



MicroRNA-101 suppresses SOX9-dependent tumorigenicity and promotes favorable prognosis of human hepatocellular carcinoma

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ABSTRACT

We previously showed that high expression levels of SOX9 correlate with hepatocellular carcinoma (HCC) progression. However, the exact role that SOX9 plays in HCC remains unclear. In this study, we firstly confirmed that miRNA-101 directly targets SOX9 in HCC. Ectopic expression of miR-101 significantly inhibited HCC cell proliferation and tumorigenicity by targeting SOX9. Moreover, the down-regulation of miR-101 in clinical HCC tissues correlates with tumor aggressiveness and poor prognosis. Therefore, miR-101 may suppress HCC tumor progression by down-regulating SOX9. MiR-101 may be a potential prognostic marker and therapeutic target for HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent malignancies and the third leading cause of cancer-related deaths worldwide [1]. The distribution of HCC is unbalanced throughout the world, with the highest incidence in Asia and Sub-Saharan Africa, especially in China. As a notoriously aggressive solid tumor, HCC is characterized by fast infiltrating growth, early metastasis, high-grade malignancy, and poor prognosis. This cancer often develops as a consequence of underlying liver diseases such as hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, and is almost associated with cirrhosis [2]. At present, surgical hepatic resection and liver transplantation are the only curative treatment modality to confer survival benefit in HCC patients. However, the overall 5-year survival rate for HCC patients is still only 5% [3]. Therefore, it is necessary to elucidate the molecular mechanisms underlying HCC progression and identify novel therapeutic targets in order to improve the clinical outcome of patients with this cancer.

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MicroRNAs (miRNAs), a class of genes encoding small RNA molecules (19–22 nucleotides), play important roles in regulating protein expression by inhibiting translation or inducing mRNA degradation by binding to the 3'-untranslational region (3'-UTR) of target mRNAs [4]. Beyond the involvement in diverse physiological and pathological processes, the increasing evidences have well demonstrated that deregulation or dysfunction of miRNAs can contribute to carcinogenesis and cancer development [5]. The roles of miRNAs in different cancers may be dependent on cancer type [6]. Some miRNAs may act as oncogenes overexpressing in cancers and contributing to the transformed phenotypes. These oncogenic miRNAs function by suppressing tumor suppressor genes. Other miRNAs may act as tumor suppressors down-regulating in cancers and allowing the expression of oncogenes. As far as HCC is concerned, accumulating evidence indicates that deregulation of miRNAs can contribute to HCC development by influencing cell growth, apoptosis, migration, or invasion. Aberrant miRNA expression has also been demonstrated to associate with clinical features of HCC, such as stage, differentiation, prognosis, and response to adjuvant therapy [7,8]. Therefore, it is necessary to identify more miRNAs as prognosis predictor or therapeutic target for this cancer.

The transcription factor SOX9 (sex determining region Y [SRY] related high-mobility group box 9) is a member of the SRY (sex

determining region Y) box gene superfamily [9]. It is essential in sex determination, chondrogenesis, respiratory epithelium development, melanocyte differentiation, and the differentiation of Paneth cells in the gut [10]. Recent studies have detected the expression of SOX9 in multiple tissues during embryogenesis, including cartilage, neural crest, notochord, kidney, pancreas, and endocardial cushions of the heart [11]. Besides these findings, SOX9 has also been demonstrated to play a role in a variety of malignancies. For example, Wang et al. [12] detected the expression of SOX9 in prostate cancer cells contributes to tumor growth and invasion; Aleman et al. [13] found that SOX9 hypermethylation in primary bladder tumours was present more than half of the cases and was significantly associated with tumour grade and overall survival; Malki et al. [14] shown that the embryonic male prostaglandin D synthase/SOX9 pathway was expressed at both the RNA and protein levels in different types of human ovarian tumors, pointing to SOX9 as a possible diagnostic marker for ovarian carcinomas. Especially, the previous study of our group detected the up-regulation of SOX9 at mRNA and protein levels in clinical HCC tissues compared with their adjacent non-neoplastic tissues [15]. Our data also indicated that high SOX9 expression is associated with advanced tumor progression and poor clinical outcome of HCC patients [15]. On the basis of these findings, the aim of the present study was to elucidate the mechanism of SOX9 plays in HCC.

2. Materials and methods

2.1. Cell culture

Human HCC cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA, USA) and was cultured in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibico, USA), 2 mM L-glutamine and antibiotics. Normal human liver cell line HL-7702 was obtained from the American Type Culture Collection (Manassas, VA, USA) and was maintained in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibico, USA). Two cell lines were both maintained at 37 °C in a humidified chamber supplemented with 5% CO₂.

2.2. Patients and tissue samples

The study was approved by the Research Ethics Committee of 302nd Hospital of PLA, Beijing, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

A total of 130 self-pairs of HCC specimens and adjacent non-neoplastic liver tissues were snap-frozen in liquid nitrogen and stored at –80 °C following surgery for qRT-PCR assay. All the tissues were obtained from 130 patients with primary HCC who underwent a curative liver resection at the 302nd Hospital of PLA, Beijing, China. These patients were diagnosed as HCC between 2001 and 2006. None of the patients recruited in this study had chemotherapy or radiotherapy before the surgery. HCC diagnosis was based on World Health Organization (WHO) criteria. Tumor differentiation was defined according to the Edmondson grading system. Liver function was assessed using the Child-Pugh scoring system. Tumor staging was determined according to the sixth edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer. The clinicopathological features of 130 patients are summarized in Table 1.

The median follow-up period was 8.6 years. Postoperative surveillance included routine clinical and laboratory examinations every third month, computed tomography scans of the abdomen, and radiographs of the chest every third month. After 5 years, the examination interval was extended to 12 months.

Table 1

Clinicopathological features and the expression of miRNA-101 in 130 hepatocellular carcinoma patients.

Clinicopathological features	Case	MiRNA-101 expression frequency (n, %)		P
		High	Low	
<i>Age (years)</i>				
≤50	72	33 (45.83)	39 (54.17)	NS
>50	58	22 (37.93)	36 (62.07)	
<i>Gender</i>				
Male	96	40 (41.67)	56 (58.33)	NS
Female	34	15 (44.12)	19 (55.88)	
<i>Tumor stage</i>				
T1	23	23 (100.00)	0 (0)	0.02
T2	40	20 (50.00)	20 (50.00)	
T3	52	12 (23.08)	40 (76.92)	
T4	15	0 (0)	15 (100.00)	
<i>Tumor grade</i>				
G1	31	31 (100.00)	0 (0)	0.008
G2	76	24 (31.58)	52 (68.42)	
G3	23	0 (0)	23 (100.00)	
<i>Growth pattern</i>				
Trabecular	101	44 (43.56)	57 (56.44)	NS
Non-trabecular	29	11 (37.93)	18 (62.07)	
<i>Cirrhosis</i>				
Yes	86	36 (41.87)	50 (58.13)	NS
No	44	19 (43.18)	25 (56.82)	
<i>Underlying liver disease</i>				
Alcoholic	25	11 (44.00)	14 (56.00)	NS
Hepatitis B	49	20 (40.82)	29 (59.18)	
Hepatitis C	35	15 (42.86)	20 (57.14)	
Unknown	21	9 (42.86)	12 (57.14)	

2.3. Target prediction

An online program Target-Scan (release human 6.2) was used for predicting miRNAs that might target SOX9.

2.4. EGFP-miR-101 expression vector and cellular transfection

We obtained the commercial Pre-miR-101 expression vector (human pMIR101-1PA-1) with the following sequence: 5'-TGC CCT GGC TCA GTT ATC ACA GTG CTG ATG CTG TCT ATT CTA AAG GTA CAG TAC TGT GAT AAC TGA AGG ATG GCA-3', Pre-miR-145 expression vector as a positive control with the following sequence: 5'-CAC CUU GUC CUC ACG GUC CAG UUU UCC CAG GAA UCC CUU AGA UGC UAA GAU GGG GAU UCC UGG AAA UAC UGU UCU UGA GGU CAU GGU U-3', and the same negative-vector as a negative control from System Bioscience (Mountain View, CA). A fluorescent marker (GFP) is also present to monitor cells that are positive for transfection. HepG2 cells were transfected through Fugene transfecting agents (Roche) with hsa-miR-101 vector, hsa-miR-145 vector (positive control), and negative control (NC) following the manufacturer's instructions, respectively.

2.5. qRT-PCR

The qRT-PCR analysis for miRNA was performed according to the similar protocol of the previous studies. Briefly, total RNA, including miRNA, was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After that, the total RNA was reversely transcribed using the corresponding RT Primer and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). U6 small RNA was used as an internal control for normalization and quantification of the target miRNA expression. The specificity of amplification was confirmed by melting curve analysis and also by running PCR products on agarose gels

(3%). Relative quantification of target miRNA expression was evaluated using the comparative cycle threshold (CT) method. All experiments were done in triplicate. Mean normalized gene expression \pm S.E. was calculated from independent experiments.

2.6. RNA interference and cellular transfection

HepG2 cells were seeded into 6-well plates and incubated overnight, and then transfected using Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. The siRNAs against SOX9 (siSOX9_2) and the control siRNA were synthesized by QIAGEN China Co., Shanghai, China. For every 10^5 cells, 0.5 μ g SOX9 siRNA or control siRNA was diluted and mixed with 3 μ l transfection reagent. After mixing and incubation for 30 min, the transfection mixture was added to the cells. After 6 h, medium was changed to growth medium. HepG2 cells were used for functional assay 24 h after transfection.

2.7. Western blot

The Western blot protocol and semiquantitative analysis were carried out following the protocol of our previous study. SOX9 antibody (rabbit polyclonal antibody, dilution 1:50, Santa Cruz Biotechnology Inc., USA) was used, and GAPDH antibody (CW0266, dilution 1:1,000, CoWin Biotech) was used as internal control. All experiments were done in triplicate. Mean normalized gene expression \pm S.E. was calculated from independent experiments.

2.8. Luciferase reporter assay

The miR-101-targeted gene was evaluated by using a luciferase reporter assay in HepG2 cells following the protocol of the previous studies. Briefly, the human SOX9 3'-UTR luciferase reporter construct was generated by cloning SOX9 mRNA 3'-UTR sequence into the 3'-UTR region of the pGL3 luciferase reporter vector (Promega Corporation, Madison WI, USA). The miR-101 target site-deleted SOX9 3'-UTR luciferase reporter construct was generated by PCR fragments of SOX9 3'-UTR luciferase reporter construct lacking the target site and ligated. HepG2 cells were cultivated in 24-well plates and co-transfected using Fugene (Roche) with 100 ng of pGL3-SOX9-miR-101 constructs, 10 ng miR-101 mimic or NC mimic, and 2 ng pRL-SV40 RLuciferase vector (Promega). The luciferase activity assay was performed 24 h after transfection using the dual-luciferase reporter assay system (Promega Corporation, Madison WI) according to the manufacturer's instructions.

2.9. In vitro cell proliferation assay

The in vitro cell proliferation of HepG2 cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method following the protocol of the previous studies. Briefly, cells were seeded into 96-well plates and transfected. At 48 h after transfection, 20 μ l MTT solution (Sigma, USA) was added into the culture medium for 4 h incubation. Then, the medium was replaced by 0.1 ml of DMSO (Sigma, St. Louis, MO, USA) and plates were shaken at room temperature for 10 min. The absorbance of each sample was recorded at 490 nm. All experiments were done in triplicate. Mean normalized gene expression \pm S.E. was calculated from independent experiments.

2.10. In vitro invasion and migration assay

Cell invasion and migration were respectively analyzed by Matrigel coated and uncoated transwell cell culture chambers (8 μ m pore size, Millipore, Billerica, MA, USA). Briefly, 48 h after transfection, cells were resuspended in serum-free medium, and

200 μ l of the cell suspension (4×10^4 cells) was seeded into the upper chambers. Medium with 5% FBS was added into the lower chambers as a chemoattractant. After 40 h of incubation, the cells that did not migrate or invade after 24 h were removed from the upper face of the filters by scrubbing with a cotton swab, after which the membrane was fixed with 4% formaldehyde for 10 min at room temperature and stained with 0.5% crystal violet for 10 min. Finally, the migratory and invasive cells were manually counted at 200 \times magnification from ten different fields of each filter. All experiments were done in triplicate. Mean normalized gene expression \pm S.E. was calculated from independent experiments.

2.11. Statistical analysis

The software of SPSS version 13.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. Continuous variables were expressed as $\bar{X} \pm s$. Fisher's exact test and the χ^2 test were performed to assess associations between miR-101 expression and clinicopathological parameters. The Kaplan–Meier method was used for survival analysis, and differences in survival were estimated using the log-rank test. A multivariate survival analysis was performed for all parameters that were significant in the univariate analyses using the Cox regression model. Differences were considered statistically significant when P was less than 0.05.

3. Results

3.1. MiRNA-101 directly targets SOX9 in HCC tissues

Our previous study performed by qRT-PCR, immunohistochemistry, and Western blot has demonstrated that SOX9 is up-regulated in human HCC tissues at both mRNA and protein levels [15]. In the present study, using miRNA target predicting program (TargetScan human 6.2), there was only one miRNA–miR-101 which is hypothesized as a candidate miRNA that targets SOX9 (Fig. 1A), but other known direct regulators of SOX9, such as miR-145 [16,17], were not predicted. Different from other approaches, TargetScan requires perfect complementarity to the seed region of a miRNA and then extends these regions to unravel complementarity outside the region [18]. Thus, it features an efficient reduction in the false-positive rate but, because of its required strict complementarity in the seed region, loosely conserved targets and those containing wobble pairings are more likely to be missed [19].

Then, we performed qRT-PCR, transient transfection of the HCC cell line HepG2 with miR-101 vector plasmid, and luciferase reporter assay to test our hypothesis. In order to validate the specificity of this test system, hsa-miR-145 vector was used as the positive control because it has been demonstrated to be a direct regulator of SOX9 by previous studies [16,17]. According to the results of qRT-PCR analysis, miR-101 and miR-145 (positive control) expression in HCC tissues were both significantly lower than those in adjacent non-neoplastic liver tissues [for miR-101: HCC tissues vs. normal liver tissues: 0.87 ± 0.45 vs. 1.74 ± 0.78 , $P < 0.001$, Fig. 1B; for miR-145: HCC tissues vs. normal liver tissues: 0.82 ± 0.39 vs. 1.79 ± 0.62 , $P < 0.001$, Fig. 1C].

To verify our predicted target of hsa-miR-101, we transfected HepG2 cells with hsa-miR-101 vector, hsa-miR-145 vector (positive control), negative vector (NC), and blank control culture medium (mock). Western blot analysis showed that, at 24 h post-transfection, the overexpressions of hsa-miR-101 and hsa-miR-145 both resulted in a significant decrease of endogenous SOX9 protein levels, compared with cells transformed with NC or mock (HepG2 cells transfected with hsa-miR-101 vector vs. those

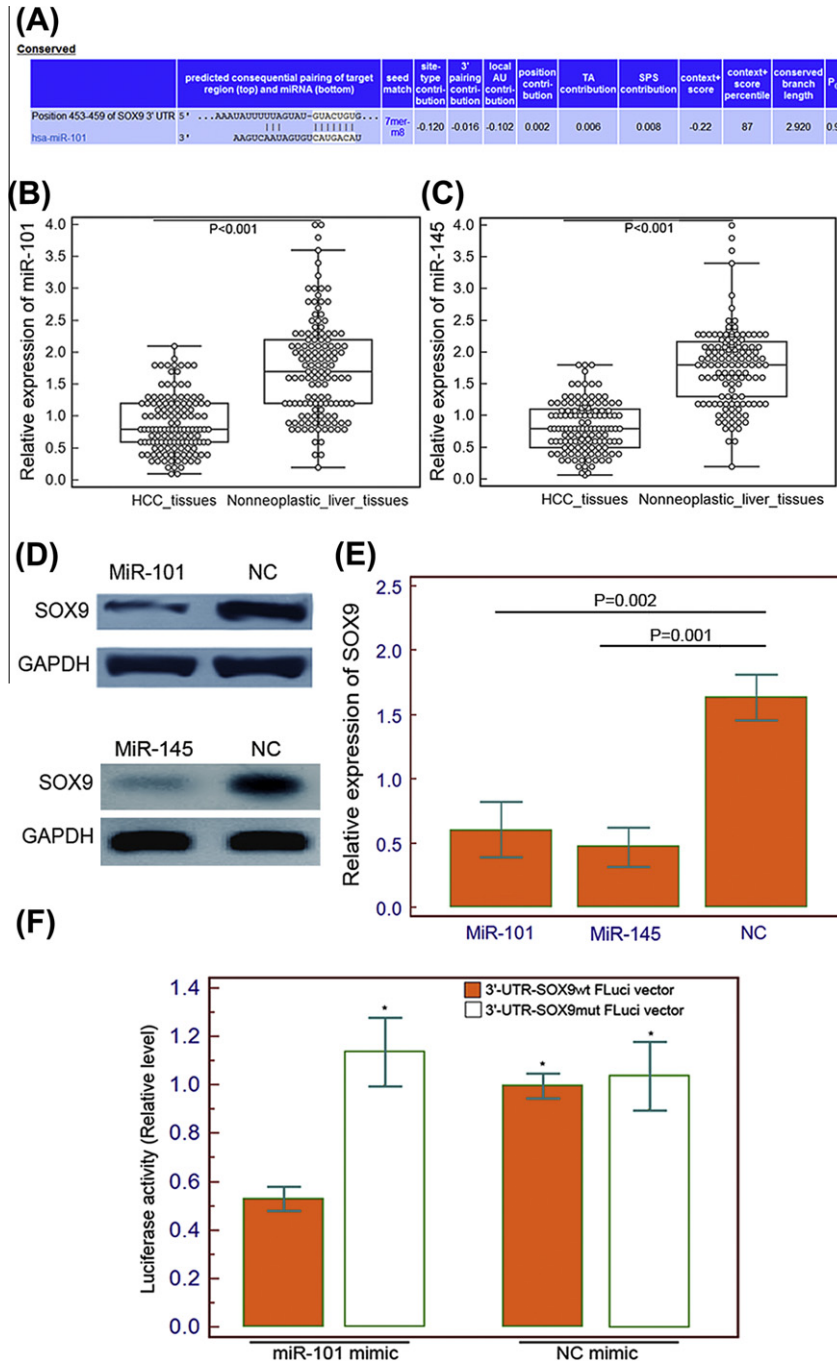


Fig. 1. MiRNA-101 (miR-101) directly targets SOX9 in hepatocellular carcinoma (HCC) tissues. (A) MiR-101 is predicted to target SOX9. (B) and (C) MiR-101 and miR-145 are both downregulated in human HCC tissues detected by qRT-PCR analysis. (D) and (E) The relative expression levels of SOX9 protein in HepG2 cells detected by Western blot analysis at 24 h post-transfection of miR-101 and miR-145 vectors, respectively. GAPDH was used as an internal loading control. Negative control vector (NC). (F) Luciferase report assay was performed to confirm the miR-101 binding target. The luciferase activity was detected after co-transfection of FLuci vector (3'-UTR-SOX9wt FLuci vector or 3'-UTR-SOX9mut FLuci vector), miR-101 mimic or negative mimic into HepG2 cells. * $P < 0.001$, comparison with HepG2 cells co-transfected with 3'-UTR-SOX9wt FLuci vector and miR-101 mimic.

with negative vector: 0.60 ± 0.20 vs. 1.63 ± 0.15 , $P = 0.002$; HepG2 cells transfected with hsa-miR-145 vector vs. those with negative vector: 0.49 ± 0.07 vs. 1.63 ± 0.15 , $P = 0.001$; Fig. 1D and E). As well as the over-expression of miR-101, SOX9 knockdown by SOX9 siRNA markedly reduced the endogenous SOX9 protein levels of HepG2 cells (Fig. 2).

Furthermore, to verify whether SOX9 was a direct target of miR-101, the luciferase reporter assay was performed by co-transfection of miR-101 and a luciferase reporter plasmid containing the

3' UTR of human SOX9. Luciferase activity was detected at 48 h after the co-transfection of FLuci vector (3'-UTR-SOX9wt FLuci vector or 3'-UTR-SOX9mut FLuci vector), miR-101 mimic or NC mimic, and RLuci vector in HepG2 cells. As shown in Fig. 1E, the luciferase activity was decreased to 0.53 in HepG2 cells co-transfected with 3'-UTR-SOX9wt FLuci vector and miR-101 mimic compared with 3'-UTR-SOX9mut FLuci vector and NC mimic (Fig. 1F).

Taken together, these observations suggested that miR-101 directly target SOX9.

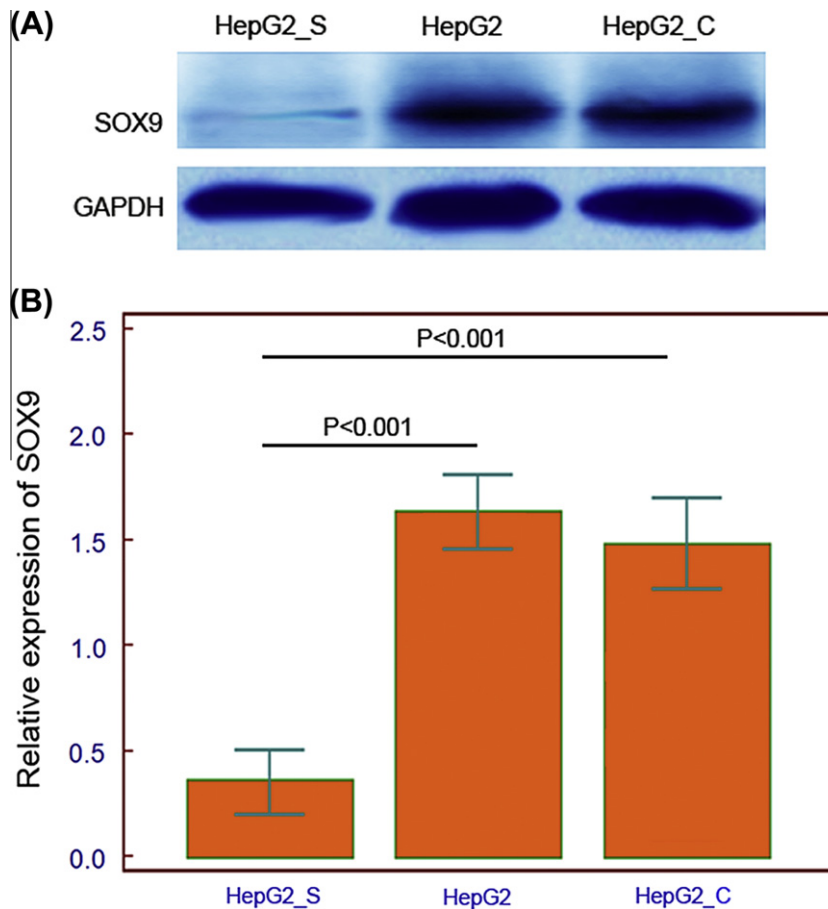


Fig. 2. Down-regulation of SOX9 by siRNA interference. Western blot analysis was performed to detect the expression levels of SOX9 protein in SOX9 siRNA-transfected (HepG2-S), control siRNA-transfected (HepG2-C) and non-transfected (HepG2) cells. GAPDH was used as an internal loading control.

3.2. MiR-101 inhibits cell proliferation of human HCC cells in vitro by targeting SOX9

The down-regulation of miR-101 in HCC tissues comparing with adjacent non-neoplastic liver tissues prompted us to determine whether miR-101 functions as a tumor suppressor. Compared with normal human liver cell line HL-7702, the expression level of miR-101 in HCC cell line HepG2 was markedly decreased (HepG2 vs. HL-7702: 2.17 ± 0.31 vs. 3.20 ± 0.36 , $P = 0.001$; Fig. 3A). In line with the results of miR-145 (positive control, $P = 0.02$), the enforced expression of miR-101 significantly inhibited cell proliferation of HepG2 cells ($P = 0.02$; Fig. 3B). Meanwhile, HepG2 cells expressing SOX9 siRNA showed significant decrease in cell proliferation, compared to HepG2 cells transfected with control siRNA ($P = 0.01$, Fig. 3C). To further verify whether miR-101 inhibited cell proliferation of HCC cell line through SOX9, we made use of HCC cell line HepG2 cells in which SOX9 was knocked down by SOX9 siRNA transfection. As shown in Fig. 3D and E, the enforced expression of miR-101 significantly inhibited cell proliferation of HepG2 cells transfected with control siRNA ($P = 0.02$, Fig. 3D) but failed to do so in HepG2 cells transfected with SOX9 siRNA ($P > 0.05$, Fig. 3E). Therefore, miR-101 may inhibited cell proliferation of HepG2 cells by targeting SOX9.

3.3. MiR-101 inhibits human HCC cells migration and invasion in vitro by targeting SOX9

To determine the function of miR-101 on tumorigenicity of HCC cells, we performed the in vitro invasion and migration assay by

establishing miR-101-expressing HCC transfectants with hsa-miR-101 vector. In line with the results of miR-145 (positive control), enforced expression of miR-101 inhibited the migratory ability of HCC cells as indicated by the decrease in migrated cells in the Transwell cell migration assay ($P = 0.002$, Fig. 4A). A similar result was observed in the cell invasion assay ($P = 0.004$, Fig. 4A). As well as the findings of miR-101 overexpression, SOX9 siRNA transfection also significantly inhibited the cell migration and invasion of HepG2 cells compared with the control cells at 48 h after transfection (both $P = 0.002$, Fig. 4B). To further verify whether miR-101 inhibited the cell migration and invasion of HCC cell line through SOX9, we made use of HCC cell line HepG2 cells in which SOX9 was knocked down by SOX9 siRNA transfection. As shown in Fig. 4C and D, the enforced expression of miR-101 significantly inhibited the cell migration and invasion of HepG2 cells transfected with control siRNA (both $P = 0.004$, Fig. 4C) but failed to do so in HepG2 cells transfected with SOX9 siRNA (both $P > 0.05$, Fig. 4D). Therefore, miR-101 may suppress HCC metastasis via negatively regulating the migratory and invasive abilities of HCC cells by targeting SOX9.

3.4. Down-regulation of miR-101 associates the advanced tumor progression of human HCC

To evaluate whether miR-101 expression was associated with clinicopathological features of patients with HCC, we analyzed the association of miR-101 expression with T stage, tumor grade, presence of cirrhosis, underlying liver disease including alcohol abuse, viral hepatitis B and C, sex, and age (Table 1). Based on

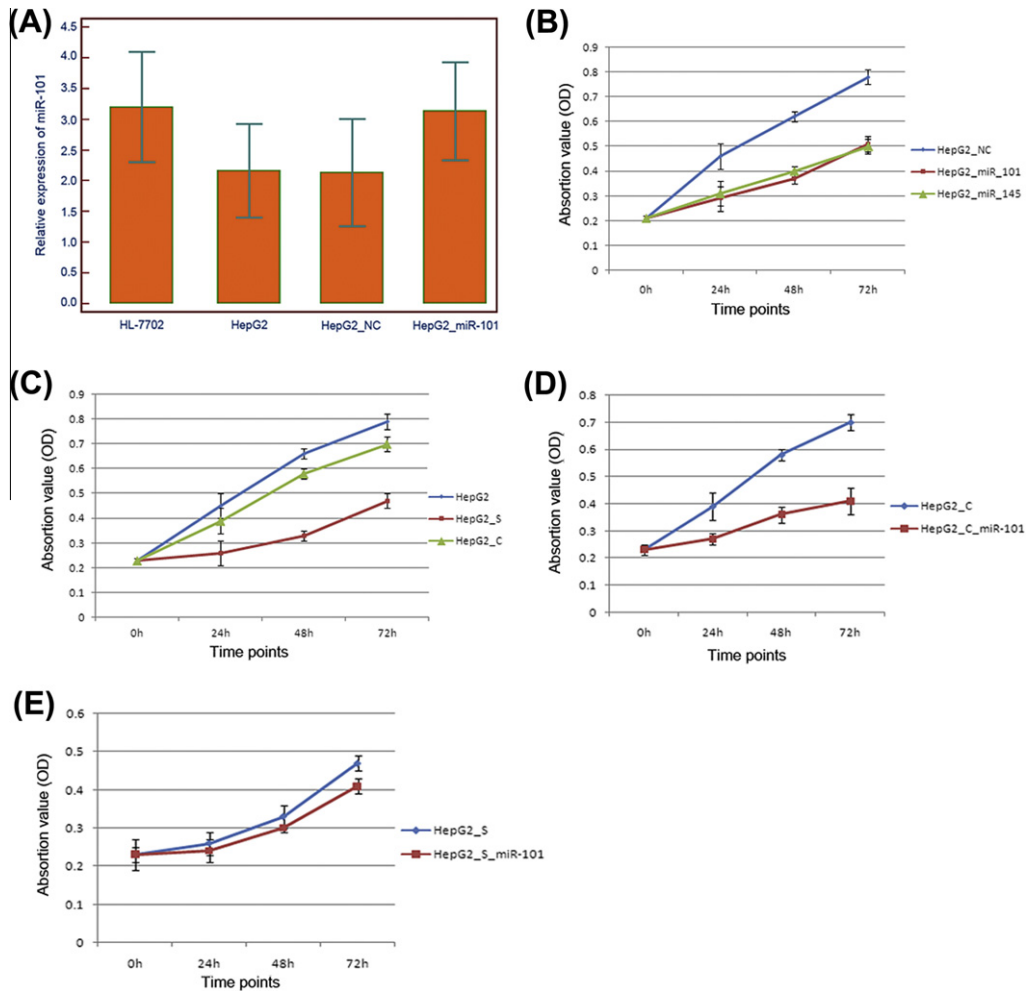


Fig. 3. miRNA-101 (miR-101) inhibits cell proliferation of hepatocellular carcinoma (HCC) cells in vitro by targeting SOX9. (A) miR-101 expression in normal human liver cell line HL-7702, HCC cell line HepG2, and HepG2 cells transfected with miR-101 (HepG2_miR-101) or negative control (HepG2_NC); (B) Growth curves of HepG2 cells following transfection with miR-101 (HepG2_miR-101), miR-145 (HepG2_miR-145, positive control) or negative control (HepG2_NC); (C) Growth curves of SOX9 siRNA-transfected (HepG2-S), control siRNA-transfected (HepG2-C) and non-transfected (HepG2) cells; (D) Growth curves of HepG2 cells transfected with control siRNA (HepG2-C), and HepG2 cells transfected with control siRNA and miR-101 (HepG2-C-miR-101); (E) Growth curves of HepG2 cells transfected with SOX9 siRNA (HepG2-S), and HepG2 cells transfected with SOX9 siRNA and miR-101 (HepG2-S-miR-101).

the median value (0.80) of miR-101 expression, all patients were divided into two groups including group with low expression of miR-101 ($n = 75$) and group with high expression of miR-101 ($n = 55$). As the results, the down-regulation of miR-101 was more frequently found in the higher tumor stage (T3–4) and tumor grade (G3) than in the lower tumor stage (T1–2, $P = 0.02$) and tumor grade (G1–2, $P = 0.008$), respectively.

3.5. Down-regulation of miR-101 associates poor prognosis of patients with HCC

Five-year disease-free survival was observed in 30 (23.08%) patients, whereas in 100 (76.92%) patients, disease recurred, and 88 (67.69%) even died during a 5-year follow-up period. We observed a trend that 5-year disease-free survival in the group with low miR-101 expression was significantly poorer than that in the group with high miR-101 expression ($P = 0.01$, log-rank test; Fig. 5A). Additionally, the Kaplan–Meier plot of 5-year overall survival curves stratified by miR-101 expression was shown in Fig. 5B. A significant relationship was found between miR-101 expression and 5-year overall survival ($P = 0.006$, log-rank test, Fig. 5B). Furthermore, in a multivariate Cox model, including tumor size, tumor stage, tumor grading, presence of cirrhosis, gender, age,

and miR-101 expression, we found that miR-101 expression was an independent poor prognostic factor for both 5-year disease-free survival (hazards ratio [HR] = 2.56, 95% confidence interval [CI] = 1.32–5.69, $P = 0.02$, Table 2) and 5-year overall survival (HR = 3.27, CI = 1.18–6.92, $P = 0.01$, Table 2) in HCC.

4. Discussion

HCC is a complex and heterogeneous disease with multiple underlying pathogenic mechanisms caused by various risk factors. It is of great significance to identify novel and effective molecular markers for HCC diagnosis and treatment assessment. Here, the main findings of the present study are as following five points. Firstly, the miRNA target prediction program TargetScan was used to predict an miRNA—miR-101 as a candidate regulator for SOX9 expression. Using human HCC cell line HepG2 transfected with miR-101 overexpression vector, we further validated that SOX9 was down-regulated at protein level by the proposed target miRNA, miR-101, which was also confirmed by the luciferase reporter assay, suggesting that miRNA-101 directly targets SOX9 in HCC tissues. Secondly, the expression level of miR-101 was decreased both in clinical HCC tissues and HCC cell line in vitro. Thirdly, the down-regulation of miR-101 in clinical HCC tissues was associated

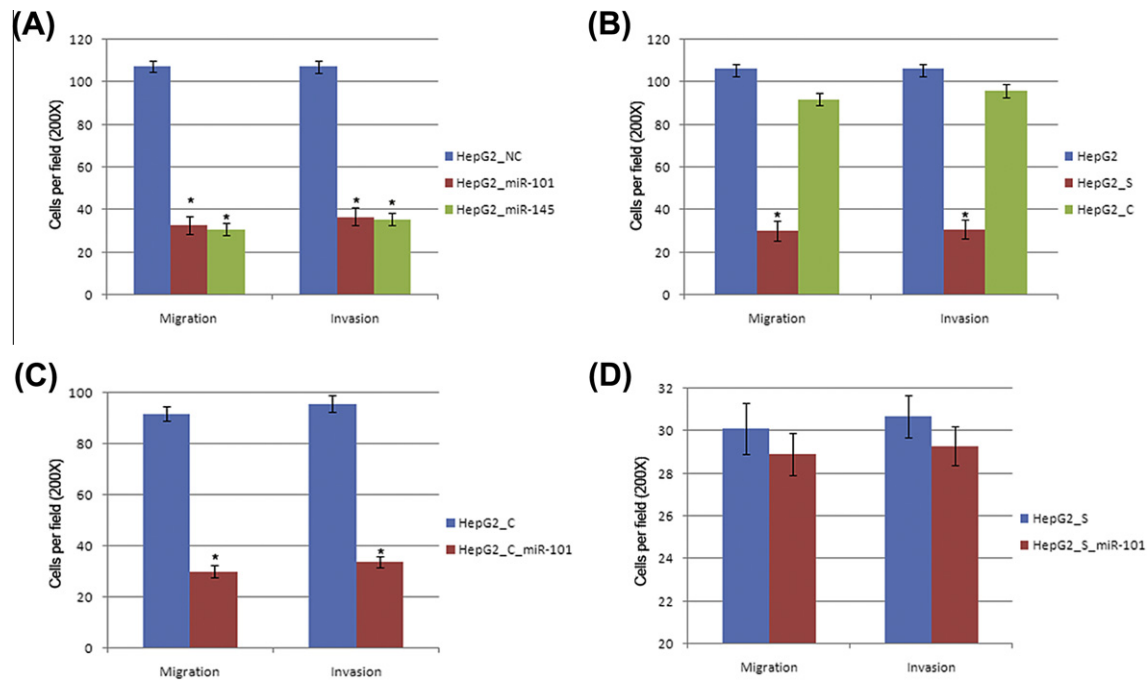


Fig. 4. MiRNA-101 (miR-101) inhibits human hepatocellular carcinoma (HCC) cell migration and invasion by targeting SOX9. (A) Transwell migration assay and Matrigel invasion assay of HepG2 cells transfected with miR-101 vector (HepG2_miR-101), miR-145 (HepG2_miR-145, positive control) or negative control (HepG2_NC) vector showed that the over-expression of miR-101 and miR-145 both caused a two- to threefold decrease in cell migration and invasion. "*" refers to $P < 0.05$ when compared with HepG2_NC group. (B) Transwell migration assay and Matrigel invasion assay of SOX9 siRNA-transfected (HepG2-S), control siRNA-transfected (HepG2-C) and non-transfected (HepG2) cells. "*" refers to $P < 0.05$ when compared with HepG2_C group. (C) Transwell migration assay and Matrigel invasion assay of HepG2 cells transfected with control siRNA (HepG2-C), and HepG2 cells transfected with control siRNA and miR-101 (HepG2-C-miR-101). (D) Transwell migration assay and Matrigel invasion assay of HepG2 cells transfected with SOX9 siRNA (HepG2-S), and HepG2 cells transfected with SOX9 siRNA and miR-101 (HepG2-S-miR-101). Enforced expression of miR-101 significantly inhibited the cell migration and invasion of HepG2 cells transfected with control siRNA (HepG2-C-miR-101 vs. HepG2-C) but failed to do so in HepG2 cells transfected with SOX9 siRNA (HepG2-S-miR-101 vs. HepG2-S). "*" refers to $P < 0.05$ when compared with HepG2-C.

with the advanced tumor progression and poor prognosis of HCC patients. Fourthly, enforced expression of miR-101 and knockdown of SOX9 by siRNA both inhibited the cell proliferation and tumorigenicity of HCC cell line in vitro. Finally, miR-101 overexpression did not inhibit the cell proliferation and tumorigenicity of HCC cells in vitro when SOX9 was knocked down. These results suggest that SOX9 direct regulator miR-101 is the potential tumor suppressor miRNA for HCC.

MiRNAs have been demonstrated to regulate multiple cellular pathways by modulating the expression of various target genes [20]. MiRNA profile can reveal prospective features in cancer. Especially in HCC, dozens of miRNAs have been reported to be involved in tumorigenesis and tumor progression. However, the functions and real targets of miRNAs were largely unknown. In the present study, we focus on miR-101, a tumor suppressive miRNA [21]. The down-regulation of miR-101 has been found in a variety of human malignancies including breast cancer, prostate cancer, bladder cancer, epithelial ovarian carcinoma, liver cancer, colon cancer, lung cancer and gastric cancer [22–26]. This miRNA functions its tumor-suppressive effects by inhibiting cell migration, invasion, proliferation, and promoting apoptosis by regulating different oncogenes in different cancer type. Semaan et al. [27] revealed that the overexpression of miR-101 in epithelial ovarian carcinoma cells may increase apoptosis, decreased cellular proliferation, invasiveness, and reduced growth of tumor xenografts by inhibiting the interaction of EzH2 with p21waf1/cip1 promoter; Wang et al. [28] indicated that the ectopic expression of miR-101 significantly inhibits cellular proliferation, migration and invasion of gastric cancer cells by targeting EZH2, COX-2, Mcl-1 and FOS. Especially in HCC, research done by Su et al. [29] in 2009 demonstrated that ectopic expression of miR-101 dramatically

suppressed the ability of hepatoma cells to form colonies in vitro and to develop tumors in nude mice. They also found that miR-101 could significantly reduced the endogenous protein level of Mcl-1, whereas the miR-101 inhibitor obviously up-regulated Mcl-1 expression and inhibited cell apoptosis. In the same year, both luciferase reporter assay and western blot analysis done by Li et al. [30] demonstrated that enforced expression of miRNA-101 in HCC represses the expression of an oncogene FOS, a key component of the activator protein-1 (AP-1) transcription factor. The current study demonstrated that SOX9 is another direct target of miR-101 in HCC cells. As intra-hepatic metastasis is the major characteristic of HCC, it is necessary to identify molecular events contributing to HCC metastasis to find an effective therapeutic treatment for metastatic HCC. Here, we demonstrated that miR-101, as well as miR-145 which has been demonstrated to be a direct regulator of SOX9 by previous studies [16,17], were functionally involved in suppressing HCC cell migration and invasion, which was supported by both clinical data and cell culture studies. In clinical samples, miR-101 was dramatically down-regulated in HCC with high tumor stage and grade compared with HCC with low tumor stage and grade, and low miR-101 expression in tumors was associated with poor survival of HCC patients. Regarding to miR-145, our data also showed its downregulation in HCC compared with adjacent non-neoplastic liver tissues, which was consistent with the findings of Karakatsanis et al. [31]. In cell culture experiments, over-expression of miR-101 and miR-145 both resulted in decreased HCC cell proliferation and cell motility. More importantly, we confirmed that miR-101 functions as a tumor suppressor for HCC by targeting SOX9.

In conclusion, our data offer the first convinced evidence that miR-101 may suppress tumor progression of HCC through

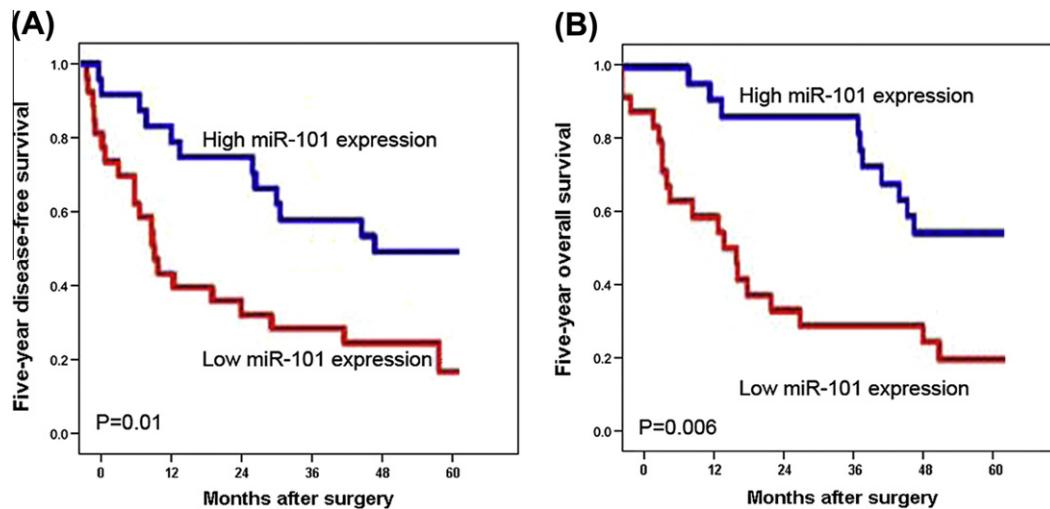


Fig. 5. Kaplan–Meier survival curves for miR-101 expression in hepatocellular carcinoma (HCC) patients. The HCC patients with low miR-101 expression showed significantly shorter disease-free survival ($P = 0.01$, A) and overall survival ($P = 0.006$, B) rates than those with high miR-101 expression.

Table 2

Multivariate survival analysis of five-year overall and disease-free survival in 130 patients with hepatocellular carcinoma.

Features	Five-year overall survival			Five-year disease-free survival		
	HR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>
Age	1.13	0.32–3.52	0.19	1.54	0.32–3.74	0.13
Gender	1.19	0.35–3.86	0.14	1.56	0.36–3.83	0.13
Tumor size	1.93	0.69–4.06	0.06	1.95	0.62–4.27	0.06
Tumor stage	2.88	1.37–5.20	0.01	2.69	1.39–6.01	0.01
Tumor grade	1.56	0.61–4.09	0.08	1.55	0.61–4.47	0.09
Presence of cirrhosis	1.92	0.74–4.10	0.06	1.92	0.79–4.22	0.06
MiR-101 expression	3.27	1.18–6.92	0.01	2.56	1.32–5.69	0.02

down-regulating SOX9. MiR-101 may be a potential prognostic marker and therapeutic target for HCC.

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