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Original Research Article

Jatrorrhizine inhibits liver cancer cell growth by targeting the expressions of miR-221-3p and miR-15b-5p

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Abstract

Purpose: *To investigate the effect of jatrorrhizine on hepatic cancer cell proliferation and its mechanism of action.*
Methods: *Jatrorrhizine-mediated changes in cell viability were measured using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, while apoptosis induction was evaluated by flow cytometry. Transwell assay was used for the measurement of cell invasion, whereas cell migration was assessed by wound healing assay. The protein expression of Axin2 was determined with western blotting assay.*
Results: *Jatrorrhizine significantly ($p < 0.049$) suppressed the viability of HepG2 and HCCLM3 cells in the concentration range of 0.5 to 16.0 µM. Treatment of HepG2 and HCCLM3 cells with 4.0 µM jatrorrhizine markedly suppressed cell invasion, when compared to untreated cells ($p < 0.0493$). Jatrorrhizine significantly promoted the apoptosis of HepG2 and HCCLM3 cells at 48 h, relative to untreated cells, but 16.0 µM jatrorrhizine markedly suppressed the expressions of miR-221-3p and miR-15b-5p ($p < 0.0493$). Moreover, jatrorrhizine significantly up-regulated the protein expressions of Axin2 in HepG2 and HCCLM3 cells at 48 h ($p < 0.0493$).*
Conclusion: *Jatrorrhizine inhibits the proliferation, and suppressed the invasiveness and migration of HepG2 and HCCLM3 liver cancer cells, but increases their apoptosis. Moreover, it down-regulates the expressions of miR-221-3p and miR15b-5p and promotes Axin2 protein expression in HepG2 and HCCLM3 cells. Therefore, jatrorrhizine is a potential drug candidate for the treatment of liver cancer.*

Keywords: *Jatrorrhizine, Liver cancer, HepG2, HCCLM3, Apoptosis, Axin2, MiR-221-3p, MiR-15b-5p*

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INTRODUCTION

Liver cancer is a frequently diagnosed gastrointestinal tract tumor which ranks third in cancer-related mortality world-wide [1]. The survival of liver cancer patients after hepatic resection is unsatisfactory because of fast tumor recurrence and metastasis [2]. It has been demonstrated that the pathogenesis of liver cancer is highly complicated due to the involvement of multiple genes and proteins [3].

MicroRNAs (miRNAs) which are endogenously present in cells, are non-coding and single-stranded molecules made up of 18 - 25 nucleotides. They function in RNA silencing and

post-transcriptional regulation of gene expression [4]. It has been reported that the expressions of miR-181 family members were elevated in liver cancer stem cells extracted from α-fetoprotein (AFP)-positive hepatic carcinoma samples [5]. Comparison of miR-21 levels in serum of liver carcinoma patients, chronic hepatitis cases and normal volunteers revealed markedly up-regulated miR-21 expression in hepatic cancer patients [6]. It has been reported that miR-221 expression was abnormally increased in different kinds of cancers such as hepatic, breast and gastric carcinomas [7]. This indicates that miR-221 plays important role in the pathogenesis of liver cancer, and may act as prominent target for the treatment of different cancers. Another member of miR-181 family, miR-15b has also been linked to multiple kinds of cancers, for example, proliferation of glioma cells in the form of miR15b/HOTAIR/p53 regulatory loop [8]. It suppressed the expression of cysteine-rich protein, thereby promoting the proliferation of prostate cancer cells [9].

Jatrorrhizine (Figure 1) and many other related compounds are present in *Tinospora capillipes* Gagnep [10-12]. Studies have revealed the mitigating effect of jatrorrhizine and its prominent bioactivity profile against several disorders [10-12]. Jatrorrhizine and related compounds exert many pharmacological effects such as anticancer, anti-microbial and anti-parasitic properties [10-12]. The current study was carried out to investigate the effect of jatrorrhizine on hepatic cancer cell proliferation, and the associated mechanism of action.

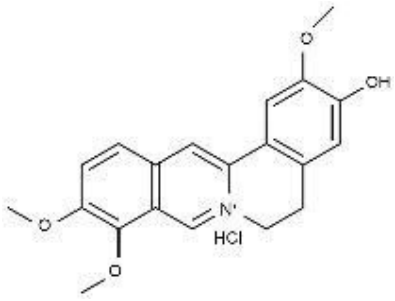

Figure 1: Chemical structure of jatrorrhizine

EXPERIMENTAL

Cell culture

The HepG2 and HCCLM3 cell lines were procured from the American Type Culture Collection (Chinese Academy of Sciences). The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 10 % FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL) in a 5 % CO_2 incubator at 37 ˚C.

Cell proliferation assay

The cell lines were separately seeded in 96-well plates at a density of 2 x 105 cells/well and cultured for 24 h, followed by 48-h incubation with jatrorrhizine at doses of 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 µM at 37 °C. Thereafter, 20 µL of MTT solution (5 mg/mL) was added to each well of the plate, and further incubation of cells was carried out for 4 h. The resultant formazan crystals were solubilized with dimethyl sulfoxide (DMSO, 150 µL). The well plates were shaken, and the absorbance of each well was read at 487 nm using a scanning multi-well spectrophotometer. The viability of each cell line was calculated from the absorbance values.

Flow cytometry

The HepG2 and HCCLM3 cells were plated, each at a density of 2 x 10^6 cells/well, and treated with 16.0 µM jatrorrhizine for 48 h, followed by washing in PBS. For apoptosis determination, the cells were fixed overnight in 70 % methyl alcohol at 20 ˚C prior to washing in PBS. Thereafter, the cells were stained with Annexin V/PI and examined using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell invasion assay

The inhibitory effect of jatrorrhizine on invasiveness of cells was determined using 24-well Transwell plates (8 mm pore size). Coating of chamber inserts with 200 mg/mL Matrigel was followed by drying overnight under sterile conditions. The HepG2 and HCCLM3 cells were treated with 16.0 µM jatrorrhizine for 48 h, after which 2 x 10^6 cells were put in the top chamber in RPMI-1640 medium. The lower chamber contained RPMI-1640 along with 20 % FBS. The cells were incubated for 48 h, followed by cleaning of the top chamber with cotton swab and subsequent fixing of cells in methyl alcohol. Then, the cells were stained with hematoxylin and eosin for 25 min, and the invaded cells on the underside of the membrane were counted under a light microscope (Olympus Corporation, Tokyo, Japan).

Wound healing assay

The cells were seeded in 6-well plates at a density of 2 x 10^5 cells/well, and were cultured for 24 h to >95 % confluence. A wound was scratched at the middle of the cellular monolayer

of each cell line, using the tip of a 100-ml plastic micropipette. Free cells were washed off with PBS, after which the the cells were incubated for 48 h with 16.0 µM jatrorrhizine at 37 ˚C. Wound healing was measured by calculation of width of the original scratch using NIH Image version 1.62 programs. The measurement was made by randomly capturing images of five selected fields.

Western blotting

The HepG2 and HCCLM3 cells seeded at density of 2 x 10^6 cells/well were incubated with 16.0 µM jatrorrhizine for 48 h. Then, total protein content of the cells was extracted with RIPA lysis buffer along with protease inhibitors, in accordance with the instructions from the manufacturer. Each cell lysate was subjected to centrifugation at 12000 x g for 30 min, and the protein content of the supernatant was determined using the bicinchoninic acid method. The proteins were resolved on 12 % SDS-polyacrylamide gel electrophoresis (30-µg protein samples per lane), followed by transfer onto PVDF membrane which was blocked by treatment with 5 % skimmed milk and TBS for 2 h. Then, the membrane was incubated overnight at 4 ºC with primary antibodies against Axin2 (catalog no. ab32197 Abcam; dilution 1:1,000) and β-actin (catalog no. ab37168 Abcam; dilution 1:5,000), after which the membrane was washed twice with TBS + Tween-20. Then, the membrane was incubated for 2 h with horseradish peroxidase-conjugated secondary antibody diluted 1:4,500 (Sigma-Aldrich) at room temperature. The bands were visualized using enhanced chemiluminescence system, with GAPDH as loading control.

Quantitative polymerase chain reaction (qPCR)

Total RNA from 16.0 µM jatrorrhizine treated cells was extracted at 48 h using TRIzol® (Invitrogen) as per instructions of the manufacturer. The SYBR Premix Ex Taq (Takara Bio, Inc.) system was used for reverse transcription for 25 min at 15 ˚C, for 25 min at 41 ˚C and for 6 min at 84 ˚C. For qPC reaction SYBR Premix Ex Taq along with cDNA (1 µL) samples from ABI-7500 system was used. In PCR the thermocycling set up used consisted of 2 min at 93 ˚C and then 32 cycles for 25 s at 93 ˚C, for 28 s at 57 ˚C and for 28 s at 70 ˚C. Then level of U6 small RNA was considered as internal reference. The sequence of primers used was: MiR-15b-5p sense, 5'-ATG AAC TTT CTC TGT CTT GG-3' and antisense, 5'-CAG TGC GTG TCG TGG AGT-3'; miR-221-3p sense, 5'-CGG CTA CAT TGT CTG CCT G-3' and antisense, 5'-CAG TGC GTG TCG TGG AGT-3'; and U6 sense, 5'-CGC TTC GGC AGC ACA TAT AC-3' and anti-sense, 5'-AAC GCT TCA CGA ATT TGC GT-3'. For calculation of relative miR-221-3p and miR-15b-5p expression 2-ΔΔCq method was used [13].

Statistical analysis

Data are expressed as mean ± standard deviation of independently performed triplicate experiments, and data analysis was done with SPSS 17.0 (SPSS, Inc.). Multiple group data comparisons were made using one-way ANOVA and Student-Newman-Keuls test. Differences were taken as statistically significant at $p < 0.05$.

RESULTS

Jatrorrhizine inhibited the proliferation of HepG2 and HCCLM3 cells

Proliferative changes produced in HepG2 and HCCLM3 cells by jatrorrhizine at doses of 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 µM were measured at 48 h (Figure 2). Jatrorrhizine significantly suppressed the viabilities HepG2 and HCCLM3 cells in the concentration range of 0.5 to 16.0 µM ($p < 0.049$). The viabilities of HepG2 cell cultures treated with jatrorrhizine at doses of 0.5 and 16.0 µM were 93 and 17 %, respectively. Treatment with jatrorrhizine at doses of 0.5 and 16.0 µM suppressed HCCLM3 cell viability to 95 and 21 %, respectively.

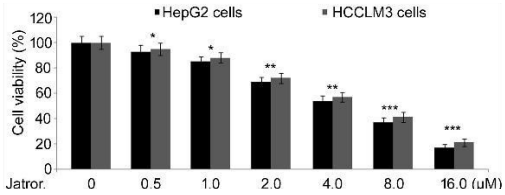

Figure 2: Effect of jatrorrhizine on viabilities of HepG2 and HCCLM3 cells. Jatrorrhizine at indicated concentrations was added to the cultures of cells and incubation was performed for 48 h. Viability changes induced by jatrorrhizine were measured using MTT assay. *P < 0.0497; **p < 0.0189; ***p < 0.0093, vs. control cells

Jatrorrhizine inhibited the invasion and migratory potential of HepG2 and HCCLM3 cells

In HepG2 and HCCLM3 cells, treatment with jatrorrhizine at a dose of 4.0 µM significantly suppressed their invasion, when compared to untreated cells ($p < 0.0493$; Figures 3 A and 3B). Treatment of HepG2 cells with 4.0 µM

jatrorrhizine for 48 h suppressed their invasive potential to 32 %, relative to 100 % in untreated cells. Treatment of HCCLM3 cells with 4.0 μM jatrorrhizine suppressed invasion to 36 %, relative to 100 % in control cells. Jatrorrhizine treatment of HepG2 and HCCLM3 cells for 48 h markedly reduced wound healing ($p < 0.0493$), when compared to control cells (Figures 4A and 4B). Wound healing in 4.0 μM jatrorrhizine-treated HepG2 and HCCLM3 cells decreased to 21 and 26 %, respectively, relative to control cells.

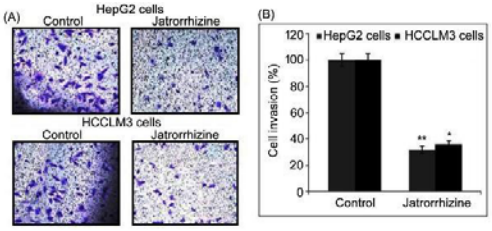

Figure 3: Effect of jatrorrhizine on invasion of HepG2 and HCCLM3 cells. Treatment with 4.0 μM jatrorrhizine for 48 h was followed by determination of invasion of HepG2 and HCCLM3 cells using Transwell assay. *P < 0.0497 and **p < 0.0195 vs. control cells

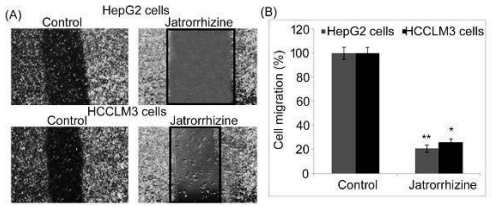

Figure 4: Effect of jatrorrhizine on migration potential of HepG2 and HCCLM3 cells. Treatment with 4.0 μM jatrorrhizine for 48 h was followed by determination of migration of HepG2 and HCCLM3 cells using wound healing assay. *P < 0.0497 and **p < 0.0195 vs. control cells

Jatrorrhizine induced apoptosis of HepG2 and HCCLM3 cells

Treatment with jatrorrhizine significantly enhanced apoptosis of HepG2 and HCCLM3 cells at 48 h, relative to untreated cells (Figure 5). In 16.0 μM jatrorrhizine-treated HepG2 and HCCLM3 cells, apoptosis increased to 68.29 and 64.61 %, respectively at 48 h. In control HepG2 and HCCLM3 cells, apoptosis was observed in 1.78 and 2.43 % cells, respectively.

Jatrorrhizine inhibited expressions of miR-221-3p and miR-15b-5p in HepG2 and HCCLM3 cells

In HepG2 and HCCLM3 cells, jatrorrhizine treatment caused marked reductions in expression levels of miR-221-3p and miR-15b-5p, relative to control cells (Figure 6). Treatment of HepG2 and HCCLM3 cells with 16.0 μM jatrorrhizine for 48 h led to marked down-regulation of the expressions of miR-221-3p and miR-15b-5p.

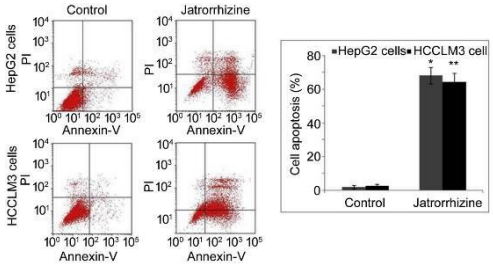

Figure 5: Effect of jatrorrhizine on apoptosis of HepG2 and HCCLM3 cells. Treatment with 16.0 μM jatrorrhizine for 48 h was followed by measurement of apoptosis. *P < 0.0497, vs control cells

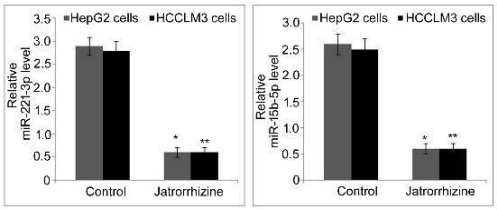

Figure 6: Effect of jatrorrhizine on mRNA expression levels of miR-221-3p and miR-15b-5p. The HepG2 and HCCLM3 cells were treated with 16.0 μM jatrorrhizine for 48 h, followed by determination of mRNA expressions of miR-221-3p and miR-15b-5p with RT-PCR. *P < 0.0497 vs control cells

Jatrorrhizine up-regulated Axin2 expression in HepG2 and HCCLM3 cells

In jatrorrhizine-treated HepG2 and HCCLM3 cells, the expression of Axin2 protein was assayed with western blotting (Figure 7). Jatrorrhizine significantly enhanced Axin2 protein expression in HepG2 and HCCLM3 cells at 48 h, relative to untreated cells.

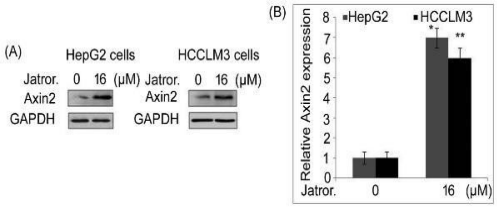

Figure 7: Effect of jatrorrhizine on Axin2 protein expression. HepG2 and HCCLM3 cells were treated with 16.0 μM jatrorrhizine for 48 h, followed by assay of Axin2 protein expression using western blotting. *P < 0.0497, **p < 0.0096 vs control cells

DISCUSSION

Liver cancer is considered the third most common tumor of the gastrointestinal tract, and it is associated with high mortality worldwide [1,14]. It has been demonstrated that miRNAs play crucial role in the pathogenesis of liver cancer and enhancement of its aggressiveness [15]. Studies have reported that in liver cancer tissues, miR-221 and miR-15b levels were abnormally elevated and demonstrated oncogenic characteristics by enhancing tumor growth and progression [16,17]. Indeed, the expressions of miR-221-3p and miR15b-5p have been reported to be associated with several types of cancers. The oncogenic property of miRNA-221-3p in gastric cancers is associated with down-regulation of PTEN expression [18].

Metastatic potential in gastric cancers is promoted by miRNA-221-3p via interaction with progestin and adipoQ receptors, thereby acting as gastric cancer biomarker [19]. Although the mechanism by which miRNAs enhances tumor growth is not clearly understood, miR-221 and miR15b are considered important targets for cancer inhibition and diagnosis. In the present study, the proliferation capacities of HepG2 and HCCLM3 cells were suppressed concentration-dependently by jatrorrhizine. Moreover, in jatrorrhizine-treated HepG2 and HCCLM3 cells, invasiveness and wound healing abilities were inhibited, when compared to control. The present study demonstrated that levels of miR-221-3p and miR-15b-5p were markedly increased in HepG2 and HCCLM3 cancer cells. However, jatrorrhizine treatment markedly suppressed the expressions of miR-221-3p and miR-15b-5p in HepG2 and HCCLM3 cells, when compared to control. These findings indicate that jatrorrhizine mediated inhibition of viabilities of HepG2 and HCCLM3 cells by targeting the expressions of miR-221-3p and miR-15b-5p.

Axin2 acts a negative regulator of Wnt/β-catenin pathway and degrades complexes of β-catenin after de-phosphorylation [20]. It is an anti-oncogene which regulates tumor growth by interacting with various proteins such as transmembrane receptors, T-cell factors and Wnt [20].

It has been revealed that digestive tract tumors and melanomas in humans are linked to the mutations in the Axin2 gene [21,22]. In the present study, Axin2 protein expressions in HepG2 and HCCLM3 cells were significantly up-regulated by jatrorrhizine, relative to untreated cells which showed very weak expression of Axin2 protein.

CONCLUSION

This study has demonstrated that jatrorrhizine inhibits the proliferation of HepG2 and HCCLM3 liver cancer cells, increases their apoptosis and suppresses their invasiveness and migration. Furthermore, it down-regulates the expressions of miR-2213p and miR-15b-5p, but up-regulates protein expression of Axin2 in HepG2 and HCCLM3 cells. Therefore, jatrorrhizine offers a potential therapeutic strategy for the management of liver cancer.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Maolin Wan - conceived and designed the study; Bo Deng and Maolin Wan – collected, analyzed the data and wrote the manuscript. Both of the authors read and approved the manuscript for publication.

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