miR-451: Potential role as tumor suppressor of human hepatoma cell growth and invasion

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Abstract. Malignant hepatoma is the leading cause of morbidity and mortality in primary liver cancer. MicroRNAs are widely accepted to act as tumor regulators to mediate tumorigenesis. Recently, miRNA-451 (miR-451) has attracted increasing attention due to its critical roles in the development of several types of cancers. Unfortunately, its function and underlying mechanism(s) of action in hepatoma remain unclear. In this study, a significant downregulation of miR-451 was observed in hepatoma cell lines. Its overexpression by administration of miR-451 mimics decreased cell viability and promoted cell apoptosis, indicating a critical role of miR-451 in cell growth. Further mechanistic analysis suggested that miR-451 overexpression accelerated cell death in a caspase-3-dependent manner, as pretreatment with its inhibitor z-VAD-fmk notably attenuated miR-451-induced cell apoptotic rates. Moreover, miR-451 upregulation abrogated cell invasive ability, accompanied with the decrease of matrix metalloproteinase-9 (MMP-9) expression levels, which may contribute to miR-451-triggered cell apoptosis. Taken together, these results reveal a prominent role of miR-451 as a tumor suppressor regulating hepatoma cell growth and invasion in a caspase-3- and MMP-9-dependent manner. Thus, our research supports this promising therapeutic agent against hepatoma.

Introduction

Hepatocellular carcinoma (HCC) is the most common aggressive carcinoma of liver and ranks as the sixth most common malignancy with an annual incidence of >560,000 deaths (1,2). Despite recent advances made in the diagnosis and clinical treatment of this tumor, the global mortality rates of HCC is still high and the prognosis of patients is very poor, with a dismal 10% 5-year overall survival rate (2,3). Substantial research has been performed to investigate the etiology of hepatoma; however, the accurate molecular mechanism underlying the pathogenesis and progression of HCC remains undefined. Therefore, to better understand the mechanism associated with hepatoma progression is vital for explore novel therapeutic strategies for hepatoma patients.

MicroRNAs (miRNAs) are known to be highly conserved, small non-coding RNA molecules with 18-24 nucleotides in length. As a new class of gene regulators, they can inversely regulate gene transcription or translation by interacting with the 3'-untranslated region (3'-UTR) of a target gene (4). Emerging evidence has confirmed the abnormal expression of miRNAs in various cancers, including glioma, non-small cell lung cancer, colon cancer and pancreatic cancer (5-7). It has been reported that miRNAs can regulate the multiple biological processes from cell proliferation, apoptosis, invasion to metastasis and survival (8,9). When downregulated, miRNAs may act as oncogenes by the targeted inhibition of tumor-suppressor gene expression, while their increase ranks as tumor suppressor affect in tumorigenesis (10). Among them, miRNA-451 (miR-451) has attracted increasing attention due to its critical roles in the development and progression of several types of tumors, such as nasopharyngeal carcinoma, esophageal carcinoma and glioma (11-13). MiR-451 gene is located on human chromosome 17 at 17q11.2, and its dysregulated expression has been widely confirmed in malignancies. In nasopharyngeal cancer, miR-451 expression levels are significantly decreased in nasopharyngeal cells and tissues; while its upregulation inhibited cell viability, migration, invasion and therefore results in the suppression of xenograft tumor growth, indicating a pivotal role of miR-451 in the initiation and progression of nasopharyngeal cancer (11). Furthermore, increased expression of miR-451 inhibits glioblastoma cell proliferation and induces cell apoptosis (14). However, the functions and mechanism of miR-451 in hepatoma development and progression is still unclear.

In this study, we found that miR-451 was downregulated in various hepatoma cells, and it as a negative regulator of cell growth and invasion. Moreover, the underlying molecular mechanism was also explored in the present investigation.

Materials and methods

Reagents and antibodies. The general caspase inhibitor (z-VAD-fmk) was obtained from Beyotime Institute of

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Technology (Shanghai, China). The polyclonal antibodies against human caspase-3 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-human MMP-9 antibodies were from Chemicon (Temecula, CA, USA).

Cell culture. Human liver cancer cell lines HepG2, HCCLM3 and SNU449 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). HepG2 and HCCLM3 cells were cultured according to the recommendations in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 μg/ml streptomycin and penicillin. SNU449 and immortalized normal human liver cell lines L02 (ATCC) were maintained in complete RPMI-1640 medium supplemented with 10% fetal calf serum. L02 cells were used as 'normal' controls for HCC analysis. All the cells were cultured in a humidified atmosphere at 37˚C with 5% CO_2.

Oligonucleotide transfection. To specifically induce miR-451 expression in HCCLM3 and SNU449, the miRIDIAN™ miR-451 mimics were introduced. The miR-451 mimics and scrambled control microRNA sequences were used as previously described (14) and obtained from GenePharma (Shanghai, China). For transfection, $1x10^6$ cells were seeded into 24-well plates and grown overnight until 50-80% confluence. After washing, 0.4 nmol microRNA mimics were mixed with 15 μl Geneporter 2 Transfection reagent (GTS, San Diego, CA, USA), followed by the transfection into cells for 6 h. The medium was replaced with fresh medium for 48 h; the expression of miR-451 was then confirmed by quantitative PCR.

Real-time PCR. Total RNA from cells was extracted using the RNAiso plus kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Then, the isolated RNA was reverse-transcribed to synthesize the first strand cDNA with the oligo(dT)18 primer using the cDNA synthesis kit (Fermentas). To assess the expression levels of miR-451 in hepatoma cells, the obtained cDNA was used as a template to perform PCR amplification using SYBR® Premix Ex Taq™ II kit (Takara). The specific primers for miR-451, U6 and MMP-9 were used as described previously (14,15). Each 20 μl reaction system comprised 2 μl of cDNA, 10 μl SYBR Premix Ex Taq II, 10 μmol/l of both sense and antisense primers. For normalization, β-actin and U6 were used to normalize mRNA and miRNA, respectively. Each experiment contained at least three replicates, and the results were calculated according to the method $2^{-\Delta\Delta Ct}$.

MTT assays. To evaluate cell proliferation, the MTT assay was performed. Briefly, cells were seeded into 96-well plates at a density of $1x10^5$ cells/well. After treatment with miR-451 and miR-control, ~20 μl MTT reagent (5 mg/ml) was added into each well and then incubated for a further 6 h at 37˚C. Then, the supernatant was replaced with 200 μl isopropanol to dissolve formazan production. Cell viability was assessed by measuring the absorbance of MTT at 590 nm using a micro-ELISA reader (Bio-Rad). All samples were performed in triplicate and the results are presented as the percentage of growth inhibition.

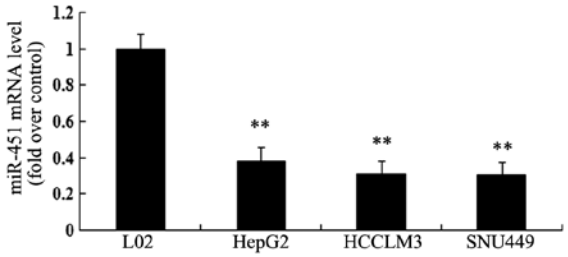

Figure 1. Expression of miR-451 in human hepatocarcinoma cell lines. To validate the expression levels of miR-451 in hepatoma cells, four HCC cell lines were used, including HepG2, HCCLM3 and SNU-449 cells. The immortalized normal human liver cell line L02 (ATCC) was used as control. The mRNA levels of miR-451 were examined by RT-PCR. $^{**}P<0.01$ versus L02 cells.

Quantification analysis of live and dead cells. After transfection with miR-451 mimics or control microRNA, cells were collected and rinsed with PBS three times. Then, cells were incubated with PBS solution consisting of 2 μM calcein AM and 4 μM PI in the dark for 20 min. Followed by washing, PBS was added to resuspend cells. The fluorescence of calcein AM and PI was analyzed by flow cytometry (Becton-Dickinson), and separately represented as the viable cells and dead cells.

Flow cytometry analysis of apoptosis. To quantitatively evaluate the rate of apoptosis, Annexin V-propidium iodide (AV-PI) staining was performed. Briefly, after pretreatment with caspase inhibitor z-VAD-fmk, the transfected cells were harvested and washed three times with PBS. Then, cells were centrifuged for 10 min, followed by resuspension in 500 μl of binding buffer including 5 μl FITC-conjugated Annexin V. Following incubation for 10 min in the dark, 5 μl of PI was added. Ultimately, all specimens were assessed by flow cytometry with a FACSCalibur using the CellQuest software (BDIS), and all the results are shown as a percentage of total cells counted.

DNA fragmentation assay. Following stable transfection with miR-451 mimics or control microRNA, cells were collected by centrifugation at 1500 rpm for 5 min. Then, cells were rinsed with PBS for three times, followed by the fixation with 4% paraformaldehyde for 30 min. After washing with PBS, the DNA-specific fluorescent dye Hoechst 33258 (Molecular Probes, Inc., Eugene, OR, USA) was added at 37˚C for 0.5 h to highlight the characteristic morphological changes of apoptosis in HCC cells. After centrifugation, cells were resuspended in PBS to observe the nuclear morphology under a fluorescence microscope.

Invasion assay. Invasiveness of HCC cells were measured by a modified Boyden chamber (BD Bioscience, Bedford, MA, USA). Cells overexpressing miR-451 were treated with antibodies against MMP-9 or control for 4 h, and then were seeded in the upper compartment. The medium including 10% fetal bovine serum was added into the lower compartment. Forty-eight hours later, cells that passed through the lower side of the membrane were stained with hematoxylin and eosin (Sigma) and quantified by counting six high-powered fields in the center of each well.

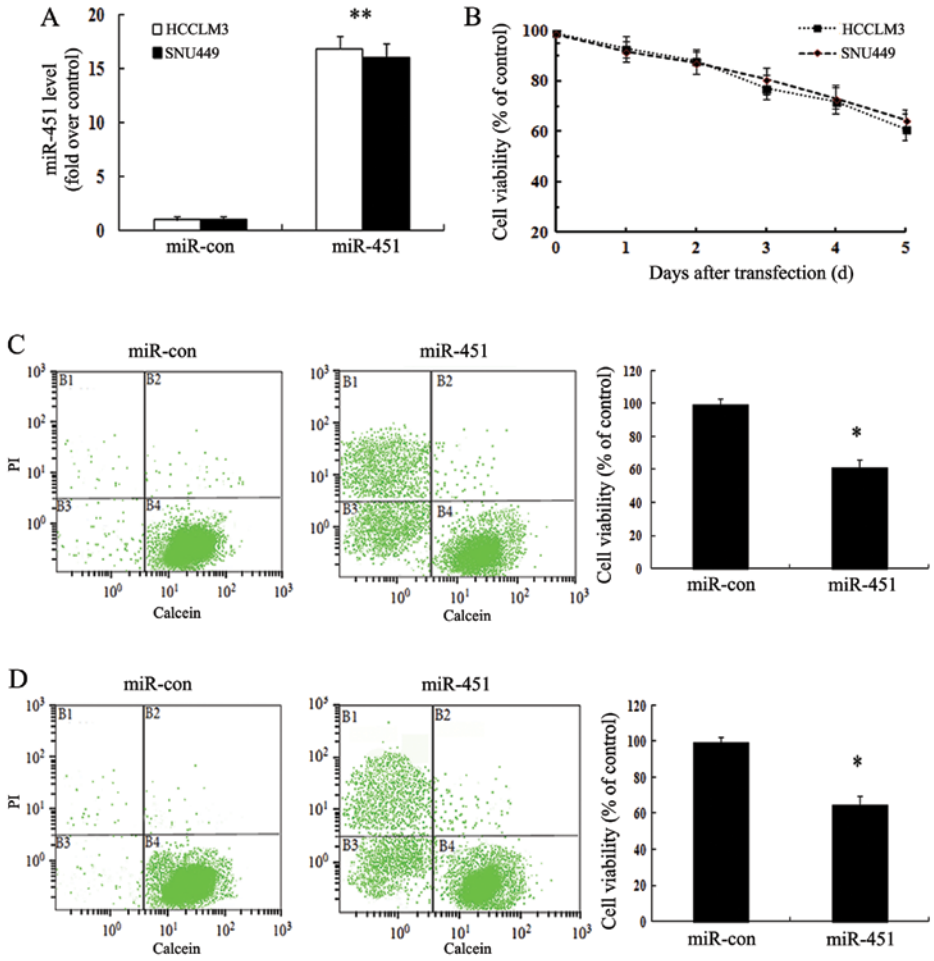

Figure 2. Effect of miR-451 on hepatoma cell viability. To investigate the function of miR-451 on cell growth, cells were transfected with miR-451 mimics and scrambled control microRNA, respectively. The corresponding transfection effective was detected by RT-PCR (A). The effect of miR-451 overexpression in HCCLM3 and SNU449 cell viability was evaluated by MTT assay (B). After transfection with miR-451 mimics, cells were incubated with 2 μM calcein and 4 μM PI in the dark for 20 min to determine the number of live/dead cells. The fluorescence of calcein AM and PI was analyzed in HCCLM3 (C) and SNU449 (D) by flow cytometry. *P<0.05 versus miR-con groups. **P<0.01.

Western blotting. Total protein from HCC cells were extracted by RIPA lysis buffer (Beyotime, Nantong, China) and quantified using the BCA assay (Pierce, Rockford, IL, USA). After electrophoresis with 9% SDS-PAGE, the targeted proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Following blocking with buffer containing 5% non-fat dry milk in Tris-buffered saline with Tween, the membranes were incubated with anti-caspase-3 and MMP-9 antibodies for 1 h. Then, cells were washed with TBST and cultured with the HRP-conjugated secondary antibodies for 1 h. The LumiGLo reagent (Pierce) was introduced to visualize the bound antibodies. The protein expression levels were normalized by β-actin.

Statistical analysis. All the results are presented as mean ± SD. The Student's t-test was used to assess the statistical significance of differences between groups. The data were analyzed by SPSS 11.0. P<0.05 was considered statistically significant.

Results

Expression of miR-451 is decreased in HCC cell lines. Expression of miR-451 is significantly downregulated in several tumors and exerts a vital role in the progression of cancer. However, the related research on miR-451 in hepatoma remains poorly understood. In this study, we examined miR-451 expression in three hepatoma cell lines (HepG2, HCCLM3 and SNU44). As shown in Fig. 1, the expression levels of miR-451 were obviously lower in three HCC cell lines than in the immortalized normal human liver cell line L02. Collectively, these results suggested a dramatical downregulation of miR-451 in HCC cells.

Overexpression of miR-451 abrogates SNU449 cell viability. Though research has been performed to explore the function of miR-451 in cancer, its effect on hepatoma remains undefined. To investigate the effect of miR-451 on the progression of hepatoma, we successfully upregulated the expression levels of miR-45 in HCCLM3 and SNU449 cells by the transfection with miR-21 mimics as detecting by RT-PCR (Fig. 2A). Further MTT analysis confirmed that miR-451 overexpression attenuated the growth of HCCLM3 and SNU449 (Fig. 2B). Moreover, cell growth inhibitory effect was obviously increased with the gradually increasing transfection times. To further corroborate the above results, we introduced the fluorescent probes calcein AM and PI to

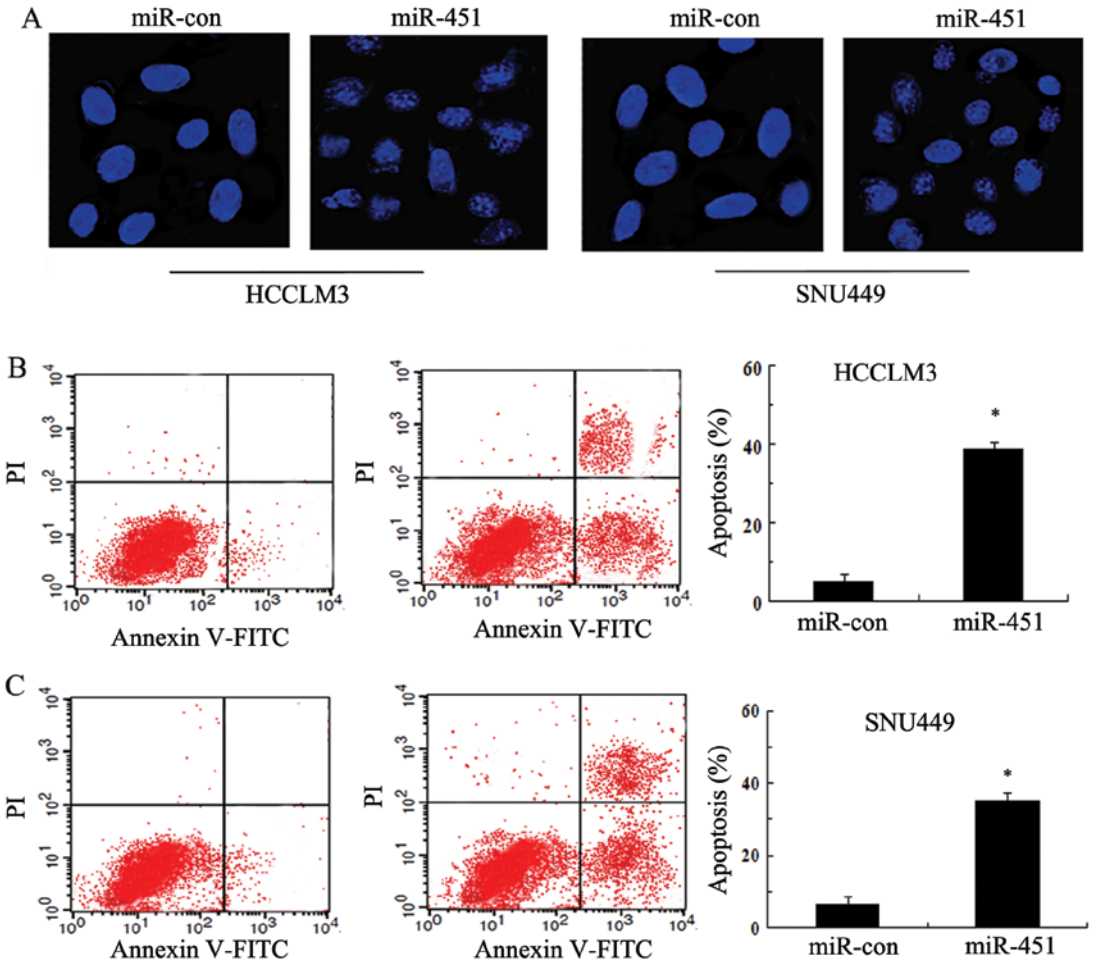

Figure 3. miR-451 overexpression promotes hepatoma cell apoptosis. After overexpression of miR-451 in HCC cells, Hoechst 32258 was used to highlight the nuclear morphology changes under a fluorescence microscope (A). To further analyze the effect of miR-451 on cell apoptosis, cells were stained with Annexin V/PI. The apoptotic rates were analyzed in HCCLM3 (B) and SNU449 (C) via flow cytometry. *P<0.05.

analyze the live/dead cells. Compared with the control group, the increased fluorescent signals of PI in regions B1 and B3 and reduced calcein AM signals in region B4 were examined, indicating that fewer live cells were observed after miR-451 upregulation in HCCLM3 (Fig. 2C). Consistently, a similar inhibitory effect of miR-451 in cell viability was also validated in SNU449 (Fig. 2D). Together, these data indicated that miR-451 overexpression attenuated HCC cell viability.

MiR-451 enhances cell apoptosis in SNU449 cells. It is known that DNA fragmentation and loss of plasma membrane asymmetry are characteristics of cell death (13). To explore whether cell apoptosis is associated with HCC cell growth inhibition induced by miR-451, we observed the morphological changes in the nuclei by Hoechst 33258 staining. As shown in Fig. 3A, miR-451 overexpression triggered a significant increase in nuclear shrinkage and DNA fragmentation, implying a critical role of miR-451 in hepatoma cell apoptosis by disrupting the nuclear morphology. Furthermore, the induction of apoptosis was further manifested by Annexin V-FITC and PI staining. After transfection with miR-451 mimics, a dramatical upregulation in the number of apoptotic cells was observed; and the apoptotic rate was 38.21% in HCCLM3 (Fig. 3B) and 34.69% in SNU449 (Fig. 3C), respectively. Therefore, these results revealed that miR-451 upregulation induced HCC cell apoptosis.

Caspase-3 is responsible for miR-451-induced cell apoptosis. Caspase-3 has been reported to be the major effector in apoptotic pathways, and its cleavage is a characteristic of cell death. The fact that miR-451 can induce hepatoma cell apoptosis is corroborated above. However, the accurate molecular mechanism of action remains unclear. Therefore, we investigated the effect of caspase-3. As shown in Fig. 4A, miR-451 transfection strikingly induced the expression of cleaved caspase-3 levels, compared with control group. To further confirm the involvement of caspase-3 in miR-451-induced apoptosis in HCC cells, we blocked the expression of caspase-3 with a general caspase inhibitor z-VAD-fmk, after which we observed a remarkable decrease in miR-451-induced apoptotic rate from 38.21 to 18.72% in HCCLM3 and from 34.98 to 19.6% in SNU449 (Fig. 4B), suggesting that miR-451 overexpression may induce hepatoma cell apoptosis in caspase-3-dependent manner.

Cell invasive ability was impeded by miR-451 mimic transfection. To investigate whether miR-451 is associated with hepatoma cell invasion, we transfected HCCLM3 and SNU449

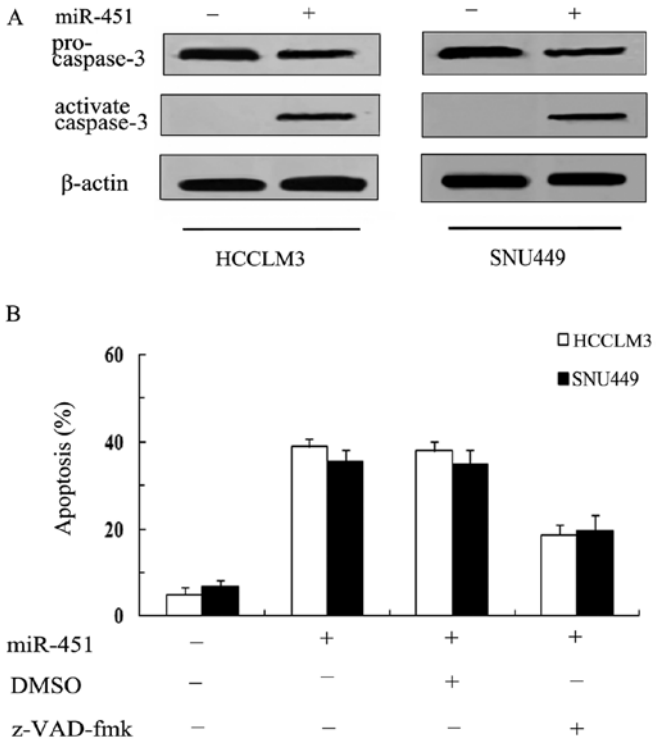

Figure 4. The elevated expression of miR-451 induces cell apoptosis in caspase-3-dependent manner. After treatment with 20 μM z-VAD-fmk or DMSO, the protein levels of caspase-3 were assessed by western blotting (A). After staining with Annexin V/PI, flow cytometry was used to measure the apoptotic rate (B). *P<0.05 versus control group. #P<0.05 versus miR-451 and DMSO groups.

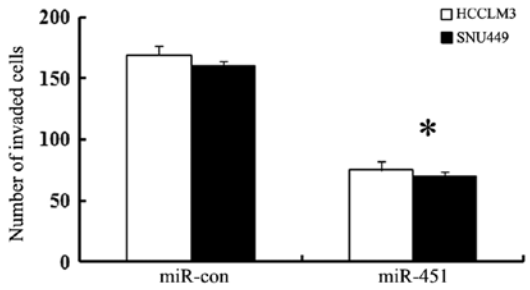

Figure 5. miR-451 upregulation inhibited hepatoma cell invasion. Following stable overexpression of miR-451, or not, the corresponding cell invasive ability was evaluated by Transwell analysis. *P<0.05.

cell lines with miR-451 oligonucleotide mimics or control. Approximately 48 h later, cell invasive ability was analyzed by Transwell assay. As shown in Fig. 5, the number of HCCLM3 cells invading through the Matrigel after miR-451 transfection with mimics was remarkablely attenuated from 169 to 75. Similar reduction in cell invasive activity was observed in miR-451-overexpressed SNU449 cells. Hence, these data indicated a potential negative regulatory effect of miR-451 on HCC cell invasion.

MiR-451 transfection attenuates cell invasion by MMP-9. The extracellular matrix metalloproteinase MMP-9 is overexpressed in various cancers and critical for the invasive potential

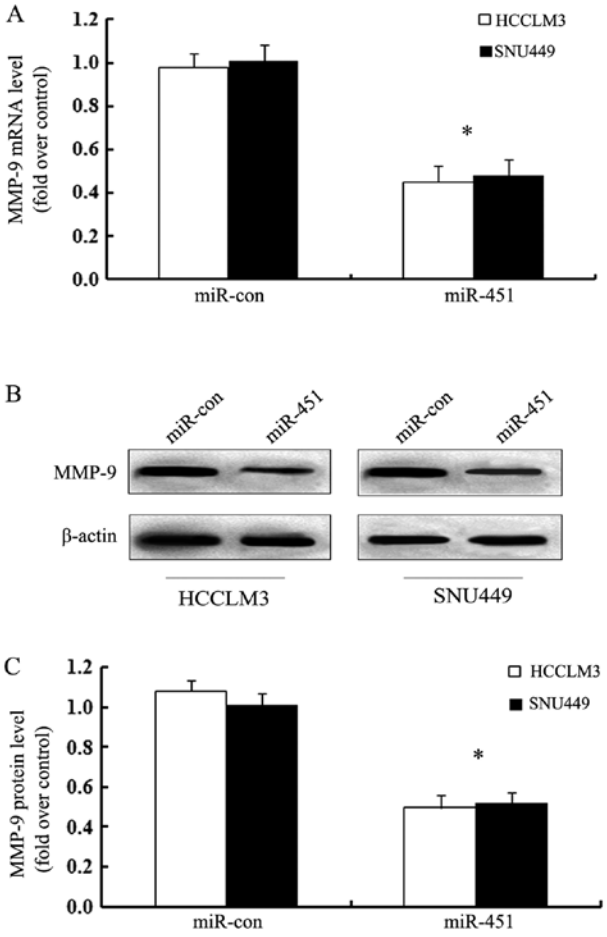

Figure 6. The overexpression of miR-451 attenuated the expression of MMP-9. Following transfection with miR-451 mimic or control miRNA, the mRNA levels of MMP-9 were confirmed by RT-PCR assay (A). The protein levels of MMP-9 (B) and its quantification analysis (C) were explored by western blotting. *P<0.05.

of tumors, including hepatoma (16,17). To further address the molecular mechanism involved in miR-451-inhibited HCC cell invasion, we explored MMP-9 as a potential target. As shown in Fig. 6A, obvious downregulation of MMP-9 mRNA was identified in HCC cell when transfected with miR-451 mimics. Simultaneously, the corresponding decrease in MMP-9 protein levels was also confirmed by western blotting (Fig. 6B). Quantitative analysis suggested that overexpression of miR-451 resulted in 0.495-fold decrease of MMP-9 in HCCLM3 and 0.52-fold in SNU449 than in the control (Fig. 6C). These data suggested that miR-451 may inhibit hepatoma cell invasion by MMP-9 expression.

Discussion

Hepatocellular carcinoma (HCC) ranks as the most common type of primary lethal neoplasm of liver cancer and is considered to be the third leading cause of cancer-related deaths worldwide (1). The high rate of invasion and metastasis results in >560,000 deaths worldwide each year (3). Therefore, to explore the mechanisms related to the progression and new biomarkers for the malignant potential of hepatocellular cancer are urgently needed.

MicroRNAs are known as a group of small non-coding RNAs possessing critical roles in multiple physiological processes including cell proliferation, differentiation, apoptosis and development (18,19). During the past decade, accumulating evidence has demonstrated a prominent role of miRNAs in the development and progression of various cancers by acting as oncogenes and tumor suppressor genes (10,20). It has been reported that many tumor suppressor miRNAs, such as miR-302b, exert a reverse response to cancer cell proliferation, growth, angiogenesis, invasion and metastasis signaling (21). Among them, miR-451 has received increasing attention due to its important function in the development of several cancer, including nasopharyngeal carcinoma, esophageal carcinoma and glioma (11,13,14). In this study, our results revealed a notable downregulation of miR-451 in HCC cells, consistent with its abnormal expression in nasopharyngeal carcinoma and glioma cells. Moreover, overexpression of miR-451 effectively decreased HCC cell viability, indicating that miR-451 upregulation induced cell death. According to these results miR-451 might be as a novel tumor suppressor regulating the progression of HCC.

It is widely accepted that many antitumor agents influence growth inhibition effect on malignant cells through inducing apoptosis, which is characterized with DNA fragmentation and loss of plasma membrane asymmetry (22-24). In the present study, transfection of miR-451 mimics promoted significantly DNA fragmentation and morphological changes in HCC cells. Moreover, the elevated miR-451 expression enhanced the apoptotic rates, implying that cell apoptosis was involved in miR-451-induced hepatoma cell growth inhibition. However, how miR-451 induces cell apoptosis remains to be further explored.

Caspase-3 is a downstream effector of cysteine-aspartic acid protease participating in programmed cell death, and its sequential activation plays an important role in the execution-phase of cell apoptosis (25,26). Numerous studies have demonstrated that caspase-3 is overexpressed in diverse malignancies, including HCC (27). Therefore, we speculate that miR-451 could induce cell apoptosis by caspase-3 signaling. To address this hypothesis, we analyzed the expression levels of caspase-3 in miR-451-transfected HCC cells. As expected, miR-451 overexpression significantly induced the expression of activated caspase-3. When blocking its activity with the specific caspase inhibitor z-VAD-fmk, the miR-451-triggered cell apoptotic rate was dramatically abrogated. Therefore, we can conclude that miR-451 induced cell death via caspase-3-regulated pro-apoptotic pathways.

miR-451 has been confirmed to be frequently downregulated in various cancers, including breast cancer, nasopharyngeal carcinoma and lung cancer (7,12,14). Importantly, its dysregulation is associated with tumor progression, including cell proliferation, growth, migration and invasion (11,28). It is known that the higher invasion and metastasis is the main cause of death in HCC patients. Therefore, to better understanding the role of miR-451 in the development of hepatoma, we further analyzed its function in HCC cell invasion. In this study, overexpression of miR-451 dramatically impeded the invasive ability of HCC cells, indicating a vital role of miR-451 in the progression of HCC. However, its underlying mechanism is still undefined.

Proteases of the matrix metalloproteinase (MMP) such as MMP-9 execute the pivotal function in the breakdown of collagen IV in basement membrane and extracellular matrix (ECM), a crucial step in tumor invasion and metastasis (29,30). Accumulating evidence has shown that MMP-9 is elevated in several types of human cancers including breast cancer, non-small cell lung cancer and gastric cancer (31-33). Furthermore, its high expression is also observed in hepatoma (16). MMP-9 is known as a key regulator of ECM remodeling and related to the poor prognosis due to its important function in tumor invasion and metastasis (16,34). The fact that miR-451 could attenuate HCC cell invasion has been confirmed herein. To further clarify the underlying mechanism, we investigated MMP-9 in miR-451-regulated hepatoma cell invasion. After transfection with miR-451 mimics, the expression levels of MMP-9 mRNA and protein were notably reduced, implying that miR-451 may inhibit HCC cell invasion in MMP-9-dependent manner.

In conclusion, our study showed obvious downregulation of miR-451 in human hepatocarcinoma cells. High expression levels of miR-451 inhibited hepatoma cell growth by suppressing cell proliferation and enhancing cell apoptosis in caspase-3-dependent manner. Additionally, miR-451 overexpression attenuated HCC cell invasion by MMP-9 expression. Accordingly, our study illustrates a potential role of miR-451 in the development and progression of hepatoma, and supports this promising therapeutic agent for future development in anti-hepatoma therapy.

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