Long Noncoding RNA KCNQ1OT1 Induces Resistance of HCC Cells To Cisplatin Through Regulating The miR-26a/CCND2 Molecular Axis

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Abstract

Objective: To explore the molecular mechanism by which LncRNA KCNQ1OT1 regulated the miR-26a/CCND2 molecular axis to participate in the resistance of Hepatocellular carcinoma(HCC) cells to cisplatin.

Methods: Cancer tissue and corresponding para-carcinoma tissue specimens were collected from 25 HCC patients with complete data admitted from January 2018 to December 2018 at The Transplantation Center of the Third Xiangya Hospital. Then, the expression levels of KCNQ1OT1, miR-26a and CCND2 in HCCtissues and cell lines were detected through qRT-PCR. Meanwhile, the sensitivity of HCC cells to cisplatin was examined through Transwell and Annexin V-FITC/PI double staining flow cytometry. Further, the targeted relationships among KCNQ1OT1, miR-26a and CCND were verified through dual-luciferase reporter gene assay, and the regulatory relationships were detected through Western blotting and qRT-PCR.

Results: KCNQ1OT1 was highly expressed in HCC tissues and cisplatin-resistant cell lines; meanwhile, over-expression of KCNQ1OT1 promoted the resistance of Huh7/CDDP cells to cisplatin. Dual-luciferase reporter gene assay verified that, KCNQ1OT1 targeted miR-26a and down-regulated its expression level. miR-26a suppressed Huh7/CDDP cell proliferation and invasion, while promoting their apoptosis, thus down-regulating the promoting effect of KCNQ1OT1 on the cisplatin resistance of HCC cells. miR-26a negatively regulated CCND2 expression, while KCNQ1OT1 down-regulated the suppression of miR-26a on CCND2 to promote Huh7/CDDP cell proliferation and invasion and to suppress apoptosis, thereby up-regulating the resistance of HCCcells to cisplatin.

Conclusions: LncRNA KCNQ1OT1 regulates the miR-26a/CCND2 molecular axis to induce the resistance of HCC cells to cisplatin.

Objective

Hepatocellular carcinoma(HCC) is a common digestive system malignancy, and its morbidity ranks the 2th place in China(1) . Chemotherapy is one of the major treatments for HCC which remarkably reduces HCC metastasis and recurrence, and improves the HCC prognosis. However, the development of chemoresistance reduces the sensitivity of HCC to chemotherapeutics, and results in treatment failure(2,3). Consequently, it is of great significance to illustrate the potential mechanism of HCC chemoresistance and to search for the novel therapeutic target. In recent years, Long noncoding RNA(LncRNA) has become the hotspot in life science research, especially in oncology(4-6). Recent studies report that some LncRNAs may serve as the biomarkers and therapeutic targets for tumor diagnosis and prognosis(7). Lnc KCNQ1OT1 is a newly discovered LncRNA, which is located at the KCNQ1 locus(8). Lnc KCNQ1OT1 may enhance the methotrexate resistance of colorectal cancer cells by cAMP signalling pathway(9). Some scholars discover that the Lnc KCNQ1OT1 expression level is up-regulated in breast cancer, revealing that it may serve as the potential therapeutic target of breast

cancer(10). At the same time, the up-regulated Lnc KCNQ1OT1 expression level is also discovered in lung adenocarcinoma, while lnc KCNQ1OT1 knockout reduces the resistance of lung adenocarcinoma to paclitaxel(11). At present, lnc KCNQ1OT1 expresion in HCC tissues and cells has been seldom reported. Therefore, this study aimed to investigate its expression in HCC tissues and cells, as well as the clinical significance.

Materials And Methods

Subjects The surgically resected HCC tissues and para-carcinoma tissues from 25 HCC patients with complete data admitted at the Transplantation Center of the Third Xiangya Hospital from January 2018 to December 2018 were collected in this study, and were immediately preserved in liquid nitrogen. All study objects had signed the informed consent to participate in this study. Our study protocol was approved by the Ethics Committee of the Third Xiangya Hospital.

Chemicals and reagents

The siRNAs of KCNQ1OT1 and CCND2,miR-26a mimics were purchased from Shanghai JEMMA; DEME and fetal bovine serum (FBS) were bought from Biological Industries (USA); penicillin and streptomycin were obtained from Beijing Leagene Biotechnology Co., Ltd; the Annexin V-FITC/PI apoptosis detection kit was purchased from eBioscience (USA); CCK-8 kit was derived from Wuhan Huamei Bioengineering Co., Ltd; Transwell chambers were purchased from Corning (USA); DNAses, together with Lipofectamine ^{TM} 2000 and revere transcription kits were bought from TaKaRa (Japan); the high-purity total RNA rapid extraction kit was purchased from Beijing BioTeke Biotechnology Co., Ltd; total protein extraction kit, cell nuclear protein and cytoplasmic protein extraction kit, SDS-PAGE gel rapid preparation kit were provided by Bio-Rad (USA); the western blotting primary antibodies and secondary antibodies were obtained from CST (USA); and dual-luciferase reporter gene kit and reporter gene vectors were provided by Promega.

Cell culture

Human HCC cell lines (Huh7, Bel7402 and HepG2) and normal human liver cells (L02) were purchased from Shanghai Institute of Cellular Biology of Chinese Academy of Sciences. The HCC cell lines were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin within an incubator under 37 ℃ and 5% CO_2 conditions.

Construction of resistant HCCcell lines

Huh7 cells at logarithmic phase were collected and stimulated with cisplatin at the mass concentration of 0.001 μg/ml for 48 h, then, the medium was discarded, and fresh DMEM was added for further culture. The cell viability was observed, if no obvious death was observed, then those logarithmic cells were selected for repeated subculture with the gradual increase in cisplatin mass concentration. Finally, cells that stably survived at the cisplatin concentration of 1 μg/ml were the Huh7/CDDP resistant cells.

Cell transfection

Huh7 and Huh7/CDDP cells were cultured in a 6-well-cell culture plate with DMEM medium. After 24 h, KCNQ1OT1 siRNA and pcDNA-KCNQ1OT1 were transfected into Huh7 cells, KCNQ1OT1 siRNA, CCND2 siRNA, pcDNA-KCNQ1OT1 and miR-26a mimics were transfected into Huh7/CDDP cells.The cell transfection efficiencies were observed at 48 h later under the fluorescence microscope.

KCNQ1OT1 expression levels in HCC tissues and cells detected by qRT-PCR

The total RNA was extracted from tissues and the cultured cells according to the one-step TRIzol method. Subsequently, cells were treated with DNAses, and 1 μg RNA was collected to prepare cDNA through reverse transcription. Later, 1 μl reverse transcription products were collected for PCR detection, with U6 and GAPDH as the internal references. The primer sequences are listed in Table 1. The detection results were calculated according to the $2^{-\Delta\Delta Ct}$ method.

Tab.1 Primer sequences

Primer	Sequence
U6	F: 5’-GAGGCACAGCGGAACG-3’
	R: 5’-CTACCACATAGTCCAGG-3’
GAPDH	F: 5’-GGTGAAGGTCGGAGTCAACG-3’
	R: 5’-CAAAGTTGTCATGGATGHACC-3’
KCNQ1OT1	F: 5’-CCGCGTAAGCCTCATAGAAG-3’
	R: 5’-GGGAGTAGGGTGAGGAAAGG-3’
miR-26a	F: 5’-GGATTGGAGAGAAAGGCAG-3’
	R: 5’-GTGCAGGGTCCGAGGT-3’
CCND2	F: 5’-GCAGAACCTGTTGACCATCG-3’
	R: 5 ’-GCTTGCGAAGGATGTGCTC-3 ’

CCND2 protein expression in HCCcells detected by Western blotting

After protein extraction, the protein concentration was detected according to the bicinchoninic acid disodium (BCA) kit instruction. 30 μg of the total protein was separated on a 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After polyvinylidene (PVDF) membrane transfer, the proteins were blocked in 5% bovine serum albumin (BSA) for 1 h, and primary antibodies were added to incubate at 4 ℃ overnight. The Bio-rad Gel DolEZ imager was used to image the proteins, and the gray level of target protein band was analyzed using the Image J software.

HCCcell proliferation level detected by CCK-8 assay

HCC Huh7 and Huh7/CDDP cells at logarithmic phase were inoculated into 96-well plates, with 10^4 cells in each well, and each well contained 100 μl medium. At 1 h prior to detection, 10 μl CCK-8 solution was added into each well. The culture plate was incubated in the incubator for 1–4 h, and the optical density (D) at 450 nm was measured using the microplate reader, so as to analyze the HCCcell proliferation capacity.

HCCcell migration capacity detected by Transwell assay

The transfected cells were selected as the experiment group, while the non-transfected cells were chosen as the control group. Cells in each treatment group were digested with trypsin and inoculated into the 24-well plates in the Transwell chambers; then, 100 μl (density, 2×10^5/ml) cell suspension was added into the upper chamber and incubated for 48 h. Cell invasion was determined by using crystal violet staining for 15 min. Later, the chambers were washed with PBS, dried and observed under the inverted microscope (×100).

HCCcell apoptosis detected by Annexin V-FITC/PI double staining flow cytometry

Cell apoptosis was detected using the flow cytometer. In brief, Huh7 and Huh7/CDDP cells were collected from the transfection group and non-transfection group and cultured until the logarithmic phase. Then, cells were washed with PBS for twice, mixed evenly with 500 μl pre-cooling 1×binding buffer and 5 μl Annexin-V-FITC, and incubated for 15 min at room temperature in dark. Later, 2.5 μl PI was added at 5 min prior to loading for staining, and the apoptosis of Huh7 and Huh7/CDDP cells was detected after loading.

Interactions among KCNQ1OT1, miR-26a and CCND2 detected by dual-luciferase reporter gene assay

First of all, the 3'UTR target sequence of the KCNQ1OT1 candidate target molecule miR-26a or the 3'UTR target sequence of the miR-26a candidate target gene CCND2 was inserted into the downstream of firefly luciferase gene. The expression vector pcDNA-EGFP-pre-KCNQ1OT1 and its target gene miR-26a verified vector pmirGLO-KCNQ1OT1-miR − 26a 3'UTR, and the expression vector pcDNA-EGFP-pre-miR-26a and its target gene CCND2 verified vector pmirGLO-miR-26a-CCND2 3'UTR, were co-transfected into 293T cells, respectively. In addition, the empty vector was co-transfected with KCNQ1OT1 or miR-26a into cells as control. Luciferase detection was carried out in accordance with dual-luciferase reporter gene kit instructions, the firefly and renilla fluorescence intensities were detected by the microplate reader, and the renilla fluorescence intensity was used as the internal reference.

Statistical analyses

SPSS 20.0 software was adopted for statistical analysis. T test was adopted for comparisons between two groups, and GrCDDPhPad Prism 7 was employed to plot the related graphs based on the experimental data. $P < 0.05$ or $P < 0.01$ indicated statistically significant difference.

Results

KCNQ1OT1 expression levels in HCC tissues, cell lines and cisplatin-resistant HCCcell line Huh7/CDDP

qRT-PCR detection results suggested that, KCNQ1OT1 expression in HCCtissues was markedly higher than that in normal tissues ($P < 0.01$, Fig. 1A). At the same time, KCNQ1OT1 expression in HCCcell lines (Huh7, HepG2 and BIU-87) was remarkably higher than that in human liver cell L02 ($P < 0.01$, Fig. 1B); besides, that in Huh7 cells was apparently higher than that in HepG2 and BIU-87 ($P < 0.05$, Fig. 1B); and that in Huh7/CDDP cells was evidently higher than that in Huh7 cells ($P < 0.01$, Fig. 1C). Thus, it was observed that, abnormal KCNQ1OT1 expression might be related to HCCgenesis and development, as well as the cisplatin resistance.

Effect of KCNQ1OT1 on the HCCcell biological behaviors

qRT-PCR detection results (Fig. 1D,E) revealed that, after knockout, KCNQ1OT1 expression in two kinds of cells was apparently lower than that in control group ($P < 0.01$); while over-expressing KCNQ1OT1 led to higher expression levels in two kinds of cells than in control group ($P < 0.01$). CCK-8 detection results (Fig. 1F,G) demonstrated that, silencing KCNQ1OT1 remarkably suppressed the proliferation capacities of Huh7 and Huh7/CDDP cells ($P < 0.05$), while over-expressing KCNQ1OT1 remarkably promoted the proliferation capacities of Huh7 and Huh7/CDDP cells ($P < 0.05$). Transwell detection results (Fig. 1H,I) demonstrated that, compared with control group, silencing KCNQ1OT1 remarkably suppressed the invasion capacities of Huh7 and Huh7/CDDP cells ($P < 0.01$). The Annexin V-FITC/PI double staining cell apoptosis detection results (Fig. 1J,K) revealed that, silencing KCNQ1OT1 remarkably promoted the apoptosis of Huh7 and Huh7/CDDP cells ($P < 0.01$). Thus, it was found that, silencing KCNQ1OT1 notably promoted the cisplatin-induced apoptosis of Huh7 and Huh7/CDDP cells, while suppressed cell proliferation and invasion capacities.

Regulatory effect of KCNQ1OT1 on miR-26a expression

The bioinformatics database StarBase V2.0 was employed to predict that miR-26a might be the target gene of KCNQ1OT1. The prediction sequences are shown in Fig. 2A. Results of dual-luciferase reporter gene assay demonstrated that, over-expressing miR-26a markedly declined the luciferase activities ($P < 0.01$. Figure 2B); however, after co-transfection of miR-26a mimics and pmirGLO-KCNQ1OT1-MUT vector with target site mutation in cells, miR-26a lost its suppression on luciferase activity. qRT-PCR analytic results revealed that, KCNQ1OT1 over-expression markedly suppressed the miR-185-5p expression level ($P < 0.01$, Fig. 2C), while KCNQ1OT1 knockout evidently promoted miR-26a expression ($P < 0.001$, Fig. 3C). Thus, it was observed that, KCNQ1OT1 was the direct target of miR-26a, and KCNQ1OT1 negatively regulated miR-26a expression.

Effect of over-expressing miR-26a on the biological behaviors of Huh7/CDDP cells

CCK-8 detection results suggested that, miR-26a over-expression notably suppressed the proliferation activity of Huh7/CDDP cells, while miR-26a and KCNQ1OT1 over-expression simultaneously markedly

reversed the proliferation activity of Huh7/CDDP cells ($P < 0.01$, Fig. 2D). Transwell detection results demonstrated that, miR-26a over-expression dramatically suppressed the invasion capacity of Huh7/CDDP cells ($P < 0.01$, Fig. 2E), while the cell invasion capacity was not markedly changed after over-expressing miR-26a and KCNQ1OT1 at the same time when compared with control group. Flow cytometry results demonstrated that, miR-26a over-expression dramatically promoted the apoptosis of Huh7/CDDP cells ($P < 0.01$, Fig. 2F), while simultaneous transfection with miR-26a mimics and pcDNA-KCNQ1OT1 had no significant influence on the apoptosis of Huh7/CDDP cells compared with control group. Thus, it was clear that, FODX2-AS1 down-regulated miR-26a to promote the proliferation and invasion of Huh7/CDDP cells while suppressing cell apoptosis.

Regulatory effect of miR-26a on CCND2 expression

It was discovered based on bioinformatics database TargetScan prediction for miR-26a that, CCND2 was the candidate target gene of miR-26a, and miR-26a was able to bind with the 3'UTR of CCND2 (Figure.3A). Luciferase reporter gene assay verified that, miR-26a negatively regulated CCND2 expression ($P < 0.01$, Figure.3B). Western blotting results demonstrated that, miR-26a over-expression markedly inhibited the expression of CCND2 in Huh7/CDDP cells ($P < 0.01$, Figure.3C,D). Clearly, CCND2 was the target gene of miR-26a, while miR-26a negatively regulated CCND2 expression.

Effect of KCNQ1OT1 on the Huh7/CDDP cell biology through the miR-26a/CCND2 molecular axis

Western blotting results suggested that, silencing CCND2 evidently inhibited CCND2 expression, while silencing KCNQ1OT1 and CCND2 simultaneously notably reversed the CCND2 expression ($P < 0.05$, Figure.4A). CCK-8 and Transwell assays jointly verified that (Figure.4B, C), compared with control group, transfection with si-CCND2 dramatically restrained cell proliferation and invasion capacities ($P < 0.01$). However, compared with si-CCND2 transfection alone group, silencing KCNQ1OT1 and CCND2 expression at the same time remarkably restored the suppression of si-CCND2 on the proliferation and invasion capacities of Huh7/CDDP cells ($P < 0.01$). In the meantime, flow cytometry results further confirmed that (Figure.4D), transfection with si-CCND2 markedly promoted cell apoptosis ($P < 0.001$), while transfection with si-KCNQ1OT1 and si-CCND2 at the same time remarkably down-regulated the promoting effect of si-CCND2 on Huh7/CDDP cell apoptosis ($P < 0.001$). Obviously, KCNQ1OT1 down-regulated the suppression of miR-26a on CCND2 to promote Huh7/CDDP cell proliferation and invasion, but suppress cell apoptosis, thus up-regulating the cisplatin resistance of Huh7/CDDP cells.

Discussion

In recent years, LncRNA has been verified to exert a vital regulatory role in the tumor genesis and development process, as well as the resistance (12,13). Nonetheless, the roles of LncRNAs in multi-drug resistance of HCC remain unclear so far. Consequently, it is urgently needed to search for the upstream key molecules of drug resistance regulation-related genes in HCC, and to intensively investigate the molecular mechanism, with the final goal of providing more evidence for the resistance of clinical HCC treatment.

Recently, researchers have discovered a class of lncRNAs with the length of over 200 nucleotides apart from the structural noncoding RNAs and various types of small RNAs (miRNAs). LncRNAs generally contain a certain conserved sequence, but there are diverse manners to regulate gene expression, including epigenetic regulation, transcriptional regulation and post-transcriptional regulation. To sum up, LncRNAs participate in multiple biological mechanisms, such as X chromosome silencing, genomic blotting and DNA damage response, to regulate the genesis and development, as well as chemoresistance of tumor diseases, like bladder cancer, thyroid cancer, colon cancer and Hepatocellular carcinoma . Recent literature reports that, LncRNA MT1JP (14), LncRNA BCAR4 (15), and LncRNA DANCR(16) have marked effects on HCC cell proliferation, migration, and epithelial-mesenchymal transition (EMT), revealing that lncRNAs may be closely correlated with HCCgenesis and development. Nonetheless, the molecular mechanism of lncRNA KCNQ1OT1 on the cisplatin resistance of HCCcells has not been reported yet.

In the meantime, recent research discovers that, the abnormal regulation of miRNAs is recognized to be the key factor for the genesis and development of multiple diseases, including HCC. For instance, miR-26a down-regulates AURKA expression to suppress HCC ell proliferation and migration(17). Chang et al. (18) verified that, miR-26a served as an independent marker for the prognosis of HCC. At the same time, some research indicates that miR-26a markedly affects the proliferation, migration and EMT of tumor cells, such as lung cancer(19), colorectal cancer(20), pancreatic cancer(21) and bladder cancer(22) . However, the mechanism of action of miR-26a in HCChas not been reported in literature.

CCND2 (cyclinD2) is predicted through bioinformatics tool as the potential target gene of miR-26a. CCND2 is a member of the cell cycle family, and its abnormal expression may result in abnormal cell proliferation. Research finds that, CCND2 is aberrantly expressed in multiple tumor tissues, such as cervical cancer(23), colorectal cancer(24), non-small cell lung cancer (25), and ovarian cancer (26). Meanwhile, HUANG et al.(27) reported that, miR-615 specifically down-regulated CCND2 to suppress the prostate cancer cell proliferation and invasion. Moreover, miR-4317(28), miR-29b(29), and miR-146a-5p(30) have been reported to specifically down-regulate the effect of CCND2 on suppressing tumor cell proliferation and migration. To this end, this study proposes that miR-26a may specifically regulate the effect of CCND2 on mediating HCCcell proliferation and invasion.

To sum up, this study finds that KCNQ1OT1 is highly expressed in HCC tissues, cell liens and resistant Huh7/CDDP cells. Over-expressing KCNQ1OT1 remarkably promotes Huh7/CDDP cell proliferation and invasion, while suppressing apoptosis. In the meanwhile, dual-luciferase reporter gene assay verifies that KCNQ1OT1 specifically negatively regulates miR-26a expression, while miR-26a binds with the 3’UTR of CCND2 and negatively regulates CCND2 expression. Further experiment discovers that, KCNQ1OT1 down-regulates the suppression effect of miR-26a on CCND2 to promote Huh7/CDDP cell proliferation and invasion, while suppressing their apoptosis, thus up-regulating the cisplatin resistance of Huh7/CDDP cells.

Declarations

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors consent for publication.

Availability of data and materials

Not applicable

Funding

Not applicable

Authors' contributions

Qi-Fa YE contributed to the conception of the study;

Cai LI performed the experiment; contributed significantly to analysis and manuscript preparation; performed the data analyses and wrote the manuscript;

And helped perform the analysis with constructive discussions.

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Not applicable

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Figures

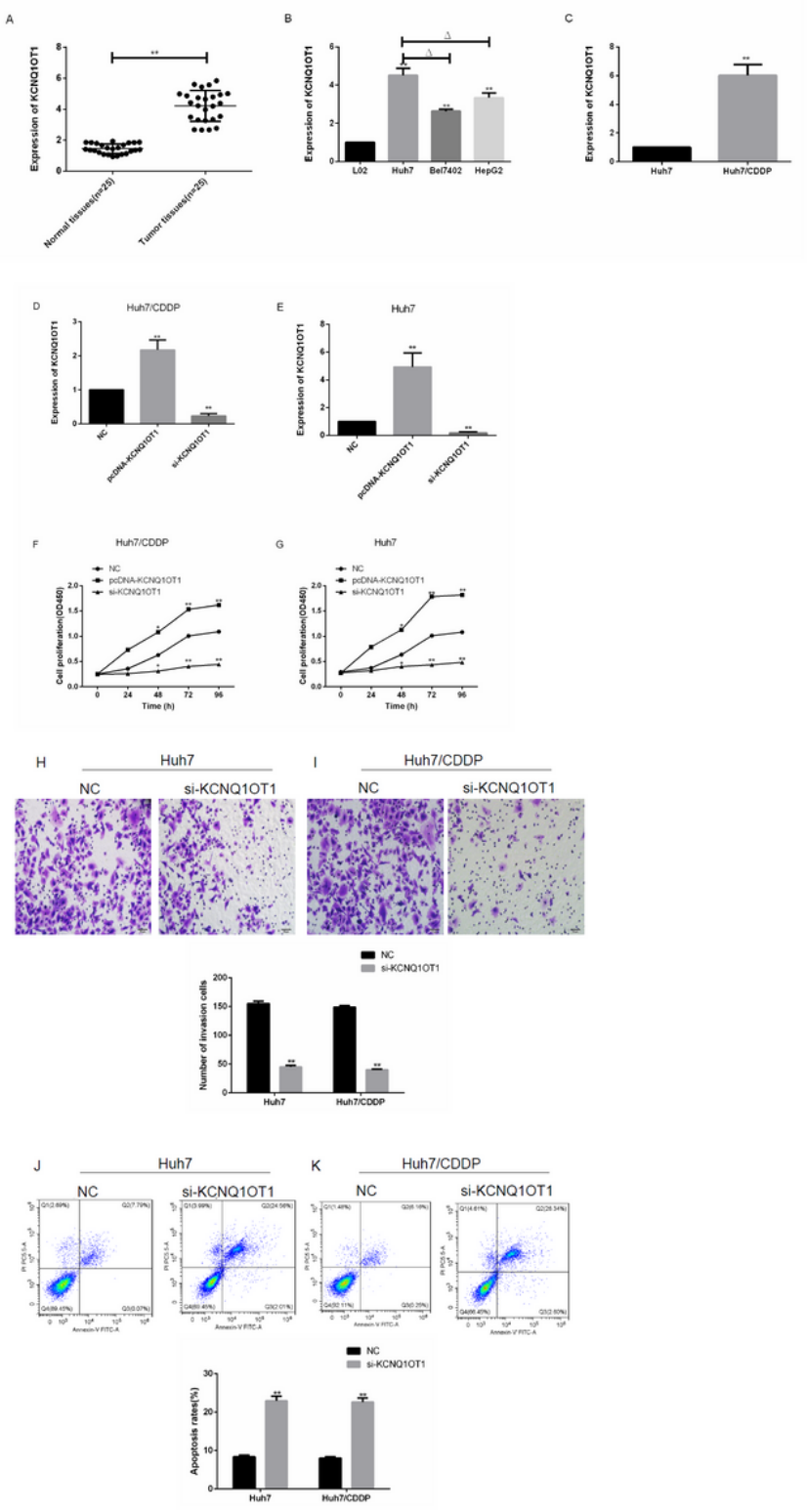

Figure 1

Expression of KCNQ1OT1 in HCCtissues ,cell lines and the effect of Silencing of KCNQ1OT1 on proliferation, invasion and apoptosis of bladder cancer. Notes: A-C: KCNQ1OT1 expression in HCCtissues, cell lines and Huh7/CDDP cells was detected by qRT-PCR, **P<0.01,△P<0.05. D and E,:KCNQ1OT1 expression in Huh7 and Huh7/CDDP cells transfections with pcDNA-KCNQ1OT1 or si-KCNQ1OT1, **P<0.01 vs NC group. F and G: Down-regulation of KCNQ1OT1 inhibits the proliferation ability of Huh7

(F) and Huh7/CDDP (G) cells. Huh7 and Huh7/CDDP were transfected with blank, pcDNA-KCNQ1OT1, and si-KCNQ1OT1 separately. CCK-8 assay was used to detect capacity of proliferation (*P < 0.05, **P < 0.01). H and I: Down-regulation of KCNQ1OT1 inhibits the invasion capability of Huh7 (H) and Huh7/CDDP (I) cells. Cell invasive ability was detected by Transwell assay (**P < 0.01). J and K: Down-regulation of KCNQ1OT1 promotes apoptosis of Huh7 (J) and Huh7/CDDP (K) cells. Apoptosis rate were detected using flow cytometry (**P < 0.01).

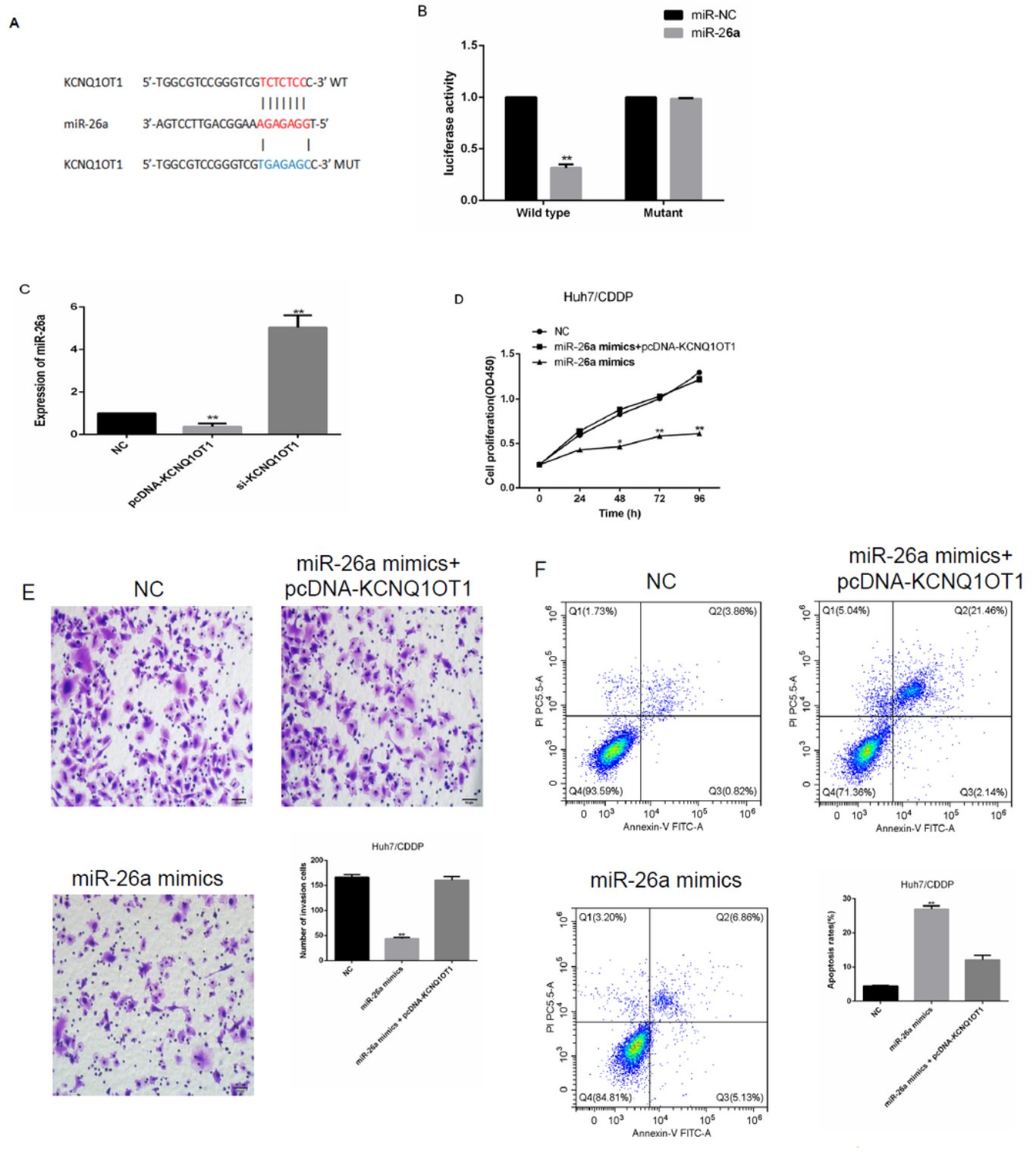

Figure 2

MiR-26a was regulated by KCNQ1OT1, suppress the proliferation and iinvasion , promote the apoptosis of Huh7/CDDP. Notes: A: The bioinformatics analysis result showed that KCNQ1OT1 had a binding site in miR-26a. B: The luciferase activity in miR-26a-wt transfected with KCNQ1OT1 was lower than that in NC group detected by dual-luciferase reporter assay (** P<0.01 vs miR-NC). C: The expression of miR-26a was detected by q RT-PCR(** P<0.01 vs NC). D: Proliferation viability of Huh7/CDDP cells was detected by CCK-8 assay; E: The invasion capability of Huh7/CDDP cells was measured by Transwell assay (×100); F: The apoptotic rate Huh7/CDDP cells was detected by flow cytometer assay(*P<0.05,** P<0.01 vs NC group or miR-26a mimics+pcDNA-KCNQ1OT1).

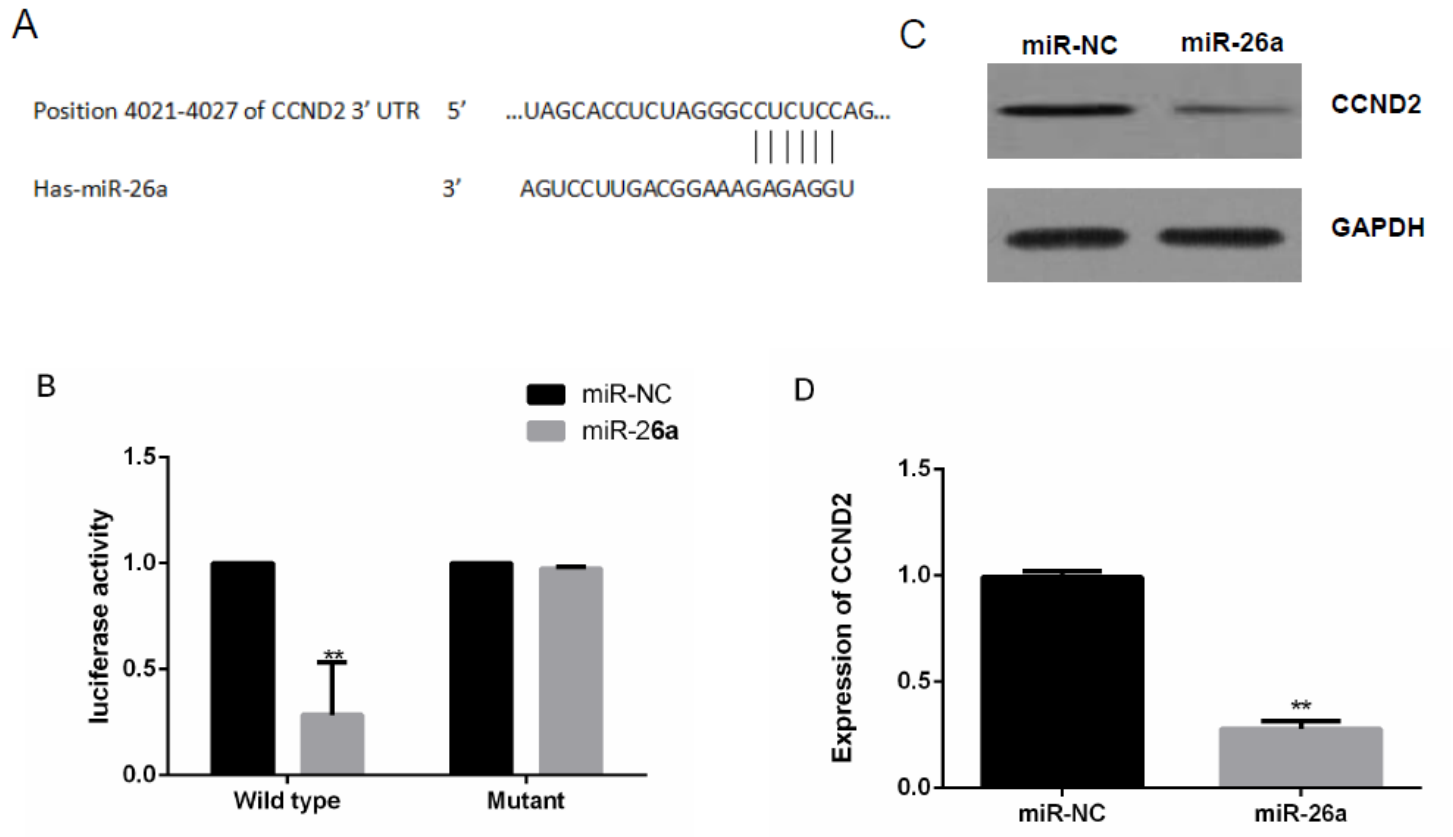

Figure 3

CCND2 was a target gene of miR-26a Notes : A: The bioinformatics analysis result showed that miR-26a had a binding site in CCND2; B: The luciferase activity in CCND2-wt cells transfected with miR-26a was lower than that in NC group detected by dual-luciferase reporter assay; C-D: The expression of CCND2 was measured by western blotting (*P<0.05,**P<0.01 vs miR-NC group).

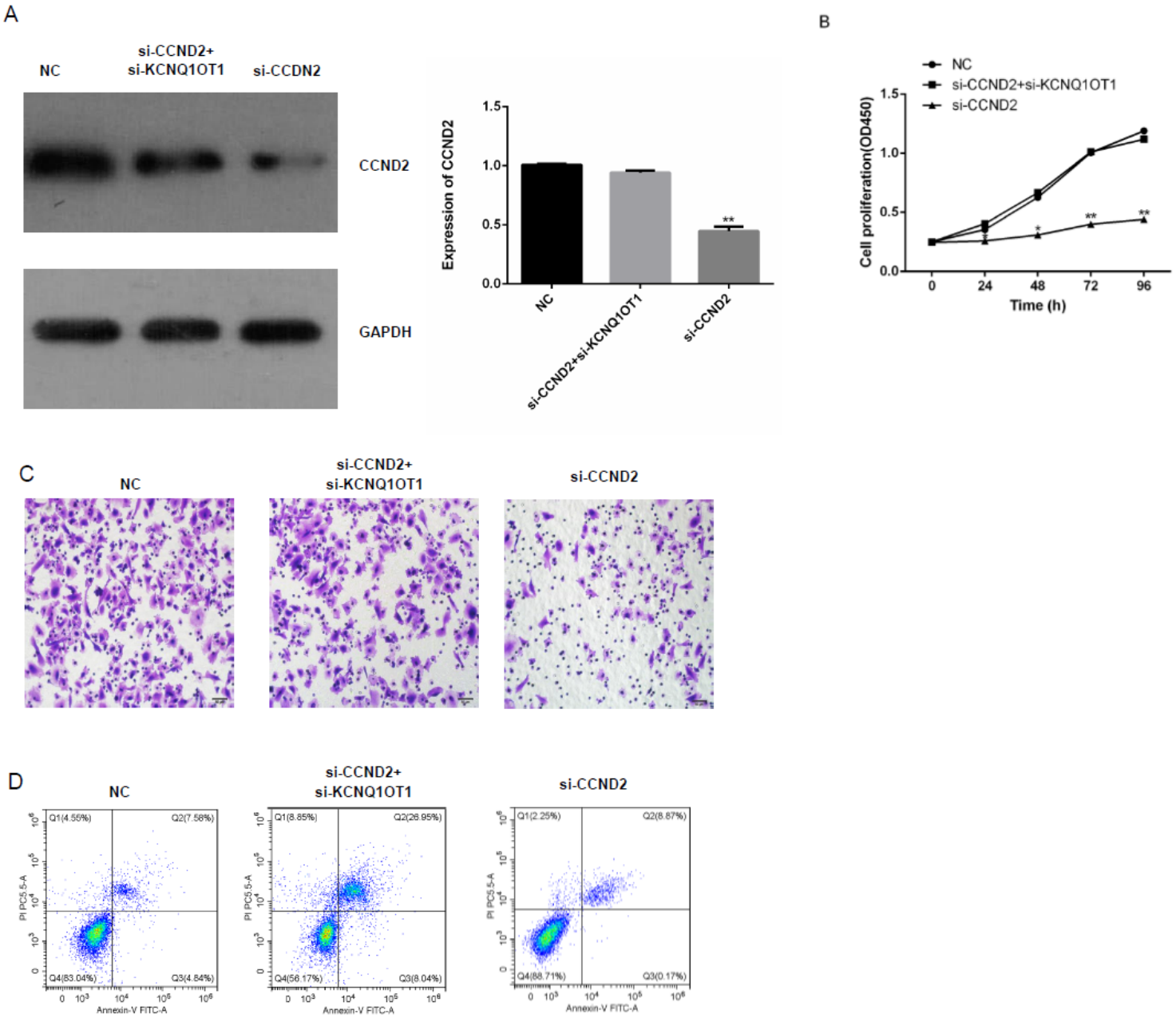

Figure 4

KCNQ1OT1 modulates biological behaviors of Huh7/CDDP cells via regulating miR-26a/CCND2 axis
Notes: A: The expression of CCND2 was detected by Western blotting; B: The cell proliferation of Huh7/CDDP cells was measured by CCK-8 assay; C: The invasion capability of Huh7/CDDP cells was measured by Transwell assay(×100); D and F: The percentage of apoptotic Huh7/CDDP cells was detected by flow cytometer assay(*P<0.05,** P<0.01 vs NC group or si-KCNQ1OT1+si CCND2 group).