Cancer Management and Research

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ORIGINAL RESEARCH

Xiaoai Jiedu Recipe suppresses hepatocellular carcinogenesis through the miR-200b-3p /Notch1 axis

This article was published in the following Dove Press journal:
Cancer Management and Research

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Purpose: Xiaoai Jiedu recipe (XJR), a formula long used by Chinese National Medical Professor Zhou Zhongying, has potent antitumor properties, but the molecular mechanism is still unclear. The aim of the study was to investigate the antitumor mechanism of XJR on hepatocellular carcinoma (HCC) by focusing on miRNA.

Methods: Three concentrations of XJR (low, middle, and high) were used to treat tumor xenograft mice models. Microarray technology was used to identify the differential expressed genes after XJR treatment, and bioinformatic tools and luciferase reporter assay to predict the potential pathways. HepG2 cells were transfected with inhibitor of miR-200b-3p to detect the effect of miR-200b-3p and Notch1 on tumor growth.

Results: XJR effectively exerted anti-HCC effect both in vitro and in vivo. MiRNA chip analysis results showed that the expression of 75 miRNAs was upregulated and 158 miRNAs was downregulated in blood from XJR-treated mice. Further validation by using real-time polymerase chain reaction (RT-PCR) assay showed that the expression of five miRNAs (miR-453, miR-200b-3p, miR-135a-1-3p, miR-1960, miR-378a-5p, and miR-466f) was consistent with the results of miRNA chip analysis. Among them, miR-200b-3p was selected as candidate for further research. Results of the MTT, migration, and wound healing assays showed that down-expression of miR-200b-3p abrogated the effect of XJR on cell growth and metastasis. Luciferase reporter assay confirmed that Notch1 was the direct target of miR-200b-3p. XJR significantly decreased Notch1 expression in HepG2 cells, whereas miR-200B-3p inhibitor abrogated the XJR-induced decrease in Notch1 expression.

Conclusion: This study indicated that XJR could effectively inhibit HCC and might exert its antitumor effect through the miR-200b-3p/Notch1 axis. These findings provided new avenues for the use of XJR for prevention and treatment of HCC.

Keywords: Xiaoai Jiedu recipe, hepatocellular carcinoma, miR-200b-3p, Notch1

Introduction

Liver cancer is one of the most common malignancies worldwide. Meanwhile it is the fifth and seventh leading cause of cancer-related death in men and women, respectively.[1] Hepatocellular carcinoma (HCC) is the most prevalent type of liver cancer, accounting for 75–85% of cases of primary liver cancer, with approximately 850 000 new cases per year worldwide.[2] Despite advances in diagnosis and treatment, overall prognosis of HCC patients remains poor, with a 5-year survival rate of less than 20%.[3] The long-term survival of cancer patients depends largely on early diagnosis and effective treatment, but most HCCs are already advanced or

metastatic at the time of diagnosis. Although surgical resection provides HCC patients with the best chance for recovery, most patients are detected too late, and only 10–20% of patients are eligible for surgical resection.[4] Therefore, we urgently need to identify alternative therapies to improve clinical survival rates.

Traditional Chinese medicine (TCM) has a long history in the treatment of complex chronic disease through a holistic manner.[5] In clinical practice, certain Chinese medicine formulae, as important sources of many chemotherapeutics and chemical anti-cancer medicine, can effectively treat and prevent malignant tumors.[6] Xiaoai Jiedu recipe (XJR), a formula long used by Chinese National Medical Professor Zhou Zhongying,[7] consists of 7 Chinese herbal medicines in a specific proportion: Hedyotis diffusa (20 g), Radix pseudostellariae (15 g), Bombyx batryticatus (10 g), Cremastra appendiculata (10 g), Centipede (3 g), Radix ophiopogonis (12 g), and Akebia trifoliata Koidz (12 g).[8] Researchers have determined that XJR can inhibit the growth and invasion of many tumors.[9] However, due to the insufficiency of research methods and complexity of components of XJR, the exact mechanism of XJR in the treatment of HCC is still unclear, which has affected its clinical application and promotion to a certain extent.

MicroRNA (miRNA) is a type of endogenous short noncoding RNA of about 21–25 nucleotides in length. Varieties of studies demonstrated that miRNAs play a key role in regulating diverse cellular biological processes and have been proven to be ideal biomarkers and targets for therapeutic intervention.[10] MiRNAs are involved in tumorigenesis and tumor progression as an oncogene or tumor suppressor. Growing evidence suggests that miRNAs play a critical role in several kinds of cancers, such as lung cancer, breast cancer, liver cancer,[11] gastric cancer, pancreatic cancer,[12] and glioma. And there are several miRNAs that play an important role in HCC, including miR-320b, miR-308-3p, miR-595, miR-885-5p, and miR-1296.[13] However, whether miRNAs play important roles in regulating XJR-induced tumor suppression is not clear. In this study, we evaluated the effects of XJR on HCC and further investigated the potential anti-cancer mechanism of XJR through comprehensive miRNA expression profiles.

Materials and Methods

Drugs and Reagents

XJR was purchased from Anhui Bozhou Traditional Chinese and Western Medicine Co., Ltd. (Anhui, China). XJR is composed of 7 different herbal ingredients: Hedyotis diffusa (20 g), Radix pseudostellariae (15 g), Bombyx batryticatus (10 g), Cremastra appendiculata (10 g), Centipede (3 g), Radix ophiopogonis (12 g), and Akebia trifoliata Koidz (12 g). Decoction of XJR (2 g/mL) was prepared according to the standard method,[8] diluted with saline (v/v, 1:1), and stored at 4°C for later use. Cisplatin was purchased from the Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China). MTT, dimethyl sulfoxide (DMSO), PBS, FBS, penicillin/streptomycin, and the other reagents for cell culture were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Luciferase reporter plasmids were purchased from Genechem Co., Ltd (Shanghai, China). Antibodies for Notch1 (b8925) and β-actin (20,536-1-AP) were purchased from Abcam (Cambridge, UK). BCA Protein Assay Kits were purchased from ThermoFisher Scientific (Waltham, MA).

Preparation of Medicated Serum

The medicated serum was prepared according to the protocol.[8] Briefly, 24 adult Sprague-Dawley rats (6～8 weeks; 220～250g) were divided into XJR (N = 12) and control (N= 12) groups. The rats were fasted for 12 hours. The XJR group rats were administrated intragastrically with XJR decoction of 9 g/kg body weight/day for 3 days, and control group rats were administrated intragastrically with physiological saline. One hour after the last administration, blood sample was collected through retinal venous plexus and centrifuged to separate serum. The collected serum was inactivated at 56°C for 30 min and then sterilized using a 0.22 lm of filter. The prepared medicated serum was stored at –80°C for future use.

Animal Study

These animal experiments were conducted in accordance with the approved guidelines of the Experimental Animal Ethical Committee of Nanjing University of Chinese Medicine (SYXK 2014–0001). Healthy athymic BALB/c-nu male mice (4～6 weeks; 20±2 g) were obtained from Changzhou Cavens Experimental Animal Co., Ltd (Changzhou, China) and raised under specific pathogen-free conditions with free access to water and food. Mice were kept at a controlled temperature of 25°C and humidity of 65% in a 12:12 h light/dark cycle. H22 cells (5×10^6 per mouse) were subcutaneously injected into the flank of mice to establish the HCC xenograft model. Mice were

evaluated for tumorigenesis daily, and tumor diameter >3mm was considered as tumor formation on day 7.

When the tumor reached 5–6 mm in diameter, the mice were randomly divided into five groups (ten mice per group): control group (NC, equal volume of physiologic saline), XJR low group (XJRL, 1 g/kg body weight/day), XJR middle group (XJRM, 3 g/kg body weight/day), XJR high group (XJRH, 9 g/kg body weight/day), and positive group (PC, 1 mg/kg body weight/day cisplatin). Mice were administrated intragastrically once a day for 10 days with the corresponding dose of agents. Mice were sacrificed at 24 h after the last intragastric administration, and tumors and blood were harvested for further analysis. Tumor volume can be calculated based on the formula of volume=(width^2×length)/2 commonly used in previous reports.[14]

Histopathology

Tumor samples of each group were fixed with 4% paraformaldehyde, embedded in paraffin, dehydrated with gradient ethanol, and sectioned into slices. Then, the sections were stained with hematoxylin-eosin (H&E) and the pathological changes of tumor tissues assessed under an optical microscope by an experienced physician.

MiRNA Microarray Analyses

The microarray analysis was performed by KangChen Corporation (Shanghai, China) and was used to detect miRNA profiling in blood with XJRH treatment using the miRCURY LNA Array system (Vedbaek, Denmark). Total RNA was extracted using Trizol (Invitrogen, USA) and miRVana miRNA Isolation Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. The concentration was measured using the NanoDrop1000 (Thermo Scientific, Wilmington, DE, USA) at 260/280 nm absorbance, and the samples were labeled using the miRCURY™ Power labeling kit. Then each array was hybridized on the miRCURY™ Array (v.18.0). Following the washing steps, the chip was scanned by the GenePix 4000B microarray scanner (Affymetrix, CA, USA). The raw data were normalized using the median normalization, and the differentially expressed (DE) miRNAs were filtered through fold change. The miRNAs with fold change >2 or <0.5 and $p<0.05$ were considered as significant DE miRNAs.

Total RNA Extraction and Quantitative RT-PCR

HCC lines (SMMC7721, HepG2, bel7402, and H22) and normal hepatocyte LO2 cells were purchased from the Chinese Academy of Sciences Shanghai Branch Cell Bank (Shanghai, China). Total RNA was isolated using Trizol reagent (Invitrogen, CA, USA) and was reverse-transcribed into cDNA using a reverse transcription kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol.[15] The primers for miRNAs were provided by Sangon Biotech (Shanghai, China), and the corresponding primer sequences used are shown in Table 1. Quantitative RT-PCR assay was carried out using miScript PCR Starter kit (Qiagen, Germany) according to the manufacturer's protocol. The relative miRNA expression level was normalized to U6.

The primers for Notch1 and GAPDH were also obtained from Sangon Biotech (Shanghai, China). Primer sequences for the genes are shown as follows: Notch1-F, 5′-GAGAAGGGAAGTTGAACGAGC-3′ and Notch1-R,

Table 1 The Primer Sequences for Genes

miRNA	Primer Sequence
miR-453	5′- GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTGCAAG −3′
miR-3471	5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAATGCC −3′
miR-200b-3p	5′- GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCTACCT-3′
miR-135a-1-3p	5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCGCCAC −3′
miR-1960	5′- GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCAGACA-3′
miR-466f	5′- GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACATGC-3′
miR-669c-5p	5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACACAC-3′
miR-669k-5p	5′- GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGCACAC-3′
miR-206-3p	5′- GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCCACAC-3′
miR-468-3p	5′- GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCAGACA −3′
U6	5′- GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGCTG-3′

Abbreviation: miRNA, microRNA.

5′-CACATGGCAACATCTAACCC-3′; GAPDH-F, 5′-TTTGGTATCGTGGAAGGAC-3′ and -R (5′-AAAGGTGGAGGAGTGGGT-3′). RT-PCR assay was performed using SYBR Green Master Mix (CloudSeq, Shanghai, China) with the ViiA™ 7 Real-time PCR System (Applied Biosystems). Relative expression of mRNA was normalized to GAPDH and analyzed according to the $2^{-\Delta\Delta Ct}$ method.

Cell Transfection

Cell transfection was utilized to manipulate the expression of miR-200b-3p with Lipofectamine 2000 reagent (Invitrogen, CA). The short hairpin RNA (shRNA) against miR-200b-3p and negative control (sh-NC) were supplied by Shanghai Genechem Co., Ltd (Shanghai, China). According to the manufacturer's specifications, HepG2 cells were transfected with Lipofectamine 2000 in OptiMem to inhibit miR-200b-3p expression.

Cell Proliferation Assays

HepG2 cells were seeded in 96-well plates at 5×10^3/pore and incubated overnight to allow cell attachment. Cells were divided into four groups: control group (NC), XJR group (cells treated with XJR, medicated serum), anti-miR-shRNA group (cells were treated with anti-miR-200b-3p-shRNA plasmids), XJR+anti-miR-shRNA group (cells were treated with XJR and anti-miR-200b-3p-shRNA plasmids). After treatments, cells were further incubated with MTT for 4 h. Then, 150 μL DMSO was added to each well. The optical density (OD) was measured with a multifunctional microplate reader (PerkinElmer Inc., Waltham, MA, USA) at a wavelength of 492 nm.

Cell Migration and Invasion Assay

To determine the role of XJR and miR-200b-3p on HCC cell migration, we used wound healing assay. As above, cells were divided into four groups: control group (NC), XJR group (cells treated with XJR, medicated serum), anti-miR-shRNA group, and XJR+anti-miR-shRNA group. Wound healing assay was performed to determine cell migration. HepG2 cells were seeded in 6-well plates and grown to form a monolayer. After reaching 80% confluence, the cell layer was scraped off using a sterile cell scraper to form a scratch, resulting in a wounded culture. After the residual cells were washed with PBS, we measured the distance traveled by the cells between the two boundaries of the cell area at 0 and 48 h, respectively.

Cell Invasion Assay

The cell invasion assay kit (Neuro Probe, Gaithersburg, USA) was used to assess the capability of cell invasion as described.[16] Cells were treated as above. After collecting the cells from different groups, we prepared cell suspensions without FBS at the density of 2×10^5 cells/mL and added 200 μL of the cell suspension to the upper chamber of transwell. A quantity of 500 μL of medium containing 10% FBS was added to the lower chamber. After 24 h of incubation, non-invading cells on the top of the membrane were removed with cotton swabs. The cells on the bottom of the membrane were collected then fixed in 10% formaldehyde solution and stained using 0.5% polycrystalline violet. After the cells were air-dried, they were observed and photographed under an inverted microscope. The number of cells was recorded in five fields of view without overlap. Experiments were performed in triplicate.

Target Prediction of MiR-200b-3p Gene

The target gene of miR-200b-3p was predicted using the overlapped data from TargetScan prediction software (http://www.targetscan.org/) and miRBase prediction software (http://www.ebi.ac.uk) for the construction of miRNA-gene interaction network.

Luciferase Reporter Assay

The entire wild-type and mutant 3′-untranslated region (UTR) of Notch1 fragment were provided by Shanghai Genechem Co., Ltd (Shanghai, China) and inserted into the pmirGLO firefly luciferase-expressing vector (Promega, WI, USA). The luciferase reporter vectors together with miR-200b-3p–mimics or the negative control vector were transfected into HepG2 cells with Lipofectamine 2000 transfection reagent (Thermo Fisher). After 48 h, the dual-luciferase® reporter assay system (Promega) was used to measure the luciferase activity according to the manufacturer's instructions.[17] Renilla luciferase activity served as the normalized control reporter.

Western Blot Analysis

Cells were homogenized in RIPA protein lysis buffer to extract total protein, and the protein concentration was quantitated with BCA protein assay (Invitrogen, CA, USA). Equal amounts of protein (300 ng) were separated by electrophoresis on 10% SDS-PAGE gel and transferred onto PVDF membrane. The membranes were blocked in 5% BSA for 2 h, then incubated with Notch1 (1:800, Cat:

ab8925) and β-actin (1:1000, Cat: # 20,536-1-AP) antibodies at 4°C overnight. After being washed with PBS, the membranes were probed with Goat Anti-Rabbit secondary antibody (1:10,000, Cat: # ad6721). Membranes were visualized by enhanced chemiluminescence. Image Lab software 4.1 (Bio-Rad, CA, USA) was used for densitometry analysis.

Statistical Analysis

Statistical analyses were performed using Student's *t*-test or one-way ANOVA analysis with SPSS version 18.0 (IBM, Chicago, IL, USA). All data were presented as mean ± standard deviation (SD) for at least three independent experiments. $P < 0.05$ was defined to be statistically significant.

Results

In Vivo Antitumor Efficiency of XJR

To better demonstrate the therapeutic effect of XJR on HCC, we used H&E staining to examine the histopathology changes of tumor tissues. As shown in Figure 1A–E, for the mice from XJRH group, it is observed that the structure of tumor tissues suffered more apparent extensive damage, such as intercellular spaces being widened irregularly, severe necrosis, and nucleus shrinkage with pyknosis.

The tumors in the NC groups grew rapidly. The XJRL, XJRM, and PC inhibited tumor growth to a certain extent. The XJRH had the strongest inhibitory effect on tumor growth (Figure 1F), which was consistent with the observation of H&E staining. Thus, considering the antitumor effect, we chose XJRH for further analysis.

MiRNA Microarray Analyses After XJRH Treatment

Our data showed that XJR can significantly inhibit the growth of liver cancer, but the genes involved in its mechanism were still a mystery. We used microarray analysis to evaluate the DE miRNAs in blood of the XJRH group relative to NC group. MiRNAs that exhibited fold changes >2 or <0.5 and with $p<0.05$ were selected as the DE miRNAs. Our result indicated that 233 miRNAs were DE in blood after XJRH treatment (75 upregulated and 158 downregulated) (Supplementary Table S1). A differential RNA expression cluster heat map clearly distinguished the DE miRNAs (Figure 2A). Red probes indicated the miRNAs which expressed higher than that of NC group after XJRH treatment, and green probes indicated the miRNAs which expressed lower than that of NC group. In the volcano plot (Figure 2B), red dots in the top right and top left indicated the up- and down-regulation of miRNAs expression in blood after XJRH treatment compared with NC group, respectively.

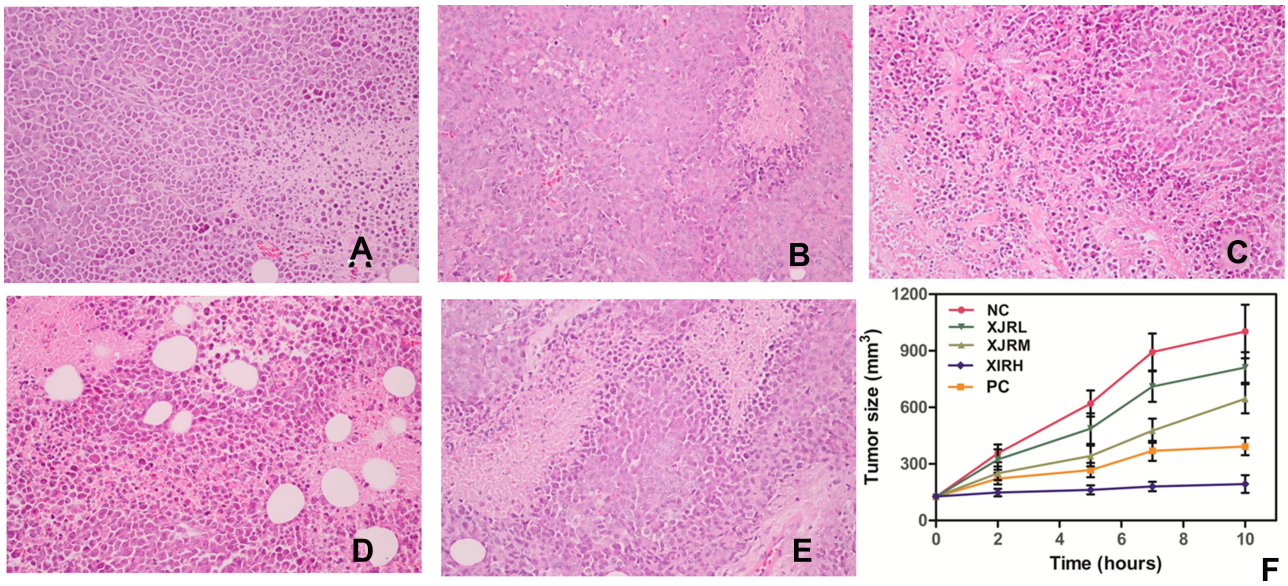

Figure 1 In vivo antitumor efficiency of XJR. Histopathological examination of tumor in (**A**) NC group, (**B**) XJRL group, (**C**) XJRM group, (**D**) XJRH group, and (**E**) PC group. The tissues were stained by H&E (400×). (**F**) The tumor volume of mice after different treatments.

Abbreviations: XJR, Xiaoai Jiedu recipe; NC, control group; XJRL, low dose of Xiaoai Jiedu recipe; XJRM, middle dose of Xiaoai Jiedu recipe; XJRH, high dose of Xiaoai Jiedu recipe; PC, positive group.

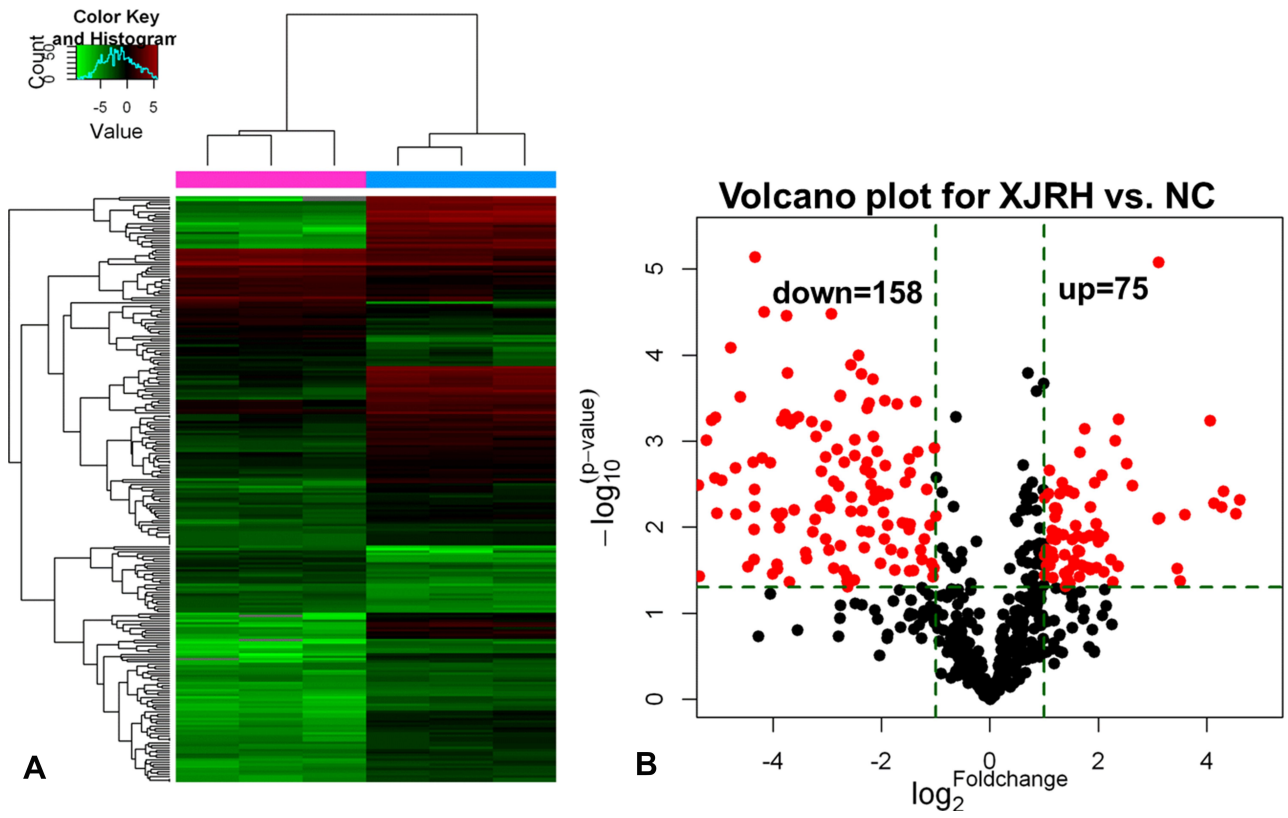

Figure 2 DE miRNAs in blood of XJRH group relative to NC group detected by microarray analysis. (**A**) Hierarchical cluster heat map of the 75 upregulated and 158 downregulated miRNAs. (**B**) Volcano plot of the DE miRNAs. Horizontal blue line indicates $p<0.05$.
Abbreviation: DE, differentially expressed.

Validation of DE MiRNAs

Based on the fold change, we chose five significantly upregulated miRNAs (miR-453, miR-3471, miR-200b-3p, miR-135a-1-3p, and miR-1960) with fold change >17 and five significantly downregulated miRNAs (miR-466f, miR-669c-5p, miR-669k-5p, miR-206-3p, and miR-468-3p) with fold change <0.0013 as candidates, and further verified by quantitative RT-PCR analysis. The results showed that the expression of miR-453, miR-200b-3p, miR-135a-1-3p, and miR-1960 were significantly higher in blood of XJRH group than those of NC group ($p<0.05$) (Figure 3A, C–E), and miR-466f was significantly lower than NC group ($p<0.05$) (Figure 3F). There was no significant difference expression of miR-3471, miR-669c-5p,

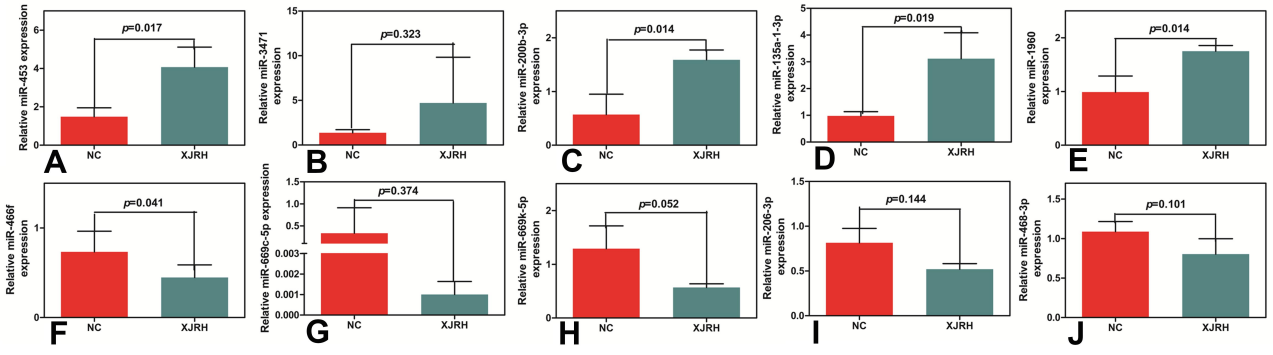

Figure 3 (**A–E**) The relative levels of the selected significantly upregulated miRNAs (miR-453, miR-3471, miR-200b-3p, miR-135a-1-3p, and miR-1960) and (**F–J**) the downregulated miRNAs (miR-466f, miR-669c-5p, miR-669k-5p, miR-206-3p, and miR-468-3p) in blood of NC group and XJRH group were confirmed using RT–PCR. The miRNAs levels were normalized to U6.
Abbreviations: XJRH, high dose of Xiaoai Jiedu recipe; NC, control group; miRNA, microRNA.

miR-669k-5p, miR-206-3p, and miR-468-3p between XJRH group and NC group ($p>0.05$) (Figure 3B, G–J). Through reference research, looking for genes with a greater correlation with the antitumor effect of XJR, we finally determined miR-200b-3p for further research.

We also analyzed the expression level of miR-200b-3p in cells and tumor tissues by RT-PCR.

HCC cells showed low expression of miR-200b-3p. The expression of miR-200b-3p in all four HCC lines (SMMC7721, HepG2, bel7402, and H22) was lower than that in normal hepatocyte LO2 cells (Figure 4A), and miR-200b-3p expression level was significantly upregulated in the tumor tissue of the XJRH-treated group compared with that of the control group (Figure 4B). The results suggested that miR-200b-3p might be a potential target for treating HCC as a tumor suppressor. Thus, considering the expression of miR-200b-3p and proliferation rate of cells, HepG2 was selected for in vitro studies.

Low Expression of MiR-200b-3p Abrogated the Effect of XJR on Cell Proliferation, Migration, and Invasion

After transfection of anti-miR-shRNA and NC-shRNA lentivirus into HepG2 cells for 48 hours, stable red fluorescence signal was observed (Figure 5A). And the effective transfection of anti-miR-shRNA into cells were confirmed by PCR as illustrated in Figure 4C.

MTT assay was utilized to assess the effect of XJR on HCC cell proliferation and its mechanism. Results of the MTT assay showed that XJR decreased proliferation of HepG2 cells, while low expression of miR-200b-3p abrogated the inhibitory effect of XJR on cell proliferation (Figure 5B).

The wound healing assay and transwell assay also showed that XJR decreased migration and invasion of HepG2 cells, but the low expression of miR-200b-3p abrogated the effect of XJR on cell migration (Figure 5C and D) and invasion (Figure 5E and F). XJR could inhibit the proliferation, migration, and invasion of HCC cells, and miRNA-200b-3p displayed a crucial role in the anticancer effect of XJR.

Notch1 is a Direct Target of MiR-200b-3p

Based on the targeting algorithm, we found the 3′-UTR of Notch1 located in position 736–743, which contained the miR-200b-3p binding sequences, and Notch1 was selected for further study. A luciferase reporter assay was performed to determine whether Notch1 is the direct target gene of miR-200b-3p in HepG2 cells. We cloned the wild-type and mutant fragments of Notch1 3′-UTR into a luciferase reporter vector (Figure 6A). As shown in Figure 6B, compared with the negative control (Scrambled group), the luciferase activity was significantly decreased in the wild-type Notch1 3′-UTR-transfected group, but the luciferase activity in the mutant Notch1 3′-UTR-transfected group was not obviously changed. The results suggested that miR-200b-3p could directly bind to the 3′-UTR of Notch1.

Transfection of Inhibitor of MiR-200b-3p Altered Notch1 Expression

To consolidate our findings, Western blotting was performed to measure the Notch1 protein level in different groups of HepG2 cells. We found XJR decreased Notch1 protein expression in HepG2 cells, whereas miR-200B-3p inhibitor reversed the XJR-induced decrease in Notch1 expression (Figure 6C and D). Furthermore, the expression level of Notch1 mRNA was validated in HepG2 cells by RT-PCR. XJR significantly decreased Notch1 mRNA expression in HepG2 cells, whereas miR-200B-3p inhibitor abrogated the XJR-induced decrease in Notch1 mRNA expression (Figure 6E).

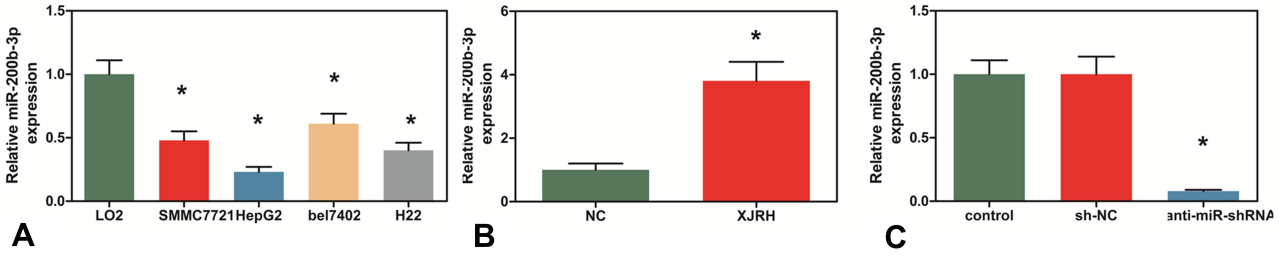

Figure 4 The relative levels of miR-200b-3p (**A**) in all four HCC lines (SMMC7721, HepG2, bel7402, and H22) and normal hepatocyte LO2 cells, (**B**) in tumor tissue of XJRH-treated group and control group, (**C**) and after transfection was confirmed using qRT-PCR. The miRNAs levels were normalized to U6. *$p<0.05$.
Abbreviations: HCC, hepatocellular carcinoma; miRNA, microRNA; qRT-PCR, quantitative RT-PCR.

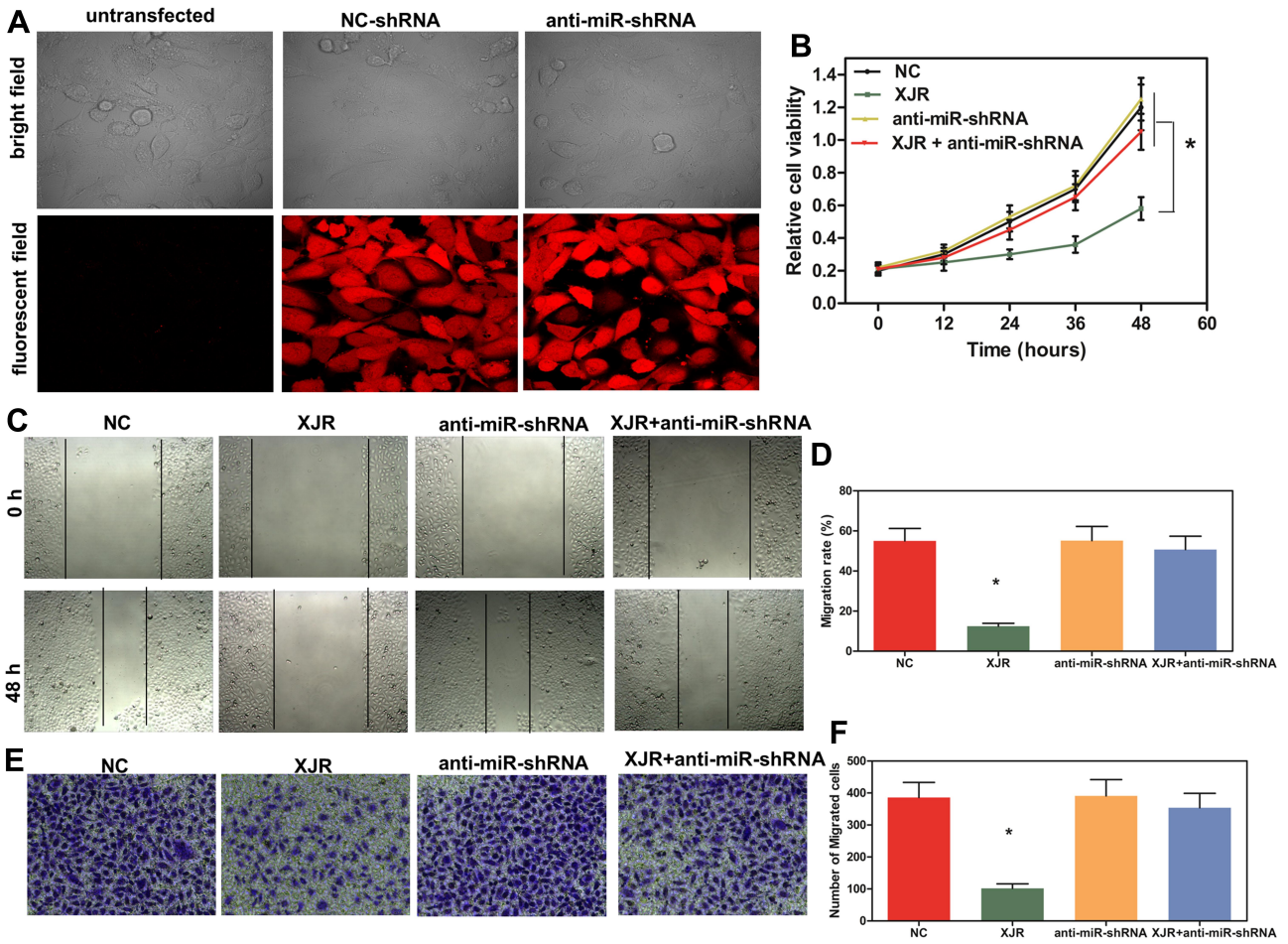

Figure 5 Effect of XJR on tumor growth and the role of miR-200b-3p. (**A**) Transfection efficiency. The red fluorescence signal of HepG2 cells transfected with NC-shRNA and anti-miR-shRNA lentivirus respectively (×100). (**B**) Cell viability was determined by MTT assay in HepG2 cells under XJR with or without anti-miR-shRNA treatment. (**C, D**) Wound healing assay in HepG2 cells under XJR with or without anti-miR-shRNA treatment. (**E, F**) Transwell assay in HepG2 cells under XJR with or without anti-miR-shRNA treatment. *p<0.05.
Abbreviations: XJR, Xiaoai Jiedu recipe; NC, control group.

Discussion and Conclusion

HCC is the most common type of primary liver cancer, since most patients are already in advanced or metastasized stages at the time of initial diagnosis, which causes serious health problems worldwide.[18] Although various treatment strategies have been developed, including surgical resection, trans-arterial chemoembolization,[19] molecular-targeted drugs,[20] radiofrequency ablation, and liver transplantation, the prognosis of HCC remains unsatisfactory.[21] XJR is an effective antitumor prescription created by traditional Chinese medicine master Zhou Zhongying, through years of clinical practice experience.[8] In our experiments, XJR showed efficacy against HCC in vivo, but the underlying antitumor mechanisms of XJR at the molecular level is still unclear. Microarray technology as one of the most popular cutting-edge technologies is widely used in drug screening, disease diagnosis and treatment, life science, and other fields.[22] In order to investigate the inhibitory effect and molecular mechanisms of XJR on HCC, microarray technology was used to study the changes of HCC genes between control and XJR-treated groups. MiRNAs are involved in the occurrence, development, metastasis, and recurrence of HCC by upregulating or downregulating gene expression levels. In this study, we used bioinformatics technology to examine miRNA changes in HCC between control and XJR-treated groups, and a software platform was used to construct a miRNA-gene regulatory network to study the molecular mechanism of XJR against HCC.

In this study, hierarchical clustering analysis results showed that 233 miRNAs (75 upregulated and 158 downregulated) were indicated to be DE in blood between control and XJR-treated groups, indicating the potential involvement of these miRNAs in the antitumor effect of XJR. Further validation showed that the expression of five

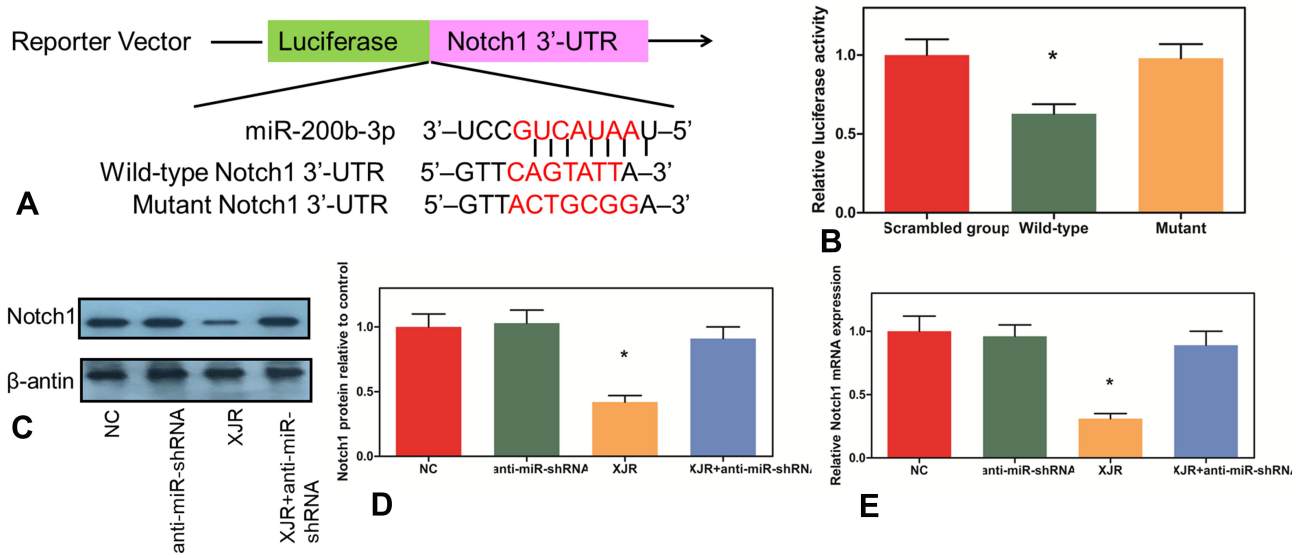

Figure 6 XJR downregulates Notch1 through miR-200b-3p in HepG2 cells. Notch1 is a direct target gene of miR-200b-3p. (**A**) Schematic representation of miR-200b-3p binding sequence in the Notch1 3′-UTR with wild-type and mutant form. The seed sequences and mutated nucleotides are in red font. (**B**) Compared with the control group, the luciferase activity of HepG2 cells transfected with wild-type Notch1 3′-UTR was reduced, and the luciferase activity of HepG2 cells transfected with CDK1 3′-UTR mutant was not significantly changed. (**C, D**) Western blot analyses of Notch1 protein in HepG2 cells. (**E**) Validation of Notch1 mRNA expression by RT-PCR. *$p<0.05$.
Abbreviations: XJR, Xiaoai Jiedu recipe; NC, control group.

miRNAs (miR-453, miR-200b-3p, miR-135a-1-3p, miR-1960, miR-378a-5p, and miR-466f) was consistent with the results of miRNA chip analysis. Through reference research, looking for genes with a greater correlation with the antitumor effect of XJR, we finally determined miR-200b-3p for further research.

The role of miR-200b-3p in cancer is mainly considered as tumor suppressor.[23,24] Studies demonstrated that miR-200b-3p played an important role in the inhibition of cancer stem cell renewal and differentiation, suppression tumor metastasis and EMT, and reversal of chemoresistance in various tumor types.[25,26] Abnormal expression of miR-200b-3p has been found in in a variety of cancers, and low expression of miR-200b-3p was thought to be associated with adverse survival in cancer patients.[27] MiR-200b-3p inhibited metastasis of breast cancer by activating RhoA and downregulating ARHGAP18.[28] By targeting the LIMK1/CFL1 pathway, miR-200b-3p could suppress growth and metastasis of breast cancer cells.[29] MiR-200b-3p suppressed metastasis of renal cell carcinoma by targeting LAMA4.[30] Overexpression of miR-200b-3p could inhibit the proliferation and cell cycle of gastric cancer cells.[31] In our in vitro study, we found XJR could inhibit the proliferation, migration, and invasion of HepG2 cells, while downregulating the expression of miR-200b-3p could abrogate the effect of XJR on HepG2 cells, indicating that miRNA-200b-3p has a crucial role in the anti-cancer effect of XJR.

Notch1 is considered as an oncogene, plays a key role in the biological processes of most cancers, and is a potential target in cancer treatment.[32,33] High expression of Notch1 was associated with tumor progression, metastasis, and poor overall survival rate.[34] Using bioinformatic tools, we predicted that Notch1 was a potential target gene for miR-200b-3p, and the results of luciferase reporter assay further evidenced that Notch1 was the direct target of miR-200b-3p. Moreover, the application of inhibitors of miR-200b-3p changed Notch1 expression and the XJR-induced decrease of Notch1 expression in HepG2 cells.

In conclusion, the present study indicated that XJR could effectively inhibit HCC and XJR might exert its anti-cancer effect through the miR-200b-3p/Notch1 axis. These results provided new evidence for the application of XJR in preventing HCC carcinogenesis, which may open new avenues for prevention and treatment of HCC.

Funding

This work is supported by the National Natural Science Foundation of China (Grant No. 81973737, 81473608, and 81804058), Chinese Medicine Science and Technology Project of Wuxi Administration of Traditional Chinese Medicine (No. ZYKJ201914), and The Open Projects of

the Discipline of Chinese Medicine of Nanjing University of Chinese Medicine Supported by the Subject of Academic priority discipline of Jiangsu Higher Education Institutions (Grant No. ZYX03JG002 and ZYX03KF003).

Disclosure

The authors report no conflicts of interest in this work.

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