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MicroRNA-32 Regulates Development and Progression of Hepatocellular Carcinoma by Targeting ADAMTS9 and Affects Its Prognosis

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEFG 1 Shengmian Li
BCDEF 1 Tingting Li
BCDEF 1 Xiaoming Li
B 1 Yue Yao
B 1 Xiaojia Jiang
A 2 Lianmei Zhao
A 2 Wei Guo

1 Department of Gastroenterology and Hepatology, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, P.R. China
2 Research Center, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, P.R. China

Corresponding Authors: Lianmei Zhao, e-mail: lianmeizhmail@163.com, Wei Guo, e-mail: guowei7303@163.com
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Background: MicroRNA-32 (miR-32) induces cell proliferation and metastasis in hepatocellular carcinoma (HCC), but the detailed mechanisms of miR-32 in regulating oncogenesis and development of HCC have not been clarified. The aim of this study was to investigate the effects of miR-32 on HCC and its clinical pathological significance, as well as to determine the functional connection between miR-32 and ADAMTS9 in HCC.

Material/Methods: Quantitative RT-PCR was used to assess the expression levels of miR-32 in HCC tissues, adjacent non-cancerous tissues, and liver cancer cell lines. *In vitro* cell proliferation, migration, and invasion assays were performed to confirm the biological functions of miR-32. Quantitative RT-PCR, Western blot analysis, and luciferase reporter assays were used to evaluate the role of miR-32 in the regulation of ADAMTS9.

Results: miR-32 was highly expressed in HCC tissues compared with corresponding adjacent non-cancerous tissues. Over-expression of miR-32 was also found in 3 human liver cancer cell lines: SMMC-7721, Huh7, and HepG2. Moreover, increasing expression of miR-32 in HCC tissues was related to shorter overall survival. *In vitro* over-expression of miR-32 promoted cell proliferation, migration, and invasion; however, the under-expression of miR-32 revealed the opposite effects. Dual-luciferase reporter assay indicated that miR-32 can directly bind to the 3'-UTR of ADAMTS9. Western blot analysis showed that over-expression of miR-32 decreased expression of ADAMTS9 protein. Rescue tests further verified the connection between miR-32 and ADAMTS9.

Conclusions: Our data indicate that miR-32 accelerates progression in HCC by targeting ADAMTS9, and the abnormal expression of miR-32 is correlated with prognosis and could become a potential therapeutic target.

MeSH Keywords: ADAM Proteins • Carcinoma, Hepatocellular • Cell Proliferation • MicroRNAs • Prognosis

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Background

Hepatocellular carcinoma (HCC) is the sixth most common cancer, and is currently the second most common cause of cancer death worldwide, leading to nearly 746 000 deaths in 2012 [1,2]. Although several conditions, including preneoplastic liver lesions, hepatitis virus infection, alcohol abuse, and aflatoxin exposure, are associated with carcinogenesis [3], the mechanism of hepatocarcinogenesis remains unclear.

MicroRNAs (miRNAs) are a class of small, endogenous, non-coding RNAs and consist of 19–24 nucleotides, which have high evolutionary conservation [4]. They have 2 forms, pre-miRNAs and mature miRNAs, but only the mature miRNAs play a crucial biological role [5], through binding to the 3'-untranslated regions (3'-UTRs) of target mRNA. Several studies also have demonstrated that ectopic expression of miRNAs might contribute to tumor processes, such as HCC and NSCLC [6,7]. miR-32 is located on the 14th intron of gene *C9orf5* [8]. miR-32 has been proved to be an important regulator in oncogenesis and it may serve as an oncogene in colorectal cancer and breast cancer [9,10]. In contrast, it also may act as a tumor suppressor in non-small cell lung cancer and oral squamous cell carcinoma [11,12]. A previous study found that increased expression of miR-32 in HCC [13]. However, the detailed mechanisms by which miR-32 regulates tumorigenesis and progression of HCC have not been defined.

ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) protein comprises new types of metalloproteinase that depend on Zn^{2+} and widely exists in mammals [14,15]. ADAMTS can inhibit tumor metastasis and is a good prognosis indicator in breast tumors and colorectal cancer [16–18]. ADAMTS9 is a member of the ADAMTS family and is a newly discovered tumor-suppressor gene. It plays an important role in tumorigenesis and progression in a variety of cancers, such as gastric cancer [19], esophageal squamous cell carcinoma, and nasopharyngeal carcinoma [20]. A study also has shown that ADAMTS9 methylation is associated with prognosis in HCC [15].

In this study, we explored the correlation between miR-32 and ADAMTS9 in HCC. To the best of our knowledge, it is the first report on this topic. We demonstrated over-expression of miR-32 in liver cancer cell lines and tissues. In addition, the over-expression of miR-32 promotes liver cancer cell proliferation, migration, and invasion. We also proved that ADAMTS9 is a direct and functional target of miR-32, and miR-32 down-regulates ADAMTS9 expression by directly targeting its 3'-UTR. Over-expression of ADAMTS9 markedly weakened the effects of miR-32 on the proliferation, migration, and invasion. Our data reveal that miR-32 may be a potential therapeutic target for HCC.

Material and Methods

Human tissue specimens

Hepatocellular carcinoma tumor tissues and para-cancerous cirrhosis tissues were collected from 80 HCC patients who underwent curative resection with informed consent between March 2010 and September 2016 in the Department of Hepatobiliary Surgery of the Fourth Hospital of Hebei Medical University. We enrolled 80 patients (75 male and 5 females) aged from 25 to 73 years (53.9±9.5). According to Child-Pugh classification of liver function, 77 of 80 cases had the liver function of Child-Pugh class A and 3 cases had Child-Pugh class B. According to the American Joint Committee on Cancer (AJCC) standard [21], among the 80 patients, 54 patients belonged to stage I or II and the others belonged to stage III or IV. This group of patients was HBsAg-positive in 65 cases, HBsAg-negative in 13 case, and HCV-Ab positive in 2 cases. None of the subjects had undergone preoperative treatments such as chemotherapy, radiotherapy, or other antineoplastic treatments. The diagnosis of HCC tissues and adjacent cirrhosis tissues were confirmed by at least 2 pathologists. All patients were followed up, and the deadline was to January 2017.

Cell lines and cell culture

Human liver cancer cell lines SMMC-7721, Huh7, and HepG2, and 293T cell lines were from Hebei Medical University. All the cells digested and amplified using 0.25% Trypsin-EDTA (Solarbio) treatment per 2 or 3 days were incubated with 10% fetal calf serum, 1% penicillin-streptomycin DMEM high-glucose medium (Hyclone), and RPMI-1640 medium (Gibco) at 37°C in 5% CO_2. Then, the cells in logarithmic growth phase were used for further experiments.

Total RNA extraction

Total RNA extraction was performed according to the manufacturer's instructions. The cells were rinsed twice with PBS before adding Trizol (1 ml) (JieRui, Shang hai); the EP tube was placed on ice for 5 min and then we added 200 µl trichloromethane. Then, the EP tube was shaken for 15 s and placed on ice for 10 min. The supernatant was collected after 15 min of centrifugation at 8800 rpm and transferred to a new 1.5 ml RNase-free EP tube. The RNase-free EP tube containing 0.5 ml of the isopropanol was held for 10 min at -20°C. The supernatant was centrifuged at 8800 rpm for 15 min, rinsed with 75% ethanol, and centrifuged again. Then, the ethanol was dried out. The sedimentary RNA was dissolved in 20 µl DEPC sterile water overnight and read at OD260/OD280 with an ultraviolet spectrophotometer. Sedimentary RNA can be stored at –80°C for a long time.

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Quantitative real-time PCR

The expression of miR-32 was examined by RT-PCR with specific RT and PCR using U6 as control. The primer of miR-32 for reverse transcription (RT) was: 5'-GTCGTATCCAGTGCAGGGTCCGA GGTATTCGCACTGGATACGACTGCAACTT-3'. The primer of miR-32 for PCR was 5'-GTGCAGGGTCCGAGGTATT-3' (upstream primer); 5'-GCCGCTATTGCA CATTACTAAGTT-3' (downstream primer). The expression of ADAMTS9 mRNA was detected by RT-PCR. β-actin served as control. The primer of ADAMTS9 for RT-PCR was the random primer in the kit. The primer of ADAMTS9 for PCR was 5'-CATAATGAACAGGATGGGCCT-3' (forward primer); 5'-TTGACCACATCCAGGGGTTG-3' (reverse primer). Quantitative PCR was performed on an MX3005P Real-time PCR Instrument (Agilent) using Real-time PCR Mixture Reagent (Promega) according to the manufacturer's protocol. The relative expression levels were quantified by the $2^{-\Delta\Delta CT}$ method.

Cell transfection

The human HCC cell line Huh7 was transfected instantaneously with the plasmid of miR-32-mimic and the miR-32-inhibitor using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells in logarithmic phase were seeded in 6-well plates (1×10^6 cells per well). All cells were cultured at 37°C in a 5% CO_2 incubator. After transfection, the cells were cultured in an incubator for 6 h. RNA was extracted after incubation in a serum-containing medium for an additional 24 h.

Western blotting assay

After rinsing 3 times with PBS, the monolayer cells were lysed in ice-cold lysis buffer for 30 min. After centrifugation at 12 000 rpm for 5 min and removing any cellular debris, 50 μg of the sample was mixed with sample loading buffer and boiled for 5 min. Proteins were separated by 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes and blocked with 5% skimmed milk/TBST for 2 h at room temperature. The whole-cell lysates were examined using the primary antibodies rabbit of anti-human ADAMTS9 (Abcam, USA) at a dilution of 1: 1000 and mouse anti-human β-actin IgG (Proteintech, USA) at a dilution of 1: 5000; the secondary antibodies goat anti-rabbit IgG and goat anti-mouse IgG (Proteintech, USA) were examined at a dilution of 1: 3000 by ECL chemiluminescence reagent.

Immunohistochemistry

The specimens were fixed in 10% formalin and embedded in paraffin. Five serial 4-μm sections were cut from the tissue blocks. The deparaffinized sections were stained with the antiADAMTS9 antibody (Sigma-Aldrich, St Louis, MO, USA) at a dilution of 1: 250 overnight at 4°C, followed by incubation with a biotinylated secondary anti-mouse IgG antibody for 1 h at room temperature. The sections were subsequently incubated with horseradish peroxidase (HRP)-conjugated streptavidin and were developed using 3, 3'-diaminobenzidine (DAB). The evaluation of ADAMTS9 staining was performed by 2 pathologists with knowledge of the clinical data of patients, as previously described. The percentage of positive tumor cells was graded as follows: 0=0%, 1=1–25%, 2=26–50%, and 3=more than 50%. Immunostaining intensity was rated as follows: 0=none, 1=weak, 2=moderate, and 3=intense. The specimen was considered immunopositive if the score when multiplying the percentage of positive cells with the intensity score is equal to or greater than 1.

Luciferase reporter assay

The 3'-UTR of ADAMTS9 mRNA contains a putative region which can bind to miR-32, and the region of 3'-UTR of ADAMTS9 was amplified by PCR. Recombinant vectors ADAMTS9-wt and ADAMTS9-mut were obtained. For the luciferase reporter assay, 293T cells in logarithmic growth phase were seeded onto 24-well plates at 37°C in a 5% CO_2 incubator for 20 h. Co-transfection of the recombinant vectors (ADAMTS9-wt and ADAMTS9-mut) and miRNA (miR-32-mimics or NC-mimics) was carried out in 293T cells using Lipofectamine 2000 (Invitrogen). After co-transfection for 24 h, cells were collected and washed twice with PBS. The luciferase activity was determined using a dual-luciferase reporter assay system (Promega).

MTS assay

MTS assay was done as previously described [22]. Huh7 cells in logarithmic growth phase were transfected, collected, and diluted, and then the cells were seeded onto 96-well plates (2000 cells/100 μl/well) and cultured in an incubator. The cells were harvested at different time points (0, 12, 24, 48, 72, and 96 h) for growth assay using MTS kit (Promega G111A) following the manufacturer's protocol, and the absorption was read at 492 nm.

Clonogenic assay

Clonogenic assay was performed according to the manufacturer's protocol. Cells were seeded into 6-well cell culture plates (200 cells/well) at 37°C. After incubation for 10 days, cells were washed twice with PBS and fixed with 4% formaldehyde for 15 min. Next, fixed colonies were washed with water and stained with crystal violet (1 ml) for 3 min. Finally, the crystal violet dye was washed with water. The number of colonies was counted under a microscope.

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Transwell assay

Cells were seeded into the Transwell chamber (1×10^6/well). Serum-free medium (500 µl) was added to the upper chamber of the Transwell insert, and RPMI-1640 medium (600 µl) containing 20% FBS was added to the lower chamber of the Transwell insert. After culturing at 37°C in a humidified 5% CO_2 incubator for 12–18 h, the inserts were washed twice with PBS. Then, the lower chamber membranes were fixed in 600 µl paraformaldehyde (4%) for 15 min. The inserts were washed again and the lower chamber was left to dry naturally. The number of cells penetrating across membrane was counted under a microscope.

Wound healing assay

Transiently transfected cells in logarithmic growth phase were seeded onto 6-well plates. After transfection for 24 h, the monolayers were wounded with a pipette. After wounding, the detached cells were removed by washing with PBS and cultures was incubated in fresh medium without fetal bovine serum (FBS). Wound closure was monitored by microscopy at 0 h, 24 h, and 48 h. Migration activity was calculated as the mean distance between the edges of 3 points.

Statistical analysis

All statistical analyses were carried out using GraphPad Prism 5.0 and SPSS 22.0 for Windows. The differences between the 2 groups were analyzed using the *t* test. The pairwise comparison between HCC tissues and adjacent tissues was performed with the Wilcoxon test. The chi-square test was used to assess the correlation of miR-32 expression with clinical characteristics. Univariate survival analysis was compared using the Kaplan-Meier method. P<0.05 was considered as statistically significant.

Results

Up-regulation of miR-32 in HCC tissues and cell lines and correlation between its expression and prognosis

To investigate the potential involvement of miR-32 in hepatocellular carcinoma, the expression level of miR-32 was first validated in a panel of 80 HCC tissues and adjacent non-cancerous tissues by qRT-PCR. As Figure 1A shows, miR-32 expression in HCC tissues was significantly higher than that in the corresponding adjacent non-cancerous tissues (p=0.001). Similarly, miR-32 expression was markedly increased in 3 human liver cancer cell lines – SMMC-7721, Huh7, and HepG2 – but no statistically significant difference was found among these cell lines (Figure 1B). These results revealed that the expression of miR-32 was up-regulated in HCC tissues and cell lines.

We found that the prognosis of HCC patients was significantly correlated with ectopic expression of miR-32. The median survival for patients with miR-32 high expression was 18 months and it was 30 months for patients with miR-32 low expression (p=0.044) (Figure 1C). The correlation between miR-32 expression levels and clinical pathological characteristics is demonstrated in Table 1. The expression level of miR-32 was not associated with any specific clinical pathological characteristics, but the prognosis of HCC patients was significantly correlated with ectopic expression of miR-32.

miR-32 promotes HCC cell proliferation, migration and invasion

To selectively over-express or under-express miR-32, mature miR-32-mimics or miR-32-inhibitor were transfected into Huh7 cells. The qRT-PCR method was performed to confirm the increased expression of miR-32 after miR-32-mimics transfection (p=0.027) and decreased expression of miR-32 following miR-32-inhibitor transfection (p=0.020) (Figure 2A). MTS assay showed that over-expression of miR-32 strongly stimulated cell proliferation (48 h, 72 h, and 96 h, all p<0.0001), while cell proliferation was remarkably impaired in miR-32-inhibitor-transfected cells compared with controls (72 h and 96 h, both p<0.0001) (Figure 2B). Over-expression of miR-32 tended to promote colony formation of Huh7 cells, but significantly decreased colony formation of Huh7 cells was found after inhibiting miR-32 expression (p=0.042) (Figure 2C). The effect of miR-32 on the migration of Huh7 cells was estimated by the wound healing assay and Transwell assay, which revealed that over-expression of miR-32 significantly stimulated cell migration (p=0.0002; p=0.0073) and under-expression of miR-32 suppressed cell migration (p<0.0001; p=0.0025) (Figure 2D, 2E). Taken together, our results show that miR-32 promotes the proliferation and migration of HCC cells *in vitro*.

ADAMTS9 suppresses HCC cell growth, migration, and invasion

We further investigated the potential targets of miR-32 by TargetScan software (*http://www.targetscan.org*). Bioinformatics analyses revealed the binding of miR-32 with ADAMTS9 mRNA 3'-UTR (Figure 3A) and predicted that ADATMS9 was a potential target of miR-32. As shown in Figures 3B and 3C, the mRNA and protein expression of ADAMTS9 was significantly down-regulated in miR-32 transfected cells compared with the miR-32-NC group (p=0.0118), whereas it was up-regulated in miR-32-inhibitor transfected group (p<0.0001). We then analyzed the expression and functions of ADAMTS9 in HCC. HCC tissues and corresponding adjacent non-cancerous tissues were examined for expression of ADAMTS9 protein by immunohistochemistry. Expression of ADAMTS9 protein was down-regulated in HCC tissues compared to corresponding adjacent

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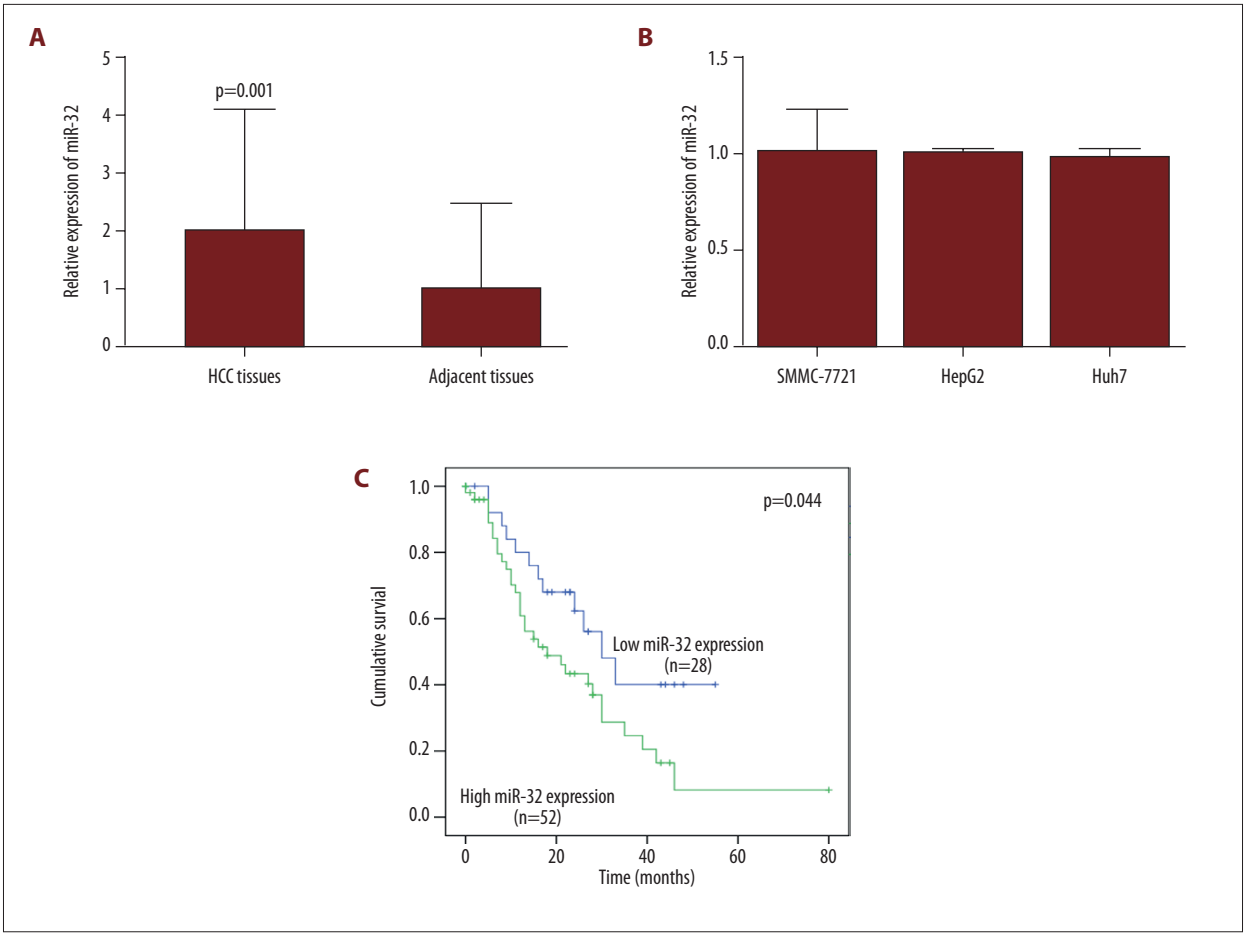

Figure 1. Expression of miR-32 in HCC tissues and cell lines and correlation between its expression and prognosis. (**A**) Expression level of miR-32 in HCC tissues was significantly increased compared to corresponding adjacent non-cancerous tissues. (**B**) Expression of miR-32 was markedly increased in 3 human liver cancer cell lines – SMMC-7721, Huh7, and HepG2 – and no statistically significant difference was found among these cell lines. (**C**) Survival curve for overall survival of HCC patients referring to different expressions of miR-32. Increasing expression of miR-32 in HCC tissues was significantly related to poor outcome.

non-cancerous tissues and in normal liver tissues (32.89% *vs.* 71.05% *vs* 90%, Figure 3D), which suggests that ADAMTS9 suppresses oncogenesis and progression in HCC. P-cDNA3.1-ADAMTS9 was then constructed and transfected into Huh7 cells. Expression of ADAMTS9 protein was determined by Western blotting assay. As Figure 3E shows, the expression of ADAMTS9 protein was significantly increased in cells transfected with p-cDNA3.1-ADAMTS9 as compared with the control cells (p=0.021). MTS assay further confirmed that the viability of proliferation of cells with ADAMTS9 over-expression was decreased compared to that of the control group after 48 h post-transfection in Huh7 cells (48 h, p=0.0044; 72 h, p=0.0409) (Figure 3F). Meanwhile, the cell migration ability of Huh7 cells transfected with p-cDNA3.1-ADAMTS9 was significantly inhibited compared with the control group as shown by wound healing assay (p<0.0001) (Figure 3G). The following Transwell assay demonstrated that over-expression of ADAMTS9 remarkably suppressed the invasion of Huh7 cells (p=0.0198) (Figure 3H). Altogether, these data strongly demonstrate that over-expression of ADAMTS9 inhibits the proliferation, migration, and invasion ability of Huh7 cells.

Up-regulation of ADAMTS9 weakens the effect of miR-32 on HCC cells proliferation, migration, and invasion

To further validate the contribution of ADAMTS9 to biological effects of miR-32, we evaluated the impact of up-regulation of ADAMTS9 on Huh7 cells proliferation, migration, and invasion. Western blotting assay showed that expression of ADAMTS9 was lower in Huh7 cells transfected with miR-32-mimics (p=0.0016), while the expression of ADAMTS9 in Huh7 cells co-transfected with miR-32-mimics and ADAMTS9 was significantly elevated (p=0.0006) (Figure 4A). Subsequently, wound healing assay (p=0.0002) and Transwell assay (p=0.0025)

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Table 1. The relationship between the expression of miR-32 and the clinical pathological characteristics in HCC patients.

Clinical information	Expression of miR-32		P
	Down	Up	
Age (years)			0.082
<60	23	33 (58.9%)	
≥60	5	19 (79.2%)	
Sex			0.809
Male	27	48 (64.0%)	
Female	1	4 (80%)	
Child-Pugh classification			1.000
A	27	50 (64.9%)	
B	1	2 (66.7%)	
Tumor size (cm)			0.457
<5	8	11 (57.9%)	
≥5	20	41 (67.2%)	
AFP (μg/L)			0.622
≤400	14	23 (62.2%)	
>400	14	29 (67.4%)	
Tumor number			0.975
Single	24	43 (64.2%)	
Multiple	4	9 (69.2%)	
Tumor metastasis			0.156
Yes	5	17 (77.3%)	
No	23	35 (60.3%)	
HBsAg-/HCV-Ab-			0.546
Positive	22	45 (67.2%)	
Negative	6	7 (53.8%)	
Clinical stage			0.582
I~II	20	34 (63.0%)	
III~IV	8	18 (62.9%)	

confirmed that over-expression of ADAMTS9 weakened the effects of miR-32 in inducing migration and invasion in Huh7 cells (Figure 4B, 4C), and cell growth was inhibited in cells co-transfected with miR-32-mimics and ADAMTS9 compared with cells transfected with miR-32-mimics (Figure 4D). These results indicate that over-expression of miR-32 is attenuated through the ADAMTS9-mediated signaling pathway, which affects proliferation, migration, and invasion of HCC cells.

miR-32 down-regulates ADAMTS9 expression by directly targeting its 3'-UTR

We further constructed a double-luciferase reporter gene vector. The results showed that the luciferase activity was markedly decreased in the PmiR-ADAMTS9-wt group transfected with miR-32 compared to the group transfected with miR-32-NC; moreover, compared with the PmiR-ADAMTS9-mut group, the luciferase activity was also reduced significantly in the PmiR-ADAMTS9-wt group when both groups were transfected with miR-32 simultaneously (p=0.005) (Figure 5). The dual-luciferase reporter assay revealed that miR-32 directly targets the ADAMTS9 3'-UTR by using 293T cells.

Discussion

Accumulating evidence has demonstrated that miRNAs are not only tumor suppressors, but also act as oncogenes, and play an important role in the tumorigenesis and development of HCC [6]. A number of studies have suggested that miRNAs bind directly to their target genes to regulate cell growth, cell cycle, or apoptosis in HCC. For example, miR-21 expression is increased and can promote the tumorigenesis of HCC by regulating cellular processes by directly targeting PTEN, PDCD4, and TIMP3 [6,23,24]. In addition, miR-99a is markedly down-regulated in HCC tissues and cell lines and acts as a tumor suppressor by inhibiting IGF-1 R and mTOR pathways [25]. Importantly, knocked-down expression of miR-99a in HCC tissues markedly correlated with shorter survival of HCC patients, and miR-99a is an independent predictor for the prognosis of HCC patients [25].

In this study, we focused on miR-32 to investigate its effects on HCC. We first studied the expression of miR-32 in HCC tissues and cell lines. We found that miR-32 was up-regulated in HCC tissues compared to corresponding adjacent non-cancerous tissues. miR-32 expression was also markedly increased in 3 human liver cancer cell lines: SMMC-7721, Huh7, and HepG2. Yan et al. also confirmed that up-regulation of miR-32 expression induces hepatogenesis and progression [13]. As shown in the present study, over-expression of miR-32 strongly stimulated cell proliferation, migration, and invasion at the cytological level, whereas under-expression of miR-32 inhibited the proliferation, migration, and invasion. Importantly, the increased expression of miR-32 in HCC tissues was associated with poor overall survival. These data indicate that the biological function of miR-32 in regulating HCC serves as one of the oncomirs. Dysfunction of miR-32 has been reported in several types of cancers. miR-32 is up-regulated in gastric cancer cells [4], colorectal cancer [9], kidney cancer [26], prostate cancer [27], breast cancer [10], and in multiple myeloma [28], and can aggravate tumorigenesis by regulating proliferation

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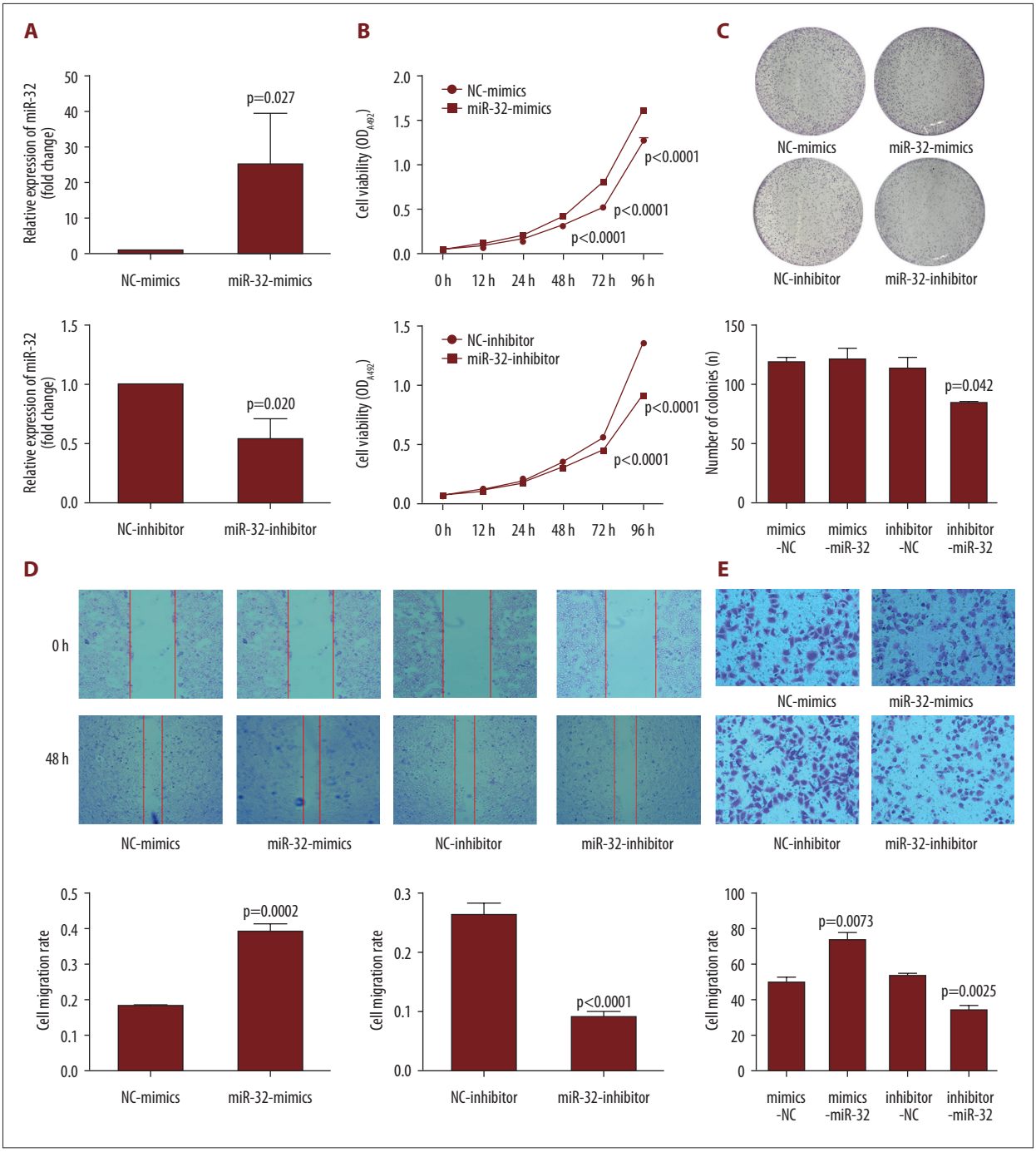

Figure 2. Effects of miR-32 on biological behaviors of Huh7 cells. (**A**) Increased miR – 32 expression in Huh7 cells transfected with miR – 32-mimics and decreased miR – 32 expression in Huh7 cells transfected with miR-32-inhibitor were confirmed by qRT-PCR. (**B**) MTS assay showed that miR-32 strongly stimulated cell proliferation, while cell proliferation was significantly impaired in cells transfected with miR-32 inhibitor compared with controls. (**C**) Clonogenic assay validated effect of miR-32 on cell proliferation. (**D**) Migration and invasion ability of cells in miR32-mimics group was significantly higher as measured by wound healing assay, while in the miR32inhibitor group it was less than that of the negative group. (**E**) Transwell assay showed migration and invasion in Huh7 cells transfected with miR – 32-mimics and in cells transfected with miR-32-inhibitor.

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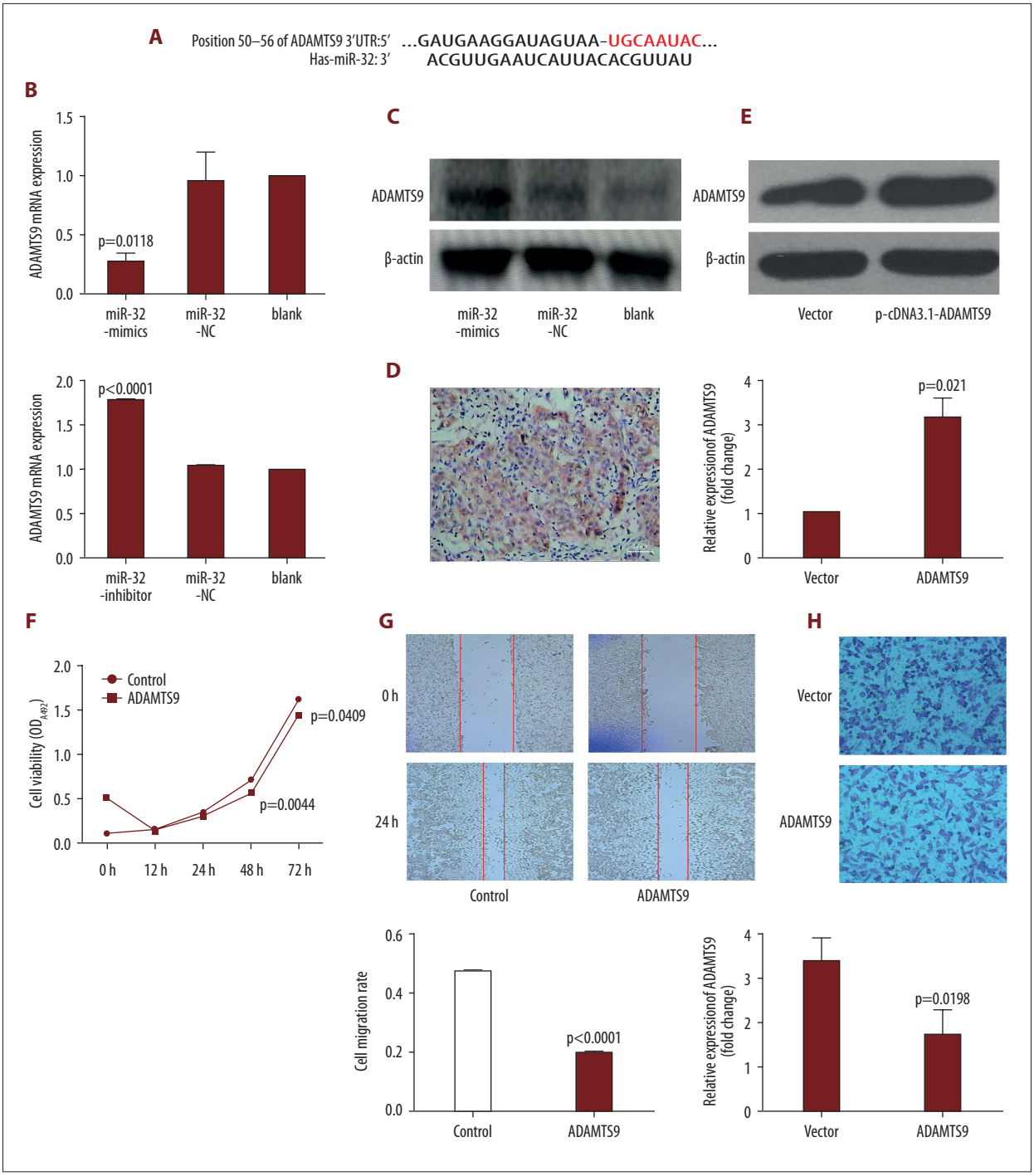

Figure 3. Effect of ADAMTS9 on growth and migration in HCC cells. (**A**) The miR-32 binding site in ADAMTS9 mRNA 3'-UTR predicted by TargetScan. (**B**) The relative expression of ADAMTS9 mRNA was significantly down-regulated in miR-32 transfected cells compared with the miR-32-NC group, whereas it was up-regulated in the miR-32-inhibitor transfected group. (**C**) Western blotting showed that expression of ADAMTS9 protein level was increased in HCC cells transfected with miR-32- inhibitor. (**D**) Immunohistochemical detection of ADAMTS9 expression in hepatocellular carcinoma. Appearance of granular stain in the cytoplasm was defined as positive expression. (**E**) Western blotting showed expression of ADAMTS9 protein was significantly increased in cells transfected with p-cDNA3.1-ADAMTS9. (**F**) Proliferation was inhibited in Huh7 cells transfected with p-cDNA3.1-ADAMTS9 as measured by MTS assay. (**G**) Cell migration and invasion were suppressed in Huh7 cells transfected with p-cDNA3.1-ADAMTS9 as determined by wound healing assay. (**H**) Transwell assay demonstrated that over-expression of ADAMTS9 significantly suppressed the invasion of Huh7 cells.

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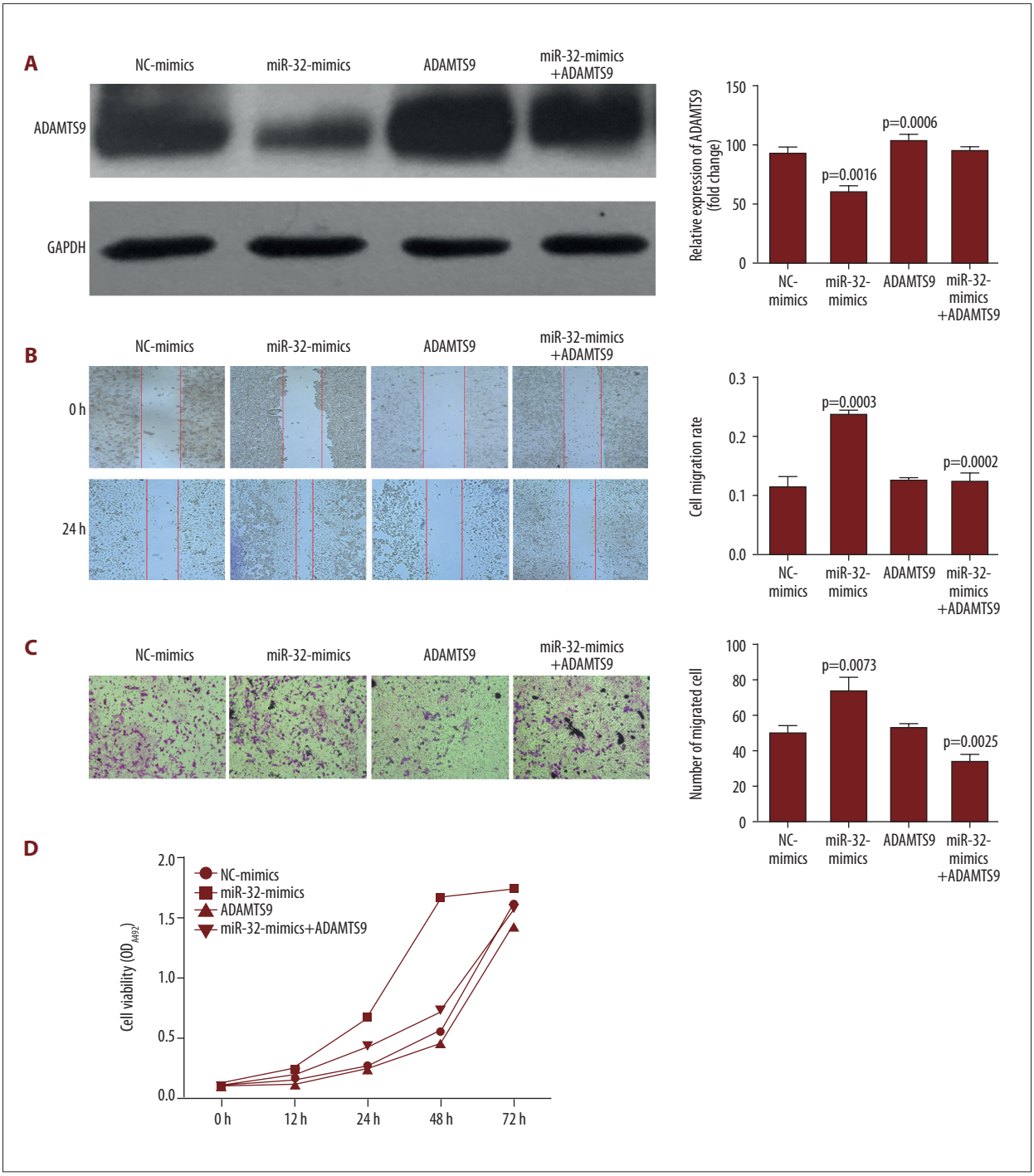

Figure 4. Effect of ADAMTS9 to biological effects of miR-32. (**A**) Expression of ADAMTS9 in Huh7 cells co-transfected with miR-32 and ADAMTS9 was significantly increased compared with cells only transfected with miR-32. (**B, C**) Wound healing and Transwell assays revealed that migration and invasion ability of Huh7 cells co-transfected with miR-32 and ADAMTS9 decreased compared with cells only transfected with miR-32, and was increasing compared with cells only transfected with ADAMTS9. (**D**) MTS assay confirmed that cell proliferation in Huh7 cells co-transfected with miR-32 and ADAMTS9 was inhibited compared with cells only transfected with miR-32, but it was increased in cells only transfected with ADAMTS9.

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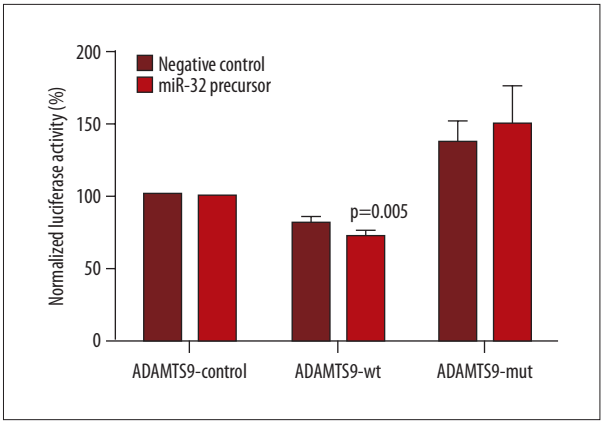

Figure 5. miR-32 targeted 3'-UTR of ADAMTS9. The relative luciferase activity in the PmiR-ADAMTS9-wt group transfected with miR-32 and transfected with miR-32-NC.

and invasion by connecting with corresponding target genes. Thus, we further investigated the potential targets of miR-32 by use of TargetScan software and predicted that ADATMS9 was a potential target of miR-32.

ADAMTS9 is a novel tumor-suppressor gene that plays an important role in the development and progression of tumors. Lo et al. reported that ADAMTS9 contributes an important function that inhibits angiogenesis and tumor growth in esophageal and nasopharyngeal carcinoma tumors [20]. Du et al. reported that ADAMTS9 is a functional tumor suppressor through inhibiting the AKT/mTOR pathway, and hypermethylation of ADAMTS9 associated with poor survival in gastric cancer patients [19]. In the present study, over-expression of ADAMTS9 inhibited proliferation, migration, and invasion in HCC cells. Moreover, expression of ADAMTS9 was decreased in HCC tissues compared to normal liver tissues. These results show that ADAMTS9 can act as tumor suppressor in hepatogenesis and progression. Importantly, miR-32 was found to be up-regulated in HCC tissues, and is accompanied by reduced expression of ADAMTS9. It would be useful to find the probe to use in defining the correlation between miR-32 and ADAMTS9 in HCC.

To further confirm the inverse association between the expressions of miR-32 and ADAMTS9, we transfected the cells *in vitro* with miR-32 and found that expression of ADAMTS9 was decreased and expression of ADAMTS9 was up-regulated in HCC cells co-transfected with miR-32 and ADAMTS9. Moreover, cell growth was inhibited in HCC cell co-transfected with miR-32 and ADAMTS9 compared with cells transfected with miR-32. Wound healing assay and Transwell assay also showed that up-regulation of ADAMTS9 weakened the effects of proliferation, migration, and invasion abilities of miR-32 on HCC cells. These studies suggest an inverse association between miR-32 and ADAMTS9 expressions, and the effects of oncogenic miR-32 could be attenuated through the ADAMTS9-mediated signal pathway. Therefore, we suspect that ADAMTS9 is a possible target of miR-32. The luciferase reporter assay further demonstrated that its down-regulation was mediated by directly binding miR-32 to the 3'-UTR of ADAMTS9, as the alteration of this region abolished this effect. These findings suggest that miR-32 suppresses ADAMTS9 protein expression by directly binding to the 3'-UTR of ADAMTS9 mRNA to regulate HCC cell proliferation and metastasis. Furthermore, *in vivo* research also confirmed that over-expression of miR-32 promoted the growth, migration, and invasion of HCC. Our results provide novel insight into the mechanisms by which miR-32 induces tumorigenesis and metastasis in HCC, at least partly by targeting anti-oncogene ADAMTS9.

Conclusions

In conclusion, miR-32 contributes to down-regulation of ADAMTS9 and regulates the proliferation, migration and invasion abilities of HCC cells. These findings demonstrate that miR-32 may act as an oncogene in hepatocellular carcinoma, partly via directly targeting ADAMTS9. Importantly, the abnormal expression of miR-32 may be correlated with prognosis in patients with HCC. miR-32 may become a potential candidate for treatment of hepatocellular carcinoma.

Conflict of interest

None.

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