miR-23b inhibits proliferation of SMMC-7721 cells by directly targeting IL-11

TIANPENG JIANG¹, ZHI HUANG¹², SHUAI ZHANG³, WEIJIE ZOU¹, LEI XIANG¹, XIAOWEN WU¹,
YAPING SHEN⁴, WEIXIN LIU⁴, ZHU ZENG⁵, ANSU ZHAO⁵, SHI ZHOU¹³ and QINGFAN ZENG⁴

¹Department of Interventional Radiology, The Affiliated Hospital of Guizhou Medical University; ²The Key Laboratory of Medical Molecular Biology, Guizhou Medical University, Guiyang, Guizhou 550002; ³Department of Interventional Radiology, The Affiliated Cancer Hospital of Guizhou Medical University; ⁴Department of Interventional Radiology, The Affiliated Baiyun Hospital of Guizhou Medical University; ⁵School of Biology and Engineering, Guizhou Medical University, Guiyang, Guizhou 550001, P.R. China

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Abstract. Hepatocellular carcinoma (HCC) is the third leading cause of cancer-associated mortality in the 21st century. microRNA (miR)-23b has been shown to be involved in the pathogenesis of many cancers, including breast and prostate cancer. However, the role of miR-23b in HCC remains unclear. The present study revealed a negative correlation between miR-23b expression in HCC tissues and progression of carcinomas. Compared to normal tissues, miR-23b expression was significantly downregulated in HCC tissues, whereas the expression of interleukin (IL)-11 and IL-11 receptor α (IL-11Rα) was significantly upregulated, indicating that miR-23b expression is negatively correlated with IL-11 and IL-11Rα expression. In addition, miR-23b inhibited proliferation and promoted apoptosis of SMMC-7721 cells. This effect was mediated by IL-11, which was found to be the direct target of miR-23b in this study. These results indicated that miR-23b regulates IL-11 and IL-11Rα expression, and might act as an anti-oncogenic agent in the progression of HCC by directly downregulating IL-11 expression.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, and is a primary malignancy that originates in the liver; it is frequently observed in patients with chronic liver diseases, such as virus infections and cirrhosis (1,2). The incidence of HCC is increasing yearly worldwide; in 2016, it accounted for 224,390 new cases and 27,170 deaths in the United States (3,4). Despite the enormous progress made in the diagnosis and treatment of HCC, the 5-year survival rates of patients with HCC remain dissatisfactory. Even in patients with early-stage HCC, who undergo surgery, the 5-year survival rate is only 47.53% (5,6). The levels of serum α-fetoprotein have been considered as a biomarker of early-stage HCC; however, the detection sensitivity of α-fetoprotein is only 39-65%, and its detection specificity is also very low (7,8). Owing to the lack of effective biomarkers of HCC, most patients are often diagnosed at advanced stages, and are not eligible for curative therapies (9,10). Traditional treatments for patients with HCC mainly include surgery and chemotherapy, which have limited efficacy (11,12). Therefore, it is essential to explore new promising therapeutic targets for treatment of such patients.

Non-coding RNAs (ncRNAs) are RNA transcripts that do not code for proteins. These play essential roles in numerous biological processes, including gene expression, development, and differentiation (13-15). MicroRNAs (miRNAs/miRs), an important subsets of ncRNAs, are characterized by their small size (20-22 nucleotides) and are single-stranded (16,17). miRNAs are usually involved in the regulation of gene expression by directly binding the target mRNA, resulting in degradation of target mRNA or suppression of its translation (18). miRNAs can also potentially regulate other diverse physiological processes, including lipid metabolism, cell apoptosis, immune reaction, and DNA repair (8,19). Due to its suppressive role in many cancers, miR-23b has been identified as a biomarker and a diagnostic and therapeutic agent in various cancers, such as ovarian cancer (20). Recent studies have suggested a crucial role of miR-23b in HCC (21). However, the underlying mechanism must be better understood.

Correspondence to: Dr Shi Zhou, Department of Interventional Radiology, The Affiliated Hospital of Guizhou Medical University, 28 Guiyi Street, Guiyang, Guizhou 550002, P.R. China
E-mail: doctorzhoushi@yeah.net

Dr Qingfan Zeng, Department of Interventional Radiology, The Affiliated Baiyun Hospital of Guizhou Medical University, 108 Gangyu Street, Guiyang, Guizhou 550002, P.R. China
E-mail: gake585936@sohu.com

¹Contributed equally

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Interleukin (IL)-11 is a multifunctional cytokine, widely expressed in various tissues, including the gut, brain, and liver (22). Previous investigations have shown that IL-11 is involved in the development of several malignant tumors, including HCC (23). It signals via IL-11 receptor (IL-11R) composed of a ligand-binding α subunit (IL-11Rα) and a β subunit, responsible for signal transduction (23,24).

In this study, we observed decreased expression of miR-23b and increased expression of IL-11 in HCC tissues, leading to the hypothesis that IL-11 expression might be regulated by miRNA. Using an HCC cell line, this is the first study to confirm that IL-11 is a direct target of miR-23b; IL-11 may contribute to the decreased cell proliferation and enhanced cell apoptosis mediated by miR-23b.

Materials and methods

Patients and tissue samples. We collected 20 pairs of human HCC tissues and corresponding normal tissues from patients with HCC at the Affiliated Hospital of Guizhou Medical University (Guiyang, China). The patient information is detailed in Table I. Written informed consent was obtained from all patients, and ethical approval was granted by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University. All tissue samples were stored at -80°C.

Cell culture. Human HCC cell lines (SMMC-7721, LM3, and Hep3B) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All HCC cell lines were routinely cultured in complete Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS) and 100 units/ml of penicillin or streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and incubated in a humidified incubator with 5% CO₂ at 37°C.

Cell transfection. SMMC-7721 cells were seeded in 6-well plates at a concentration of 1x10⁵ cells/well and cultured in a medium without antibiotics. After 24 h of culture, the cells were transfected with miR-23b mimic, inhibitor, or the corresponding negative controls (NCs), using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Additionally, pcDNA-IL-11 plasmid or siRNA was also transfected into SMMC-7721 cells, according to the manufacturer's instructions.

Immunohistochemistry. The HCC and normal tissues were prepared using the Histostain-Plus kit (MRBiotech, Emeryville, CA, USA), according to the protocol provided by the manufacturer. The tissues were cut into 4-μm thick sections. Deparaffinized sections were incubated with normal bovine serum for 2 h at room temperature, and then incubated overnight at 4°C with primary antibodies against IL-11 or IL-11Rα (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). These sections were incubated with a biotinylated conjugated secondary antibody at room temperature for at least 2 h, and the reaction signals were detected using diaminobenzidine (DAB; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), as per the instructions of the manufacturer.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from the HCC tissues, the corresponding normal tissues, and HCC cell lines was extracted using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), as per the manufacturer's protocol; cDNA was then synthesized from the extracted RNA by reverse transcription, using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). mRNA expression levels were detected using the SYBR-Green PCR Master Mix kit (Takara Biotechnology Co., Ltd., Dalian, China). The PCR reaction steps were as follows: 95°C for 30 sec as the first step in a loop, followed by 40 cycles at 95°C for 5 sec and 60°C for 34 sec as the second step. The primers of miR-23b, IL-11, IL-11Rα, GAPDH, and U6 (miRNA as internal loading control) were designed and obtained from Sangon Biotech Co., Ltd. (Shanghai, China). The primer sequences were as follows: GAPDH, 5'-CCT CGTCTCATAGACAAGATGTG-3' and 5'-GCGTGATAGCTA CATGGAACATG-3'; U6, 5'-CTCGCTTCGGGCAACA-3' and 5'-AACGCTTCGAGATTTGATGT-3'; miR-23b, 5'-GCC GCTGTAACATCTCACT-3' and 5'-GTGCAGGTCGAGT-3'; IL-11, 5'-GCGTACGAGTTGAGGAC-3' and 5'-TTGCACACATACACCCGGCTGT-3'; and IL-11Rα, 5'-ACTTCTGTCTGAACTGT-3' and 5'-GGACTGAC TCGTACGACAT-3'. The mRNA expression levels of IL-11 and IL-11Rα were normalized to those of GAPDH, while the mRNA expression levels of miR-23b were normalized to those of U6. Analysis of relative gene expression data using qPCR and the 2ΔΔCt method (25).

Colony formation assay. The transfected SMMC-7721 cells were seeded in 6-well plates and maintained in DMEM containing 10% FBS for 2 weeks. These cells were then fixed with methanol and stained with 0.1% crystal violet. The number of colonies was manually counted.

Cell apoptosis analysis. The apoptosis rate of the transfected SMMC-7721 cells was measured by Annexin-V-propidium iodide (PI) double staining (BD Biosciences, Franklin Lakes, NJ, USA) and flow cytometry analysis. The transfected SMMC-7721 cells were collected and re-suspended in 500 ml binding buffer containing 5 ml Annexin V-fluorescein isothiocyanate (FITC) and PI at a density of 1x10⁵ cells/ml. After incubation at room temperature under light-protection for about 20 min, cells were analyzed via flow cytometry.

Western blotting. SMMC-7721 cells transfected with miR-23b mimics, mimic NC, inhibitor, and inhibitor NC were lysed by radioimmunoprecipitation assay buffer (RIPA) buffer [0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1 mM MgCl₂, 10 mM Tris-HCl, pH 7.4], and then disrupted with an ultrasonic cell disruptor on ice. After high-speed centrifugation, the debris was removed and the supernatant was collected. The total protein concentration was quantified using a protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA), as per the manufacturer's instructions. Proteins (50 μg) were separated on 10% SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Non-specific sites were blocked by immersing the membranes in 5% non-fat dry milk (w/v) for 2 h at room temperature. Membranes were incubated overnight with primary antibodies...
against IL-11 (1:500; Santa Cruz Biotechnology, Inc.) or IL-11Rα (1:500; Santa Cruz Biotechnology, Inc.) at 4°C. After washing in Tris-buffered saline, the membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. GAPDH was used as an internal control, and signals were detected by enhanced chemiluminescent reagents.

**Luciferase assay.** HEK 293 cells were seeded into a 12-well plate (3x10⁵ cells/well) and transfected with 100 ng/ml UTR or mutant UTR luciferase reporter construct using Lipofectamine 2000. After co-transfection with miR-23b mimic or inhibitor or the controls, the cells were collected and luciferase activities were measured. The luciferase activity was normalized to the internal control renilla luciferase activity.

**The Cancer Genome Atlas (TCGA) data query.** To examine the relationship between miR-23b and IL-11 expression in human HCC tissues, we referred to the TCGA data portal (https://tcga-data.nci.nih.gov/tcga) for all HCC samples, and matched normal tissues with the miR-23b and IL-11 expression data available. We filtered a data set for samples having expression data for miR-23b and IL-11. Statistical analysis was performed using SPSS spearman.

**Statistical analysis.** All results were represented as mean ± standard error of the mean (SEM) of at least three independent tests, and statistical significance was analyzed by GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS software. Comparisons between two groups were performed by Student's t-test or one-way analysis of variance with the Student-Newman-Keuls test. The Pearson's correlation algorithm was used to analyze the correlation coefficient and the significance between the expression of miR-23b and IL-11. P<0.05 was considered to indicate a statistically significant difference.

### Results

**miR-23b expression is downregulated, whereas the expression of IL-11 and IL-11Rα is upregulated in HCC tissues.** To investigate whether miR-23b, IL-11, and IL-11Rα are associated with the progression of HCC, we examined the expression levels of IL-11 and IL-11Rα in HCC and corresponding normal tissues, by immunohistochemical analysis. We observed that IL-11 and IL-11Rα expression levels were higher in HCC tissues, compared to those in normal tissues (Fig. 1A). To confirm this result, RT-qPCR was performed to measure the expression level of miR-23b, IL-11, and IL-11Rα in HCC and normal tissues. Results showed that miR-23b expression was lower in HCC tissues than that in normal tissues, and the expression of IL-11 and IL-11Rα was higher in HCC tissues than that in normal tissues (Fig. 1B). A more comprehensive analysis with TCGA database showed that in HCC tissues, miR-23b expression was significantly increased, whereas IL-11 expression was significantly decreased, compared with that in normal tissues (Fig. 1C and D). Furthermore, after analyzing the results obtained from RT-qPCR and TCGA database, it was noted that IL-11 expression decreased with the increase in miR-23b level (Fig. 1E and F). These findings indicated that there is an inverse correlation between miR-23b and IL-11 expression.

In addition, we analyzed the relationship between the expression level of miR-23b and the clinicopathologic features (age, sex, tumor size, tumor staging, and vascular invasion) of each patient with HCC. Correlation between miR-23b expression and vascular invasion of HCC was observed (P=0.016; Table I).

**IL-11 and IL-11Rα expression in SMMC-7721 cells is downregulated by miR-23b.** To determine the expression of miR-23b, IL-11, and IL-11Rα in HCC cell lines, we measured the expression levels of miR-23b in the HCC cell lines, SMMC-7721, LM3, and Hep3B. Our results showed that miR-23b expression was relatively higher in the Hep3B cell lines, and lower in the LM3 cell line, compared to that in SMMC-7721 cell line (Fig. 2A). We selected SMMC-7721 cells for further study, to measure the production of IL-11 and IL-11Rα after transfection with miR-23b mimics or inhibitor. The results of RT-qPCR showed that the levels of miR-23b were increased, whereas those of IL-11 and IL-11Rα were decreased in SMMC-7721 cells transfected with miR-23b mimics, compared to those observed on transfection with the negative control miR-mimic (NC miR-mimic). The expression levels of miR-23b were lower, whereas those of IL-11 and IL-11Rα were higher in SMMC-7721 cells transfected with miR-23b inhibitor, compared to those in cells transfected with negative control-inhibitor (NC-inhibitor) (Fig. 2B). In addition, similar results were observed for western blot analysis; the production of IL-11 and IL-11Rα was downregulated by miR-23b mimics, and upregulated by miR-23b inhibitor in the transfected

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**Table I. Associations between miRNA-23b expression level and clinicopathological variables of 20 cases of HCC.**

| Clinicopathological variables | miRNA-30c expression | P-value |
|------------------------------|----------------------|---------|
| Age (years)                  |                      |         |
| <50                          | 4                    | 5       | 0.767  |
| ≥50                          | 6                    | 5       |         |
| Sex                          |                      |         |
| Male                         | 5                    | 6       | 0.738  |
| Female                       | 4                    | 5       |         |
| Tumor size (cm)              |                      |         |
| <5                           | 7                    | 8       | 0.773  |
| ≥5                           | 3                    | 2       |         |
| Tumor staging (BCLC)         |                      |         |
| A                            | 5                    | 4       | 0.801  |
| B                            | 4                    | 6       |         |
| C                            | 0                    | 1       |         |
| Vascular invasion            |                      |         |
| Yes                          | 3                    | 9       | 0.016* |
| No                           | 6                    | 2       |         |

*P<0.05. miRNA, microRNA; BCLC, The Barcelona Clinic Liver Cancer staging system; HCC, hepatocellular carcinoma.*
SMCC-7721 cells, compared to that in cells transfected with the corresponding NCs (Fig. 2C and D). These results showed that miR-23b inhibited IL-11 and IL-11Rα expression.

miR-23b inhibits proliferation and promotes apoptosis of SMCC-7721 cells. To explore the role of miR-23b in HCC progression, we performed the colony formation assay and cell apoptosis analysis to measure cell proliferation and apoptosis, respectively, in SMCC-7721 cells transfected with miR-23b mimics or inhibitor. The results of the colony formation assay showed that the number of colonies was significantly lower in cells transfected with miR-23b mimics compared to that in cells transfected with the corresponding NCs (Fig. 2C and D). These results showed that miR-23b inhibited IL-11 and IL-11Rα expression.

Figure 1. Expression levels of miR-23b, IL-11, and IL-11Rα in HCC tissues. (A) Immunohistochemical analysis was performed to detect the expression of IL-11 and IL-11Rα in HCC and corresponding normal tissues (magnification, x200). (B) The relative expression of miR-23b, IL-11, and IL-11Rα in HCC and normal tissues (n=20) was measured by the RT-qPCR. (C) Relative expression of miR-23b was examined by comparing the sample data with data in TCGA database. (D) Relative expression of IL-11 was examined by comparing the sample data with data in TCGA database. Correlation analysis of miR-23b and IL-11 expression was performed using (E) RT-qPCR and (F) data in the TCGA database. miR, microRNA; IL, interleukin; HCC, hepatocellular carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
transfected with miR-23b inhibitor than that in cells transfected with NC-inhibitor (Fig. 3A). Cell apoptosis analysis showed that the apoptosis rate was significantly higher in SMMC-7721 cells transfected with miR-23b mimics than that in cells transfected with NC miR-mimics, and was significantly lower in SMMC-7721 cells transfected with miR-23b inhibitor than that in cells transfected with NC-inhibitor (Fig. 3B). These results indicated that miR-23b might exert...
anti-oncogenic effects on the development and progression of HCC.

**IL-11 is a direct target of miR-23b.** To confirm that IL-11 is a direct target of miR-23b, a luciferase reporter assay system was used. The complete 3'UTR of IL-11 mRNA was amplified and cloned into the 3'UTR of luciferase gene in the reporter vector UTR. Using the UTR vector as a template, a vector of mutant UTR was constructed containing the 3'UTR of IL-11 with point mutations, as shown in Fig. 4A. As expected, the UTR luciferase activity was significantly decreased and increased after co-transfection with mimics and inhibitor, respectively, compared to that observed on co-transfection with the corresponding NC. Additionally, the luciferase activity was rescued by the mutation of the miR-23b recognition site (Fig. 4B). These results indicated that IL-11 is a direct target of miR-23b.

**miR-23b promotes cell proliferation and inhibits cell apoptosis by targeting IL-11.** To explore whether IL-11 inhibition by miR-23b affects cell proliferation or apoptosis, the colony formation and apoptosis assays were employed, respectively. As shown in Fig. 5A, the colony numbers were significantly decreased after transfection with miR-23b mimics, whereas a significant increase was observed after transfection with miR-23b inhibitor. The inhibitory and promoting effects on cell growth were significantly suppressed by IL-11 overexpression with pcDNA-IL-11 transfection and IL-11 knockdown with siDNA transfection, respectively. Using flow cytometry, increased cell apoptosis was observed after transfection with miR-23b mimics, whereas cell apoptosis was significantly inhibited by IL-11 overexpression with pcDNA-IL-11 transfection (Fig. 5B). On the contrary, decreased cell apoptosis was noted with miR-23b inhibitor, and it was significantly inhibited after IL-11 knockdown by siDNA. These results indicated that miR-23b can promote cell apoptosis and inhibit cell proliferation by targeting IL-11.

**Discussion**

East Asia and Middle Africa have the highest occurrence rates of HCC in the world, and the HCC incidence and mortality rates are more than twice in males than those in females (26,27). Previous studies have indicated that virus infection is one of the principle causes of HCC development, and hepatitis B (HBV) and C (HCV) virus infections account for approximately 60 and 33%, respectively, of the total cases of HCC in most developing countries (28-30). Recently, increasing evidence has shown that the aberrant expression of miRNAs emerges as a key indicator of changes in the corresponding expression of genes that may be involved in virus replication (31,32). It was reported that interferon-β upregulates the expression of many cellular miRNAs; among these, 8 miRNAs may bind to the HCV genomic RNA with their specific sequences (33). Although the precise roles of miR-122 in virus infection are not very clear, several studies have demonstrated its participation in the replication of HCV. For instance, Lanford et al reported that the liver-abundant miRNA, miR-122, is indispensable for the accumulation of HCV RNA in cultured liver cells, and that inhibition of miR-122 expression in chimpanzees results in significant suppression of HCV viremia (34). In addition, miRNA expression in HCC tissues is altered, compared to that in normal tissues, and these miRNAs might serve as critical diagnostic biomarkers or potential therapeutic targets for HCC treatment (35,36). Upregulated expression of miR-184...
was observed in HCC tissues, which was believed to exert anti-apoptotic and pro-proliferative effects on HCC cells, suggesting that miR-184 serves as an oncogenic agent in the development and progression of HCC (37). Downregulated expression of miR-335 was observed in HCC tissues and cell lines; this miRNA inhibited the proliferation and migration of HCC cells by directly targeting Rho-kinase 1 (ROCK1) (38). However, whether miR-23b is involved in HCV infection and HCC progression remains unknown. It is essential to elucidate the role of miR-23b in liver cancer to facilitate the development of new potential therapeutic targets.

miR-23b is an important member of the miR-30 family, which consists of 5 distinct and highly conserved miRNAs, namely, miR-30a, miR-30b, miR-23b, miR-30d, and miR-30e (39). The miRNA, miR-23b, is encoded by two genes (miR-23b-1 and miR-23b-2), providing more flexibility for tissue-specific expression, and more adaptability to the changing environment (40). A large number of studies have showed that alterations in miR-23b expression have been implicated in numerous malignant tumors, including those of the breast and prostate (41,42). However, only a few studies on the effects of miR-23b on HCC progression have been reported.

As a multifunctional protein, IL-11 is involved in diverse cell signaling pathways in normal cells and various pathologies (43,44). Its action is mediated by IL-11Rα. Recently, IL-11 has been reported to be released directly by some cancer cells, including breast, colon, and lung cancer cells (45-47). Likely, IL-11Rα was reported to be overexpressed and associated with some cancers, such as prostate

Figure 5. Effect of miR-23b on the proliferation and apoptosis of SMMC-7721 cells was mediated by IL-11. The proliferation (A) and apoptosis (B) of SMMC-7721 cells were determined by colony formation assay and flow cytometry, respectively, after transfection with miR-23b mimics, miR-23b inhibitor, pc-DNA-IL-11, or si-IL-11. *P<0.05, **P<0.01, ***P<0.001. IL, interleukin.
cancer (48), osteosarcoma (49), and gastric carcinoma (23). Furthermore, it was revealed that the expression of IL-11 and IL-11Rα can be updated by the same regulator, such as IL-13 (50), and can resultantly contribute to the pathogenesis together. It has been shown that IL-11 can promote tumor development by regulating downstream protein expression or activating downstream pathways, such as the STAT and MAPK pathways (23,24). Additionally, IL-11 acts on T- and B-cells and macrophages, exhibiting anti-inflammatory effects by reducing the production of pro-inflammatory cytokines, such as TNF-α, IL-β, and IFN-γ (51). This might result in the attenuation of inflammatory diseases and promote tumorigenesis (52). However, the exact mechanism by which IL-11 promotes HCC development needs to be further evaluated. In our study, we found that miR-23b expression was lower in HCC tissues than in the corresponding normal tissues. We also found that the expression levels of IL-11 and IL-11Rα were upregulated in HCC tissues, compared to those in normal tissues. To investigate the correlation between miR-23b and IL-11 and IL-11Rα, we measured the expression level of IL-11 and IL-11Rα in the HCC cell line, SMMC-7721, transfected with either miR-23b mimics or miR-23b inhibitor. Our results showed that miR-23b mimics significantly inhibited the production of IL-11 and IL-11Rα in SMMC-7721 cells, whereas transfection with miR-23b inhibitor obviously increased their production. Furthermore, the inverse correlation between miR-23b and IL-11 was confirmed by a comprehensive analysis with TCGA database. The luciferase assay showed that IL-11 is a direct target of miR-23b. In addition, we explored the effect of inhibition of IL-11 and IL-11Rα by miR-23b on the HCC cell line by various in vitro experiments, including colony formation assay and apoptosis analysis. Our results indicated that miR-23b significantly inhibited the proliferation of SMMC-7721 cells. Additionally, miR-23b promoted apoptosis in SMMC-7721 cells. But the effects of the miR-23b on the proliferation and apoptosis only investigated on SMMC-7721 cells, and we planned to be used more HCC cells for investigating the role miR-23b in the future.

In conclusion, we revealed that miR-23b regulates IL-11 and IL-11Rα expression. Furthermore, it was identified as an anti-oncogene molecule that inhibits HCC growth by directly inhibiting IL-11 expression in HCC cells, suggesting that miR-23b might serve as a potential therapeutic target for the treatment of patients with HCC. In future studies, we aim to further elucidate the mechanism by which miR-23b regulates the expression of IL-11 and IL-11Rα in HCC.

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Availability of data and materials
All data generated or analyzed during this study are included in this article.

Authors’ contributions
TPJ, ZH, SZ, WJZ, LX, XWW, YPS, WXL, ZZ, ASZ, SZ and QFZ conceived and designed the study; TPJ, ZH, SZ and WJZ performed the experiments; TPJ and ZH wrote the paper; TPJ and ZH reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate
Written informed consent was obtained from all patients, and ethical approval was granted by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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