

Original Article

miR-101-3p sensitizes hepatocellular carcinoma cells to oxaliplatin by inhibiting Beclin-1-mediated autophagy

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Abstract: Background: Increasing evidence has shown that autophagy can contribute to drug resistance. Whether microRNA-101-3p (miR-101-3p) participates in oxaliplatin (OXA) resistance via modulating Beclin-1-mediated autophagy in hepatocellular carcinoma (HCC) has not been reported. Methods: OXA-resistant Huh7 cells (Huh7/OXA) or HepG2 cells (HepG2/OXA) and OXA-sensitive Huh7 or HepG2 cells were treated with OXA in various concentrations. The expressions of miR-101-3p and Beclin-1 were monitored using qRT-PCR. Western blot was used to evaluate cell autophagy. Cell viability and the IC50 of OXA were determined using an MTT assay. Cell apoptosis was evaluated by flow cytometry. A luciferase reporter assay was introduced to confirm the relationship between miR-101-3p and Beclin-1. Results: miR-101-3p was decreased in HCC resistant tissues and cells. Moreover, an increased expression of miR-101-3p reduced cell viability and the IC50 of OXA, and it promoted cell apoptosis in Huh7/OXA and HepG2/OXA cells. miR-101-3p negatively modulated the expression of Beclin-1. Interestingly, the overexpression of Beclin-1 receded the effect of the ectopic expression of miR-101-3p in OXA-resistant HCC cells. In OXA-sensitive Huh7 and HepG2 cells, OXA significantly increased the expressions of LC3 and Beclin-1, and it decreased the abundance of p62. Furthermore, OXA markedly blocked cell viability, which was exacerbated by the introduction of the autophagy inhibitor CQ. Additionally, the elevated expression of miR-101-3p suppressed cell autophagy by inhibiting the expression of LC3 and Beclin-1 and facilitating the expression of p62. Conclusion: miR-101-3p is responsible for the sensitivity of HCC cells to OXA by inhibiting Beclin-1-mediated autophagy.

Keywords: miR-101-3p, Beclin-1, autophagy, oxaliplatin, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related deaths worldwide [1]. Chemotherapy is one of the most important treatment modalities for advanced HCC [2]. Previous studies have shown that oxaliplatin (OXA) exerts a significant anticancer effect in HCC [3]. However, drug resistance is still an obstacle for the treatment efficacy of chemotherapy [4, 5]. Autophagy is an important factor which promotes tumor chemoresistance in a variety of cancers, including HCC [6-9]. Therefore, it is necessary to elucidate the underlying molecular mechanisms of autophagy in the process of OXA resistance in HCC.

MicroRNAs (miRNAs), a class of small noncoding RNAs, are expressed aberrantly in HCC [10]. It is well documented that miRNAs play signifi-

cant roles in carcinogenesis and cancer progression through their modulation of cellular processes such as proliferation, metastasis, apoptosis, and autophagy [11-13]. Researchers have revealed that miRNAs are implicated in the chemoresistance of HCC [14, 15]. For example, an enhanced expression of miR-122 in resistant Huh7 cells reverses doxorubicin resistance through the inhibition of the pyruvate kinase isoenzyme M2 and the induction of apoptosis in doxorubicin-resistant cancer cells [16]. Moreover, the involvement of the activation of autophagy in chemoresistance is also associated with miRNAs. In a previous study, miR-30a sensitized tumor cells to cis-di-chloro-diamine platinum via decreasing Beclin-1-mediated autophagy [17]. Tan et al. found that miR-409-3p decreases chemotherapy-induced autophagy in a manner dependent on Beclin-1, thus enhancing the chemosensitivity of colon

cancer cells [18]. In HCC cells, the overexpression of miR-26 blocks doxorubicin-induced autophagy and promotes apoptosis, leading to a decrease in the resistance to chemotherapy [9]. Although miRNAs have been widely reported to be linked to the chemotherapy of HCC, many miRNAs and their underlying mechanisms in this process have still not been fully investigated. miR-101-3p is lowly-expressed in lung cancer A549 and H1299 cells. Long non-coding RNA metastasis-associated lung adenocarcinoma transcript 1 promotes cisplatin resistance by sponging miR-101-3p and enhancing myeloid cell leukemia 1 expression [19]. MiR-101-3p has been shown to be downregulated in HCC tissues and cells and could be a potential diagnostic marker of HCC [20]. Those findings indicate that miR-101-3p may play an important role in the chemotherapy of HCC. Moreover, miR-101-mediated autophagy inhibition sensitizes breast cancer cells to 4-hydroxytamoxifen [21], demonstrating that miR-101 may be a potential autophagy inhibitor. Although many researchers have explored the functions of miR-101-3p in HCC, the exact role of miR-101-3p in OXA resistance and the regulation of autophagy must still be elucidated.

In this study, we sought to explore the role of miR-101-3p in HCC cell resistance to OXA and its underlying molecular mechanisms.

Materials and methods

Clinical samples

We evaluated 42 tissue samples from oxaliplatin-sensitive patients and 28 HCC tissue samples from oxaliplatin-resistant HCC patients at the Gansu Provincial Cancer Hospital. All patients enrolled in this study signed an informed consent before the tissue samples were collected. The tissue sample acquisition was approved by the Review Board of the Hospital Ethics Committee of the Gansu Provincial Cancer Hospital.

Cell culture and transfection

The human HCC cell lines Huh7 and HepG2 and the human normal liver cell line LO2 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium

(Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) in a 5% CO₂ atmosphere at 37°C. Huh7 and HepG2 cells were treated with OXA (HaoRan Biological Technology Co., Ltd., Shanghai, China) in different concentrations (0, 0.5, 1, 2, 5, and 10 µM). Oxaliplatin-resistant HCC Huh7 cells (Huh7/OXA) and HepG2 cells (HepG2/OXA) were transfected with miR-101-3p mimic (miR-101-3p), negative control miR-NC, pcDNA 3.0 vector (vector), Beclin-1 overexpression plasmid (Beclin-1). Subsequently, the transfected OXA-resistant cells were exposed in OXA with increasing concentrations (0, 1, 5, 10, 20, 40, and 80 µM). The transfected or OXA-treated cells were used for the following experiments.

MTT assay

The MTT reagent (Sigma) was used to analyze cell proliferation, and Dimethyl sulfoxide was used to dissolve the blue crystals. Cell viability was measured with absorbance at 490 nm. The 50% growth inhibition (IC₅₀) of OXA was also assessed. The experiment was conducted three times.

QRT-PCR assay

The expressions of miR-101-3p and Beclin-1 were measured by qRT-PCR. Trizol reagent (Beyotime, Shanghai, China) was used to extract total RNAs and then the RNAs were reversely transcribed into complementary DNA using a TaqMan® MicroRNA Reverse Transcription kit (Biosystems, Foster City, CA, USA). The reverse transcription was conducted according the instructions of the Prime Script™ RT reagent kit (Takara, Dalian, China) to quantify the expression of the mRNAs. After that, PCR was determined using a SYBR Green kit (Takara). The relative expression levels of miR-101-3p (normalized to U6) and Beclin-1 (normalized to GAPDH) were calculated using the 2^{-ΔΔCt} method. The sequences of the primers used are as follows: miR-101-3p-F, 5'-GCCGCCACCATGGTG-AGCAAGG-3', miR-101-3p-R, 5'-AATTGAAAAA-GTGATTAAATT-3'; U6-F, 5'-CTCGCTTCGGCAGC-ACATA-3', U6-R, 5'-CTCGCTTCGGCAGCACATA-3'; Beclin-1-F, 5'-AGCTGCCGTATACTGTTCT-3', Beclin-1-R, 5'-TGTGTCTTCAATCTTGCTT-3'; GAPDH-F, 5'-ATCACCATCTTCCA-GGAGCG-3', GAPDH-R, 5'-GTTCTTCCACCACTTCGTCC-3'.

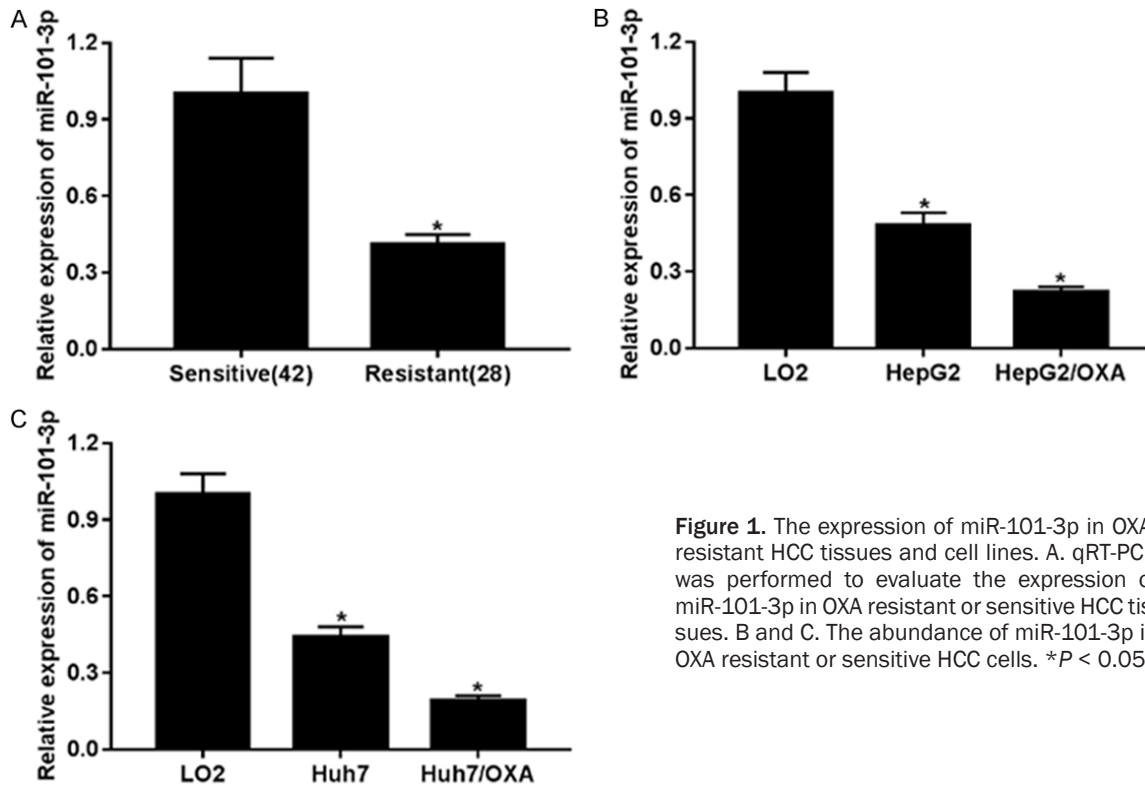


Figure 1. The expression of miR-101-3p in OXA-resistant HCC tissues and cell lines. A. qRT-PCR was performed to evaluate the expression of miR-101-3p in OXA resistant or sensitive HCC tissues. B and C. The abundance of miR-101-3p in OXA resistant or sensitive HCC cells. * $P < 0.05$.

Cell apoptosis assay

The cells were harvested and then washed with a phosphate buffered solution and incubated with Annexin-V and PI (BD Biosciences, Franklin Lakes, NJ, USA) at room temperature for 15 min in the dark. Then, the cell apoptosis was measured using a flow cytometer FACSCalibur (BD Biosciences). The experiment was done three times.

Luciferase reporter assay

The wild type 3'UTR sequence of the Beclin-1 (Beclin-1-Wt) luciferase reporter plasmid or the muted 3'UTR sequence of the Beclin-1 (Beclin-1-Mut) plasmid and miR-101-3p mimic or miR-NC were co-transfected into Huh7/OXA and HepG2/OXA cells. The luciferase activity was evaluated using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western blot assay

Briefly, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Roche, Mannheim, Germany). The membranes were subsequently blocked

with 5% skim milk and then incubated overnight at 4°C with antibodies against Beclin-1, p62 and LC3. After that, the membranes were incubated with a secondary antibody. The proteins were visualized using enhanced chemiluminescence (Beyotime). The antibodies used in the current study were purchased from Abcam (Cambridge, MA, USA).

Statistical analysis

Data were presented as the mean \pm standard deviation (SD). All statistical analyses were carried out using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The differences were evaluated using Student's *t* test or a one-way ANOVA. *P* values less than 0.05 were considered statistically significant.

Results

miR-101-3p is downregulated in OXA-resistant HCC tissues and cell lines

First, 42 of the HCC sensitive tissues and 28 of the HCC resistant tissues were subjected to a qRT-PCR analysis. We found that miR-101-3p was drastically downregulated in the HCC OXA-resistant tissues (**Figure 1A**). Also, the expres-

sion of miR-101-3p was reduced in the Huh7 and HepG2 cells compared to the cells in the HCC normal liver cell line LO2. As expected, the miR-101-3p expression levels were similarly decreased in the HCC OXA-resistant cells Huh7/OXA and HepG2/OXA (**Figure 1B** and **1C**). Therefore, we thought that miR-101-3p may be involved in the OXA-resistance in HCC.

miR-101-3p overexpression inhibits OXA resistance in HCC cells

The IC₅₀ was subsequently determined in different cell lines. As shown in **Figure 2A**, the Huh7/OXA and HepG2/OXA cells had a higher IC₅₀ of OXA than the HCC sensitive cells did. To investigate the effect of miR-101-3p on the OXA-resistance of HCC, we strongly elevated the level of miR-101-3p in the HCC OXA-resistant cells. The results of the transfection efficiency showed that the introduction of the miR-101-3p mimic effectively increased the level of miR-101-3p in the HCC OXA-resistant cells (**Figure 2B**). Moreover, cell viability and the value of the IC₅₀ of OXA were also examined in this experiment. It was observed that the overexpression of miR-101-3p significantly enhanced the sensitivity of HCC OXA-resistant cells to OXA (**Figure 2C-F**). In addition, the cell apoptosis rate was evidently higher in the miR-101-3p-overexpressed Huh7/OXA and HepG2/OXA cells than the rate of the cells transfected with miR-NC (**Figure 2G** and **2H**). The data indicated that miR-101-3p could reduce the resistance of HCC cells to OXA.

miR-101-3p suppresses the expression of Beclin-1 in HCC Huh7/OXA and HepG2/OXA cells

Beclin-1 was identified as a potential target of miR-101-3p because miR-101-3p possesses binding sites with Beclin-1 (**Figure 3A**). Then, a luciferase reporter assay was employed to validate the relationship between Beclin-1 and miR-101-3p. The results demonstrated that the miR-101-3p mimic notably decreased the luciferase activity of the Beclin-1-Wt group, but it had no significant impact on the luciferase activity of the Beclin-1-Mut group (**Figure 3B** and **3C**). Moreover, the mRNA level of Beclin-1 was lower in cells with the introduction of the miR-101-3p mimic than it was in the cells transfected with miR-NC (**Figure 3D**). A Western blot analysis further disclosed that the protein level of Beclin-1 was repressed in the miR-101-3p

group compared with the protein level of the miR-NC group (**Figure 3E**).

miR-101-3p-induced inhibition on oxaliplatin-resistance in HCC is mediated by Beclin-1

To further test the involvement of Beclin-1 in the miR-101-3p-mediated repression of OXA resistance in HCC, we co-overexpressed the miR-101-3p and Beclin-1 in OXA-resistant HCC cells. The ectopic expression of miR-101-3p significantly dampened the protein level of Beclin-1, which was regained by the introduction of the Beclin-1 overexpression plasmid (**Figure 4A** and **4B**). An MTT assay further demonstrated that the forced expression of Beclin-1 strikingly rescued the effects of the miR-101-3p upregulation on the IC₅₀ of OXA (**Figure 4C** and **4D**). Additionally, the overexpression of the miR-101-3p-induced apoptosis was also reduced by increased levels of Beclin-1 (**Figure 4E** and **4F**).

Autophagy is required for oxaliplatin-resistance via the miR-101-3p/Beclin-1 axis

Due to the involvement of Beclin-1 in miR-101-3p-mediated inhibition on the OXA-resistance of HCC, whether Beclin-1-induced autophagy is responsible for miR-101-3p-mediated inhibition on the OXA-resistance of HCC was also explored in the following experiments. Western blot analysis showed that the ratio of LC3II/LC3I and the expression of Beclin-1 were elevated, but the level of p62 was decreased in the HCC sensitive cell HepG2 (**Figure 5A-C**). Meanwhile, the same phenomenon was also observed in the Huh7 cells (**Figure 5D-F**). In the HCC OXA-sensitive or OXA-resistant cells, CQ-mediated autophagy inhibition exacerbated the OXA-mediated suppression of cell viability (**Figure 5G** and **5H**), suggesting that the inhibition of autophagy may contribute to the sensitivity of HCC cells to OXA. In addition, we further demonstrated that the overexpression of miR-101-3p significantly enhanced the protein level of p62, but it decreased the protein levels of LC3 and Beclin-1 (**Figure 5I-L**). These data demonstrated that miR-101-3p has an role in the OXA resistance of HCC cells by means of its modulation of Beclin-1-mediated autophagy.

Discussion

OXA has previously been reported to have efficacy in HCC [3]. However, chemoresistance is a

miR-101-3p sensitizes HCC cells to oxaliplatin

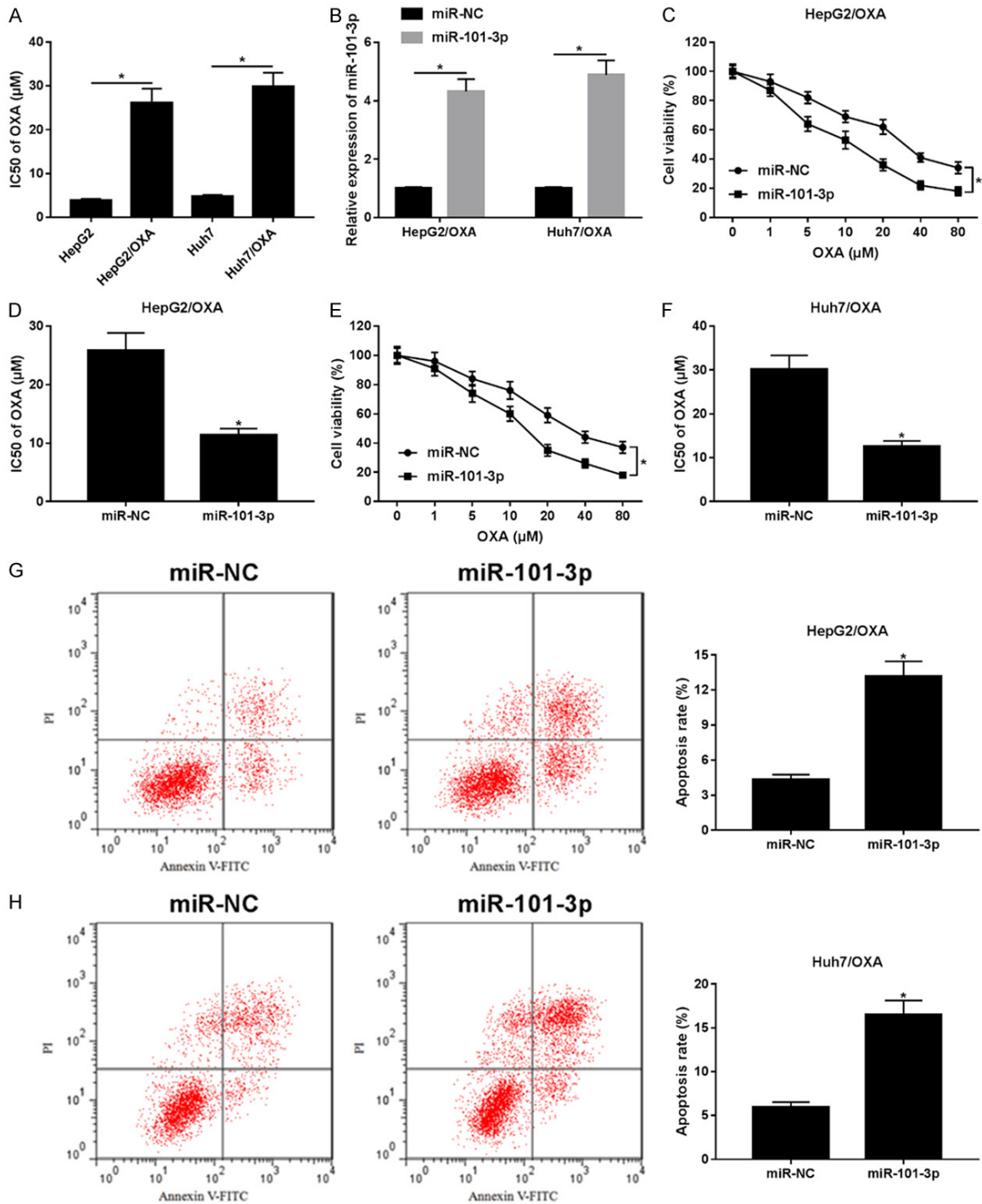


Figure 2. The effect of miR-101-3p overexpression on OXA resistance of HCC cells. A. An MTT assay was conducted to analyze the IC₅₀ of OXA. B. The expression of miR-101-3p in cells transfected with an miR-101-3p mimic or miR-NC. C and D. Cell viability and the IC₅₀ of OXA were detected in HepG2/OXA cells of the miR-101-3p mimic or the miR-NC group. E and F. Cell viability and the IC₅₀ of OXA were measured in the Huh7/OXA cells of the miR-101-3p mimic or the miR-NC group. G and H. Cell apoptosis was examined using flow cytometry. **P* < 0.05.

bottleneck of HCC in response to OXA treatment. Therefore, it would be helpful to develop more effective treatment strategies by understanding the mechanisms of resistance to OXA.

Extensive research has revealed the involvement of miR-101-3p in the development of cancers, including HCC. For instance, miR-101 could enhance the sensitivity of the OS cell line

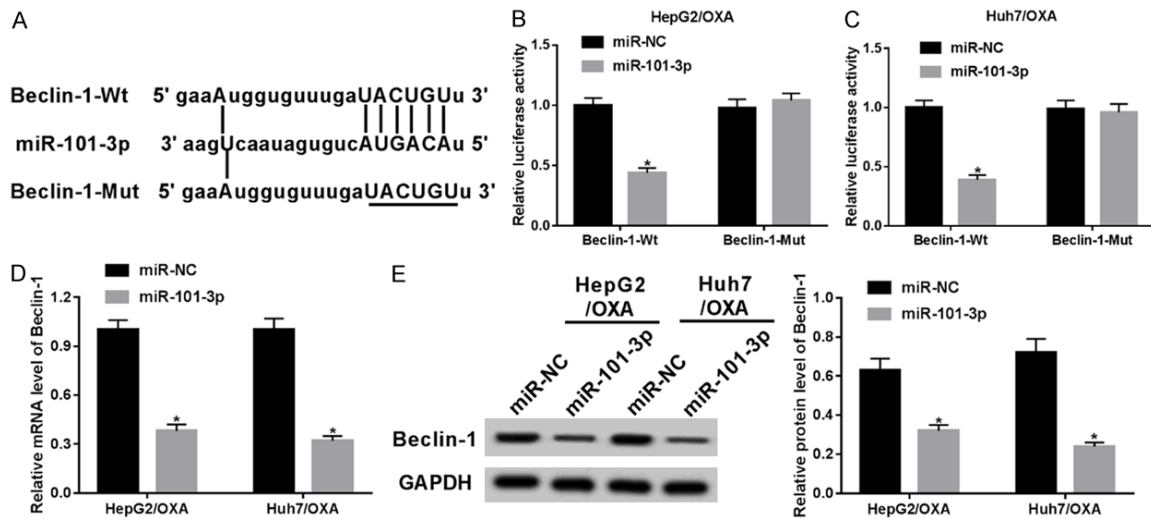


Figure 3. The effect of miR-101-3p on the expression of Beclin-1 in HCC Huh7/OXA and HepG2/OXA cells. **A.** The binding sites between miR-101-3p and Beclin-1 were predicted by starBase. **B** and **C.** The luciferase activity was measured in Huh7/OXA and HepG2/OXA cells co-transfected with Beclin-1-Wt or Beclin-1-Mut luciferase reporter plasmids and the miR-101-3p mimic or miR-NC. **D.** The mRNA level of Beclin-1 was quantified in cells transfected with the miR-101-3p mimic or miR-NC. **E.** The protein levels of Beclin-1 in Huh7/OXA and HepG2/OXA cells. * $P < 0.05$.

U-2 through blocking autophagy [22]. Moreover, the expression of miR-101 is reduced in human HCC tissues and hepatoma cell lines. miR-101 inhibits colony formation and promotes apoptosis and suppresses tumorigenicity via targeting Mcl-1 in HCC cells [23]. The results of a study by Cao et al. also indicated the low expression of miR-101 in HCC tissues and further demonstrated that increasing the miR-101 level could induce the inhibition of HepG2 cell proliferation, migration and invasion abilities [24]. Xu et al. [25] further supported the tumor suppressor role of miR-101 in HCC because the upregulation of miR-101 inhibits proliferation, invasion, colony formation, and cell cycle progression by binding to the oncogene polycomb group protein enhancer of zeste homolog 2. It has been reported that miR-101 is involved in the radiation therapy or chemotherapy of HCC and miR-101 could be delivered to the tumor site and sensitize the tumor to radiation [26]. Moreover, miR-101 represses autophagy and synergizes with either doxorubicin or fluorouracil to induce apoptosis in HCC cells [25]. Although researchers have provided evidence that miR-101-3p is related to HCC chemoresistance by the modulation of cell autophagy in HCC, it is unclear whether miR-101 is associated with the OXA resistance of HCC. Here, a low expression of miR-101-3p was observed in HCC OXA-resistant tissues or cells. Moreover, miR-101-3p upregu-

lation enhanced the sensitivity of HCC cells to OXA by inhibiting cell proliferation and inducing apoptosis. These data imply that miR-101-3p may participate in the chemoresistance of OXA.

We identified Beclin-1 as a target of miR-101-3p and then disclosed that this autophagy-related protein was curbed by the upregulation of miR-101-3p in HepG2/OXA and Huh7/OXA cells, suggesting that it plays a role in chemoresistance. The results of our rescue-of-function experiment revealed that the gain of Beclin-1 abolished the miR-101-3p-mediated promotion of the sensitivity of HCC to OXA. It has been shown that Beclin-1 is involved in maintaining the balance of autophagy and apoptosis because Beclin-1-mediated autophagy hampers the expression and function of cleaved caspase-8 to protect against the cadmium-induced activation of apoptosis [27]. The role of Beclin-1-mediated autophagy could play a vital role in many cancers. Hou. et al. reported that miR-17-5p could increase the radio-sensitivity of glioma cells by the inhibition of Beclin-1-mediated autophagy [28]. In pediatric leukemia cells, Wu et al. [29] analyzed cell viability, apoptosis as well as autophagy, during the oxidative stress (OS). Their findings indicated that Beclin-1 is a direct target of miR-93 and miR-93 enhances the sensitivity of cells to OS during chemotherapy by Beclin-1-mediated autophagy. In addi-

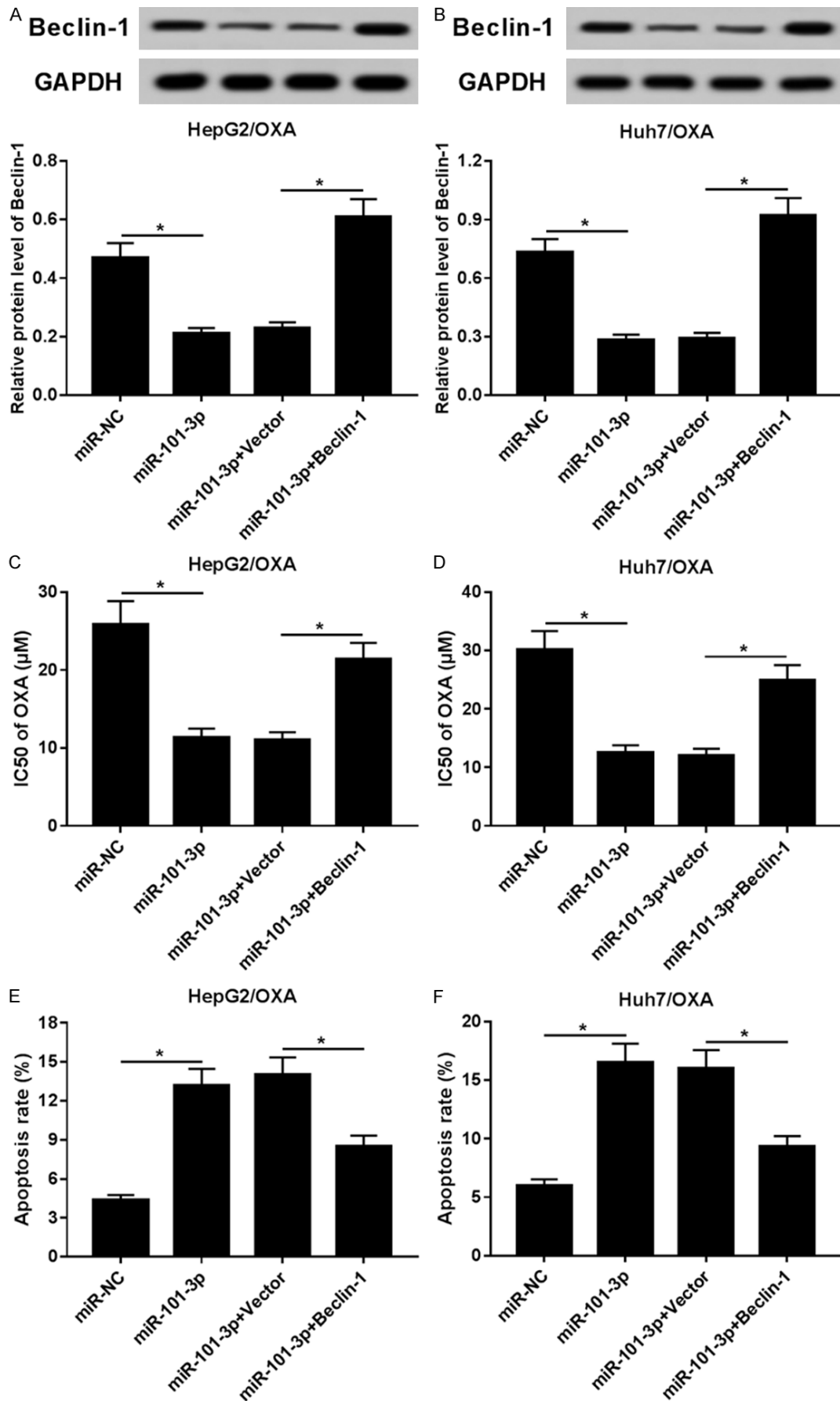


Figure 4. The effect of Beclin-1 on the miR-101-3p-induced inhibition on oxaliplatin-resistance in HCC. A and B. Huh7/OXA and HepG2/OXA cells were introduced with a miR-101-3p mimic, an miR-NC, miR-101-3p + vector, and miR-101-3p + Beclin-1 and western blot were used to examine the protein expression of Beclin-1. C and D. An MTT assay was employed to assess the IC50 of OXA. E and F. Cell apoptosis was determined by flow cytometry. **P* < 0.05.

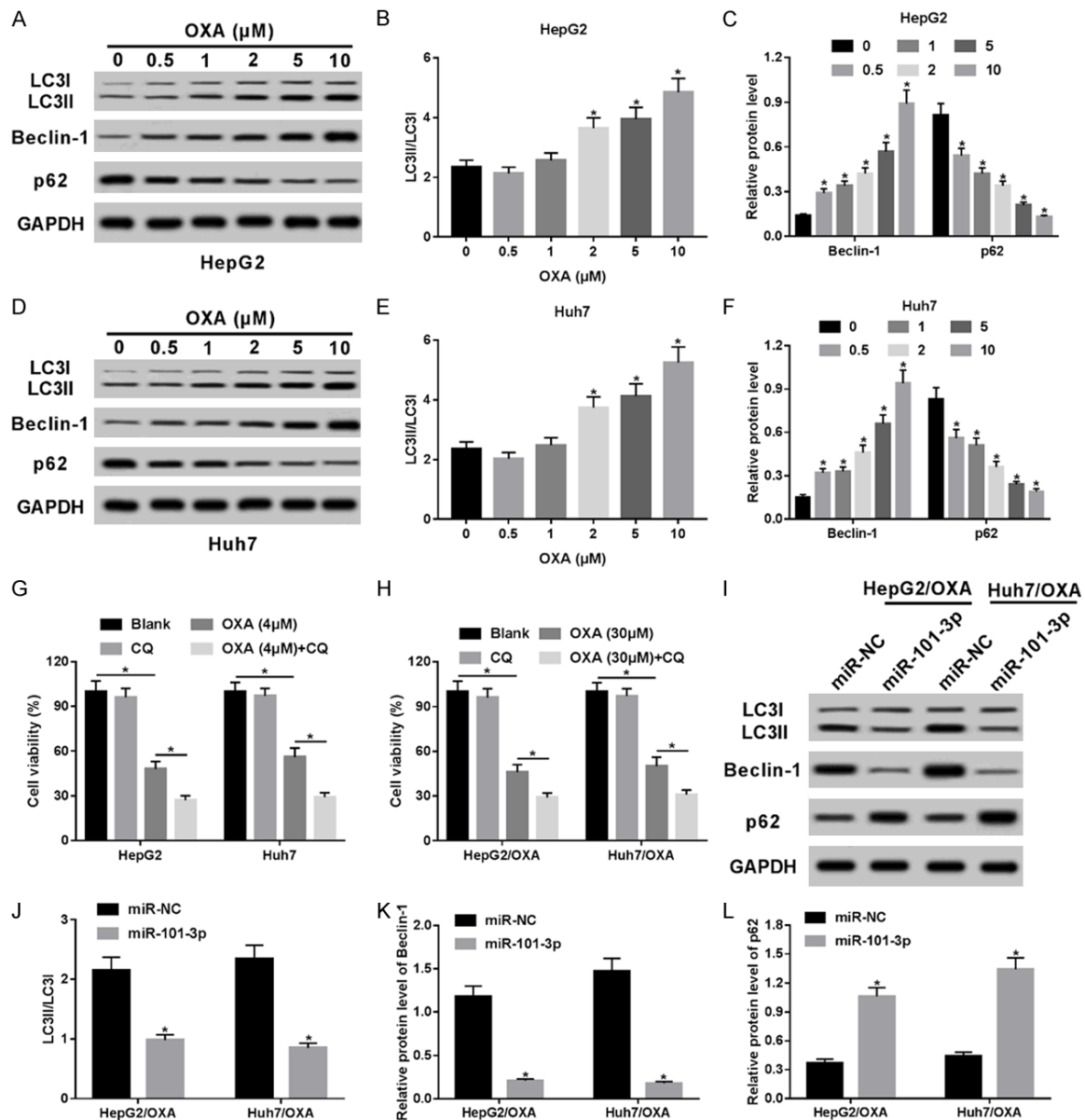


Figure 5. The involvement of the miR-101-3p/Beclin-1 axis in oxaliplatin-resistance in HCC. A-F. Western blot was applied to examine the expressions of autophagy-related proteins in OXA-mediated HepG2 or Huh7 cells. G and H. Cell viability in HepG2, HepG2/OXA, Huh7 cells, and the Huh7/OXA cells of the Blank, OXA, CQ, or OXA + CQ group. I-L. The protein expression levels of autophagy-related proteins in Huh7/OXA and HepG2/OXA cells. **P* < 0.05.

tion, it was found that the activation of Beclin-1-mediated autophagy by the miR-449a/CDGSH iron sulfur domain 2 axis contributes to the repression of cell proliferation in glioma cells [30]. Consequently, we thought that Beclin-1-mediated autophagy may also be a vital factor

in regulating the sensitivity of HCC cells to OXA. Notably, we disclosed that OXA induced autophagy in HCC sensitive cells via the regulation of LC3, Beclin-1, and p62. Moreover, the transfection of the autophagy inhibitor CQ could enhance the OXA-induced proliferation inhibition

in HCC cells, revealing that the inhibition of autophagy increases the sensitivity of HCC cells to OXA. The data of the present study further manifested that the enhanced expression of miR-101-3p contributes to the promotion of autophagy. Taken together, our investigation uncovered that miR-101-3p impedes cell viability and enhances apoptosis via the modulation of Beclin-1-mediated autophagy, thus resulting in the inhibition of the resistance of HCC cells to OXA. However, our study only explored the role of miR-101-3p in vitro. Its function should be validated in xenograft tumors in future studies.

In conclusion, our results supported the hypothesis that miR-101-3p is correlated with the OXA resistance of HCC cells via Beclin-1-mediated autophagy, expounding the molecular basis of the OXA resistance of HCC and providing a potential target for decreasing the chemoresistance of HCC to OXA.

Acknowledgements

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Disclosure of conflict of interest

None.

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