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Original Research

Effect of lncRNA KCNQ1OT1 on autophagy and drug resistance of hepatocellular carcinoma cells by targeting miR-338-3p

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Abstract: The current experiment aimed to investigate the effects of lncRNA KCNQ1OT1 on the proliferation, autophagy and drug resistance of hepatocellular carcinoma cells, as well as the potential molecular mechanism. Hepatocellular carcinoma SK-HEP-1 cells and DDP resistant SK-HEP-1/DDP cells were treated with cisplatin (DDP) of different concentrations (1 nmol/L, 2 nmol/L, 4 nmol/L, 8 nmol/L, 16 nmol/L). The survival rate of SK-HEP-1 and SK-HEP-1/DDP cells was determined by the CCK8 method. QRT-PCR was used to detect the levels of lncRNA KCNQ1OT1 and miR-338-3p in normal hepatocyte HH01, hepatocellular cell SK-HEP-1 and hepatoma cisplatin-resistant cell SK-HEP-1/DDP. Western blot was carried out to detect the expression levels of autophagy-related protein Beclin1 and proliferation-related protein P21 in cells. A dual-luciferase reporter assay system was performed to validate the relationship between KCNQ1OT1 and miR-338-3p. After the treatment of 1 nmol/L, 2 nmol/L, 4nmol/L, 8nmol/L and 16nmol/L cisplatin (DDP), the survival rate of SK-HEP-1/DDP cells is higher than that of SK-HEP-1 cells. The level of lncRNA KCNQ1OT1 was increased successively in HH01, SK-HEP-1 and SK-HEP-1/DDP cells, while miR-338-3p was decreased successively. Silencing lncRNA KCNQ1OT1 or over-expressing miR-338-3p combined with 16nmol/L DDP treatment reduced the survival rate of SK-HEP-1/DDP cells and up-regulate levels of P21 and Beclin1 proteins. LncRNA KCNQ1OT1 targeted and negatively regulated the expression of miR-338-3p. Inhibition of miR-338-3p reversed the effect of silencing lncRNA KCNQ1OT1 on survival, autophagy and cisplatin sensitivity of SK-HEP-1/DDP cell. LncRNA KCNQ1OT1 targets miR-338-3p to regulate the survival rate and autophagy of SK-HEP-1/DDP cells and improve the cisplatin sensitivity of SK-HEP-1/DDP cells. LncRNA KCNQ1OT1 is a potential molecular target for hepatocellular carcinoma.

Key words: Hepatocellular carcinoma; lncRNA KCNQ1OT1; miR-338-3p; Survival rate; Autophagy; Cisplatin sensitivity.

Introduction

Autophagy plays a key role in maintaining cell homeostasis, which can resist the malignant transformation of cells in healthy cells but is temporarily inhibited in hepatocellular carcinoma (1). Autophagy plays a role in the occurrence, metastasis, targeted therapy and drug resistance of hepatocellular carcinoma (2). Beclin1 is an autophagy-specific gene that mediates or regulates the formation and maturation of autophagosomes, which has increased protein expression during autophagy (3). Studies have shown that long-chain non-coding RNA KCNQ1OT1 has up-regulated expression in a variety of cancers, which relates to poor prognosis and drug resistance of cancer. Through starBase prediction, this study found that miR-338-3p has a binding site with KCNQ1OT1. miR-338-3p has down-regulated expression in hepatocellular carcinoma, which relates to cancer staging and metastasis (4). However, the expression and role of KCNQ1OT1 and miR-338-3p in cisplatin-resistant hepatocellular carcinoma cells, their relationship and their effects on cisplatin resistance in hepatocellular carcinoma cells remain unknown.

This project mainly studies the effects of lncRNA KCNQ1OT1 and miR-338-3p on survival rate, autophagy and cisplatin sensitivity of hepatocellular carcinoma resistant cell SK-HEP-1/DDP in the hope of providing new research directions for cisplatin sensitivity in hepatocellular carcinoma cells.

Materials and Methods

Materials

Human normal hepatocellular carcinoma HH01 and hepatocellular carcinoma cell line SK-HEP-1 were purchased from ATCC; Trypsin and RNA extraction reagent TRIzol were purchased from Sigma-Aldrich, USA; cisplatin (DDP) was purchased from Beijing Solarbio Science & Technology Co., Ltd. Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from Hyclone, USA; CCK8 kit was purchased from Shanghai Beyotime Biotechnology Co., Ltd.; si-KCNQ1OT1 and negative control si-NC, miR-NC, miR-338-3p mimics (miR-338-3p), pcDNA and pcDNA-KCNQ1OT1, anti-miR-NC, anti-miR-338-3p and primers were purchased from Shanghai GenePharma Co., Ltd. Lipofectamine 2000 transfection reagent, real-time PCR kit, reverse transcription kit (RT-PCR) were purchased from Bao Bioengineering (Dalian) Co., Ltd.; BCA protein detection kit was purchased from Jiangsu KeyGen Biotech

Co., Ltd.; Dual-Luciferase Reporter Assay System was purchased from Promega, USA; Beclin1, P21 and GAPDH antibodies were purchased from Santa Cruz, USA; Real-time PCR instrument was purchased from Bio-Rad, USA.

Method

Cell culture and establishment of SK-HEP-1/DDP drug-resistant cell line

SK-HEP-1 cells were cultured in an RPMI-1640 medium containing 10% FBS, 100U/mL penicillin, -100 μg/mL streptomycin in a 37°C, 5% CO2 incubator. According to literature (5), SK-HEP-1 cells were continuously induced with cisplatin at a large dose and low concentration to produce cisplatin resistance. The cells in the logarithmic growth phase were cultured in RPMI-1640 medium containing 16nmol/L cisplatin. The culture solution was discarded after 24 hours. After being washed 3 times, the cells were cultured with fresh culture solution and repeatedly passaged by medium changes. After the cells restored the logarithmic growth phase, 4nmol/L (IC50) DDP culture solution was used for screening passage cells for 5 times. After IC50 measurement, culture solution containing16nmol/LDDP was used for screening passage cells for 7 times in consecutive 5 months to obtain cisplatin-resistant hepatocellular carcinoma cell SK-HEP-1/DDP. Then, SK-HEP-1/DDP cells were cultured in RPMI-1640 culture solution containing low-concentration cisplatin (0.01 nmol/L).

CCK8 test to detect cell survival rate

SK-HEP-1 and SK-HEP-1/DDP cells were seeded in a 96-well microplate at 3×10^3 cells/well, 100 μL cells per well. After overnight culture, DDP at a final concentration of 1 nmol/L, 2 nmol/L, 4nmol/L, 8nmol/L, 16nmol/L was added to the culture solution, cultured for 72h, added with 10 μL CCK8 solution for each well, and cultured for 2h. Absorbance (A) value at 450 nm was measured using a microplate reader. Survival rate% = A value of the experimental group/A value of the control group×100%.

Real-time PCR detection of lncRNA KCNQ1OT1 and miR-338-3p expression

Total RNA of HH01, SK-HEP-1 and SK-HEP-1/DDP cells was extracted using TRIzol reagent, and then cDNA was synthesized according to instructions on the reverse transcription PCR kit. cDNA was reacted according to the instructions of Real-time PCR to detect ncRNA KCNQ1OT1 and miR-338-3p contents. The primers are as follows: miR-338-3p upstream primer:5'-CCTCCTATTTCCAGCATCAGTG-3', downstream primer: 5'-TATGCTTGTTCTCGTCTCTGTGTC-3'; lncRNA KCNQ1OT1 downstream primer: 5'-CTTTGCAGCAACCTCCTTGT-3', downstream primer 5'-TGGGGTGAGGGATCTGAA '; U6 upstream primer 5'-ATTGGAACGATACAGAGAAGATT-3 ', downstream primer 5'- GGAACGCTTCACGAATTTG-3'; GAPDH upstream primer 5'-GCACCGTCAAGGCTGAGAAC-3 ', downstream primer 5 '-TGGTGAAGACGCCAGTGGA-3'. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method.

Cell transfection

SK-HEP-1/DDP cells in the logarithmic growth phase were seeded into a 6-well plate at 2×10^6 cells/mL and transfected when the cells were fused into one layer. According to the instructions on Lipofectamine 2000 Transfection Kit, vectors or fragments like si-NC, si-KCNQ1OT1, miR-NC, miR-338-3p, pcDNA, pcDNA-KCNQ1OT1, si-KCNQ1OT1+anti-miR-NC, si-KCNQ1OT1+anti-miR-338-3p were transfected into SK-HEP-1/DDP cells and respectively recorded as a si-NC group, si-KCNQ1OT1 group, miR-NC group, miR-338-3p group, pcDNA group, PcDNA-KCNQ1OT1 group, si-KCNQ1OT1+anti-miR-NC group, si-KCNQ1OT1+ anti-miR-338-3p group. Cells were collected 48h after transfection.

Western blot experiment

SK-HEP-1/DDP cells of each group treated in 1.2.3 and 1.2.4 were collected, smudged to collect proteins and detect the concentration. After SDS-PAGE, membrane transfer, it was blocked at room temperature for 2h with the skim milk powder, added with the primary antibody (Beclin1 1: 1000, P21 1: 1000 and GAPDH antibody 1: 3000), and incubated at 4°C overnight. Wash the membrane 3 times with PBST buffer for 5 min each, then add diluted secondary antibody, incubate for 1 h at room temperature, followed by development and photography. Protein levels were analyzed using GAPDH as an internal reference.

Dual-luciferase reporting system experiment

The constructed wild type (WT-KCNQ1OT1) and mutant (MUT-KCNQ1OT1) reporter vectors of lncRNA KCNQ1OT1 were co-transfected with miR-NC or miR-338-3p to SK-HEP-1/DDP cells according to method 1.2.4. 48 h after the transfection, the cells were collected, lysed to collect the supernatant by centrifugation. Using Renilla luciferase activity as an internal reference, the relative firefly luciferase activity was detected and calculated.

Statistical processing

The data are expressed as mean ± standard deviation($\bar{x}\pm s$) and analyzed by SPSS19.0 statistical software. An independent sample *t*-test was used for pairwise comparison, while a one-way analysis of variance was used for comparison between multiple groups. $P<0.05$ suggests a statistically significant difference.

Results

The sensitivity of hepatocellular carcinoma SK-HEP-1 and cisplatin-resistant hepatocellular carcinoma cell SK-HEP-1/DDP to cisplatin

Hepatocellular carcinoma SK-HEP-1 and cisplatin-resistant hepatocellular carcinoma cell SK-HEP-1/DDP were respectively treated with cisplatin (DDP) at concentrations of 1 nmol/L, 2 nmol/L, 4nmol/L, 8nmol/L, 16nmol/L. It was found that as the concentration increased, SK-HEP-1 and SK-HEP-1/DDP cells had gradually decreased the survival rate. At the highest concentration of 16nmol/L, SK-HEP-1/DDP's survival rate of (92.29 ± 9.23) % was significantly higher than

Table 1. The sensitivity of SK-HEP-1 and SK-HEP-1/DDP cells to cisplatin(x±s, n=9).

Drug level(nmol/L)	Survival rate (%)	
	SK-HEP-1	SK-HEP-1/DDP
0	100.00±10.00	99.81±10.01
1	90.11±9.04*	99.02±9.98
2	79.11±8.12*	97.81±9.81
4	62.54±6.31*	97.30±9.71
8	41.21±4.58*	96.19±9.68
16	32.86±3.21*	92.29±9.23
F	122.6	0.0373
P	<0.0001	0.9992

Compared to group 0,*P<0.05.

SK-HEP-1's (32.86 ±3.21) %, as shown in Table 1. This indicates that SK-HEP-1/DDP cell has significantly lower sensitivity to DDP than SK-HEP-1 cell. In subsequent experiments, DDP concentration of 16nmol/L which inhibited SK-HEP-1/DDP at a rate of about 10% was selected, which had a small effect on cells.

Expression of KCNQ1OT1 and miR-338-3p in SK-HEP-1, SK-HEP-1/DDP and HH01 cells

Detection of normal liver cell HH01, hepatocellular carcinoma cell SK-HEP-1 and cisplatin-resistant hepatocellular carcinoma cell SK-HEP-1/DDP shows that compared with HH01 cell, SK-HEP-1/DDP has the highest content of lncRNA KCNQ1OT1, followed by SK-HEP-1 cell; SK-HEP-1/DDP has the lowest content of miR-338-3p, followed by SK-HEP-1 cell, all with statistical significance (P <0.05), as shown in Table 2.

Effect of silencing KCNQ1OT1 on survival rate and autophagy of SK-HEP-1/DDP cell

Under 16nmol/L DDP treatment, compared with a si-NC group, SK-HEP-1/DDP cells in the si-KCNQ1OT1 group has reduced lncRNA KCNQ1OT1 content, significantly decreased cell survival rate, increased Beclin1 and P21 protein levels, with statistically significant difference (P <0.05), as shown in Figure 1 and Table 3. It suggests that silencing KCNQ1OT1 combined with 16nmol/L DDP treatment can inhibit the survival of SK-HEP-1/DDP cells, promote cell autophagy, and enhance cell sensitivity to cisplatin.

Table 2. Expressions of KCNQ1OT1 and miR-338-3p in SK-HEP-1, SK-HEP-1/DDP and HH01(x±s, n=9).

Group	KCNQ1OT1	miR-338-3p
HH01	1.00±0.10	1.01±0.10
SK-HEP-1	2.98±0.31*	0.37±0.04*
SK-HEP-1/DDP	3.61±0.37*#	0.12±0.01*#
F	206.1	486.2
P	<0.0001	<0.0001

Compare with HH01 group,*P<0.05; Compared with SK-HEP-1 group,#P<0.05.

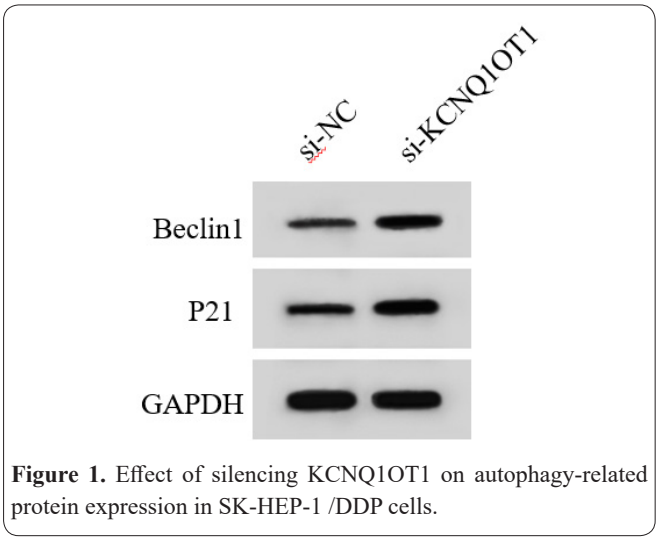

Figure 1. Effect of silencing KCNQ1OT1 on autophagy-related protein expression in SK-HEP-1 /DDP cells.

Effect of miR-338-3p overexpression on survival rate and autophagy of SK-HEP-1/DDP cells

Under 16nmol/L DDP treatment, compared with a miR-NC group, SK-HEP-1/DDP cells in the miR-338-3p group have significantly increased miR-338-3p content, significantly reduced cell survival rate, increased Beclin1and P21 protein contents, with statistically significant difference (P <0.05), as shown in Figure 2 and Table 4. It shows that miR-338-3p overexpression combined with 16nmol/L DDP can inhibit the survival rate of SK-HEP-1/DDP cells, promote cell autophagy and enhance cell sensitivity to cisplatin.

KCNQ1OT1 targets regulation of miR-338-3p expression

It is found via starBase prediction that miR-338-3p

Table 3. Effects of silencing KCNQ1OT1 on cell viability and autophagy of SK-HEP-1/DDP cells (x±s, n=9).

Group	KCNQ1OT1	Beclin1	P21	Survival rate (%)
si-NC	0.97±0.10	0.11±0.01	0.23±0.02	100.01±10.02
si-KCNQ1OT1	0.30±0.03*	0.51±0.05*	0.60±0.06*	53.18±5.12*
t	13.42	2.342	17.55	12.49
P	<0.0001	<0.0001	<0.0001	<0.0001

Compared with the si-NC group, *P<0.05.

Table 4. Effects of overexpression of miR-338-3p on cell survival and expression of autophagy-related proteins in SK-HEP-1 /DDP cells ($\bar{x}$±s, n=9).

Group	miR-338-3p	Beclin1	P21	Survival rate (%)
miR-NC	1.01±0.10	0.12±0.01	0.21±0.02	100.00±10.01
miR-338-3p	3.32±0.31*	0.59±0.06*	0.77±0.08*	47.68±4.92*
t	21.28	23.18	20.37	14.07
P	<0.0001	<0.0001	<0.0001	<0.0001

Compared with the miR-NC group, *P<0.05.

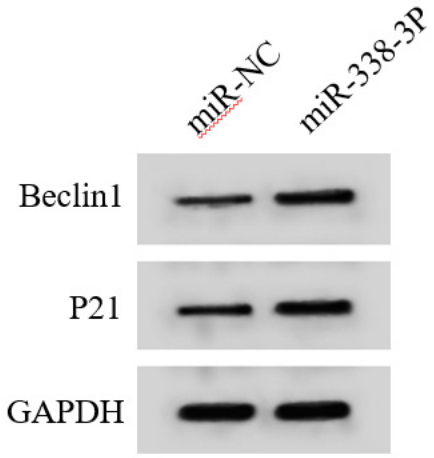

Figure 2. Effect of over-expression of miR-338-3p on autophagy-related protein expression in SK-HEP-1 /DDP cells.

sequence contains a binding site complementary to lncRNA KCNQ1OT1, as shown in Figure 3. The results of dual-luciferase reporter assay show that compared with a miR-NC group, relative activity of firefly luciferase in wild-type WT-KCNQ1OT1 is significantly reduced in a miR-338-3p group (P <0.05), while that in mutant MUT-KCNQ1OT1 is not obviously changed, as shown in Table 5. qRT-PCR results show that inhibition of KCNQ1OT1 may increase miR-338-3p content (*P* <0.05), and KCNQ1OT1 overexpression may significantly decrease miR-338-3p content (*P* <0.05), as shown in Table 6. This indicates that KCNQ1OT1 targets the negative regulation of miR-338-3p expression.

Inhibition of miR-338-3p can attenuate the effect of KCNQ1OT1 silencing on survival rate and autophagy of SK-HEP-1/DDP cells

Under 16nmol/L DDP treatment, compared with the si-KCNQ1OT1+anti-miR-NC group, SK-HEP-1/DDP cells in the si-KCNQ1OT1+anti-miR-338-3p group have decreased miR-338-3p content, increased cell survival rate, decreased Beclin1 and P21 protein expression levels, with statistically significant difference (*P* <0.05). This indicates that inhibition of miR-338-3p reverses the effect of lncRNA KCNQ1OT1 silencing on survival, autophagy and cisplatin resistance of SK-HEP-1/DDP cells (Table 7, Fig 4).

Discussion

Hepatocellular carcinoma has a high incidence worldwide, with about half of new cases in China every year. Studies have shown that autophagy plays an important role in hepatocellular carcinoma and autophagy regulators may be a treatment choice (6). Chemotherapy

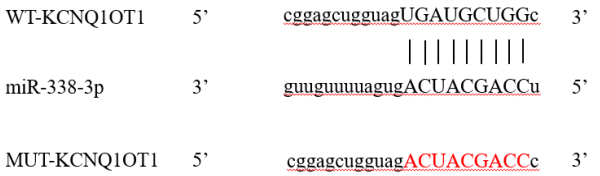

Figure 3. miR-338-3p targeted by KCNQ1OT1.

Table 5. Double luciferase reports ($\bar{x}$±s, n=9).

Group	WT-KCNQ1OT1	MUT-KCNQ1OT1
miR-NC	0.96±0.09	0.98±0.09
miR-338-3p	0.17±0.01*	0.99±0.10
t	26.17	0.33
P	<0.0001	0.745

Compared with the miR-nc group, **P*<0.05.

Table 6. KCNQ1OT1 negatively regulates miR-338-3p expression ($\bar{x}$±s, n=9).

Group	miR-338-3p
si-NC	1.01±0.10
si- KCNQ1OT1	2.45±0.25*
pcDNA3.1	1.00±0.10
pcDNA3.1- KCNQ1OT1	0.28±0.03#
F	357.4
P	<0.0001

Compared with the si-NC group, **P*<0.05; Compared with pcDNA3.1 group, #*P*<0.05.

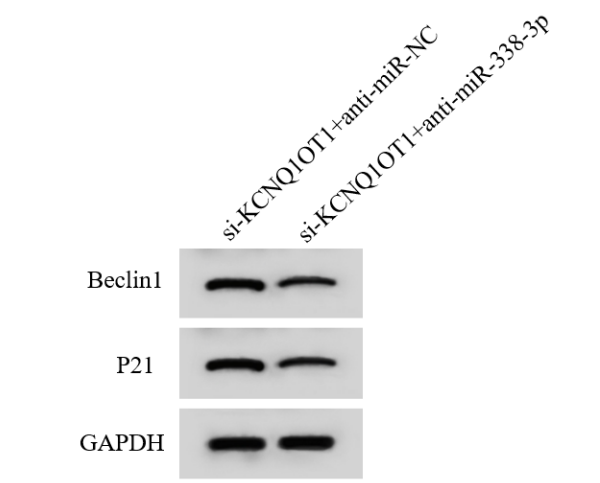

Figure 4. Effects of miR-338-3p inhibition and KCNQ1OT1silencing on autophagy-related protein expression in SK-HEP-1 /DDP cells

is the first-choice treatment for patients in the middle and advanced stage, but non-specificity of chemotherapeutic drugs and drug resistance of cancer cells may lead to treatment failures. Studies have shown that a

Table 7. Inhibition of miR-338-3p attenuated the effect of KCNQ1OT1 silencing on the viability of SK-HEP-1/DDP cells ($\bar{x}$±s, n=9).

Group	miR-338-3p	Survival rate (%)
si-KCNQ1OT1+anti-miR-NC	0.99±0.10	55.61±5.55
si-KCNQ1OT1+anti-miR-338-3p	0.27±0.03*	90.02±9.17*
t	20.689	9.631
P	<0.0001	<0.0001

Compared with si-KCNQ1OT1 + anti-miR-NC group, **P*<0.05.

Table 8. Effects of miR-338-3p inhibition and KCNQ1OT1 silencing on autophagy-related protein expression SK-HEP-1/DDP cells (x±s, n=9).

Group	Beclin1	P21
si-KCNQ1OT1+anti-miR-NC	0.52±0.05	0.61±0.06
si-KCNQ1OT1+anti-miR-338-3p	0.21±0.02*	0.27±0.03*
t	17.270	15.205
P	<0.0001	<0.0001

Compared with the si-KCNQ1OT1+anti-miR-NC group, *P<0.05.

variety of lncRNAs are associated with chemotherapy resistance in hepatocellular carcinoma (7).

Recent research reports that lncRNA KCNQ1OT1 is abnormally expressed in a variety of cancers. For instance, lncRNA KCNQ1OT1 is up-regulated in non-small cell lung cancer (NSCLC) (8) and ovarian cancer (9, 10), which is related to clinical pathology of NSCLC and proliferation and migration of ovarian cancer. LncRNA KCNQ1OT1 regulates tongue cancer proliferation and cisplatin resistance through miR-211-5p-mediated Ezrin/Fak/Src signaling; it enhances chemotherapy resistance of colon cancer to oxaliplatin by targeting miR-34a/ATG4B pathway (10, 11); it also regulates miR-760/PPP1R1B through cAMP signaling pathway, enhancing colorectal cancer cells' resistance to methotrexate (12). Other reports show that KCNQ1OT1 is significantly up-regulated in oxaliplatin-resistant hepatocellular carcinoma cell lines HepG2 and Huh7; knocking out KCNQ1OT1 can inhibit cancer cell proliferation, migration and invasion, and lower expression of drug resistance genes (MRP5, MDR1, LRP1) [13]. In this study, cisplatin-resistant hepatocellular carcinoma cell line SK-HEP-1/DDP was established to detect survival rate of hepatocellular carcinoma SK-HEP-1 and cisplatin-resistant cell SK-HEP-1/DDP after treatment with cisplatin (DDP) at different concentrations (1 nmol/L, 2 nmol/L, 4 nmol/L, 8 nmol/L, 16 nmol/L). The results showed that SK-HEP-1/DDP had higher survival rate against DDP than SK-HEP-1 cells, lncRNA KCNQ1OT1 expression was significantly up-regulated in SK-HEP-1/DDP cells, silencing KCNQ1OT1 combined with DDP treatment could reduce SK-HEP-1/DDP cell survival rate and promote cell autophagy, up-regulate the contents of P21 and autophagy protein Beclin1, and improve cell sensitivity to DDP, which are consistent with the above findings (13), indicating that lncRNA KCNQ1OT1 may regulate drug resistance in hepatocellular carcinoma.

Through starBase prediction, this study found that miR-338-3p has a binding site with lncRNA KCNQ1OT1. a miR-338-3p expression is down-regulated in prostate cancer (14), gastric cancer (15) and breast cancer (16), which is related to proliferation, metastasis and apoptosis of cancer cells. Studies have shown that MiR-338-3p enhances the sensitivity of ovarian cancer cells to cisplatin by down-regulating WNT2B (17). miR-338-3p mediates resistance of p53 mutant colon cancer cells to 5-fluorouracil by down-regulating mTOR expression (18). In hepatocellular carcinoma cells, miR-338-3p targets hypoxia-inducible factor 1α (HIF-1α) to inhibit the growth of hepatocellular carcinoma cells and enhance cell sensitivity to sorafenib (19). This indicates that miR-338-3p is related to tumor cell proliferation, metastasis and drug resistance. This study found that miR-338-3p had a significantly lower content in SK-HEP-1/DDP than in SK-HEP-1 and HH01 cells. miR-338-3 overexpression combined with DDP treatment could inhibit SK-HEP-1/DDP cell survival rate and promote cell autophagy, increasing cell sensitivity to cisplatin. Dual-luciferase reporter assay found that lncRNA KCNQ1OT1 targets negative regulation of miR-338-3p expression. It was also found that inhibition of miR-338-3p could reverse the effect of lncRNA KCNQ1OT1 silencing on the survival and autophagy of SK-HEP-1/DDP cells, which verifies that the two has targeted regulatory relationship in hepatocellular carcinoma cells (20).

This study illustrates that in DDP-resistant hepatocellular carcinoma cell SK-HEP-1/DDP, lncRNA KCNQ1OT1 expression is up-regulated, a miR-338-3p expression is down-regulated, and lncRNA KCNQ1OT1 targets miR-338-3p to regulate SK-HEP-1/DDP cell survival rate, autophagy, and cisplatin resistance. lncRNA KCNQ1OT1 may be a potential chemosensitization target for hepatocellular carcinoma.

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