Abstract. Hepatocellular carcinoma (HCC) is the fifth-most common cancer and third leading cause of cancer-related deaths worldwide. Increasing evidence indicates that dysregulation of microRNAs is often observed in HCC, and has been extensively investigated in terms of cancer formation, progression, diagnosis, therapy, and prognosis. Recently, microRNA-326 (miR-326) has been demonstrated to play important roles in multiple types of human cancer. However, the expression pattern, clinical significance, roles and regulatory mechanisms of miR-326 in HCC have yet to be elucidated. In this study, miR-326 was frequently downregulated in HCC tissues and cell lines. Low miR-326 expression was significantly associated with the TNM stage, differentiation and lymph node metastasis of HCC patients. Further functional assays demonstrated that the recovered miR-326 expression inhibited HCC cell proliferation and invasion and activated cell apoptosis in vitro. In addition, LIM and SH3 protein 1 (LASP1) was identified as a direct target gene of miR-326 in HCC. Furthermore, LASP1 was upregulated in HCC tissues and cell lines. The expression level of LASP1 mRNA was inversely correlated with that of miR-326 in HCC tissues. Moreover, LASP1 silencing elicited effects similar to miR-326 overexpression on HCC cells, and LASP1 upregulation markedly reversed the effects of miR-326 overexpression on HCC cells. These results revealed that miR-326 suppressed the progression of HCC by directly targeting LASP1. Therefore, miR-326 may be used as a potential therapeutic target for the treatment of patients with HCC.

Introduction

Hepatocellular carcinoma (HCC), which is a dominant histological type of liver cancer, is the fifth-most common cancer and third leading cause of cancer-related deaths worldwide (1). The major risk factors of HCC includes hepatitis B or C virus infection, alcohol abuse, non-alcoholic fatty liver disease, autoimmune-mediated hepatitis, primary biliary cirrhosis and exposure to carcinogens (2). Despite current advancements in diagnostic methods and treatment strategies, the average survival of HCC patients has not improved significantly (3). Tumour progression, high recurrence rate and metastasis are the major causes of cancer-related deaths in patients with HCC (4,5). The majority of HCC patients are diagnosed in late or end stages and thus deprived of optimal treatments (6). Therefore, the molecular mechanisms involved in the formation and progression of HCC should be elucidated and efficient therapeutic targets for patients with this malignancy should be developed.

MicroRNA (miRNAs) are families of short endogenous non-coding RNA molecules of approximately 19-25 nucleotides in length (7). miRNAs have been recognized as pivotal regulators of gene expression by binding to the 3'-untranslated regions (UTRs) of target mRNAs in a base-pairing manner; subsequently, induces translational repression or mRNA degradation (8,9). miRNAs also play essential roles in various biological processes, including cell proliferation, cell cycle, apoptosis, differentiation, angiogenesis and movements (10,11). More than 50% of miRNAs are located in cancer-associated genomic regions or in fragile sites; as such, miRNAs may contribute to the regulation of tumourigenesis and tumour development (12). miRNAs are abnormally expressed in various human cancers, such as breast cancer (13), bladder cancer (14), gastric cancer (15), thyroid cancer (16), and HCC (17). miRNAs upregulated in tumours perform oncogenic functions through the regulation of tumour suppressor genes; by comparison, miRNAs underexpressed in tumours may possess tumour-suppressive roles by directly targeting oncogenes (18,19). Therefore, miRNAs may be considered as promising therapeutic targets and biomarkers.

Recently, miR-326 has been reported to play important roles in multiple types of human cancer (20-23). However, miR-326 in HCC has yet to be described. Therefore, the objective or our study was to elucidate the expression pattern,
clinical significance, roles and the underlying regulatory mechanisms of miR-326 in HCC.

**Materials and methods**

**HCC tissues and cell lines.** Fresh paired HCC tissues and adjacent non-cancerous liver tissues were obtained from 54 HCC patients who underwent surgical resection at Longgang Hospital of Traditional Chinese Medicine (Guangdong, China) from April 2013 to December 2015. None of these patients were treated with radiotherapy or chemotherapy before surgery was performed. Non-cancerous tissue samples were obtained 5-10 cm away from the primary tumour. The tissue specimens were immediately snap-frozen in liquid nitrogen after surgical resection and stored at -80˚C and prior to RNA and protein extraction. This research was approved by the Ethics Committee of Longgang Hospital of Traditional Chinese Medicine, and all of the participants provided a written consent for the application of their tissues and information.

Human HCC cell lines ( HepG2, Hep3B, SMCC-7721 and Huh-7) and the wild-type hepatic cell line LO2 were purchased from Cell Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% Co2 and penicillin (100 U/ml). All cells were maintained at 37˚C in a humidified atmosphere of 5% CO2 in air.

**miRNAs, small interfering RNAs (siRNA), plasmids, and transfection.** miR-326 mimics, scrambled miRNA mimic negative control (miR-NC), siRNA against human LASP1 (LASP1 siRNA) and negative control siRNA (NC siRNA) were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). LASP1 overexpressed plasmid (pcDNA3.1-LASP1) and pcDNA3.1 empty plasmid were obtained from Shanghai Academy of Sciences (Changchun, China). Cells were transfected with miRNAs, siRNAs or plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) coated with Matrigel (BD Biosciences, Inc., San Jose, CA, USA). Transfected cells were trypsinized, collected and suspended in FBS-free DMEM medium. Subsequently, 5x104 cells per well were placed into upper chambers, while the bottom side of chambers was filled with DMEM containing 20% FBS. After 48 h incubation at 37˚C with 5% CO2, cells that had invaded the lower chamber were fixed with 4% paraformaldehyde for 30 min, Cell apoptosis was evaluated using flow cytometry (eBioscience, Inc., San Diego, CA, USA) coated with Matrigel (BD Biosciences, Inc.). After incubation at room temperature in the dark for 2 h, apoptosis was determined using a QuantoCyte Cell Apoptosis Kit (Beckman Coulter, Inc., Brea, CA, USA) coated with Matrigel (BD Biosciences, Inc.). After incubation at room temperature in the dark for 30 min, Annexin V-FITC (Invitrogen) and 10 µl propidium iodide (Invitrogen) were added to each well and incubated at 37˚C with 5% CO2 for another 2 h. Finally, absorbance was examined at a wavelength of 450 nm by using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Cell invasion assay.** Cell invasion ability was determined using transwell chambers (8-µm pores; BD Biosciences, San Jose, CA, USA) coated with Matrigel (BD Biosciences, Inc.). Briefly, transfected cells were trypsinized, collected and suspended in FBS-free DMEM. Subsequently, 10 µl of FBS-free DMEM containing 20% FBS. After 48 h incubation at 37˚C with 5% CO2, cells that had invaded the lower chamber were fixed with 4% paraformaldehyde for 30 min, Cell invasion ability was determined using Transwell chambers (8-µm pores; BD Biosciences, Inc., San Jose, CA, USA) coated with Matrigel (BD Biosciences, Inc.). After incubation at room temperature in the dark for 30 min, Cell apoptosis was evaluated using flow cytometry (Beckman Coulter, Inc., Brea, CA, USA) for 1 h, according to the manufacturer's protocol. This experiment was performed in triplicate and repeated three times.

**miRNA targets prediction.** The potential target genes of miR-326 were predicted using miRanda (http://www.microrna.org/microrna/home.do) and TargetScan (http://www.Targetscan.org/). The predicted target genes supported by both methods were selected for further analysis.

**Luciferase reporter assay.** The 3'UTR of LASP1 containing the predicted miR-326 binding site was amplified through PCR and cloned into psiCHECK2 vector (Promega Corp.) to produce psiCHECK2-LASP1-3'UTR-Wt. psiCHECK2-LASP1-3'UTR-Mut. In luciferase reporter assay, cells were...
co-transfected with luciferase reporter plasmid and miR-326 mimics or miR-NC by using Lipofectamine 2000. After 24 h of incubation, Firefly and *Renilla* luciferase activities were detected with a dual-luciferase reporter assay system (Promega Corp.). *Renilla* luciferase activity was also used for normalization.

**Western blot analysis.** Protein was extracted from tissues or cells using RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). The concentration of total protein was detected using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Equal amounts of protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking in 10% skimmed milk at room temperature for 2 h, the membranes were incubated overnight at 4°C with following primary antibodies: mouse anti-human monoclonal LASP1 antibody (cat. no. sc-374059; 1:1000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-human monoclonal GAPDH antibody (cat. no. sc-69778; 1:1000 dilution; Santa Cruz Biotechnology, Inc.). Following washing three times with Tris-buffered saline with 0.5% Tween-20, the membranes were incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (cat. no. sc-2005; 1:5000 dilution; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Protein bands were visualized using the Pierce™ ECL Western Blotting Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA), and analyzed using the Quantity One® software (Bio-Rad Laboratories, Inc.).

**Statistical analyses.** Data are presented as mean ± standard deviation. Measurement data were analysed via Student’s t-test or one-way ANOVA in SPSS 17.0 (Chicago, IL, USA). The association between miR-326 and the clinicopathological factors of HCC patients was evaluated using Pearson’s χ² test. The correlation between miR-326 and LASP1 mRNA expression was evaluated through Spearman's correlation analysis. P-values <0.05 were considered to indicate statistically significant differences.

**Results**

miR-326 is downregulated in *HCC* tissues and cell lines. To assess the roles of miR-326 in *HCC*, we measured its expression in 54 paired *HCC* tissues and adjacent non-cancerous liver tissues through RT-qPCR. The results revealed that the miR-326 expression was significantly lower in the *HCC* tissues than in the adjacent non-cancerous liver tissues (Fig. 1A, *P*<0.05). This finding was confirmed by determining the miR-326 expression in *HCC* cell lines and wild-type hepatic cell line LO2. We found that the miR-326 expression was lower in the *HCC* cell lines than in LO2 (Fig. 1B, *P*<0.05). Therefore, miR-326 may play essential roles in *HCC* progression.
Low miR-326 expression is correlated with poor HCC phenotype. To investigate the effect of miR-326 on HCC progression, we analysed the association between the low expression of miR-326 and the clinicopathological factors of HCC patients. The HCC patients were divided into two subgroups based on the median miR-326 expression of 0.56: low miR-326 group (<0.56, 28 cases) and high miR-326 group (>0.56, 26 cases). As shown in Table I, the miR-326 expression level was significantly correlated with TNM stage (P=0.025), differentiation (P=0.015) and lymph node metastasis (P=0.014). These results suggested that the decreased miR-326 expression is correlated with poor HCC phenotype.

miR-326 inhibits HCC cell proliferation and invasion and promotes cell apoptosis. We transfected HepG2 and Huh-7 cells with miR-326 mimics or miR-NC to reveal the exact roles of miR-326 in HCC and performed RT-qPCR at 48 h post-transfection to determine the transfection efficiency. Our results indicated that miR-326 was markedly upregulated in HepG2 and Huh-7 cells after the cells were transfected with miR-326 mimics (Fig. 2A, P<0.05). CCK8 assay demonstrated that the proliferation of miR-326 mimic-transfected HepG2 and Huh-7 cells was inhibited, but the proliferation of miR-NC-transfected cells was not affected (Fig. 2B and C, P<0.05). Transwell invasion assay showed that the upregulated miR-326...
expression decreased the number of invasive HepG2 and Huh-7 cells (Fig. 2D, P<0.05). We also conducted flow cytometry assay to investigate whether miR-326 can affect HCC cell apoptosis. As shown in Fig. 2E, the miR-326 overexpression significantly promoted the apoptosis of HepG2 and Huh-7 cells (P<0.05). These results suggested that miR-326 can impair HCC cell proliferation and invasion and promote apoptosis.

miR-326 directly targets LASP1 in HCC. miRanda and TargetScan were used to search for the possible targets of miR-326 and to analyse the regulatory mechanism of miR-326 in HCC. Among these potential target genes, LASP1 was selected as our target for further analysis (Fig. 3A) because it was upregulated in HCC and contributed to HCC formation.
To determine whether LASP1 is a direct target of miR-326, we co-transfected psiCHECK2-LASP1-3'-UTR-Wt or psiCHECK2-LASP1-3'-UTR-Mut into HepG2 and Huh-7 cells with miR-326 mimics or miR-NC. As shown in Fig. 3B, the recovered miR-326 expression repressed the luciferase activity of psiCHECK2-LASP1-3'-UTR-Wt in both HepG2 and Huh-7 cells (P<0.05), whereas the mutated 3'-UTR of LASP1 showed no change in its luciferase activity. RT-qPCR and western blot analysis were subsequently performed to examine the influence of miR-326 on LASP1 expression. We found that the expression of LASP1 was downregulated at mRNA (Fig. 3C, P<0.05) and protein levels (Fig. 3D, P<0.05) in HepG2 and Huh-7 cells after these cells were transfected with miR-326 mimics. Therefore, LASP1 is a direct target of miR-326 in HCC.

miR-326 is inversely correlated with LASP1 in HCC. To determine the relationship between miR-326 and LASP1 in HCC, we evaluated the expression pattern of LASP1 in HCC tissues and adjacent non-cancerous liver tissues through RT-PCR and western blot analysis. The results indicated that the mRNA and protein levels of LASP1 were higher in HCC tissues than in adjacent non-cancerous liver tissues (Fig. 4A and B, P<0.05). The LASP1 expression was also higher in HCC cell lines than in LO2 (Fig. 4C, P<0.05). Spearman’s correlation analysis revealed that the mRNA levels of LASP1 were negatively correlated with the miR-326 expression in HCC tissues (Fig. 4D; r= -0.6854, P<0.0001). These findings suggested that the upregulation of LASP1 may be caused by miR-326 downregulation in HCC.

LASP1 downregulation imitates the effects of transfection with miR-326 mimics in HCC cells. Considering that LASP1 was identified as a direct target of miR-326 in HCC, we investigated whether LASP1 mediates the roles of miR-326 in HCC by transfecting HepG2 and Huh-7 cells with LASP1...
siRNA or NC siRNA. Western blot results indicated that the siRNA-mediated LASP1 knockdown in Hep g2 and Huh-7 cells was efficient (Fig. 5A, P<0.05), and the siRNA-mediated LASP1 downregulation could inhibit cell proliferation and invasion and promote apoptosis in HepG2 and Huh-7 cells (Fig. 5B-e, P<0.05). These results further confirmed that

Figure 6. LASP1 overexpression abrogates the effects of miR-326 mimics on HCC cell proliferation, invasion and apoptosis. (A) Western blot analysis of LASP1 proteins in Hepg2 and Huh-7 cells transfected with pcDNA3.1, pcDNA3.1-LASP1 or pcDNA3.1-LASP1+miR-326 mimics. (B and C) Induced LASP1 expression could partially rescue the suppressive effects of miR-326 on Hepg2 and Huh-7 cell proliferation. (D) Inhibition of cell invasion by miR-326 mimics in Hepg2 and Huh-7 cells were markedly reversed by LASP1 overexpression. LASP1 overexpression markedly blocked the effect of miR-326 on apoptosis of (E) HepG2 and (F) Huh-7 cells. *P<0.05 compared with the respective control.
miR-326 plays tumour-suppressing roles in HCC partly by inhibiting LASP1 expression.

**LASP1 overexpression reverses the suppressive effects of miR-326 on HCC cells.** Reverse experiments were performed to determine whether LASP1 is involved in miR-326-mediated tumour-suppressing effects on cell proliferation, invasion and apoptosis. To clarify this speculation, we further transfected the miR-326 mimic-transfected HepG2 and Huh-7 cells with pcDNA3.1-LASP1 plasmid and restored their expression. Western blot analysis demonstrated that LASP1 was significantly upregulated in HepG2 and Huh-7 cells transfected with pcDNA3.1-LASP1 (Fig. 6A, P<0.05). Additionally, pcDNA3.1-LASP1 abolished the inhibited LASP1 expression caused by the miR-326 mimics in HepG2 and Huh-7 cells. The recovered LASP1 expression significantly rescued the inhibitory effects of miR-326 on HCC cell proliferation (Fig. 6B and C, P<0.05) and invasion (Fig. 6D, P<0.05). The LASP1 upregulation also markedly blocked the effect of miR-326 on cell apoptosis in HepG2 and Huh-7 cells (Fig. 6E and F, P<0.05). These results suggested that miR-326 exerted tumour-suppressive roles in HCC, at least in part, by suppressing LASP1.

**Discussion**

HCC, the most common subtype of liver cancer, is characterised by uncontrolled growth, metastatic dissemination, recurrence and drug resistance and thus considered as one of the most deadly forms of cancers (27-29). Therefore, the molecular mechanisms underlying tumorigenesis and progression of HCC should be elucidated to develop novel therapeutic methods and to improve the survival rate of patients with this malignancy. Accumulating evidence revealed that dysregulation of miRNAs is often observed in HCC, and is recently extensively investigated in terms of cancer formation, progression, diagnosis, therapy and prognosis (30-32). As such, cancer-specific miRNAs and their corresponding direct target genes essential for HCC carcinogenesis and progression should be identified to help produce promising therapeutic targets for patients with this disease.

In this study, we found that miR-326 expression was lower in HCC tissues and cell lines than in adjacent non-cancerous liver tissues and wild-type hepatic cell line LO2. miR-326 was also closely related to TNM stage, differentiation and lymph node metastasis of patients with HCC, reflecting a trend of poor prognosis. Functional assays revealed that the recovered miR-326 expression inhibited HCC cell proliferation and invasion and induced apoptosis in vitro. Hence, miR-326 played tumour-suppressing roles in HCC development. Moreover, the underlying mechanisms of miR-326 inhibiting HCC progression was investigated, and the results revealed that LASP1 was a direct and functional target of miR-326 in HCC.

miR-326 is dysregulated in human tumour subtypes. For example, miR-326 expression is decreased in glioma tissues and cell lines (33,34). Low miR-326 expression is strongly correlated with advanced pathological grade and low KPS score (35). Li et al reported that miR-326 is downregulated in gastric cancer and cell lines (36,37). miR-326 downregulation is correlated with clinical stage, tumour depth, lymph node metastasis and distant metastasis. Survival analysis indicated that miR-326 is a poor independent prognostic factor for patients with gastric cancer (36). In another study, miR-326 was decreased in osteosarcoma, and low miR-326 expression is associated with distant metastasis and advanced clinical stage. Osteosarcoma patients with reduced miR-326 expression levels likely experience a shorter survival period (38). miR-326 is also downregulated in non-small cell lung cancer (20), colorectal cancer (21), pancreatic cancer (22) and breast cancer (23). These findings suggested that the low expression pattern of miR-326 may be universal and thus a potential prognostic factor.

Abnormal miR-326 expression is involved in the malignant phenotype of cancer. In glioma, ectopic miR-326 expression suppresses cell proliferation, colony formation, invasion and metabolic activity, reduces ATP and glutathione levels, induces apoptosis, causes cell cycle arrest at the G1 phase and improves the chemosensitivity of cells to curcumin (33,34,39,40). In gastric cancer, miR-326 overexpression represses cell growth, migration and invasion (36,37). In non-small cell lung cancer, the induced miR-326 expression attenuates cell proliferation, colony formation, migration, and invasion, reverses chemoresistance, enhances cell apoptosis in vitro and decreases tumour growth in vivo (20,41-43). In osteosarcoma, the recovered miR-326 expression inhibits cell growth and metastasis (38). In colorectal cancer, miR-326 overexpression decreases cell proliferation and motility, increases cell apoptosis and promotes cell cycle arrest (21). The miR-326 upregulation sensitizes the chemosensitivity of breast cancer cells to VP-16 and doxorubicin (23). These findings suggested the fundamental role of miR-326 in tumourigenesis and development of malignant tumours and illustrate the potential of this miRNA as a therapeutic target in these types of cancer.

To further understand the underlying mechanisms of miR-326, its downstream functional targets need to be identified. Previous studies identified several miR-326 target genes, including NOB1 (33), PKM2 (34) and SMO (40) in glioma; FSCN1 (36) in gastric cancer; NSBP1 (41), CCND1 (20), Phox2a (42), ADAM17 (44) and SP1 (43) in non-small cell lung cancer; and MRP-1 (23) in breast cancer. In our study, an important molecular link between miR-326 and LASP1 in HCC was determined. Online bioinformatics analysis predicted that LASP1 was a potential miR-326 target gene. Luciferase reporter assay confirmed that miR-326 directly targeted the 3'UTR of LASP1. RT-qPCR and western blotting further showed that the miR-326 upregulation decreased the mRNA and protein expression levels of LASP1 in HCC cells. LASP1 was also upregulated in HCC tissues and negatively related to miR-326 expression level. LASP1 silencing elicited effects similar to miR-326 overexpression in HCC cells, and rescue experiments demonstrated that the LASP1 upregulation reversed the effects of miR-326 on HCC cells. These results suggested that miR-326 acted as a tumour suppressor in HCC partly by directly targeting LASP1. Hence, the direct targets of miR-326 should be identified to understand its role in HCC and to develop novel therapeutic targets.

LASP1, a member of LIM proteins and nebulin family of actin-binding proteins, was initially derived from a cDNA library of metastatic axillary lymph node from human breast cancer (45). LASP1 protein comprises three domains: an N-terminal LIM domain, a nebulin repeat domain and a...
C-terminal SH3 domain (46). The LASP1 overexpression has been implicated in several human cancers, including breast cancer (47), oesophageal squamous cell carcinoma (48), colorectal cancer (49), pancreatic cancer (50) and prostate cancer (51). LASP1 enhanced cell proliferation, migration, invasion and cell cycle progression in several types of cancer (52-54). In HCC, LASP1 is upregulated to a higher extent in tumour tissues than in adjacent non-tumourous tissues. The LASP1 expression levels are associated with hepatitis B surface antigen and serum α-fetoprotein level of HCC patients. The LASP1 expression levels are associated with hepatitis B extent in tumour tissues than in adjacent non-tumourous tissues.

Multivariate survival analysis has also indicated that LASP1 is an independent prognostic factor of patient survival (26,55). Functional assays have demonstrated that LASP1 promotes HCC cell proliferation and migration and yields aggressive phenotypes (26). Therefore, LASP1 should be investigated as a potential target to inhibit HCC.

In conclusion, our current study found that miR-326 was significantly downregulated in HCC and was correlated with an aggressive tumour phenotype. miR-326 targeted LASP1 to inhibit cell proliferation and invasion and to promote apoptosis in vitro. These results suggested that miR-326 is a prognostic biomarker and miR-326/LASP1 axis is a potential therapeutic target in patients with HCC.

References


