Original Article
MiR-26b inhibits hepatocellular carcinoma cell proliferation, migration, and invasion by targeting EphA2

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Abstract: Deregulated microRNAs (miRNAs) have been shown to play important roles in cancer progression as a result of changes in expression of their target genes. In this study, we investigated the expression of miR-16b in eight hepatocellular carcinoma (HCC) cell lines, revealed the roles of miR-26b on hepatocellular carcinoma (HCC) cell proliferation, migration, and invasion, and confirmed that EphA2 is a direct target of miR-26b. The miR-26b expression was decreased and EphA2 expression was evaluated in HCC cell lines. Luciferase assays revealed that miR-26b inhibited EphA2 expression by targeting the 3'-untranslated region of EphA2 mRNA. Overexpression of miR-26b dramatically inhibited the proliferation, invasion, and migration of HCC cells by targeting EphA2. More-over, miR-26b down-regulated *c-Myc* and *CyclinD1* expression, which was reversed by overexpressed EphA2. Taken together, our data demonstrated the mechanism of miR-26b contributed to HCC progression and implicated that miR-26b's potential in HCC therapy.

Keywords: miR-26b, hepatocellular carcinoma, EphA2, proliferation, invasion, migration

Introduction

Hepatocellular carcinoma (HCC) is a common and aggressive cancer, with an increasing incidence globally, especially in China [1-3]. Although the risk factors of HCC are well characterized, the molecular pathogenesis of this tumor type is still not pretty understood. As more and more lives of the patients are taken by HCC, it is urgent to clarify the molecular mechanisms underlying the initiation, progression and metastasis of HCC to develop novel and more effective treatment strategies against the maligance.

MicroRNAs (miRNAs), as a class of small (22-nucleotide) non-coding RNAs, have been identified to be aberrantly expressed in several human malignancies [4]. miRNAs regulate gene expression by binding to the 3'untranslated region (3'-UTR) of their target mRNAs, modulating mRNA stability and/or translation [5]. Previous studies have identified a number of miRNAs that show aberrant expression in HCC [6, 7].

For instance, miR-144 has been shown to be down-regulated in HCC tissues, and miR-141 functions as a tumor suppressor and inhibits the migration and invasion of HCC cells by targeting Tiam1 [8] and zinc finger E-box binding homeobox 2 (ZEB2) [9]. The expression level of miR-302b is dramatically decreased in clinical hepatocellular carcinoma specimens, and miR-302b HCC cell proliferation and growth in vitro and in vivo by targeting AKT2 [10]. miR-184 functions as an oncogenic regulator in hepatocellular carcinoma (HCC) by targeting INPPL1 directly [11]. Therefore, further exploration of the expression and function of miRNAs will provide insight into the pathogenesis and progression of HCC.

Erythropoietin-producing hepatocellular (EPH) receptors make up the largest family of receptor tyrosine kinases (RTKs), involving in many biological processes and playing important roles in disease and development [12]. EphA2, as a member of the Eph receptor family, is the most widely studied with respect to develop-

ment, tumorigenesis, angiogenesis, and metastasis [13]. Recently, accumulating studies showed that EphA2 represents as the potential therapeutic targets in several types of cancer, including GC [12, 14], esophageal squamous cell carcinoma [15], glioblastoma [16], head and neck squamous cell carcinoma [17], ovarian cancer [18, 19], cholangiocarcinoma [20], lung cancer [21, 22], breast cancer [23, 24], colorectal cancer [25], and prostate cancer [26, 27]. Recent works showed that EphA2 expression was prominent in highly invasive hepatoma cells, and its overexpression was significantly correlated with decreased differentiation and poor survivalfor HCC patients [28]. Yang et al. revealed that EphA2 is overexpressed in HCC, which relate to tumor progression, metastasis, and prognosis [29]. However, the molecular mechanisms underlying EphA2 overexpression in HCC are still largely unknown. Recently, accumulating of miRNAs were found to exert their biological functions by targeting EphA2, including miR-141 [30], miR-520d-3p [31], miR-200a [32], and miR-26b [33]. This evidence suggests that investigation into the precise molecular mechanisms underlying the interaction between miRNAs that target EphA2 and HCC progression could provide valuable information for the development of a novel therapeutic strategy.

In the current study, we evaluated the expression of miR-26b in 8 HCC cell lines. Furthermore, we demonstrated the effect and mechanism of miR-26b on HCC cell proliferation, invasion, and migration.

Materials and methods

Cell culture

The HCC cell lines HepG2, SMMC7721, Huh7, bel-7402, PLC, LM3, 97L, 97H and human hepatocyte line LO2 were used in this study. All cells were grown in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah, USA) and 1% penicillin/streptomycin.

RNA extraction and RT-PCR

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR) Total RNA was isolated from tissues and cell lines using the miRNeasy Mini Kit (Qiagen). The miRNA Q-PCR Detection Kit (GeneCopoeia) was used for quantification of miRNA levels according to the manufacturer's protocol. For quantification of EphA2 mRNA levels, the RT reactions were conducted with the RevertAid TM H Minus First Strand cDNA Synthesis Kit (Fermentas). qRT-PCR was performed using an ABI 7900 System (Bio-Rad). RNU6B and β-actin were used as normalizing controls for miRNA and mRNA quantification, respectively. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expression levels. The primers were as follows: miR-26b, forward primer: 5'-CCCAGTTC-AAGTAATTCAGG-3' and reverse primer: 5'-TTTG-GCACTAGCACATT-3'; EphA2, forward primer: 5'-TGGCTCACACACCCGTATG-3' and reverse primer: 5'-GTCGCCAGACATCACGTTG-3'.

Manipulation of miR-26b expression levels

The miR-26b mimics, negative control and miR-26b inhibitor were purchased from RiboBio (Guangzhou, Guangdong, China). The final concentration of transfection is 20 nM.

Dual-luciferase reporter assay

The 3'-UTR sequence of EphA2 was amplified from normal human genomic DNA (NM_004431) and subcloned into the pmirGLO luciferase reporter vector (Promega). LM3 cells (3.5×10^4) were seeded in triplicate in 24-well plates and cotransfected with wild-type (WT) or mutant (Mut) 3'-UTR vectors and miR-26b mimics or inhibitors using Lipofectamine 2000. After 48 h, the cells were assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) by following the manufacturer's instructions. The firefly luciferase activities were normalized to Renilla luciferase activity. The firefly luciferase activity of the cells that were transfected with miRNA mimics or inhibitors is represented as the percentage of activity relative to that of cells that were transfected with negative controls. All experiments were performed in triplicate.

Western blot analysis

Whole cell extracts were prepared with a cell lysis reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manual, and then, the protein was quantified by a BCA assay (Pierce, Rockford, IL, USA). Then, the protein samples were separated by SDS-PAGE (10%) and detected by Western blot using polyclonal (rabbit) anti-EphA2, anti-*c-Myc* and ant-*cyclinD1* anti-
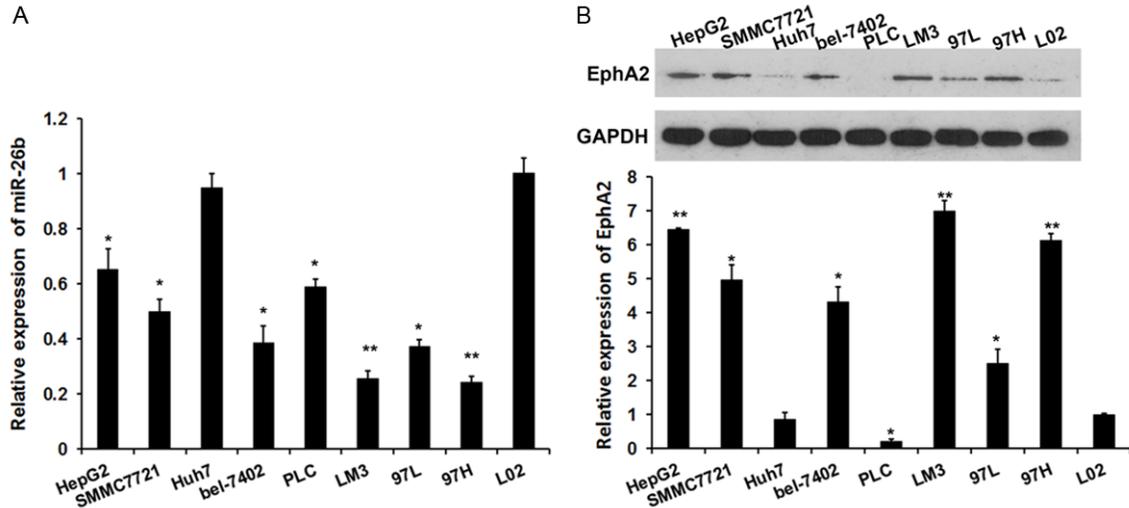
Figure 1. The expression of miR-26b and EphA2 in HCC cell lines. A. qRT-PCR analysis revealed the expression level of miR-26b in eight HCC cell lines (HepG2, SMMC7721, Huh7, bel-7402, PLC, LM3, 97L and 97H) and human hepatocyte line L02. B. Western blot analysis revealed the relative expression level of EphA2 was examined in eight HCC cell lines (HepG2, SMMC7721, Huh7,bel-7402, PLC, LM3, 97L and 97H) and human hepatocyte line L02.All experiments were repeated at least three times. Each bar represents the mean of three independent experiments. *P < 0.05 and **P < 0.01 versus L02 cell line.
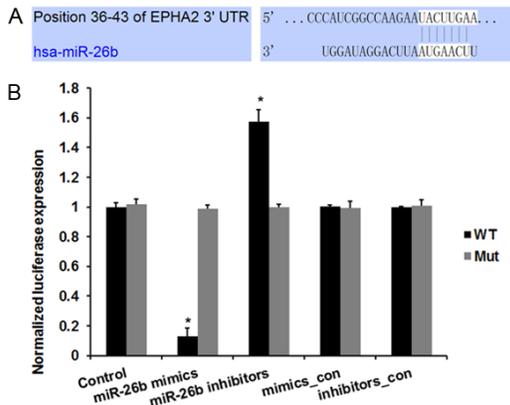

Figure 2. 26b directly targets the 3'-UTR of EphA2. A. Representative diagram of the predicted wild-type (WT) or mutant (Mut) binding site of miR-26b in the 3'-untranslated region (UTR) of EphA2 mRNA. B. The luciferase reporter plasmid containing the WT or Mut EphA2 3'-UTR was cotransfected into LM3 cells with miR-26b mimics or inhibitors. Luciferase activity of the cells was assayed at 48 h after transfection, and the values were normalized to the normal control values. *P < 0.01 (compared with the control).

body (Santa Cruz Bio-technology, Santa Cruz, CA, USA). Goat anti-rabbit IgG (Pierce, Rockford, IL, USA) secondary antibody conjugated to horseradish peroxidase and ECL detection systems (SuperSignal West Femto, Pierce) were used for detection.

Cell survival assay

Cells were seeded at 1000 per well in 96-well plates. Cell viability was evaluated using a Cell Counting Kit (CCK-8) according to the manufacturer's instruction at indicated time. All results were from three separate experiments with six replicates.

Invasion assay

The capability of cell invasion was examined by Tran swell invasion assay. Cells were cultivated to 80% confluence on the 12-well plates. Then, we observed the procedures of cellular growth at 24 h. All the experiments were repeated in triplicate. The Tran swell migration chambers were used to evaluate cell invasion. Then cells invading cells across the membrane were counted under a light microscope.

Wound healing assay

For the wound healing assay, cells were seeded in 12-well plates and grown to 90% confluence. Monolayers in the center of the wells were scraped with pipette tips and washed with PBS. Cell movement into the wound area was monitored and photographed at 0 and 24 h using a light microscope. The migration distance between the leading edge of the migrating cells
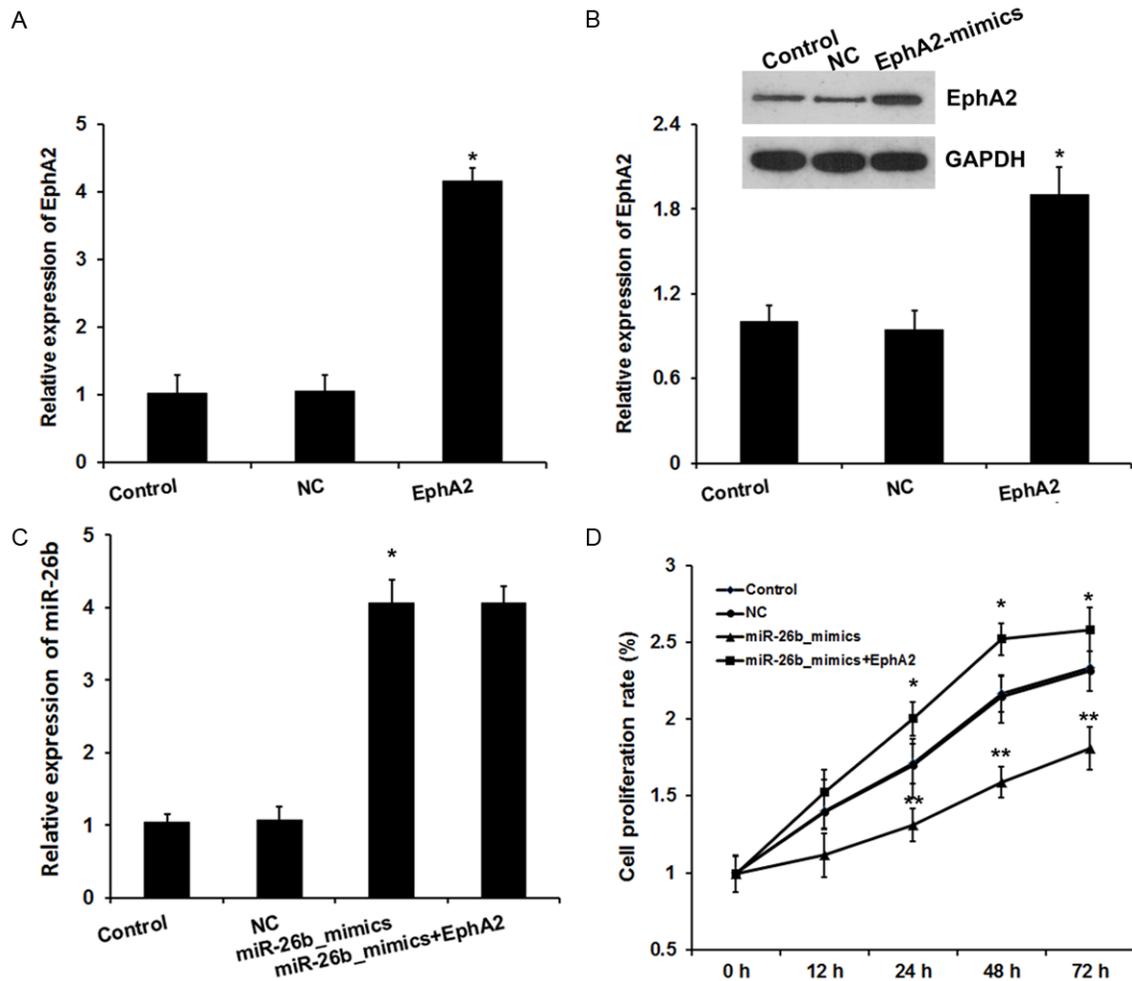
Figure 3. miR-26b inhibited cell proliferation of HCC. A. LM3 cells were transfected with EphA2 mimics. The expression of EphA2 was detected by qRT-PCR. B. LM 3 cells were transfected with EphA2 mimics. The expression of EphA2 was detected by western blot. C. LM3 cells were transfected with miR-26b mimics and EphA2 mimics. The expression of miR-26b was detected by qRT-PCR. D. The effects of miR-26b and EphA2 on cell proliferation. Error bars represent ± S.E. and *, $P < 0.01$ versus control and NC.

and the edge of the wound was compared as previous work [34].

Statistical analysis

Each experiment was repeated at least three times. Data were shown as mean ± s. d and analyzed using SPSS 18.0. Statistical comparisons between groups were analyzed using Student's t-test and a two-tailed $P < 0.05$ was considered to indicate statistical significance.

Results

Expression of miR-26b and EphA2 in HCC cells

We first employed qRT-PCR to detect miR-26b levels in HCC cell lines. Consistent with previ-

ous work [35], the result of real-time PCR analysis showed that the expression level of miR-26b was markedly downregulated in seven of the HCC cell lines (HepG2, SMMC7721, bel-7402, PLC, LM3, 97L and 97H) except in Huh7 cell line, in comparison with the expression levels in human hepatocyte line L02 (**Figure 1A**). We then assayed the EphA2 expression levels in HCC cell lines. EphA2 showed significantly higher expression in HCC cell lines (HepG2, SMMC7721, bel-7402, LM3, 97L and 97H) except Huh7 and PLC cell lines, in comparison with the expression levels in human hepatocyte line L02 (**Figure 1B**). Taken together these results indicate that miR-26b may be a tumor inhibitor and EphA2 may be an oncogenic regu-
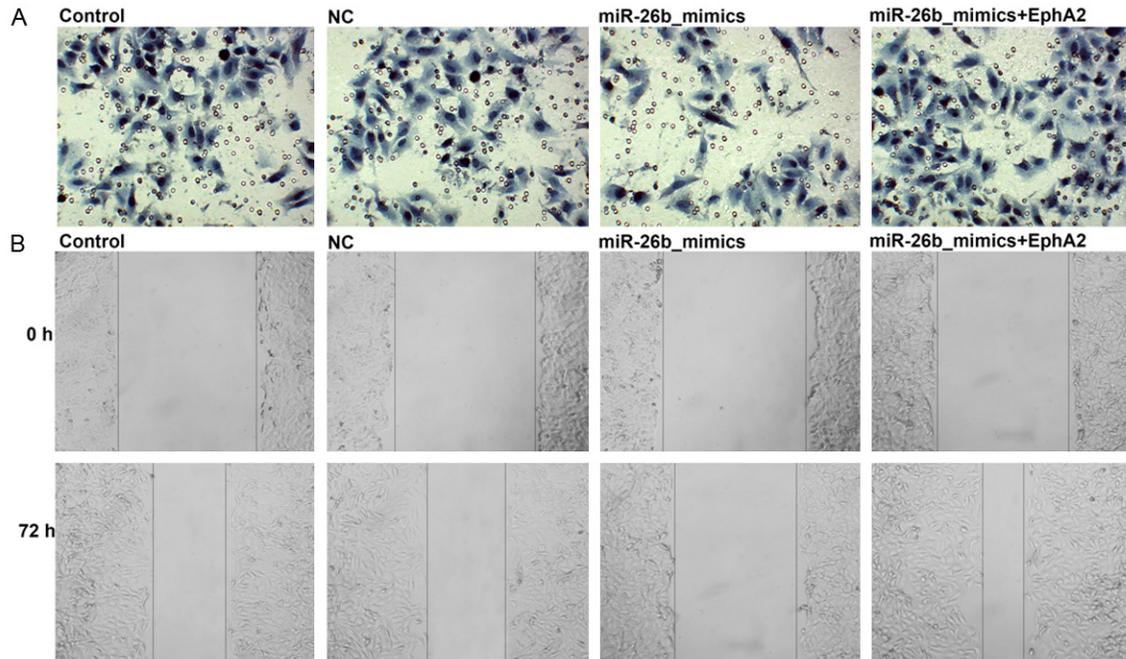
Figure 4. miR-26b inhibited invasion, and migration of HCC cells. A. The effects of miR-26b and EphA2 on cell invasion. B. The effects of miR-26b and EphA2 on cell migration.

lator in the progression of human hepatocellular carcinoma.

miR-26b directly targets EphA2 *in HCC cells*

To elucidate whether EphA2 is a potential downstream target gene of miR-26b in HCC cells, the miRNA target prediction websites www.microR-NA.org and TargetScan were used to identify a conserved miR-26b-binding site in the 3'-UTR of EphA2 mRNA. We then cloned WT or Mut target region sequence of the EphA2 3'-UTR, which was inserted into a luciferase reporter vector (**Figure 2A**). Subsequently, these reporter vectors were cotransfected with miR-26b mimics or inhibitors and negative control (mimics_con and inhibitors_con) into the LM3 cell line. As shown in **Figure 2B**, co-transfection of miR-26b mimics suppressed the luciferase activity of the reporter containing wild-type EphA2 3' UTR sequence, but failed to inhibit that of mutated EphA2 by dual-luciferase reporter assay. Inversely, luciferase activity was significantly increased in the cells transfected with miR-26b inhibitors, but did not change in the Mut 3'-UTR cells (**Figure 2B**). These data suggest that EphA2 may be a direct functional target of miR-26b in HCC.

miR-26b inhibited proliferation, invasion, and migration of HCC cells by suppressing EphA2 expression

We transfected EphA2 mimics in LM3 cells to overexpressed EphA2 protein. As shown in **Figure 3A**, mRNA level of EphA2 was greatly increased by EphA2 mimics, which was also confirmed by western blot analysis (**Figure 3B**). Moreover, qRT-PCR analysis showed that overexpressed EphA2 did not affect the expression of miR-26b. To further characterize the functional importance of miR-26b in HCC progression, we examined its effect on the proliferation of HCC cells using a CCK-8 assay. The data showed that miR-26b mimics significantly decreased the proliferation of HCC cells, which was reversed by transfected with EphA2 mimics (**Figure 3D**).

To evaluate the impact of miR-26b on cell invasion and migration, Transwell invasion and wound-healing assays were employed. The results showed that miR-26b mimics decreased the invasion of LM3 cells, which was reversed by overexpressed EphA2 (**Figure 4A**). Similar results were observed in wound-healing assays of LM3 cells (**Figure 4B**). Together, these
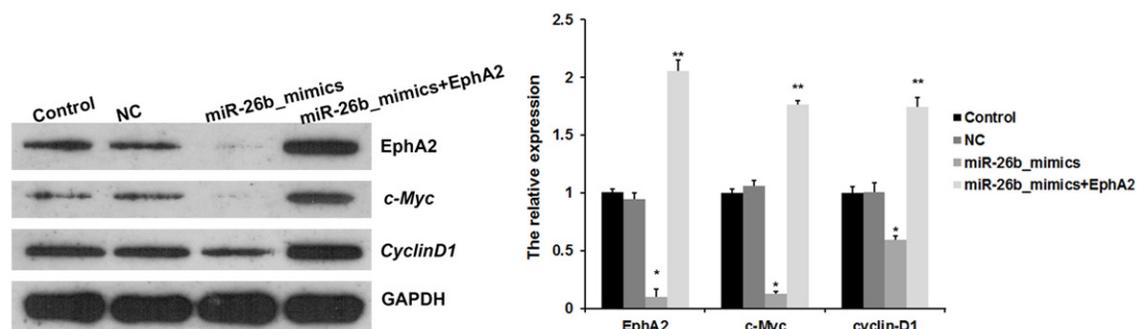
Figure 5. miR-26b downregulates the expression of *c-Myc* and *CyclinD1* by targeting EphA2. miR-26b mimics, miR-26 normal controls (NC) and EphA2 mimics (EphA2) were cotransfected into LM3 cells. Forty-eight hours later, proteins were harvested for Western blotting; GAPDH served as an internal control. Error bars represent ± S.E. *, $P < 0.01$ versus control and NC, **, $P < 0.01$ versus miR-26b_mimics.

findings demonstrate that miR-26b inhibits HCC cell proliferation, invasion, and migration in vitro by targeting EphA2.

miR-26b down-regulates c-Myc and CyclinD1 expression by targeting EphA2

Our previous study indicated that silencing of EphA2 results in decreased protein levels of *c-Myc*, and *CyclinD1*, which are oncoproteins that play an important role in cell proliferation, cell cycle regulation, and invasion, respectively [14, 36]. In the present study, Western blotting was used to investigate whether miR-26b regulates the expression of *c-Myc* and *CyclinD1* through targeting EphA2. The results revealed that the expression levels of EphA2, *c-Myc* and *CyclinD1* in cells transfected with miR-26b mimics were significantly lower than those of cells transfected with NC miRNA (**Figure 5**). These results provide further support of the inhibitory role that miR-26b plays in the proliferation, invasion, and migration of GC cells by targeting EphA2.

Discussion

Dysregulation of miRNA expression is a key factor underlying tumorigenesis. In this study, we performed qRT-PCR to investigate the expression levels of miR-26b in eight HCC cell lines. miR-26b expression levels were significantly down-regulated in seven HCC cell lines (HepG2, SMMC7721, bel-7402, PLC, LM3, 97L and 97H) compared with human hepatocyte line L02. Similar findings have been reported in several

other cancer types, including tongue squamous cell carcinoma [37], breast cancer [38] and glioma [33]. Consistent with this study, Shen et al. showed that MiR-26b expression was decreased in HCC cell lines (HepG2, MHCC97L MHCC97H, BEL-7402, Huh7, HCCC-9810, Hep-3B and QGY-7703) compared with the expression levels in normal liver tissue from three patients and was inversely correlated with the grade of HCC [35]. These results indicated that miR-26b is down-regulated in HCC.

EphA2 has been studied extensively because of its overexpression in several human cancers including melanoma, non-small cell lung cancer, breast cancer, prostate cancer and colon cancer [14, 15, 19, 22, 39]. Recently, accumulating of miRNAs were found to exert their biological functions by targeting EphA2, including miR-141 [30], miR-520d-3p [31], miR-200a [32], and miR-26b [33]. Indeed, Wu et al. confirmed that miR-26b directly target the 3'-UTR of EphB2 in glioma [33]. In this study, luciferase reporter analysis revealed that EphB2 is also a direct target of miR-26b in HCC. Recently, Shen et al. reported that miR-26b inhibits epithelial-mesenchymal transition in hepatocellular carcinoma by targeting USP9X [35]. These results indicate that the regulation of target genes by miRNAs is more complicated than previously considered. Target site recognition is primarily determined by multiple instances of seven-nucleotide core complementarity, which implies that each miRNA can influence the expression of a remarkably large number of different mRNAs [5].

Proliferation, invasion, and migration are critical steps of HCC progression and recurrence. Therefore, elucidating the pathological mechanisms underlying HCC proliferation, invasion, and migration is critical for development of effective therapies. In the present study, we showed that overexpressed miR-26b potently suppressed the HCC cells' proliferative, invasive, and migration behaviors, whereas overexpressed EphA2 reversed these behaviors. Moreover, miR-26b down-regulated the expression of EphA2, whereas EphA2 did not affect the expression of miR-26b.

Several studies have reported that the canonical Wnt/β-catenin signaling pathway target genes *c-myc* [40] and *cyclinD1* [41] are essential for cancer cell proliferation and invasion. Ectopic expression of EphA2 could induce changes in the expression levels of *c-Myc* and *CyclinD1* through the canonical Wnt/β-catenin signaling pathway [14]. In the current study, we observed that expression levels of *c-Myc* and *CyclinD1* were down-regulated by miR-26b, which was reversed by EphA2 mimics. In conclusion, these results suggested that miR-26b inhibits the proliferation, invasion, and migration of HCC cells through the EphA2-mediated-Wnt/β-catenin signaling pathway.

In summary, our study showed that miR-26b and EphA2 were down-regulated and up-regulated in HCC respectively. Moreover, ectopic expression of miR-26b inhibited HCC proliferation, invasion, and migration through targeting EphA2, providing potential new biomarkers and therapeutic targets for patients with HCC.

Disclosure of conflict of interest

None.

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