MicroRNA-132-3p regulates cell proliferation, apoptosis, migration and invasion of liver cancer by targeting Sox4

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Abstract. The present study investigated whether microRNA (miR)-132-3p targeted transcription factor SOX-4 (Sox4) for the inhibition of proliferation, migration, invasion and promotion of apoptosis in liver cancer (LC) cells. The expression of miR132-3p and Sox4 mRNA was evaluated by quantitative PCR and protein expression was determined by western blot analysis. Cell proliferation, apoptosis, migration, and invasion were assessed at different time points by the MTT assay, flow cytometry analysis, wound healing assay and Transwell migration assay, respectively. Bioinformatics prediction and luciferase assays were performed to validate and confirm Sox4 as a potential target of miR-132p. There was a reduced expression of miR-132-3p in HepG2 and Huh7 cell lines compared with HccLM3 cells. Overexpression of miR-132-3p resulted in significant inhibition of proliferation and induction of apoptosis in LC cells. Moreover, migration and invasion of HepG2 cells were suppressed by over expressing miR-132-3p. However, downregulation of miR-132-3p in Hep-G2 cells promoted cell growth, invasion and migration and inhibited apoptosis. Bioinformatics analysis predicted Sox4 as a potential target of miR-132-3p, which was further confirmed by the luciferase reporter assay. In addition, an inverse association was observed between miR-132-3p and Sox4 expression. miR-132-3p may regulate the proliferation, apoptosis, migration and invasion of HepG2 cells by targeting Sox4.

Introduction

Liver cancer (LC) is a major cause of morbidity and mortality. Hepatocellular carcinoma (HCC) is the main histological

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subtype of LC, accounting for ~90% of primary LC, and the third leading cause of cancer-related deaths (1). In 2018, the number of new HCC cases was 841,080, while the number of deaths was 781,631 (1). The early clinical manifestations of LC are not obvious, and once diagnosed about half are found at an advanced or terminal stage, while LC also has a poor prognosis and the 5-year survival rate is only 16% worldwide (2). Approximatey three-quarters of LC cases are attributed to chronic hepatitis B virus (HBV) and hepatitis C infections (3). Also, ~50% of patients with LC have HBV-associated cirrhosis and the morbidity of LC can be as high as 78% in areas where chronic HBV incidence is high (4). The risk of LC among patients with chronic hepatitis (CH) is a 100 times higher compared with patients without CH and the incidence of HBV-associated cirrhosis is also higher in patients with CH (5,6). China has a very high incidence of HBV infection with a high proportion of new cases every year and has become one of the countries with the highest incidence of LC in the world (1). Therefore, strategies that may prevent HBV infection and spread are urgently required. In addition, understanding the mechanisms of proliferation, apoptosis, invasion, and migration of LC cells is important for developing better treatment regimens for patients with LC.

Transcription factor SOX-4 (Sox4) is a 47-kDa protein and a member of the high-mobility group box transcription factor family. The DNA-binding domains of Sox4 are as follows: i) DNA-binding transcription factor activity, RNA polymerase II-specific; ii) protein heterodimerization activity; and iii) transcription regulatory region sequence-specific DNA binding (7). The Sox4 protein, with a single-box, binds with high sequence specificity to variants of the DNA sequence (A/T)(A/T)CAAAG resulting in deformation of the DNA molecule to facilitate the binding of other transcription factors capable of binding the afore mentioned DNA sequence (8). Meta-analysis data reported that Sox4 plays an important role in tumor development (9). Sox4 has been demonstrated to be highly expressed in human LC samples and to contribute to hepatocarcinogenesis by inhibiting p53-mediated apoptosis, while decreased expression of Sox4 could be a useful prognostic marker for survival after surgical resection, as low expression levels of Sox4 could significantly inhibit the growth and migration of LC cells (10). However, the exact mechanism by which Sox4 regulates LC development remains unclear.

MicroRNAs (miRs) are endogenous non-coding RNA molecules of ~22 nucleotides in length, that function as important regulators of gene expression by binding to the 3' untranslated region (UTR) of specific mRNA molecules (11). Available evidence indicates that dysregulation of miRs can contribute to tumor progression and metastasis (12,13). Human miR-132, one of the miRs that can potentially regulate the expression of various tumor suppressor genes, including p53, is located on human chromosome 17 (14,15). The expression of miR-132 in LC, osteosarcoma and colorectal cancer tissue has been demonstrated to be lower compared with normal tissue (14-16). In the present study, bioinformatics analysis predicted Sox4 as a potential target gene of miR-132 and it was hypothesized that over expression of miR-132 markedly inhibited cancer cell growth, invasion and migration as well as promoted cell apoptosis by targeting Sox4.

Materials and methods

Cell lines and culture. Human LC cell lines HepG2, Huh7 and HccLM3 were obtained from Shanghai Chinese Academy of Sciences. The cells were cultured in Eagle's Minimum Essential Medium (EMEM; Thermo Fisher Scientific Inc.) containing 10% FBS (Thermo Fisher Scientific, Inc.), 1% penicillin and streptomycin solution at 37°C in an incubator with a humidified atmosphere and 5% CO_2.

Transient transfection for HepG2 cells. miR-132 mimic (5'-ACCGUGGCUUUCGAUUGUUACU-3'), miR-negative control of the mimic (5'-CAGGUAAUCAACGCGGAGGUCA-3'), miR-132 inhibitor (5'-AGUAACAAUCGAAAGCCACGGU-3') and miR-negative control of the inhibitor (5'-CGUGGUGCUCGUGAAGGGUCGG-3') were synthesized and purified by Suzhou GenePharma Co., Ltd. All miRs above were transfected at a final concentration of 50 nmol/l using Lipofectamine® 2000 reagent (Thermo Fisher Scientific Inc.) following the manufacturer's protocols. The group with untreated cells was defined as control group. Total RNA and protein were extracted 48 h after transfection.

Reverse transcription-quantitative (RT-q) PCR. Total RNA including miRs was isolated from different LC cell lines using TRIzol® reagent (Thermo Fisher Scientific, Inc) following the manufacturer's protocol. By using a Prime Script reverse-transcribed Reagent Kit with gDNA Eraser (cat. no. RR047A, Takara Bio, Inc.), total RNA was reverse-transcribed into cDNA. Gene expression levels were determined via real-time PCR using the commercial kit (SYBR Premix Ex Taq™ II with Tli RNaseH) (cat. no. RR820A, Takara Bio, Inc.,) in an ABI PRISM 7500 system (Thermo Fisher Scientific, Inc.). Thermo cycling conditions were as follows: 95°C For 5 min; 40 cycles at 95°C for 15 sec; 56°C for 30 sec; and 72°C for 15 sec with a final extension at 72°C for 7 min. The relative quantification value for each gene was calculated by the $2^{-\Delta\Delta Cq}$ method (17) using U6 small nuclear RNA as an internal reference gene. The following human-specific primers were used: β-actin forward, 5'-AGCGAGCATCCCCCAAAG TT-3' and reverse, 5'-GGGCACGAAGGCTCATCATT-3; Sox4, forward, 5'-CAGCAAACCAACAATGCCGA-3' and reverse, 5'-GATCTGCGACCACACCATG-3'; hsa-miR-132-3p

forward, 5'-TGCGCTAACAGTCTACAGCCA-3' and loop primer, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACCGACCATG-3'; U6 forward, 5'-CGC TTCGGCAGCACATATAC-3' and reverse, 5'-AAATATGGA ACGCTTCACGA-3'; miRNA antisense strand, the Universal reverse primer, 5'-CCAGTGCAGGGTCCGAGGTATT-3'. All experiments were performed in triplicate.

Cell proliferation assay. To explore the effect of miR-132-3p on the proliferation of HepG2 cells, $3x10^3$ cells were seeded in a 96-well plate and allowed to grow overnight. The cells were then transfected with miRs or control sequences for 48 h. The purple formazan was dissolved using DMSO. MTT cell proliferation assay was performed at 24, 48 and 72 h at a wavelength of 568 nm. Experiments were performed in triplicate.

Flow cytometry for apoptosis analysis. HepG2 cells were transfected with cmiRs for 48 h. Transfected cells were harvested, washed twice in PBS and then stained with Annexin V-allophycocyanin (APC) and 7-aminoactino-mycin D (7-AAD) using the Annexin V-APC/7-AAD detection kit (Nanjing KeyGen Biotech Co., Ltd.) following the manufacturer's protocols. The apoptotic cells were positively stained by Annexin V. Each sample was analyzed using a FACSCalibur (BD Biosciences) with Cell Quest Pro software (version 5.1). All experiments were performed in triplicate.

Transwell invasion assay. To determine cell invasion, the Transwell Matrigel invasion assay was conducted using Transwell chambers precoated with Matrigel at 37°C for 5 h (BD Biosciences), following the manufacturer's instructions. Briefly, $2x10^4$ HepG2 cells transfected with miRs were suspended in 150 μl of EMEM without serum and seeded on the upper chamber. EMEM (600 μl) containing 10% FBS was added to the lower chamber. After 24 h incubation at 37°C in a 5% CO_2 incubator, cells that remained in the upper chamber were removed with cotton swabs and the penetrating cells were fixed in methanol, and then stained with 0.1% crystal violet for 20 min at room temperature. Cell invasion was quantified by counting cells on the lower surface by phase contrast microscopy using an Olympus IX51 microscope (Olympus Corporation). Quantitative analysis of invasion SPSS 17.0 software (SPSS, Inc.)

Cell wound healing assay. Migratory ability of LC cells was determined using the cell wound healing assay. HepG2 cells were plated into six-well plates without antibiotics. Then, cells were transfected with miR-132 mimic, mimic control, miR-132 inhibitor and inhibitor control. After 48 h, transfected cells were digested with 0.25% pancreatin and $5x10^5$ cells were inoculated into six-well plates so that they could form a confluent monolayer by the next day. A 200 μl pipette head perpendicular to the back of the plates was used to scratch a line, making sure that each well had at least 5 lines. Then, the cells were washed 3 times with PBS and cultured in serum-free medium at 37°C in an incubator with 5% CO_2. After 24 h, the cells were visualized and images were captured under an Olympus IX51 phase-contrast inverted microscope (Olympus Corporation). The width of cell wound closure was

measured at 0 and 24 h of the experiment. All experiments were performed in triplicate.

Western blot analysis. For protein extraction, HepG2 cells were washed twice in cold PBS and then lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology) containing a protease inhibitor cocktail (Merck KGaA). The protein concentration of cell lysates was quantified using abicinchoninic acid kit (Beyotime Institute of Biotechnology) and 40 μg of each of protein extract were separated by 10% SDS-PAGE, and then transferred to a PVDF membrane. The membranes were blocked with 5% skimmed milk diluted with Tris-buffered saline Tween-20 at room temperature for 2 h and incubated overnight at 4°C with rabbit anti-Sox4 antibody (1:500; Boster Biotechnology; cat. no. pb0654) and rabbit anti-GAPDH antibody (1:1,000; cat. no. ab9485; Abcam). The membranes were then incubated with secondary antibody (1:1,000; cat. no. A0277; Beyotime Institute of Biotechnology) for 2 h at room temperature. The protein bands were visualized using ECL plus reagents (Thermo Fisher Scientific, Inc.). The densitometry of the western blot protein bands was measured using the BandScan version 5.0 software (Glyko Biomedical, Ltd). Each sample was analyzed in triplicate.

Vector construction. The Psi-Check-Sox4 full length 3'UTR plasmid (Addgene Plasmid #26989) was digested with the *Not*I and *Xho*I enzymes to obtain the full-length 3'UTR of Sox4. The recombinant vector was termed as pYr-MirTarget (Biovector Lab, Inc.). All the constructs were verified by sequencing.

Dual-luciferase reporter assay. HepG2 cells (2x10^5/well) were seeded in 12-well plates. The next day cells were co-transfected with 1 μg pYr-MirTarget-Sox4-3'UTR reporter plasmid, 50 nmol/l of miR132-3p mimic or mimic control using Lipofectamine 2000. Then, 24 h after transfection, both Firefly and *Renilla* luciferase activities were quantified using the dual luciferase reporter system (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The relative luciferase unit (*Renilla* luciferase/Firefly luciferase) was calculated to determine the activation of the target gene. All experiments were performed at least twice.

Bioinformatic analysis. To determinewhether Sox4is a direct target of miR-132-3p, two bioinformatic software programs were used, TargetScan.human6.2 (targetscan.org/vert_61/) and microRNA.org August 2010 release (microrna. org/microrna/home.do).

Statistical analysis. Statistical analysis was performed using the SPSS 17.0 software (SPSS, Inc.). The data were presented as the mean ± standard deviation. One-way analysis of variance followed by Tukey's or Bonferroni's post hoc test was used for multiple comparisons between the groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-132-3p is downregulated in human LC cell lines. First, the basal expression of miR-132-3p was determined by RT-qPCR in three liver cancer cell lines (HepG2,
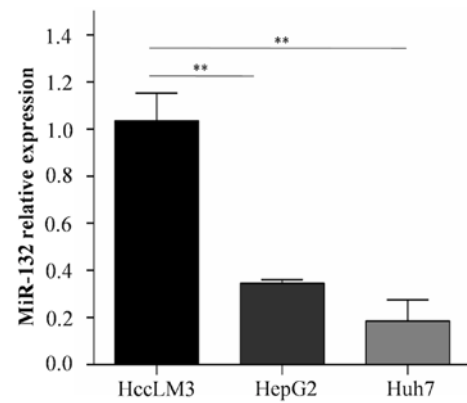

Figure 1. mRNA expression of miR-132-3p inHepG2, Huh7 and HccLM3 liver cancer cell lines. **P<0.01. miR, microRNA.

Huh7 and HccLM3). The results demonstrated significantly reduced expression of miR-132-3p in HepG2 and Huh7cell lines compared with the HccLM3 cell line (Fig. 1). Among these 3 cell lines, HepG2 was chosen for further experiments due to its common usage.

An inverse association between the expression of miR-132-3p and Sox4 in HepG2 cells. As presented in Fig. 2, the level of miR-132-3p was significantly increased following transfection with miR-132-3p mimics compared with the mimics control group confirming the successful over expression of miR-132-3p in HepG2 cells. By contrast, transfection of HepG2 cells with the miR-132-3p inhibitor decreased the expression level of miR-132-3p (Fig. 2A). In addition, the mRNA levels of Sox4 were measured in HepG2 cells as describe above. Sox4 mRNA expression was decreased in cells overexpressing miR-132-3p, whereas Sox4 mRNA expression levels were increased in cells under expressing miR-132-3p, compared with the respective control groups (Fig. 2B). Protein expression levels of Sox4 were measured in the control, mimic and inhibitor groups by western blotting. Similar results to those for Sox4 mRNA expression levels were obtained (Fig. 2C and D).

miR-132-3p inhibitscell proliferation and inducesapoptosis in HepG2 cells. After confirming the expression of miR-132-3p in HepG2 cells, the role of miR-132-3p in proliferation and apoptosis of HepG2 cells was investigated. Cell proliferation measured using the MTT assay revealedthat overexpression of miR-132-3p significantly inhibited the proliferation of HepG2 cells, whereas decreased expression of miR-132-3p promoted the proliferation of HepG2 cells at time points 48 and 72 h (Fig. 3A). To determine if proliferation was inhibited due to cell apoptosis, apoptosis in HepG2 cells transfected with miR-132-3p mimics or miR-132-3p inhibitor was also measured. Flow cytometry analysis revealed that the number of apoptotic HepG2 cells was significantly higher in cells transfected with miR-132-3p and was significantly lower in cells transfected with the miR-132-3p inhibitor, compared with their respective controls (Fig. 3B and C), thus confirming that miR-132-3p regulated the proliferation and apoptosis of HepG2 cells.

miR-132-3p inhibits invasion and migration of HepG2 cells. After transfection for 48 h, the effects of miR-132 on
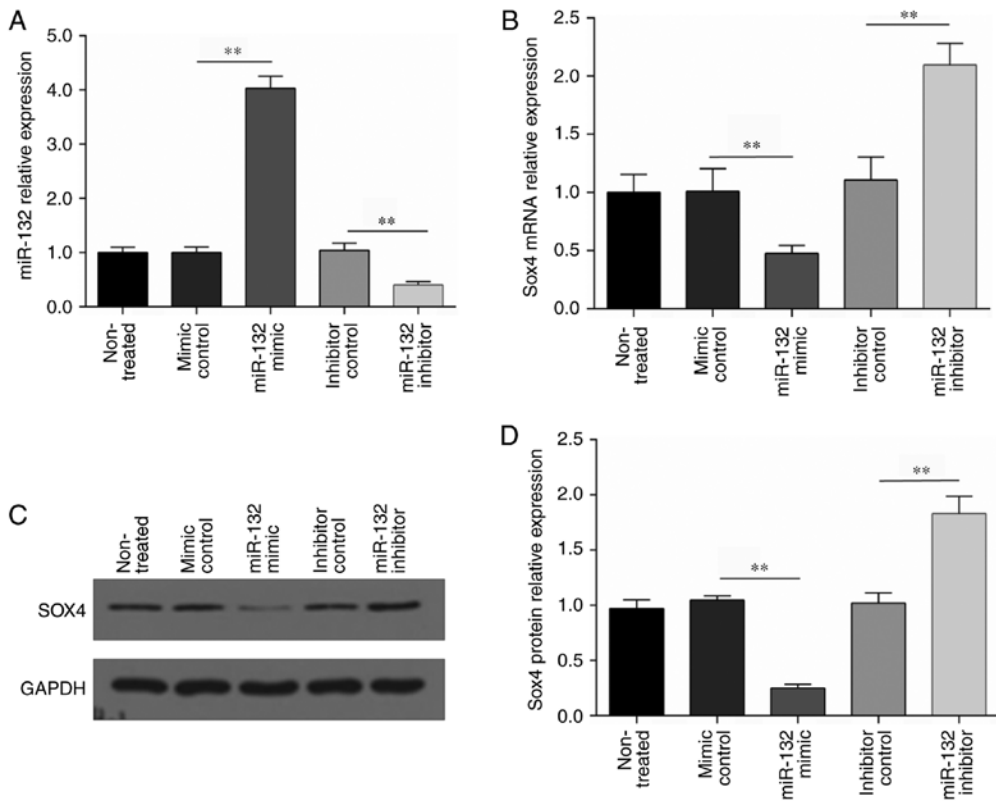
Figure 2. Expression of miR-132-3p and Sox4 in HepG2 cells in five experimental groups. (A) miR-132-3p mRNA expression. (B) Sox4 mRNA expression. (C) Sox4 protein analysis using western blotting and (D) quantification of Sox4 relative protein expression. **P<0.01. miR, microRNA; Sox4, transcription factor SOX-4.
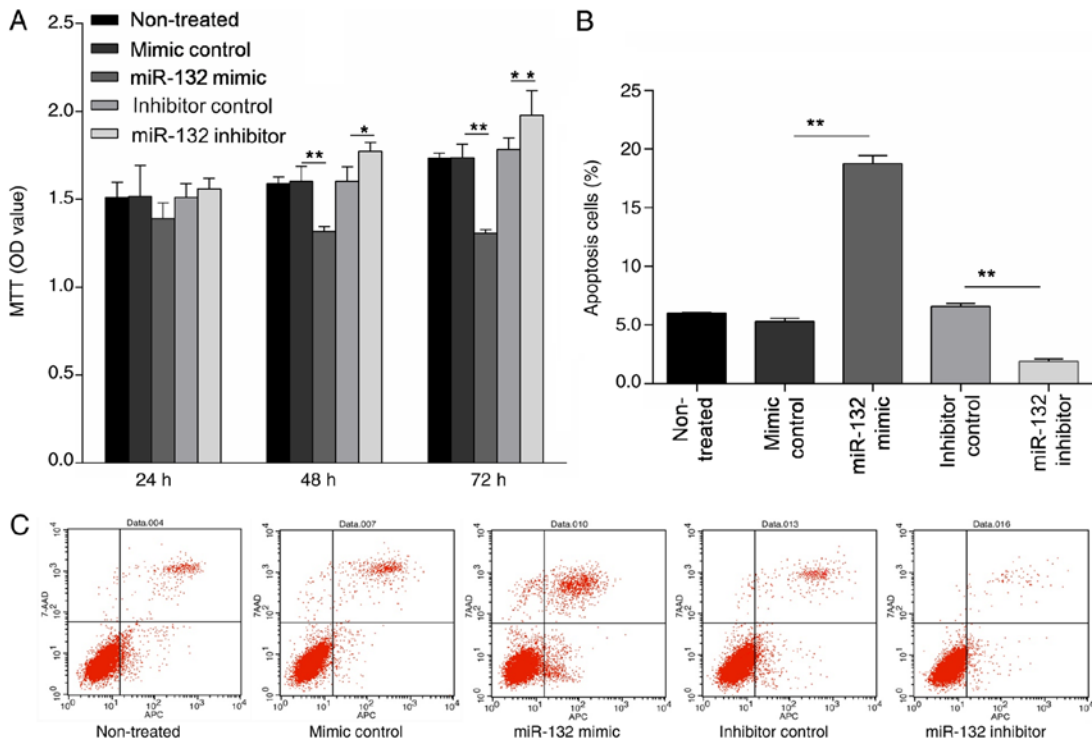

Figure 3. Effect of miR-132-3p expression on (A) proliferation and (B) apoptosis. (C) Representative flow cytometry plots for investigating apoptosis of HepG2 cells using Annexin V-7AAD staining. *P<0.05 and **P<0.01. APC, allophycocyanin; 7-AAD, 7-aminoactinomycin D; miR, microRNA.

invasion in HepG2 cells were evaluated using the Transwell invasion chamber assay. The number of invading HepG2

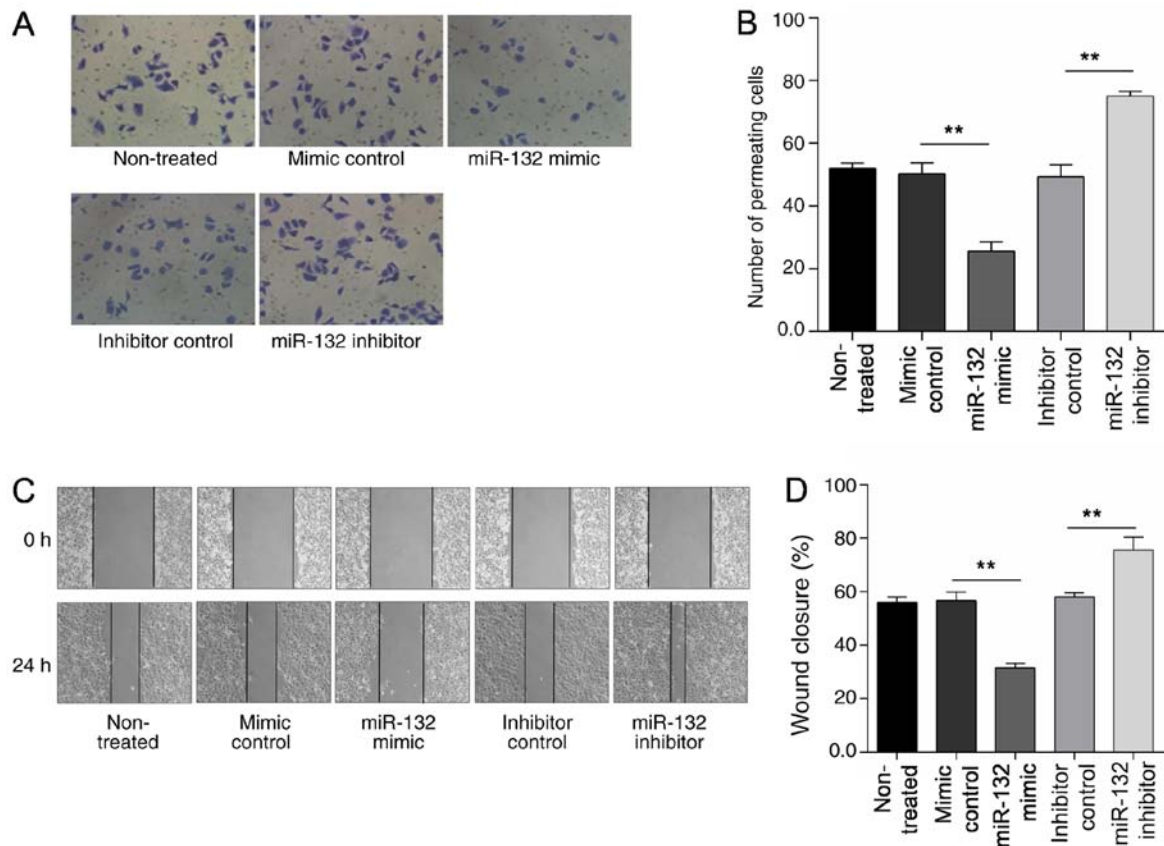cells was significantly reduced in cells transfected with miR-132-3p mimics compared with those transfected with

Figure 4. Effects of miR-132-3p on invasion and migration of HepG2 cells. (A) Representative images of invading cells of each groupand (B) quantification of invading cells. (C) Representative images of cell wound healing assay of each experimental group at 0 and 24 h. (D) Percent wound closure of each experimental group. **P<0.01. miR, microRNA.

mimic control (Fig. 4A and B). By contrast, the number of invading cells was significantly increased in HepG2cells transfected with the miR-132-3p inhibitor, indicating that miR-132-3p inhibited invasion in HepG2 cells (Fig. 4A and B). The wound healing assay results demonstrated that the percent wound closure in the miR-132-3p mimics group was significantly reduced, whereas it was significantly increased in the miR-132-3p inhibitor group compared with the inhibitor control group (Fig. 4C and D). Taken together, the results indicated that miR-132-3p inhibited the migration of HepG2 cells.

Sox4 is a direct target of miR-132-3p in LC cells. The bioinformatic analysis indicated that the Sox43'UTR harbors one conserved binding site (TargetScan.human) and two targets of miR-132-3p (microRNA.org) (Fig. 5A). These sites were at least partially complementary to a motif that is found in the seed region of the miR-132-3p. To confirm thatSox4isa direct target of miR-132-3p, the 3'UTR was cloned to the downstream of the wild type luciferase stop codon. The results revealed that overexpression of miR-132-3p significantly inhibited the luciferase activity of the pUC57-Sox4-3'UTR (Fig. 5B). Together, the results of the bioinformatics analysis and luciferase reporter assay suggested that Sox4 was a direct target of miR-132-3p in LC cells.

Discussion

Emerging evidence suggests that miR expression is dysregulated in human malignancies including LC (18). Dysregulated miRs affect the hallmarks of cancer through the regulation of tumor cell proliferation, apoptosis and other important pathological processes by targeting multiple genes and signaling pathways in LC (18). Among the miRs, miR-132-3pexpression was demonstrated to be more frequently downregulated in HBV-associated LC tissues (19). Additionally, miR-132 was found to be downregulated in LC compared with paracarcinoma and normal liver tissue (19,20). In the present study it was further confirmed that miR-132-3p expression was significantly reduced in HepG2 compared with HccLM3 cells. The mechanism underlying this decreased expression is believed to be mediated through the HBV X protein (HBx)-induced hypermethylation of the promoter of miR-132, which was previously demonstrated to be more prevalent in HBx-expressing HepG2 cells (21). Low expression of miR-132 has been observed in other types of human cancer, including colorectal, lung, cervical, breast cancers and glioma (22-25). By contrast, high miR-132 expression has been reported in gastric cancer (26). The mean level of miR-132 in LC tissues was significantly lower than that found in matched tumor-adjacent tissues and its expression was negatively associated with tumor differentiation and the TNM stage, which is a cancer staging notation system that describes the stage of a cancer which originates from a solid tumor with alphanumeric codes and lymph node metastasis (21). Additionally in the aforementioned study, miR-132 inhibited tumor growth, volume and weight (20). However the precise role and mechanism through which miR-132 exerts its effect on LC was unclear. In the present study, the biological
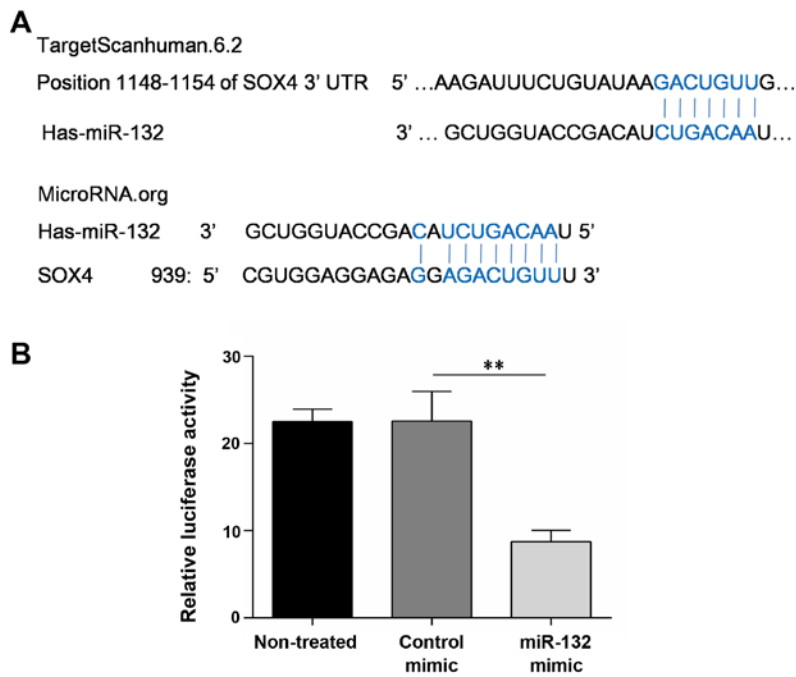
Figure 5. Sox4 is a direct target of miR-132-3p in HepG2 cells. (A) Result of bioinformatics prediction in TargetScan.human 6.2. Conserved binding site found in Sox4 3'UTR locus by TargetScan.human or by microRNA.org. (B) Relative luciferase unit (*Renilla* luciferase/Firefly luciferase) to detect the activation of Sox4 gene. **P<0.01. Sox4, transcription factor; SOX-4. miR, microRNA; 3'UTR, 3' untranslated region.

functions and mechanism by which miR-132-3p regulates LC were elucidated. Furthermore, to the best of the authors' knowledge, the current study was the first to demonstrate that Sox4 was a novel target of miR-132-3p in LC.

In the present study, miR-132-3p mimics and anmiR-132-3p inhibitor were used to modulate the expression of miR-132-3p in HepG2 cells. The results of the MTT assays demonstrated that overexpression of miR-132-3p inhibited the proliferation of HepG2 cells. Consistent with the results of the current study, Liu *et al* (20) reported that ectopic expression of miR-132 inhibited cell proliferation, colony formation, migration and invasion, as well as induced apoptosis in HepG2 cells *in vitro*. Liu *et al* also demonstrated that miR-132 inhibited LC growth and decreased cancer volume and weight *in vivo*. In addition, proliferation and colony formation of LC cells were revealed to be suppressed by the miR-132-mediated inhibition of the Akt-signaling pathway in miR-132-transfected cells (26). The present study demonstrated that high levels of miR-132 significantly induced apoptosis of HepG2 cells. However, cell proliferation was promoted and cell apoptosis was inhibited in HepG2 cells transfected with the miR-132-3p inhibitor compared with the inhibitor control group. Moreover, invasion and migration of HepG2 cells were inhibited by the overexpression of miR-132-3p, which was reversed by miR-132-3p inhibitor transfection. The molecular mechanism underlying this beneficial effect of miR-132-3p was investigated by undertaking the identification and validation of a potential target of miR-132-3p.

Sox4 expression was reported to be increased in 63.8% of human HCC tissue samples and the increased expression of Sox4 promoted HCC development (10). Since Sox4 has been demonstrated to be overexpressed in HCC and to contribute to hepatocarcinogenesis, the present study sought to determine whether Sox4 was a direct target of miR-132-3p in LC cells. This was first determined through bioinformatic analysis using two independent bioinformatic software tools. Furthermore, the present study determined that the expression of Sox4 mRNA and protein in HepG2 cells was inversely proportional to the level of miR-132-3p. These findings are consistent with previous findings in osteosarcoma and lung cancer cells indicating that Sox4 is a target of miR-132-3p (27,28). Moreover, it has been reported that miR-132-3p inhibited cell growth and metastasis in osteosarcoma cells by downregulating Sox4, and knockdown ofSox4 promoted miR-132-mediated cell growth and metastasis in osteosarcoma cells (27). Similar to the results of the present study, the expression of miR-132 in non-small cell lung carcinoma cells was decreased, and overexpression of miR-132 inhibited cell invasion and migration by targeting SOX4 (28).

Zheng *et al* (29) reported that overexpression of miR-132 in colorectal cancer cells inhibited cell invasion and migration by targeting the zinc finger E-box binding homeobox 2 gene. Another report indicated that miR-132 regulated apoptosis of glioma cells by blocking the sterol regulatory element-binding transcription factor 1 metabolic pathway related to Sirtuin 1 (30). Studies have also reported that overexpression of Sox4 was closely associated with tumor progression and metastasis (31,32). Sox4 maybe a crucial oncogene affecting tumor progression and metastasis in HCC and lung cancer (10,33). Overexpression of Sox4 has also been detected in other types of solid tumors including prostate, breast, bladder and lung cancer (7,34,35). Downregulation of the expression of Sox4 inhibited cell proliferation, metastasis and induced apoptosis in lung cancer cells (33).

Since one miR can target multiple genes, it was important to determine whether miR-132-3p targeted other genes besides Sox4 in the same and in different signaling pathways. In this respect, proliferation and colony formation of LC cells were suppressed through the miR-132-mediated inhibition of the AKT-signaling pathway in miR-132-transfected cells (26). Furthermore, miR-132

expression was inversely associated with PIK3R3 mRNA expression levels in clinical HCC tissue samples (20). Yes-associated protein 1 is also a potential target of miR-132 (19). The involvement of these targets in a similar signaling pathway in miR-132-3p-mediated regulation of LC warrants further investigation.

Taking the findings of the present study together, it can be hypothesized that miR-132-3p maybe a potential antioncogene in liver cancer. In addition, the present study demonstrates that miR-132-3p serves an important role in regulating proliferation, apoptosis, invasion and migration in LC cells. Further studies are required to evaluate the effects of miR-132-3p in animal models of LC as well as in human LC tissue specimens, blood and plasma samples. The present study provides new insights into the molecular mechanisms mediating the development of human LC. The regulation of miR-132-3p-targeted genes in patients with LC may be an efficacious therapeutic strategy. Additionally, it may enable the screening of early stage LC by determining the level of miR-132-3p in human blood.

Acknowledgements

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JH, DL, TX and SG performed the experiments. XW and YW analyzed the data. JH, JW and NC contributions to conception and design, and drafting the manuscript or revising it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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