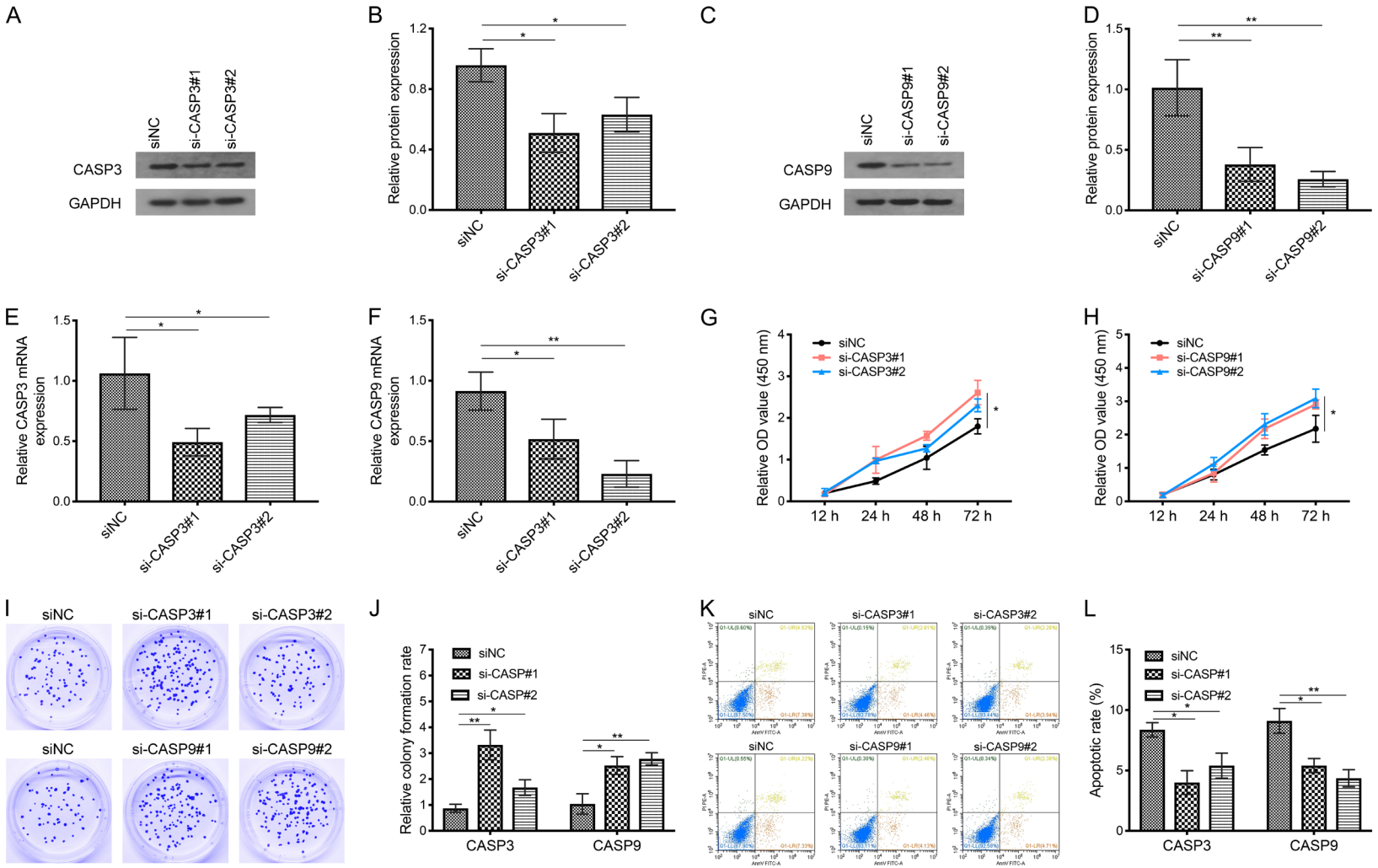

Figure 3. Inhibition of CASP3/CASP9 exhibits similar promoting effects of miR-767-3p overexpression on HCC. A-D. WB analysis showed successful knockdown of CASP3 and CASP9 using siRNAs. E, F. Confirmation of CASP3 and CASP9 knockdown by RT-qPCR analysis. G, H. Cell proliferation assay showed that inhibition of CASP3/CASP9 promoted Hep3B cell proliferation. I, J. Colony formation assay and its quantification. K, L. Flow cytometry of cell apoptosis and its quantification. *P < 0.05, **P < 0.05 compared with relative control groups. NC: Negative Control; CASP3: caspase 3; CASP9: caspase 9.

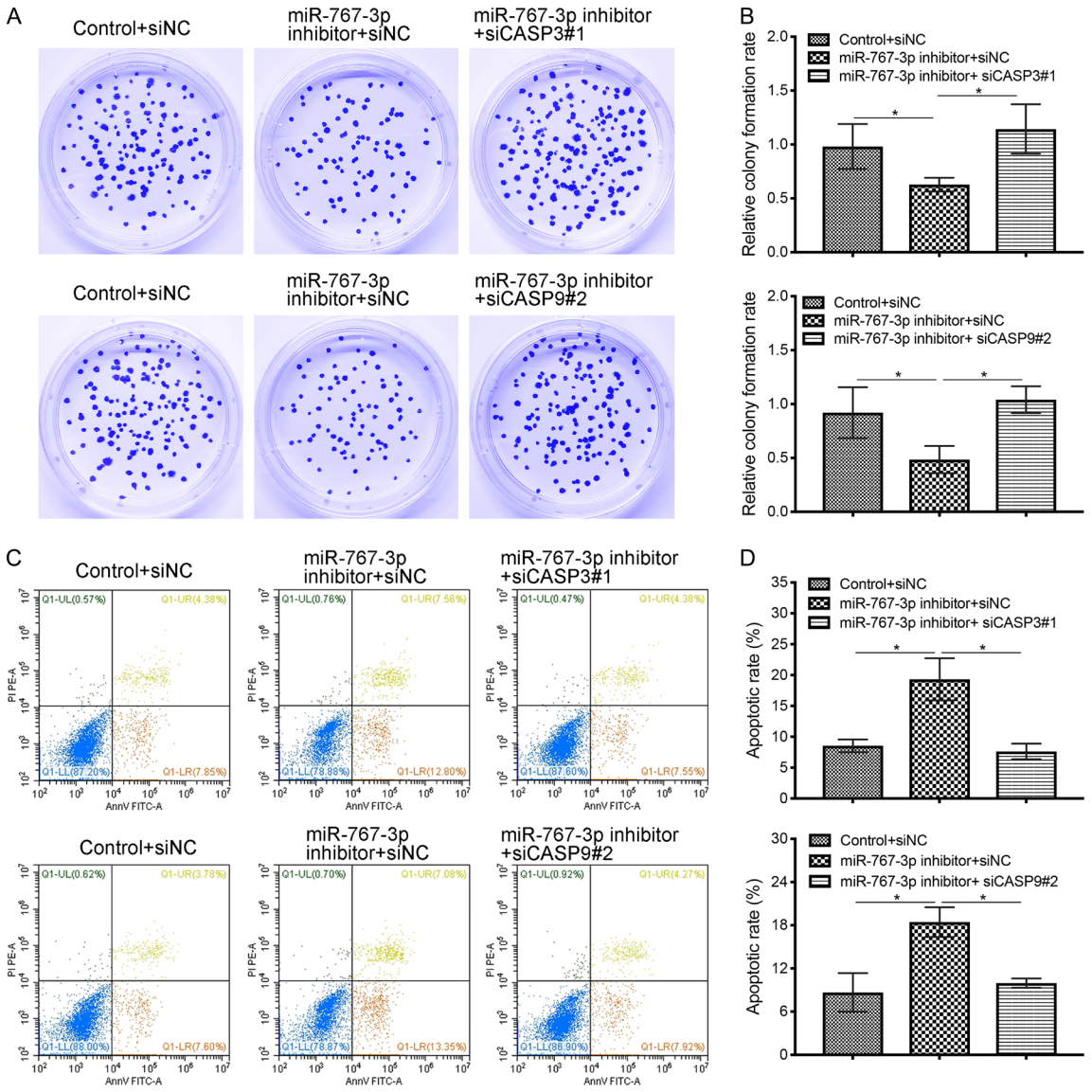

Figure 4. Knockdown of CASP3/CASP9 significantly attenuated the miR-767-3p inhibition-induced reduction of cell growth in Hep3B cells. A, B. Colony formation assay and its quantification. C, D. Flow cytometry of cell apoptosis and its quantification. *P < 0.05, **P < 0.05 compared with relative control groups. NC: Negative Control; CASP3: caspase 3; CASP9: caspase 9.

this has assisted in elucidating the molecular mechanisms of HCC contributed to the discovery of effective clinical treatment targets for HCC [19, 20]. In this study, we, for the first time, detected increased expression of miR-767-3p in clinical HCC specimens and HCC cell lines compared with non-tumor tissues and liver cells. In addition, functional studies showed that overexpression of miR-767-3p promoted HCC cell growth and inhibited HCC cell apoptosis, both in vitro and in vivo, whereas inhibition of miR-767-3p resulted in the opposite effects. These findings were determined by comparing the effects of miR-767-3p overexpression and inhibition. It was discovered that treatment with miR-767-3p led to an increase in the expression of vascular endothelial growth factor, which is considered to be one of the most significant markers of cancer, and vice versa. In addition to this, it was discovered that caspase-3 and caspase-9 are direct targets of the miR-767-3p. The overexpression of miR-767-3p had the effect of inhibiting these enzymes. When taken as a whole, miR-767-3p

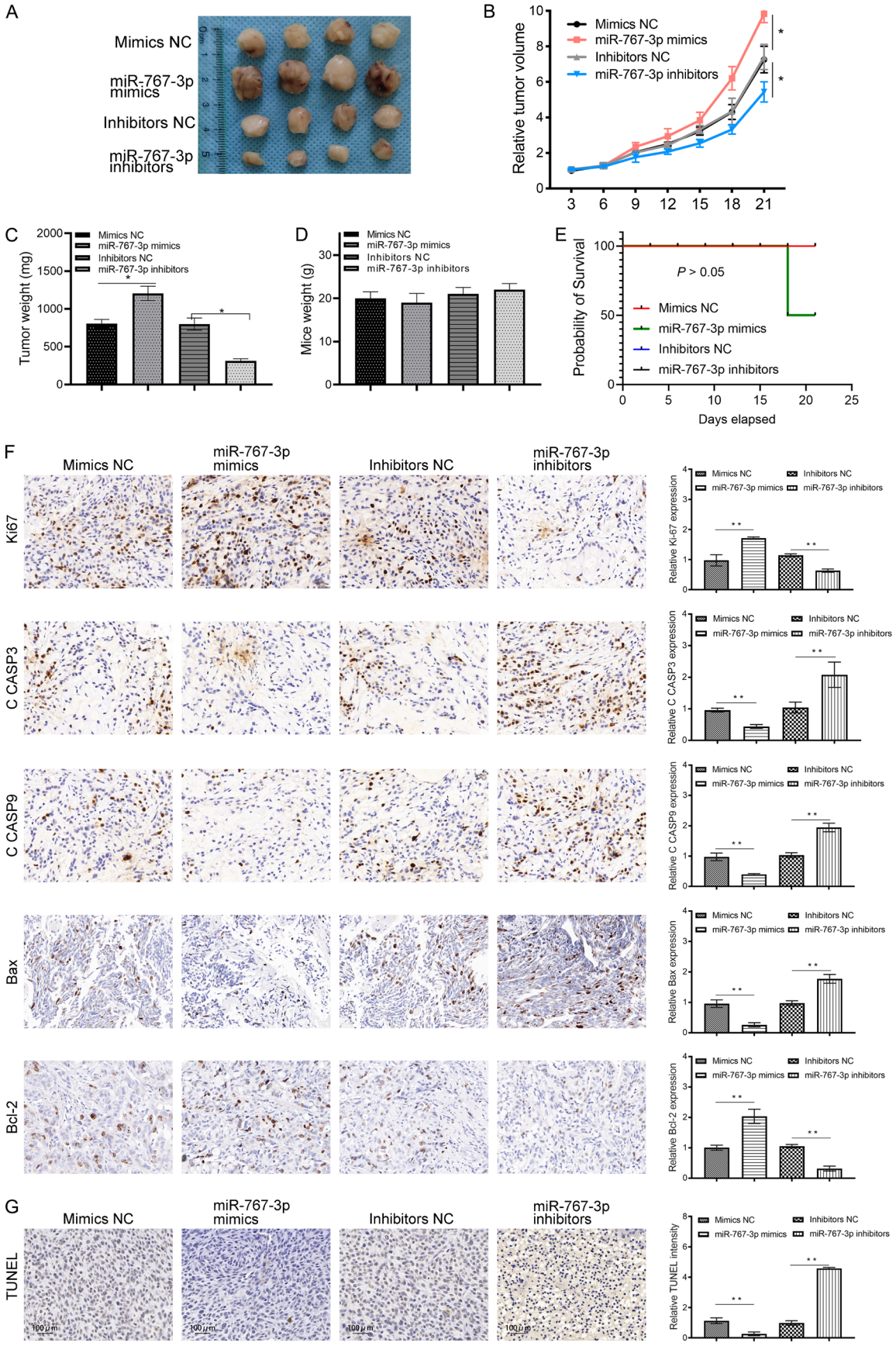
A
Mimics NC
miR-767-3p mimics
Inhibitors NC
miR-767-3p inhibitors
B
Relative tumor volume
Mimics NC
miR-767-3p mimics
Inhibitors NC
miR-767-3p inhibitors
C
Tumor weight (mg)
D
Mice weight (g)
E
Probability of Survival
P > 0.05
Days elapsed
F
Ki67
C CASP3
C CASP9
Bax
Bcl-2
Relative Ki-67 expression
Relative C CASP3 expression
Relative C CASP9 expression
Relative Bax expression
Relative Bcl-2 expression
G
TUNEL
Relative TUNEL intensity

Figure 5. miR-767-3p promotes HCC tumor growth in vivo. A. Tumors were collected at day 21 after the injection. Scale bar = 1 cm. B. Growth curves of the tumor volume changes. C. Tumor weight of mice in four groups. D. Mice weight in four groups. E. Mice survival rate of the four group. F. IHC staining of Ki-67, cleaved caspase-3/-9, Bax, Bcl-2 and its quantification. G. TUNEL staining of tissue samples and its quantification. Scale bar = 100 μm. *P < 0.05, **P < 0.05 compared with relative control groups. NC: Negative Control; IHC: immunohistochemistry.

was responsible for promoting the formation of HCC via inhibiting the caspase-3/caspase-9 pathway.

The miR-767 is a recently discovered miRNA that has been linked to cancer and plays a key role in the regulation of carcinogenesis [21-23]. Its expression is normally suppressed in most tissues, however it is observed to be active in a diverse range of human malignancies [23]. According to Zhang et al.'s findings, the level of expression of miR-767 was considerably elevated in human melanoma specimens and cells. Based on the findings of functional assessments, miR-767 was found to enhance the growth of human melanoma via inhibiting CYLD expression [21]. miR-767-3p is a recently identified member of the miR-767 family. It has been demonstrated to perform either a suppressive role or a promotional role in a variety of human malignancies. Furthermore, Simone et al. found that the expression of miR-767-3p was dramatically elevated in glioblastomas, which further indicates that miR-767-3p plays a role in the promotion of tumors [13]. According to the findings of Liu and colleagues, the levels of miR-767-3p in the serum of colorectal cancer patients were substantially higher than those seen in healthy individuals [14]. Similar results were also confirmed by another study, in which miR-767-3p was reported to be a possible diagnostic marker of colorectal cancer [11, 12].

Apoptosis in tumor cells can be induced by activating a signaling cascade that involves caspase-3 and caspase-9 [24-27]. The cleavage of PARP-1 and an increase in the activity of caspase-9 are both results of the activation of caspase-3 [28, 29]. Emerging data suggests that tumor suppression can be achieved by activation of the caspase-3/caspase-9 signaling pathway [29-34], whereas inhibition of caspase-3/caspase-9 results in tumor progression [35-37]. As a direct consequence of this, the activation of caspase-3 and caspase-9 has gained widespread recognition as an effective strategy for the treatment of human cancers [37-39]. In the current investigation, we used a variety of techniques, such as transfection, bioinformatics analyses, and a dual luciferase reporter assay, in order to characterize the independent and combined effects of miR-767-3p, caspase-3, and caspase-9 in HCC cells. This was done so that we could determine how these factors affect HCC cells independently and together. We were able to demonstrate that miR-767-3p in HCCs had direct effects on caspase-3 as well as caspase-9. The inhibition of caspase-3 and caspase-9 had the same growth-promoting effects on HCCs as the overexpression of miR-767-3p did. In addition, knockdown of caspase-3/caspase-9 significantly attenuated the miR-767-3p knockdown-induced inhibition of cell growth in HCC cells. This suggests that miR-767-3p promoted the growth of HCC tumors by inhibiting the expression of caspase-3/caspase-9. Caspase-3 and Caspase-9 are two enzymes that are involved in the caspase cascade.

Conclusion

In conclusion, we have shown data that there may be a substantial connection between miR-767-3p and the development of HCC. It is a new finding that miR-767-3p increased the proliferation and blocked the apoptosis of human HCC by decreasing the caspase-3/caspase-9 pathway. miR-767-3p might be a potential diagnostic biomarker and a therapeutic target for HCC, although further studies are needed.

Disclosure of conflict of interest

None.

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