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miR-122 enhances sensitivity of hepatocellular carcinoma to oxaliplatin via inhibiting MDR1 by targeting Wnt/β-catenin pathway

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is one of the primary causes of cancer-related death and resistance to cytotoxic chemotherapy is the major cause of mortality in HCC patients. miR-122 is a liver specific miRNA and is found to be reduced in HCC, however, the function of miR-122 in HCC chemosensitivity remains elusive.
Methods: We used qRT-PCR to measure expressions of miR-122, β-catenin and MDR1 in four HCC cell lines. And we assessed the effects of miR-122 or β-catenin on cell viability and apoptosis upon oxaliplatin (OXA) treatment by MTT assay and flow cytometry. In addition, we validated the interactions of miR-122/β-catenin and β-catenin/MDR1 by dual luciferase reporter assay and chromatin immunoprecipitation (ChIP). Western blotting was used to determine the protein levels of β-catenin, Wnt1 and MDR1. In the end, we verified the anti-tumor effect of miR-122 in vivo by using mouse tumor xenograft model.
Results: We found that miR-122 was down-regulated in HCC cells. Up-regulation of miR-122 or inhibition of Wnt/β-catenin signaling promoted HCC cells apoptosis and increased the sensitivity of HCC cells to OXA. On the molecular level, we showed that miR-122 directly targeted and suppressed Wnt/β-catenin pathway while β-catenin bound with MDR1 promoter and activated its transcription. Overexpression of miR-122 inhibited MDR1 expression via directly suppressing Wnt/β-catenin pathway.
Conclusion: Our study fully demonstrated that miR-122 inhibits MDR1 expression via suppression of Wnt/β-catenin pathway, thereby enhancing HCC sensitivity to OXA. Therefore, miR-122 could serve as a novel potential therapeutic target for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is among the major causes of cancer-related mortality, accounting for nearly 90% of total liver cancer burden (Finn, 2013; Daher et al., 2018). Owing to the difficulty of diagnosing the disease at early stages, most patients are diagnosed at late stages, when surgical resection is not feasible. Transcatheter arterial chemoembolization (TACE) or systemic chemotherapy is the main treatment for patients at advanced stages, but drug resistance is a big obstacle in further improving the postoperative outcome of HCC patients (Daher et al., 2018). As a result, the prognosis of HCC remains very poor. Understanding the underlying molecular mechanisms could help develop more effective pharmacological therapies.

Oxaliplatin (OXA) is the third-generation platinum analog, which is a cell cycle non-specific drug (Qin et al., 2013). It can covalently bind DNA and block DNA replication and transcription. OXA has been shown to have significant anti-cancer activities against many tumor cell lines, such as gastric, breast, renal, colorectal carcinomas and sarcomas, and it is the most effective chemotherapy drug for advanced HCC patients at present (Le Grazie et al., 2017; Kou et al., 2017). However, only 8.15% to 15.6% patients are sensitive to OXA, and the occurrence of chemoresistance is a huge obstacle in HCC treatment (Qin et al., 2014). Therefore, there is clear need to understand the mechanisms of chemotherapeutic resistance in HCC. Overexpression of P-glycoprotein (P-gp) is highly associated with acquired and/or intrinsic drug resistance in many tumors including HCC (Shukla et al., 2011; Tiwari et al., 2011). P-gp is encoded by the MDR1 gene (multidrug resistance receptor 1) and acts as an ATP-binding cassette (ABC) transporter. P-gp has been shown actively involved in the efflux of antineoplastic agents from cancer cells (Ambudkar et al., 2003). Nevertheless, the complex

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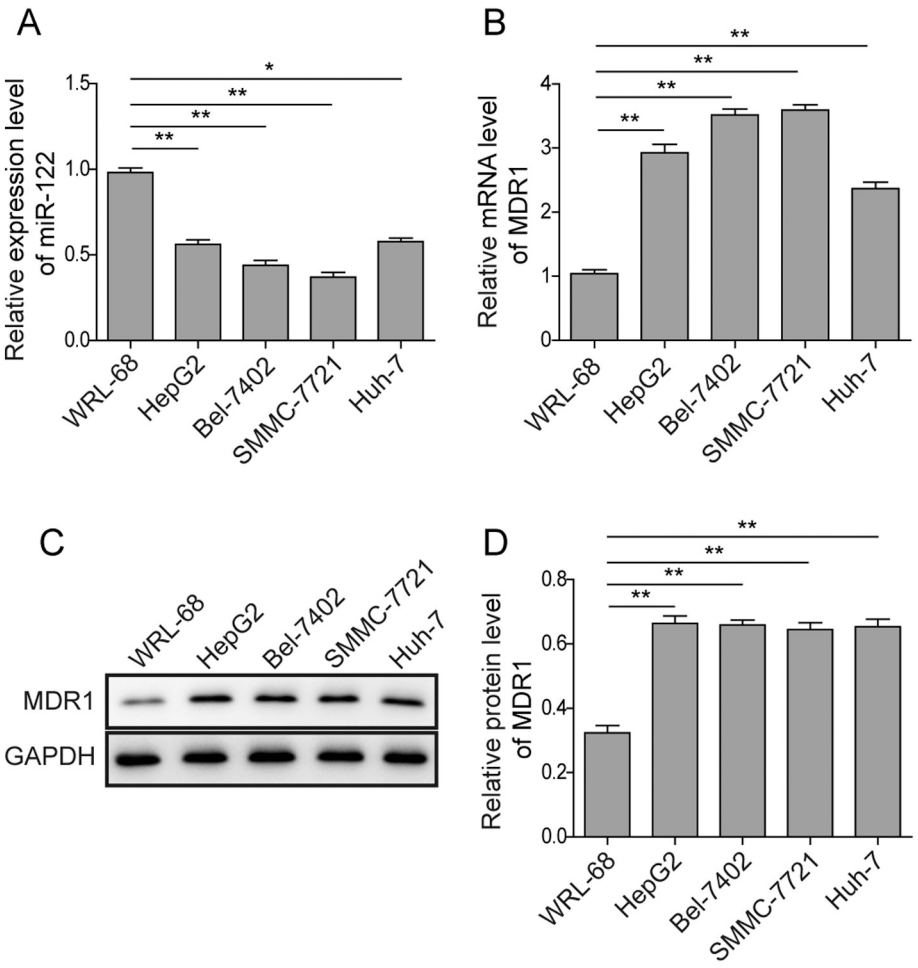

Fig. 1. miR-122 was down-regulated and MDR1 was up-regulated in HCC cells. qRT-PCR analysis of miR-122(A) and MDR1 (B) levels in HCC cells. (C) Western blot analysis of MDR1 in HCC cells. (D) Quantification of MDR1 expression in HCC cells. GAPDH was used as a loading control. All the results were shown as mean ± SD ($n = 3$), which were three separate experiments performed in triplicate. *p < .05 and **p < .01.

mechanisms underlying the effects exerted by this protein has not been fully elucidated.

MicroRNAs (miRNAs) are a class of endogenous, small and non-coding RNAs (19–25 nucleotides) (Moran et al., 2017). They negatively regulate gene expression by binding the 3′-untranslated region (3'-UTR) of target messenger RNAs (mRNAs), resulting in mRNA degradation or inhibition of translation. miRNAs play critical roles in regulating multiple cellular processes such as differentiation, proliferation, chemoresistance and metastasis (Mishra, 2012). As a liver-specific miRNA, miR-122 has been shown to play critical roles in regulating hepatocyte development, differentiation and cholesterol metabolism (Bandiera et al., 2015). In HCC tissues, it was reported that miR-122 expression was greatly reduced and the down-regulation of miR-122 has been associated with HCC development and progression (Morita et al., 2011; Coulouarn et al., 2009). Also, increasing evidence indicates that miR-122 regulates the chemosensitivity of HCC cells. Deletion of miR-122 could promote epithelial-mesenchymal transition (EMT) and HCC formation. Furthermore, ectopic expression of miR-122 in HepG2 and Hep3B cells could increase these cells' sensitivity to doxorubicin and sorafenib (Fornari et al., 2009; Bai et al., 2009). Nevertheless, the exact role of miR-122 in HCC and OXA resistance is not yet fully understood. Previous studies suggested that miR-122 could directly target Wnt/β-catenin pathway (Xu et al., 2012), and it is known that the Wnt/β-catenin signaling pathway plays a crucial role in tumor development, including regulating MDR1 expression (Grainger & Willert, 2018; Liu et al., 2016). We thus hypothesized that miR-122 regulates the chemosensitivity of HCC through modulating Wnt/β-catenin pathway, thereby affecting MDR1 expression.

In this study, we investigated the role of miR-122 in HCC. We found that miR-122 was down-regulated and Wnt/β-catenin pathway was activated in HCC. Moreover, overexpression of miR-122 and inhibition of Wnt/β-catenin signaling could repress HCC cell proliferation, promote cell apoptosis, enhance HCC cells' sensitivity to OXA and inhibit tumor growth in vivo. Mechanistically, we identified Wnt/β-catenin pathway as the downstream target of miR-122. Through directly inhibiting Wnt/β-catenin signaling pathway, miR-122 down-regulated MDR1 expression, thereby restoring chemotherapeutic sensitivity of HCC cells. Altogether, our data showed that miR-122 plays a key role in the regulation of HCC chemoresistance, which might be a promising target for future therapy development.

2. Material and methods

2.1. Cell culture and treatment

Human liver cancer cell lines (HepG2, Bel-7402, SMMC-7721 and Huh-7) and normal liver cell line (WRL-68) were obtained from the American Type Culture Collection (ATCC, USA). HepG2 and WRL-68 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific, USA) while Bel-7402, SMMC-7721 and Huh-7 cells were cultured in RPMI-1640 (Thermo Fisher Scientific, USA). The medium was supplemented with 10% fetal bovine serum (FBS, Neuromics, USA) and 1% penicillin-streptomycin (Gibco, USA) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

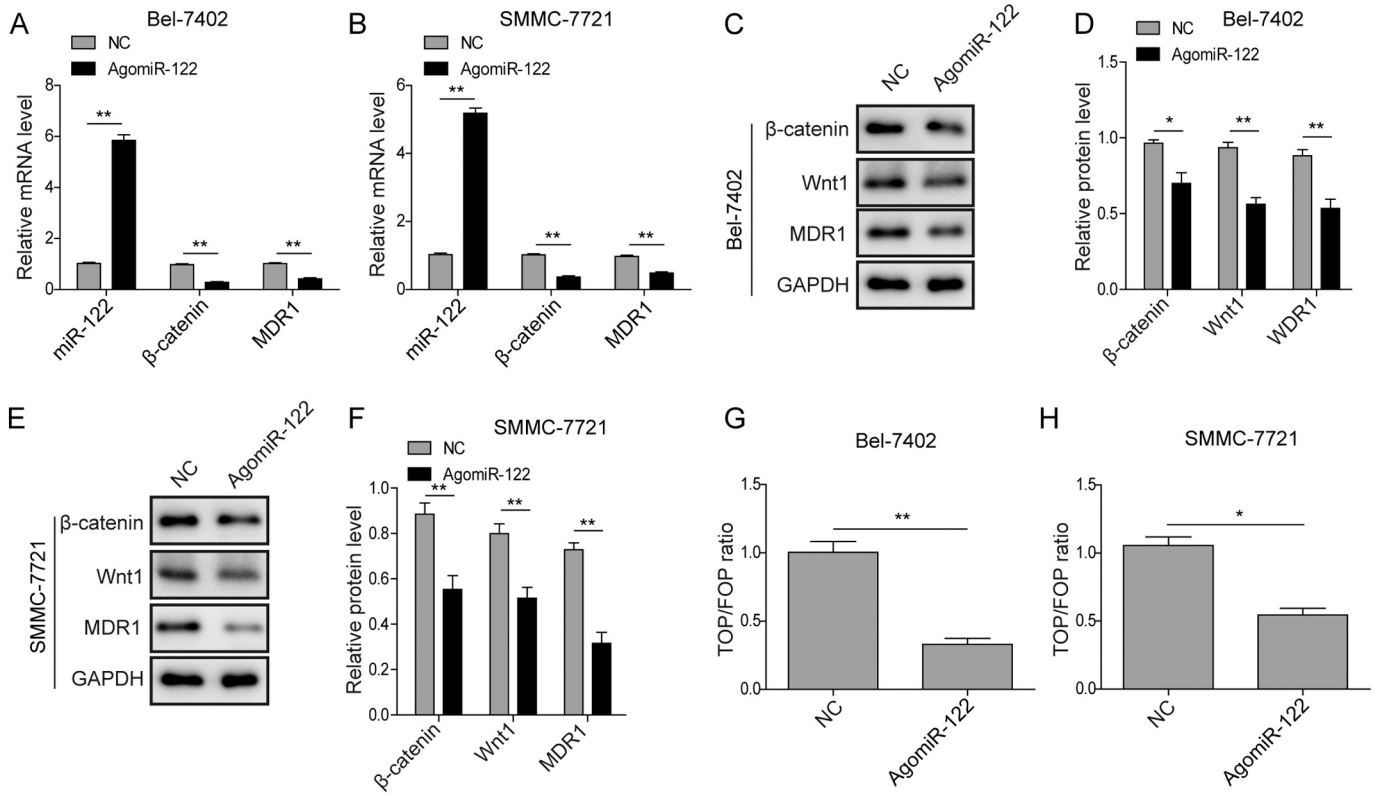

Fig. 2. miR-122 suppressed MDR1 expression by inhibiting Wnt/β-catenin pathway. qRT-PCR analysis of miR-122, β-catenin and MDR1 levels in HCC cells transfected with AgomiR-122 or negative control (NC) in Bel-7402(A) and SMMC-7721 (B) cells. Western blot analysis of β-catenin, Wnt1 and MDR1in Bel-7402(C) and SMMC-7721 (E) cells transfected with AgomiR-122 or NC. (D, F) Quantification of β-catenin, Wnt and MDR1 levels in (C, E).GAPDH was used as a loading control. Quantification of luciferase activity in Bel-7402(G) and SMMC-7721 (H) cells transfected with NC or AgomiR-12. All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate. *p < .05 and **p < .01.

Cells were treated with ciclopirox olamine (CIC, 50 μM) or OXA (0, 8, 16, 32, 64, 128 μg/mL) for 24 h as indicated.

2.2. Cell transfection

miR-122 mimics (AgomiR-122) were synthesized by GenePharma (Shanghai, China). β-catenin sequence was synthesized and sub-cloned into transfection plasmid to generate recombinant vector pcDNA3.1-β-catenin (β-catenin). β-catenin specific short hairpin RNAs (shRNA) were cloned into pSicoR vector (shβ-catenin). Transfection was performed using Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's protocol.

2.3. MTT assay

Cells were seeded in a 96-well plate at a density of 5×10^3 cells per well and then incubated at 37 °C for 24 h. 10 μL of MTT (3-(4,5-Dimethyltjiazol-2-yl)-2,5-diphenltetrazolium bromide; 5 mg/mL) were added to each well for additional 3 h of incubation at 37 °C. The reaction was terminated by adding 100 μL detergent reagent, and the absorbance values were measured at the wavelength of 570 nm.

2.4. Cell apoptosis assay

Cell apoptosis was measured by Annexin-V-FITC/propidium iodide (PI) staining using Annexin-V-FITC /PI apoptosis Detection Kit (BD Bioscience, CA) according to manufacturer's protocol. HCC cells were plated in 6-well plate and grown to 60% confluence. Then they were transfected with plasmids or AgomiR-122 as indicated. After that, cells were harvested and incubated with Annexin-V-FITC/PI for 15 min in the dark, and analyzed by flow cytometry.

2.5. Luciferase report assay

Dual-luciferase kit (Dual-Glo™ Luciferase Assay System, Promega, USA) was used to measure the transcriptional activity of β-catenin. Briefly, HCC cells (10^5 cells/well) were co-transfected with 1 μg of constitutively active vector encoding *Renilla* luciferase (Promega,USA) and 10 μg of β-catenin-responsive firefly luciferase reporter plasmid Top*Flash* (Millipore, USA) or the negative control Fop*Flash* (Millipore, USA) using Lipofectamine 2000 according to manufactures' protocol. Cells were harvested after 24 h and luciferase activity was measured in triplicate according to the manufacturer's instructions. The firefly luciferase activity was normalized tothe *Renilla* luciferase activity. To determine the function of miR-122 on β-catenin transcriptional activity, HCC cells were co-transfected with Top*Flash* or Fop*Flash* and AgomiR-122 using Lipofectamine 2000. Cells were harvested after 24 h for luciferase measurements.

To assess the regulation of MDR1 gene by β-catenin, HCC cells were seeded in 12-well plates (10^5 cells/well) and co-transfected with MDR1 promoter reporter construct (or pGL3-basic and pGL-control, as negative control and positive control respectively) with or without shβ-catenin, or β-catenin as indicated in figures. After 24 h, the co-transfected cells were lysed with Reporter Lysis Buffer, and the luciferase activity was detected using a Luciferase Reporter Gene Assay Kit (Promega, USA).

2.6. Chromatin immunoprecipitation (ChIP) assay

EZ-Magna ChIP™ G-Chromatin Immunoprecipitation Kit and the Magna Grip™ Rack (Cell Signaling Technology, USA) were used to perform ChIP assay according to the manufacturer's protocol. For each chromatin immunoprecipitation, 5 μg of anti-β-catenin or H3 antibody and 1 μL of normal mouse IgG were used. After immunoprecipitation,

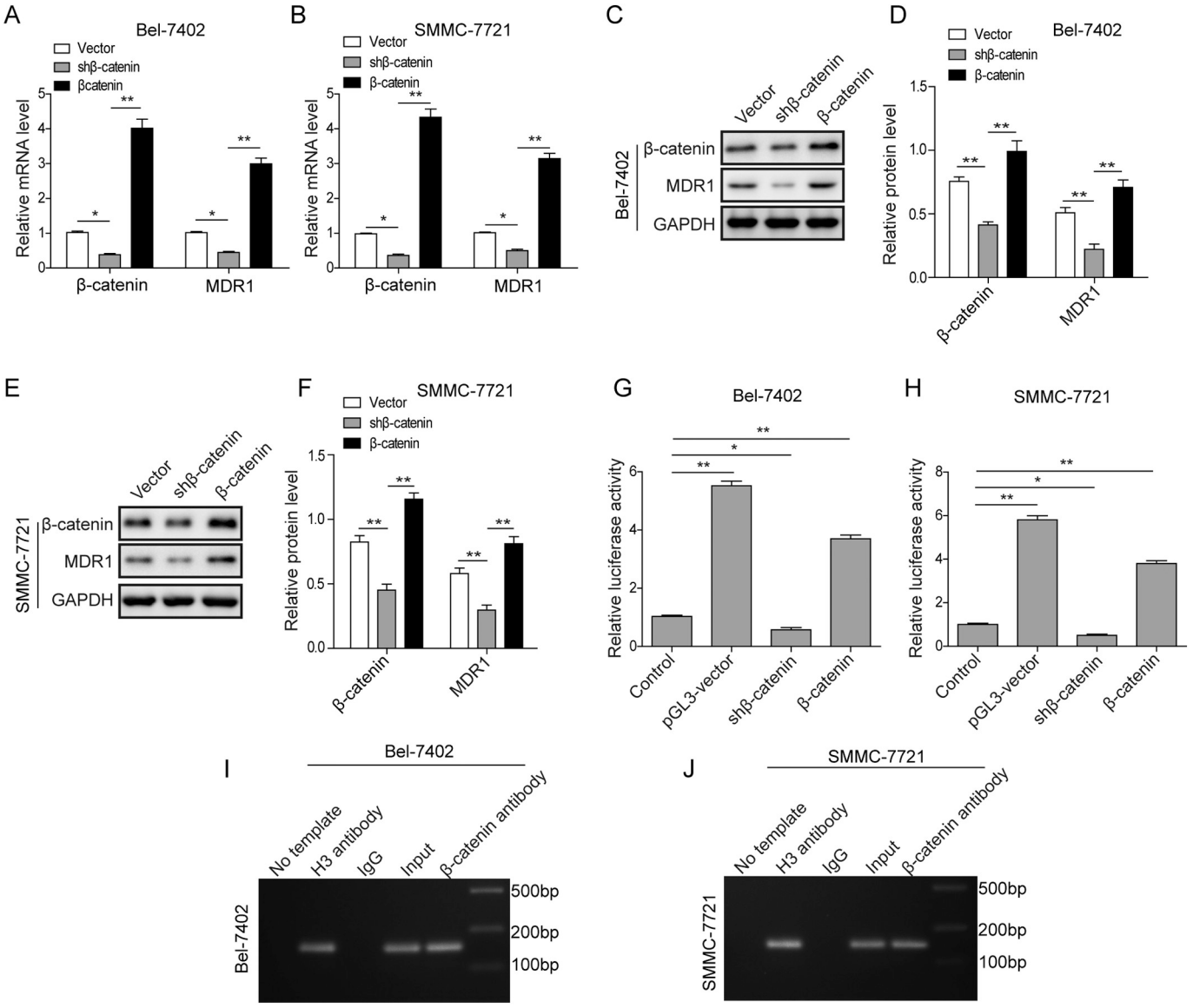

Fig. 3. β-catenin positively regulated MDR1 expression by binding MDR1 promoter. qRT-PCR analysis of β-catenin and MDR1 levels in Bel-7402 (A) and SMMC-7721 (B) cells transfected with shβ-catenin, pcDNA3.1-β-catenin or vector plasmid . Western blot analysis of β-catenin and MDR1in Bel-7402 (C) and SMMC-7721 (E) cells transfected with shβ-catenin, pcDNA3.1-β-catenin or vector plasmid. (D, F) Quantification of β-catenin and MDR1 levels in (C, E). Quantification of luciferase activity in Bel-7402 (G) and SMMC-7721 (H) cells transfected with shβ-catenin, pcDNA3.1-β-catenin or vector plasmid. β-catenin was immunoprecipitated with specific antibodies from HCC cells, followed by PCR analysis of MDR1 gene in Bel-7402(I) and SMMC-7721 (J) cells. All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate. *p < .05 and **p < .01.

chromosomal DNA was purified and analyzed using PCR to detect the MDR1 promoter region. The primers used for amplifying the MDR1 promoter region including the potential β-catenin site were as follows: forward: 5'-AGTCATCTGTGGTGAGGCTGAT-3′; reverse: 5'-TACTCGAA TGAGCTCAGGCTTC-3′.

2.7. RNA extraction and qRT-PCR

Total RNA was isolated from the cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. DNaseI was used to avoid DNA contamination. 1 μg total RNA from each sample was subjected to reverse transcription using QuantiTect Reverse Transcription Kit (Qiagen, Dutch). qRT-PCR was performed in triplicate for each sample using the Maxima SYBR Green qRT-PCR Master Mix (Toyobo, Japan). Relative expression levels of miRNA and mRNA were normalized to U6 small nuclear RNA andβ-actin, respectively. The following primers were used for analysis:miR-122 forward: 5'-TTGAA TTCTAACACCTTCGTGGCTACAGAG-3′, reverse: 5'-TTAGATCTCATTTA TCGAGGGAAGGATTG-3′; MDR1 forward: 5'-CCCATCATTGCAATAGC AGG-3′, reverse: 5'-TGTTCAAACTTCTGCTCCTGA-3′; β-catenin forward: 5'-ATGGCTACTCAAGCTGAC-3′, reverse: 5'-CAGCACTTTCAGCA CTCTGC-3′; U6 forward: 5'-CTCGCTTCGGCAGCACA-3′, reverse: 5'-AACGCTTCACGAATTTGCGT-3′; β-actin forward: 5'-AAATCTGGCACC ACACCTTC-3′, reverse: 5′- GGGGTGTTGAAGGTCTCAAA-3′.

2.8. Western Blot analysis

Total proteins were extracted from cultured cells by RIPA buffer (Beyotime Institute of Biotechnology, Nantong, China) with protease inhibitor cocktail. Protein concentration was determined by pierce BCA protein Assay (Thermo Fisher Scientific, USA). Equal amounts of protein samples (30 μg) were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore, USA). The membranes were blocked for 1 h at room temperature. Subsequently, primary antibodies were added

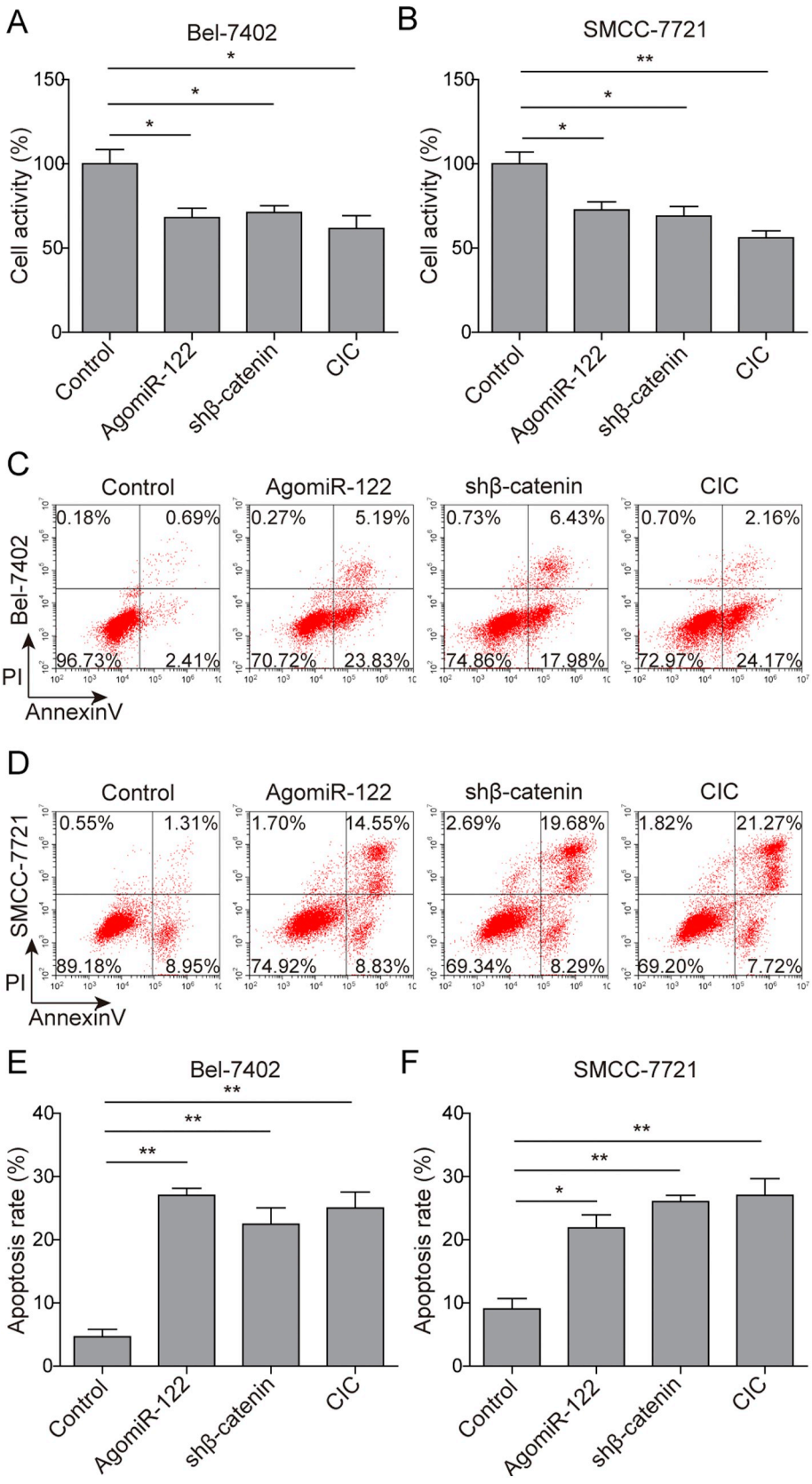

Fig. 4. Overexpression of miR-122 and inhibition of β-catenin inhibited HCC cell proliferation and promote apoptosis. Cell viability was measured by MTT assay in Bel-7402 (A) and SMMC-7721 (B) cells transfected with AgomiR-122, shβ-catenin, or treated with CIC for 24 h. Cell apoptosis was determined by Annexin-V/PI staining and flow cytometry analysis in Bel-7402 (C) and SMMC-7721 (D) cells transfected with AgomiR-122, shβ-catenin or treated with CIC for 24 h. (*E*-F) Quantifications of cell apoptosis percentage in (C-D). All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate. *p < .05 and **p < .01.

and incubated overnight at 4 °C. Selected proteins were detected with specific antibodies. The following antibodies were used: MDR1 (1:2000, Abcam, USA), β-catenin (1:2500, Abcam, USA), Wnt1 (1: 5000, Abcam, USA) and GAPDH (1:5000, Abcam, USA). Following washes with TBST, the membranes were incubated with a secondary antibody (Anti-Mouse and Anti-Rabbit IgG [H + L] antibodies diluted by 1: 1000, KPL, USA) for 1 h at room temperature. Protein bands were visualized using the ECL kit (Promega, USA).

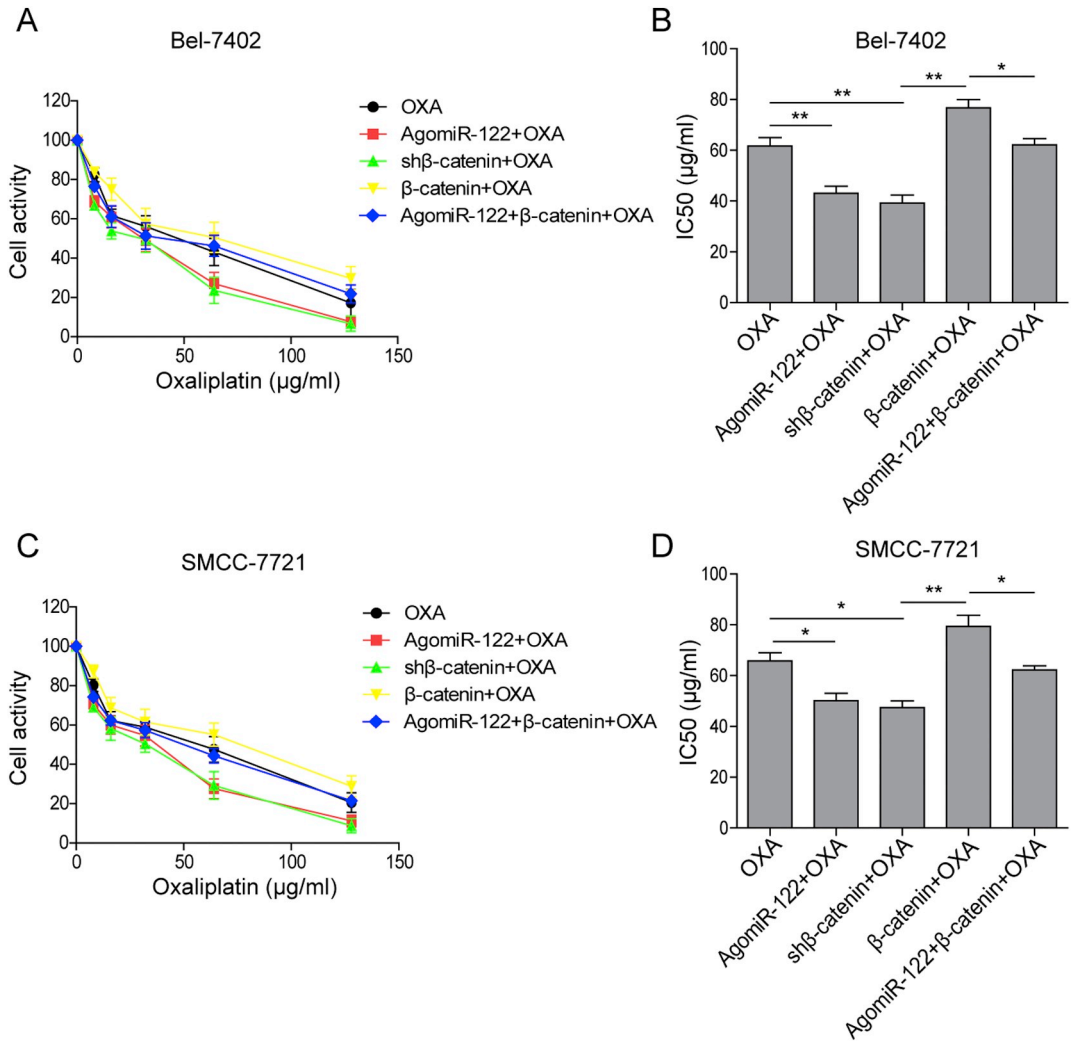

Fig. 5. miR-122 promoted HCC chemosensitivity to OXA. Cell viability was measured using a MTT assay to calculated the IC_{50} values of OXA in Bel-7402(A) and SMMC-7721 (C) cells transfected with AgomiR-122, shβ-catenin, pcDNA3.1-β-catenin and AgomiR-122 + β-catenin, followed by various concentrations of OXA treatment. (B,D) Quantification of IC_{50} in (A, C).All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate. *p < .05 and **p < .01.

2.9. Nude mice xenograft experiments

6-week-old nude mice were acquired from SJA Laboratory Animal Co., Ltd. (Hunan, China; $n = 21$). Experiments were approved by the Animal Care and Use Committee of school of medicine, university of electronic science and technology of Chengdu. Nude mice were implanted with unilateral subaxillary subcutaneous injection of 5×10^6 HCC cells to induce tumors. Mice were divided randomly into three groups (i.e. control group, OXA treatment group, AgomiR-122 + OXA group, seven mice per group). The mice received an intraperitoneal injection of OXA (10 mg/kg) with or without AgomiR-122 starting from the third week every four days as previously described(Lee et al., 2015; Xu et al., 2017). Tumor volume was measured every five days for 30 days and calculated according to the formula: tumor volume (mm^3) = $0.5 \times (W)^2 \times (L)$, where L represents the length and W represents the width. 30 days later, the mice were sacrificed and the tumor weight was measured.

2.10. Statistical analysis

All experiments were performed at least three times in triplicate. Data were presented as Mean ± SD. All statistical analysis was performed in GraphPad Prism 5. Statistical significance was determined by unpaired two-tailed Student *t*-test or one-way ANOVA as indicated in the figure legends. A statistical significance was defined when $p < .05$.

3. Results

3.1. miR-122 is down-regulated while MDR1 is up-regulated in HCC cells

To investigate the functions of miR-122 in HCC, we first examined miR-122 and MDR1 levels in HCC cell lines using qRT-PCR. Compared to normal liver cell line WRL-68, we detected a significantly lower expression of miR-122 in all four HCC cell lines (HepG2, Bel-7402, SMMC-7721 and Huh-7) (Fig. 1A). As expected, both MDR1 mRNA and protein level were also much higher in HCC cell lines than in normal liver cells (Fig. 1B-1D). Together, these data indicated that miR-122 expression is inhibited and negatively associated with MDR1 level in HCC. Given that miR-122 level was lowest in Bcl-7402 and SMMC-7721 cells, we thus used those two cell lines for further studies.

3.2. miR-122 inhibits MDR1 expression via targeting Wnt/β-catenin pathway in HCC cells

It has been reported that Wnt/β-catenin pathway has critical roles in chemoresistance and regulating MDR1 expression in many types of cancers (Arend et al., 2013). Additionally, previous studies also indicated

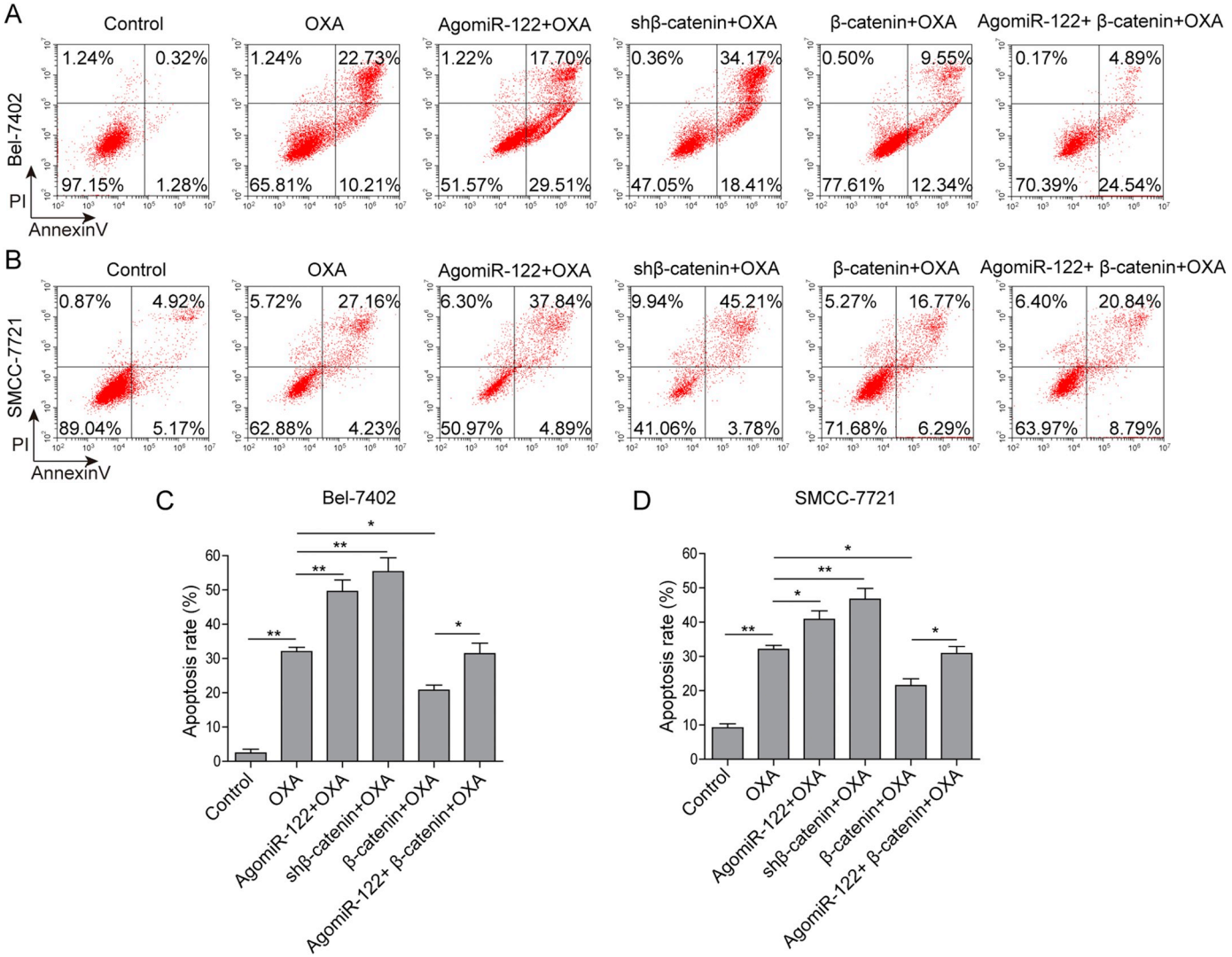

Fig. 6. miR-122 increased HCC cell apoptosis induced by OXA. Cell apoptosis was determined by Annexin-V/PI staining and flow cytometry analysis in transfected Bel-7402 (A) and SMMC-7721 (B) cells with OXA treatment. (C-D) Quantifications of cell apoptosis percentage in (A-B). All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate. *p < .05 and **p < .01.

that miR-122 acted as a negative regulator of Wnt/β-catenin pathway (Xu et al., 2012). Therefore, we speculated that miR-122 may regulate MDR1 expression in HCC by modulating Wnt/β-catenin signaling. To validate this hypothesis, we first examined Wnt/β-catenin pathway activity after miR-122 overexpression in two HCC cell lines. As shown in Fig. 2A-2B, β-catenin and MDR1 mRNA levels were both decreased in HCC cells transfected with AgomiR-122 compared with NC control (Fig. 2A-2B). Further, we detected lower expressions of β-catenin, Wnt1 and MDR1 in miR-122 overexpression cells (Fig. 2C-F), suggesting that miR-122 could inhibit Wnt/β-catenin signaling pathway. Next, we used the TOPflash/FOPflash luciferase reporter system to directly test the interaction between miR-122 and β-catenin. We observed a significantly lower luciferase activity in cells transfected with AgomiR-122 than that in cell transfected with NC (Fig. 2G-2H). Taken together, these results showed that miR-122 directly targets Wnt/β-catenin pathway and may inhibit MDR1 expression through Wnt/β-catenin pathway.

3.3. β-catenin directly binds to MDR1 promoter and increases MDR1 expression

Wnt/β-catenin pathway has been shown to regulate MDR1 expression in multiple cancers including cholangiocarcinoma and ovarian cancer (Arend et al., 2013; Zhou et al., 2017). To verify this regulation in HCC, we manipulated β-catenin level through shRNA or overexpression and measured how these manipulations affected MDR1 expression in HCC cell lines. First, we confirmed that cells transfected with shβ-catenin or β-catenin had lower or higher expressions of β-catenin, respectively (Fig. 3A-3F). Using qRT-PCR, we found that knockdown of β-catenin decreased MDR1 mRNA level while overexpression of β-catenin significantly increased MDR1 mRNA level (Fig. 3A-3B). Consistently, using western blot analysis, we saw similar effects (Fig. 3C-3F). β-catenin shRNA down-regulated β-catenin and MDR1 expressions while β-catenin overexpression up-regulated β-catenin and MDR1 levels (Fig. 3C-3F). Next, we used luciferase assay to directly examine the interaction between β-catenin and MDR1. We found that the luciferase activity in cell transfected with shβ-catenin was significantly lower than vector group, while β-catenin overexpression significantly increased the luciferase activity, suggesting that β-catenin increases MDR1 transcription activity (Fig. 3G-3H). Furthermore, ChIP assay results demonstrated that β-catenin was found to occupy the endogenous MDR1 promoter in HCC cells (Fig. 3I-3J). All together, the above results indicated that Wnt/β-catenin pathway promotes MDR1 expression by binding to its gene promoter.

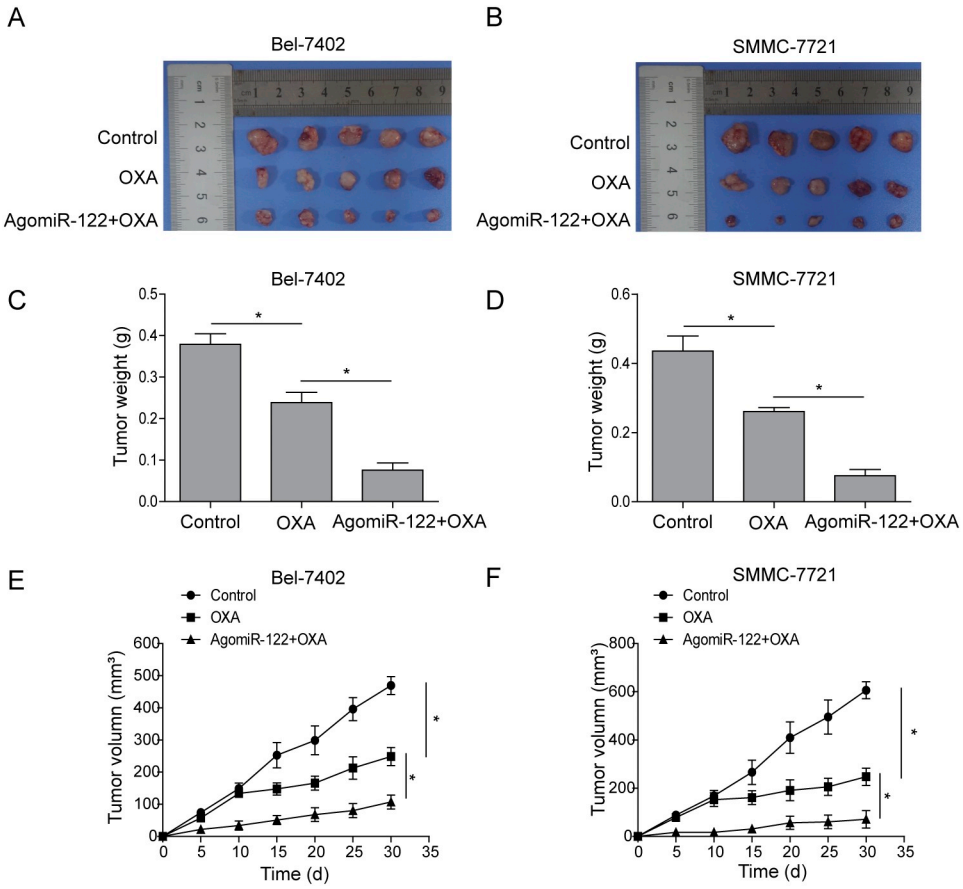

Fig. 7. miR-122 enhanced the antitumor effect of OXA in vivo. (A-B) Representative images of tumors in mice bearing HCC xenograft tumors after transfection of AgomiR-122 with or without OXA treatment. (C-D) Quantifications of tumor weight in three groups. (E-F) Quantification of tumor volume in three groups. All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate. *p < .05 and **p < .01.

3.4. Overexpression of miR-122 and inhibition of Wnt/β-catenin signaling inhibit HCC cell proliferation and promote apoptosis

To directly examine the role of miR-122 and Wnt/β-catenin pathway in HCC cell proliferation and apoptosis, we performed MTT assay and flow cytometry analysis. MTT assay results demonstrated that overexpression of miR-122 inhibited cell proliferation (Fig. 4A-4B). Similarly, cells transfected with shβ-catenin or treated with Wnt inhibitor CIC showed reduced proliferation compared with control (Fig. 4A-4B). Further, with flow cytometry analysis, we observed that ectopic expression of miR-122 or shβ-catenin significantly increased the percentage of cell apoptosis (Fig. 4C-4E). CIC treatment increased cell apoptosis as well (Fig. 4C-4E). These data clearly demonstrated that overexpression of miR-122 and inhibition of Wnt/β-catenin pathway repress HCC cell proliferation and increase cell apoptosis.

3.5. miR-122 increases HCC cells' sensitivity to OXA via suppressing Wnt/β-catenin signaling pathway

To test whether miR-122 is involved in HCC chemosensitivity, we overexpressed miR-122 in HCC cell lines and assessed its effects on cell survival upon OXA treatment. In cytotoxicity assay, we found that overexpression of miR-122 increased cancer cells' sensitivity to OXA (Fig. 5A-5D). The IC_{50} was reduced to45.03 μg/mL from 60.34 μg/mL for Bel-7402 cells (Fig. 5A-5B). In SMMC-7721 cells, the IC_{50} went down to 48.08 μg/mL from 63.45 μg/mL. Knockdown of β-catenin also increased the sensitivity of HCC cells to OXA (Fig. 5C-5D). On contrary, overexpression of β-catenin reduced the sensitivity to OXA and reversed the effects of miR-122 overexpression (Fig. 5A-5D). Together, these data showed that miR-122 enhances the sensitivity of HCC cells to OXA through suppressing Wnt/β-catenin signaling pathway.

3.6. miR-122 promotes OXA-induced cell apoptosis by inhibiting Wnt/β-catenin signaling pathway

Next, we performed flow cytometry analysis to examine the effects of miR-122 on cancer cell apoptosis. We found that OXA treatment significantly increased the percentage of apoptosis cells in Bel-7402 and SMMC-7721 cells (Fig. 6A-6D). Moreover, overexpression of miR-122 or knockdown of β-catenin further increased the percentage of apoptotic cells upon OXA treatment. In contrast, overexpression of β-catenin reduced the effect of OXA and partially inhibited cell apoptosis induced by miR-122 (Fig. 6A-6D), indicating that miR-122 promoted cancer cell death induced by OXA via inhibiting Wnt/β-catenin pathway.

3.7. miR-122 enhances the antitumor effect of OXA in vivo

In the end, we determined whether miR-122 could sensitize HCC cells to chemotherapeutic agents in vivo. AgomiR-122 was delivered to nude mice bearing HCC cells combined with OXA treatment. Compared with control group, OXA treatment significantly reduced tumor weight and volume (Fig. 7A-7F). Moreover, miR-122 overexpression further decreased the tumor weight and volume (Fig.7A-7F). These results fully demonstrated that miR-122 up-regulation enhances the sensitivity of HCC to OXA in vivo.

4. Discussion

Despite advances in therapy and research, the prognosis of HCC for the majority of patients has not changed significantly over the past decades as a result of chemoresistance (Lohitesh et al., 2018). Exploring the mechanism of chemoresistance is the necessary prerequisite to better clinical outcomes. miR-122 has been indicated to play some roles in HCC development and lower expression of miR-122 has been reported in HCC (Bandiera et al., 2015; Tsai et al., 2012), but its

relationship with chemosensitivity remains unclear. In the present study, we further elucidated the function of miR-122 in HCC. We confirmed that miR-122 expression was significantly down-regulated in HCC cells, and overexpression of miR-122 could increase the sensitivity of HCC cells to OXA and promote OXA-induced apoptosis. Moreover, miR-122 could inhibit Wnt/β-catenin signaling pathway and decrease MDR1 expression via directly targeting β-catenin. Collectively, our study identified a novel miRNA implicated in OXA resistance of HCC, which might help provide a target for future therapeutic development.

Emerging evidence indicates that aberrant expressions of miRNA are involved in tumorigenesis and progression (Mishra, 2012; Yang et al., 2018). Many miRNA have been observed dysregulated in cancers. Additionally, they play important roles in regulating drug resistance by acting on downstream targets (Di Leva et al., 2014). As reported, reduced miR-122 levels have been related with poor prognosis and metastasis of hepatocellular carcinoma, and several targets of miR-122, including cycin G1, IGF1R and Wnt1, have been implicated in hepatocarcinogenesis, EMT, chemoresistance and angiogenesis (Bandiera et al., 2015; Nakao et al., 2014). Altogether, those studies suggested that miR-122 functions as a tumor suppressor in the liver. Here, we found that miR-122 was significantly down-regulated in human HCC cell lines, which was consistent with previous reports. miR-122 acts as a tumor suppressor gene in hepatocarcinogenesis. Overexpression of miR-122 inhibited HCC cell proliferation and increased cell apoptosis. Furthermore, miR-122 increased HCC cells' sensitivity to OXA. We also validated the antitumor activity of miR-122 by using in vivo nude mouse model. These data fully suggested that loss of miR-122 is a key mechanism that cancer cells utilize to gain chemoresistance in HCC.

The Wnt/β-catenin has critical roles in normal tissue development as well as in tumorigenesis and chemoresistance (Arend et al., 2013; Cui et al., 2012). Without Wnt stimulation, cytoplasmic β-catenin is assembled into a destruction complex and gets phosphorylated in this complex and subsequently targeted for proteasomal degradation. Stimulation by Wnt ligands inhibits the phosphorylation and thus prevents degradation of β-catenin, which then enters the nucleus and regulates the expression of downstream target genes involved in multiple cellular processes (Krishnamurthy & Kurzrock, 2018). The aberrant activation of Wnt/β-catenin pathway has been detected in many types of cancers, such as ovarian cancer (Arend et al., 2013) and intestinal cancers (Krausova & Korinek, 2014). The activation of this signaling pathway would result in aberrant expressions of many downstream proteins, including MDR1 gene, which played an important role in developing drug resistance in cancer cells (Tiwari et al., 2011). For instance, in chemoresistance breast cancer cells, the Wnt/β-catenin pathway was found activated, resulting MDR1 hyperactivity (Zhang et al., 2016). In this study, we observed enhanced Wnt/β-catenin activity in HCC cells. Inhibition of Wnt/β-catenin pathway could suppress cell proliferation and increase cell apoptosis. Furthermore, β-catenin is a downstream target of miR-122, which was consistent with previous studies. Loss of miR-122 could trigger the activation of Wnt/β-catenin signaling pathway in HCC, and up-regulation of miR-122 could inhibit Wnt/β-catenin pathway and thereby enhance sensitivity of HCC cells to OXA.

It has been reported that Wnt/β-catenin signaling could induce MDR1/P-gp expression and that inhibition of Wnt/β-catenin pathway down-regulates MDR1/P-gp expression and reverses multi-drug resistance in tumors such as cholangiocarcinoma and ovarian cancer (Shen et al., 2013; Zhang et al., 2015). Here, we saw similar results in HCC and demonstrated that β-catenin directly binds to MDR1 promoter and promotes its transcription. Taken together, our results suggested that miR-122 increases the sensitivity of HCC to OXA because of its inhibition of MDR1/P-gp expression via suppressing Wnt/β-catenin signaling in HCC.

5. Conclusions

In summary, our study indicated that miR-122 directly targets the Wnt/β-catenin pathway and subsequently inhibits the expression of MDR1, thereby increasing the sensitivity of HCC cells to OXA and promoting OXA-induced apoptosis. Targeting miR-122 may be a potential strategy to increase chemosensitivity, hence improving the prognosis of HCC.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Ambudkar, S.V., Kimchi-Sarfaty, C., Sauna, Z.E., Gottesman, M.M., 2003. P-glycoprotein: from genomics to mechanism. Oncogene 22, 7468–7485.
Arend, R.C., Londono-Joshi, A.I., Straughn Jr., J.M., Buchsbaum, D.J., 2013. The Wnt/beta-catenin pathway in ovarian cancer: a review. Gynecol. Oncol. 131, 772–779.
Bai, S., Nasser, M.W., Wang, B., Hsu, S.H., Datta, J., et al., 2009. MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. J. Biol. Chem. 284, 32015–32027.
Bandiera, S., Pfeffer, S., Baumert, T.F., Zeisel, M.B., 2015. miR-122–a key factor and therapeutic target in liver disease. J. Hepatol. 62, 448–457.
Coulouarn, C., Factor, V.M., Andersen, J.B., Durkin, M.E., Thorgeirsson, S.S., 2009. Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. Oncogene 28, 3526–3536.
Cui, J., Jiang, W., Wang, S., Wang, L., Xie, K., 2012. Role of Wnt/beta-catenin signaling in drug resistance of pancreatic cancer. Curr. Pharm. Des. 18, 2464–2471.
Daher, S., Massarwa, M., Benson, A.A., Khoury, T., 2018. Current and Future Treatment of Hepatocellular Carcinoma: an Updated Comprehensive Review. J Clin Transl Hepatol 6, 69–78.
Di Leva, G., Garofalo, M., Croce, C.M., 2014. MicroRNAs in cancer. Annu. Rev. Pathol. 9, 287–314.
Finn, R.S., 2013. Emerging targeted strategies in advanced hepatocellular carcinoma. Semin. Liver Dis. 33 (Suppl. 1), S11–S19.
Fornari, F., Gramantieri, L., Giovannini, C., Veronese, A., Ferracin, M., et al., 2009. MiR-122/cyclin G1 interaction modulates p53 activity and affects doxorubicin sensitivity of human hepatocarcinoma cells. Cancer Res. 69, 5761–5767.
Grainger, S., Willert, K., 2018. Mechanisms of Wnt signaling and control. Wiley Interdiscip Rev Syst Biol Med e1422.
Kou, P., Zhang, Y., Shao, W., Zhu, H., Zhang, J., et al., 2017. Significant efficacy and well safety of apatinib in an advanced liver cancer patient: a case report and literature review. Oncotarget 8, 20510–20515.
Krausova, M., Korinek, V., 2014. Wnt signaling in adult intestinal stem cells and cancer. Cell. Signal. 26, 570–579.
Krishnamurthy, N., Kurzrock, R., 2018. Targeting the Wnt/beta-catenin pathway in cancer: Update on effectors and inhibitors. Cancer Treat. Rev. 62, 50–60.
Le Grazie, M., Biagini, M.R., Tarocchi, M., Polvani, S., Galli, A., 2017. Chemotherapy for hepatocellular carcinoma: the present and the future. World J. Hepatol. 9, 907–920.
Lee, J.S., Oh, E., Yoo, J.Y., Choi, K.S., Yoon, M.J., et al., 2015. Adenovirus expressing dual c-Met-specific shRNA exhibits potent antitumor effect through autophagic cell death accompanied by senescence-like phenotypes in glioblastoma cells. Oncotarget 6, 4051–4065.
Liu, L.J., Xie, S.X., Chen, Y.T., Xue, J.L., Zhang, C.J., et al., 2016. Aberrant regulation of Wnt signaling in hepatocellular carcinoma. World J. Gastroenterol. 22, 7486–7499.
Lohitesh, K., Chowdhury, R., Mukherjee, S., 2018. Resistance a major hindrance to chemotherapy in hepatocellular carcinoma: an insight. Cancer Cell Int. 18, 44.
Mishra, P.J., 2012. The miRNA-drug resistance connection: a new era of personalized medicine using noncoding RNA begins. Pharmacogenomics 13, 1321–1324.
Moran, Y., Agron, M., Praher, D., Technau, U., 2017. The evolutionary origin of plant and animal microRNAs. Nat Ecol Evol 1, 27.
Morita, K., Taketomi, A., Shirabe, K., Umeda, K., Kayashima, H., et al., 2011. Clinical significance and potential of hepatic microRNA-122 expression in hepatitis C. Liver Int. 31, 474–484.
Nakao, K., Miyaaki, H., Ichikawa, T., 2014. Antitumor function of microRNA-122 against hepatocellular carcinoma. J. Gastroenterol. 49, 589–593.
Qin, S., Bai, Y., Lim, H.Y., Thongprasert, S., Chao, Y., et al., 2013. Randomized, multicenter, open-label study of oxaliplatin plus fluorouracil/leucovorin versus doxorubicin as palliative chemotherapy in patients with advanced hepatocellular carcinoma from Asia. J. Clin. Oncol. 31, 3501–3508.
Qin, S., Cheng, Y., Liang, J., Shen, L., Bai, Y., et al., 2014. Efficacy and safety of the FOLFOX4 regimen versus doxorubicin in Chinese patients with advanced

hepatocellular carcinoma: a subgroup analysis of the EACH study. Oncologist 19, 1169–1178.
Shen, D.Y., Zhang, W., Zeng, X., Liu, C.Q., 2013. Inhibition of Wnt/beta-catenin signaling downregulates P-glycoprotein and reverses multi-drug resistance of cholangiocarcinoma. Cancer Sci. 104, 1303–1308.
Shukla, S., Ohnuma, S., Ambudkar, S.V., 2011. Improving cancer chemotherapy with modulators of ABC drug transporters. Curr. Drug Targets 12, 621–630.
Tiwari, A.K., Sodani, K., Dai, C.L., Ashby Jr., C.R., Chen, Z.S., 2011. Revisiting the ABCs of multidrug resistance in cancer chemotherapy. Curr. Pharm. Biotechnol. 12, 570–594.
Tsai, W.C., Hsu, S.D., Hsu, C.S., Lai, T.C., Chen, S.J., et al., 2012. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. J. Clin. Invest. 122, 2884–2897.
Xu, J., Zhu, X., Wu, L., Yang, R., Yang, Z., et al., 2012. MicroRNA-122 suppresses cell proliferation and induces cell apoptosis in hepatocellular carcinoma by directly targeting Wnt/beta-catenin pathway. Liver Int. 32, 752–760.
Xu, K., Chen, G., Qiu, Y., Yuan, Z., Li, H., et al., 2017. miR-503-5p confers drug resistance by targeting PUMA in colorectal carcinoma. Oncotarget 8, 21719–21732.
Yang, G., Shen, T., Yi, X., Zhang, Z., Tang, C., et al., 2018. Crosstalk between long non-coding RNAs and Wnt/beta-catenin signalling in cancer. J. Cell. Mol. Med. 22, 2062–2070.
Zhang, H., Jing, X., Wu, X., Hu, J., Zhang, X., et al., 2015. Suppression of multidrug resistance by rosiglitazone treatment in human ovarian cancer cells through down-regulation of FZD1 and MDR1 genes. Anti-Cancer Drugs 26, 706–715.
Zhang, Z.M., Wu, J.F., Luo, Q.C., Liu, Q.F., Wu, Q.W., et al., 2016. Pygo2 activates MDR1 expression and mediates chemoresistance in breast cancer via the Wnt/beta-catenin pathway. Oncogene 35, 4787–4797.
Zhou, H., Lin, C., Zhang, Y., Zhang, X., Zhang, C., et al., 2017. miR-506 enhances the sensitivity of human colorectal cancer cells to oxaliplatin by suppressing MDR1/P-gp expression. Cell Prolif. 50.