miR-375/Yes-associated protein axis regulates IL-6 and TGF-β expression, which is involved in the cisplatin-induced resistance of liver cancer cells

KANRU YU[1*], HAO LI[1], ZHONGYI JIANG[1], HAN-JEN HSU[3], HSING-CHUN HSU[4], YUMEI ZHANG[5] and KUNWEI WANG[2*]

[1]Department of General Surgery, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200080; [2]Department of Endocrinology, Shanghai Tianyou Hospital, School of Medicine, Tongji University, Shanghai 200331; [3]Department of Urology, Shanghai ZhongShan Hospital, Fudan University School of Medicine, Shanghai 200123; [4]Department of Surgery, Shanghai Ruibo Clinic, Shanghai 200023; [5]Department of VIP Clinical, Shanghai East Hospital, Tongji University School of Medicine, Shanghai 200123, P.R. China

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Abstract. Chemotherapy resistance is one of the major challenges in the treatment of liver cancer (LC). The present study aimed to investigate the potential roles of Yes-associated protein (YAP), the core component of the Hippo signaling pathway, in chemoresistance of LC. YAP expression and its function in chemoresistance of LC cells were investigated. It was revealed that the expression levels and nuclear localization of YAP were increased in cisplatin (CDDP)-resistant LC (LC/CDDP) cells. The targeted inhibition of YAP using small interfering RNA or an inhibitor restored the CDDP sensitivity of LC cells. YAP overexpression was discovered to be essential for the increase of IL-6 and TGF-β expression levels in LC/CDDP cells. Furthermore, it was identified that increased mRNA stability was the primary reason for the upregulation of YAP expression in LC/CDDP cells, which was due to the downregulation of microRNA (miR)-375 expression in LC/CDDP cells. In conclusion, the findings of the present study suggested that the miR-375/YAP axis may regulate the expression levels of IL-6 and TGF-β, which may subsequently be involved in the CDDP resistance of LC cells. The current results indicated that the targeted inhibition of this axis and signaling pathway may be helpful in overcoming CDDP resistance.

Correspondence to: Dr Kunwei Wang, Department of Endocrinology, Shanghai Tianyou Hospital, School of Medicine, Tongji University, No. 528 Zhennan Road, Putuo District, Shanghai 200331, P.R. China
E-mail: wkw2021518@sina.com

*Contributed equally

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Introduction

Liver cancer (LC) was the third leading cause of cancer-associated deaths worldwide in 2016, demonstrating an increasing incidence rate (1). Notably, >50% of LC cases occur in China (2). Liver resection or transplantation is available for early stage LC, while for patients who have reached a stage beyond curative surgery, systematic chemotherapy is the primary treatment option (3). Tyrosine kinase inhibitors (TKIs), such as sorafenib, have been widely used as first-line chemotherapy treatments for LC (4). Cisplatin (CDDP) is another frontline chemotherapeutic drug used for the treatment of LC (5); it can induce the apoptosis of cancer cells via intercalating base pairs of DNA strands and inhibiting DNA/RNA synthesis (6,7). However, chemoresistance is one of the greatest challenges for the chemotherapeutic treatment of LC, leading to limited therapy efficiency and a poor prognosis (8). Therefore, it remains a priority to investigate the mechanisms involved in chemotherapy resistance to overcome this resistance and increase the efficacies of treatments.

The dysregulation of the Hippo signaling pathway has been reported in various types of cancer, including prostate, ovarian, colon, liver, lung and pancreatic cancer (9). Yes-associated protein (YAP) is the core component of the Hippo signaling pathway and is highly conserved from the fruit fly (*Drosophila*) to mammals (10). The upregulation of YAP expression has been reported in several types of human tumor, such as breast cancer (11), and has been associated with a poor prognosis of cancer progression in breast and lung cancer (12-14). Previous studies have indicated that the dysregulation of the YAP and Hippo signaling pathway is involved in the chemoresistance of cancer cells; for example, YAP promotes epithelial-mesenchymal transition and chemoresistance in pancreatic cancer cells (15), and it regulates cellular quiescence to modulate chemoresistance and cancer relapse in colon cancer cells (16). However, whether YAP is involved in the chemoresistance of LC remains to be determined. Therefore, the present study aimed to investigate the potential roles of YAP in LC chemoresistance.

Materials and methods

Cell culture. The human LC cells, HepG2, Huh-6 and Huh-7, were purchased from the American Type Culture Collection. Cells were cultured in DMEM supplemented with 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.) and maintained in a 5% CO_2 incubator at 37˚C.

To generate CDDP-resistant LC cells, cells were treated with increasing concentrations of CDDP (Sigma-Aldrich; Merck KGaA) over 6 months, with a final concentration of 1 μM, as reported previously (17,18). The resistant cells were named HepG2/CDDP, Huh6/CDDP and Huh7/CDDP, respectively.

Cell proliferation assay. Cells were plated and cultured in 96-well plates in 100 μl medium at a density of $1x10^3$ cells/well. Following treatment with increasing concentrations (0, 0.5, 1, 5, 10, 20 and 50 μM) of CDDP for 48 h at room temperature, 10 μl Cell Counting Kit-8 (Abmole Bioscience Inc.) reagent was added to each well and incubated at 37˚C for 2 h. In order to evaluate the effect of YAP, HepG2/CDDP and Huh-7/CDDP cells were pre-treated with or without 4 μM verteporfin (VP; Sigma-Aldrich; Merck KGaA; cat. no. SML0534) for 90 min at room temperature and then further treated with increasing concentrations of CDDP (0, 0.5, 1, 5, 10, 20 and 50 μM) for 48 h at room temperature. In order to investigate whether IL-6 and TGF-β were involved in YAP-regulated chemoresistance of LC cells, HepG2/CDDP cells were pre-treated with 100 ng/ml anti-IL-6 (cat. no. MAB206-SP; R&D Systems, Inc.) or anti-TGF-β (cat. no. BE0057; Bio X Cell) for 2 h at room temperature and then further treated with increasing concentrations of CDDP (0, 0.5, 1, 5, 10, 20 and 50 μM) for 48 h at room temperature. Additionally, HepG2/CDDP or Huh-7/CDDP cells were pre-treated with VP (4 μM) combined with recombinant (r)IL-6 (100 ng/ml; cat. no. 206-IL-010/CF; R&D Systems, Inc.) or rTGF-β (100 ng/ml; cat. no. 240-B-002/CF; R&D Systems, Inc.) for 2 h at room temperature, and then further treated with increasing concentrations of CDDP (0-20 μM) for 48 h at room temperature. The absorbance was measured at 450 nm using a microplate reader (ENSIGHT; PerkinElmer, Inc.) according to the manufacturer's protocol. The cell viability was calculated as the percentage of the viability of untreated control cells. Experiments were repeated ≥3 times.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and treated with DNase I (Promega Corporation) to remove the DNA contamination. RNA (1 μg) was reverse transcribed into cDNA using the cDNA Synthesis SuperMix (Beijing TransGen Biotech Co., Ltd.) according to the manufacturer's protocol. qPCR was subsequently performed using the SYBR Premix Ex Taq II kit (Takara Biotechnology Co., Ltd.) and a Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc.). The following primer sequences were used: YAP forward, 5'-GGC ATACACCTACTCAACTACGG-3' and reverse, 5'-TGG GCGGTGTAGAATCAGAGTC-3'; precursor-YAP forward, 5'-CCGGCTTGCTCTTATCAAAC-3' and reverse, 5'-GTC ATCGCTTCCCAAACATT-3'; IL-6 forward, 5'-ACTCAC CTCTTCAGAACGAATTG-3' and reverse, 5'-CCATCTTTG GAAGGTTCAGGTTG-3'; IL-10 forward, 5'-TCTCCGAGA TGCCTTCAGCAGA-3' and reverse, 5'-TCAGACAAGGCT TGGCAACCCA-3'; IL-12 forward, 5'-TGCCTTCACCAC TCCCAAAACC-3' and reverse, 5'-CAATCTCTTCAGAAG TGCAAGGG-3'; TNF-α forward, 5'-CTCTTCTGCCTGCTG CACTTTG-3' and reverse, 5'-ATGGGCTACAGGCTTGTC ACTC-3'; TGF-β forward, 5'-TACCTGAACCCGTGTTGC TCTC-3' and reverse, 5'-GTTGCTGAGGTATCGCCAGGA A-3'; MALAT1 forward, 5'-AAAGCAAGGTCTCCCCAC AAG-3' and reverse, 5'-GGTCTGTGCTAGATCAAAAGG CA-3'; and GAPDH forward, 5'-GGAGCGAGATCCCTC CAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCTCA TGG 3'. The PCR cycling conditions were 15 min at 95˚C, followed by 40 cycles for 10 sec at 95˚C, 30 sec at 60˚C and 1 sec at 72˚C, and 1 cycle of cooling for 30 sec at 50˚C.

To analyze the expression levels of miRNAs, the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to generate cDNA according to the manufacturer's protocol. The thermocycling conditions included an initial denaturation at 95˚C for 3 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 30 sec. The forward primer is the exact sequence of the mature miRNA (http://www.mirbase.org/search.shtml). The forward primer for U6 was 5'-TGCGGGTGCTCGCTTCGCAGC-3'. The reverse primer was supplied by the aforementioned kit. GAPDH and U6 were used as the internal reference genes for the normalization of mRNA and miRNA, respectively. The gene expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (19). Each sample was analyzed in triplicate.

Subcellular fractionation. The cytoplasmic and nuclear fractions of cells were prepared using the PARIS™ kit (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The protein expression levels within the cytoplasmic and nuclear fractions were analyzed by western blotting. Aliquots of cytoplasmic and nuclear fractions were also subjected to RNA isolation and RT-qPCR, as aforementioned, to analyze the subcellular localization of YAP mRNA. Transcripts of the housekeeping gene GAPDH were used for normalization, while nuclear MALAT1 RNA was selected as endogenous control for the nuclear RNA.

Western blotting. Total protein was extracted from cells using 1X RIPA lysis buffer (50 mM Tris HCl, 150 mM NaCl and 1 mM EDTA) containing a protease inhibitor cocktail (Roche Diagnostics). Total protein was quantified using a bicinchoninic acid assay kit and 20 μg protein/lane was separated by 10% SDS-PAGE. The separated proteins were subsequently transferred onto a nitrocellulose membrane (EMD Millipore) using a wet transfer apparatus. The membranes were blocked with 5% skimmed milk at room temperature for 2 h. Following the incubation with the primary antibodies at 4˚C overnight, the membranes were further incubated with the HRP-conjugated secondary antibody (cat. no. ab7090; Abcam; 1:10,000) diluted in 5% skimmed milk. Protein bands were then visualized in a gel imaging system (MG8600; Bio-Rad Laboratories, Inc.). The following primary antibodies (1:1,000; Abcam) were used: Anti-H2A.X (cat. no. ab229914), anti-YAP (cat. no. ab56701), anti-TAZ (cat. no. ab84927), anti-calpain (cat. no. ab39170)

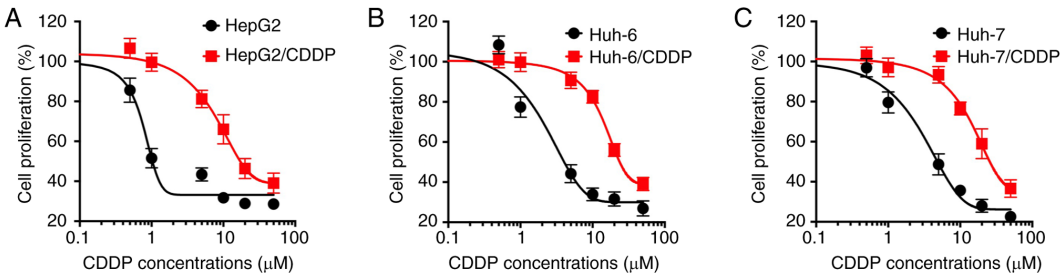

Figure 1. Establishment of LC/CDDP cells. Cell proliferation of (A) HepG2/CDDP, (B) Huh-6/CDDP or (C) Huh-7/CDDP cells and their parental cells treated with increasing concentrations of CDDP (0, 0.5, 1, 5, 10, 20 and 50 μM) for 48 h. Data are presented as the mean ± SD of three independent experiments. LC, liver cancer; CDDP, cisplatin; LC/CDDP cells, CDDP-resistant LC cells.

and anti-GAPDH (cat. no. ab229914). GAPDH was used as the loading control for normalization. The gray values were analyzed using ImageJ software (version 1.46; National Institutes of Health).

Cell transfection and treatment. The small interfering RNA (siRNA/si) negative control (si-NC; 5'-GCACAACAAGCC GAAUACA-3'), si-YAP (siYAP-1, 5'-GCGUAGCCAGUU ACCAACA-3'; siYAP-2, 5'-CAGUGGCACCUAUCACUC U-3'), miRNA control (miR, 5'-UUCUCCGAACGUGUC ACGUTT-3') and miR-375 mimics (5'-UUUGUUCGU UCGGCUCGCGUGA-3') were synthesized by Shanghai GenePharma Co., Ltd.. Upon cells reaching 50-60% confluence, the transfection was performed using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions with 20 μM of each construct or siRNA. After transfection for 6 h at 37˚C, the medium was replaced with fresh complete medium. To investigate the effect of YAP on chemosensitivity, HepG2/CDDP, Huh-6/CDDP and Huh-7/CDDP cells were transfected with si-NC or si-YAP-1 for 12 h and then further treated with increasing concentrations of CDDP (0, 0.5, 1, 5, 10, 20 and 50 μM) for 48 h.

mRNA and protein stability assay. To determine the mRNA stability, cells were treated with 5 μg/ml actinomycin D (Act-D; cat. no. A9415; Sigma-Aldrich; Merck KGaA) at 37˚C for 0, 2, 4 or 8 h. Subsequently, total RNA was collected and the target mRNA was analyzed using RT-qPCR, as aforementioned. For the protein stability assay, cells were incubated with 100 μg/ml cycloheximide (CHX) at 37˚C for 0, 2, 6 or 12 h and then protein expression was analyzed using western blotting, as aforementioned.

Immunofluorescence. Cells cultured on coverslips were washed with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. After blocking with 3% BSA in PBS containing 0.3% Triton X-100 solution at 37˚C for 1 h, cells were incubated with a primary antibody against YAP (cat. no. ab56701; 1:1,000; Abcam) overnight at 4˚C and then treated with an anti-Alexa Fluor 594 secondary antibody (1:200; R&D Systems China Co., Ltd.; cat. no. IC1420T) for 1 h at room temperature. Then, DAPI solution (5 μg/ml) was added to stain the cell nuclei for 5 min at room temperature. The fluorescence signal was observed under a confocal microscope (TCS-SP5; Leica Microsystems GmbH; magnification, x10).

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc.) and presented as the mean ± SD. The comparisons between two groups were analyzed using an unpaired Student's t-test. All experiments were performed ≥3 times independently. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of LC/CDDP cells. The CDDP sensitivity of both resistant and parental LC cells was investigated. The results revealed that the established CDDP-resistant cells were more resistant to CDDP treatment compared with their corresponding parental cells (Fig. 1). The IC_{50} values of CDDP for HepG2/CDDP and HepG2 cells were 22.8 and 3.45 μM, respectively (Fig. 1A), those for Huh-6/CDDP and Huh-6 cells were 30.6 and 5.05 μM, respectively (Fig. 1B), while the IC_{50} values of CDDP for Huh-7/CDDP and Huh-7 cells were 30.5 and 6.51 μM, respectively (Fig. 1C). The current data confirmed the successful establishment of LC/CDDP cells.

YAP expression is upregulated in CDDP-resistant LC cells. It has been previously reported that the Hippo signaling pathway regulates the progression of LC (20). Thus, the present study analyzed the expression levels of YAP and the transcriptional coactivator with PDZ-binding motif (TAZ), another important member of the Hippo signaling pathway (20), in both parental and CDDP-resistant LC cells. The protein expression levels of YAP, but not TAZ, were significantly upregulated in the HepG2/CDDP, Huh-6/CDDP and Huh-7/CDDP cells compared with in their corresponding parental cells (Fig. 2A). Furthermore, RT-qPCR analysis revealed that the mRNA expression levels of YAP were significantly upregulated in the CDDP-resistant LC cells compared with in their respective parental cells (Fig. 2B). In addition, the amount of YAP localized in both the cytosol and nucleus was increased in HepG2/CDDP cells compared with in HepG2 cells (Fig. 2C), which was confirmed by immunofluorescence staining (Fig. 2D).

YAP is involved in the CDDP resistance of LC cells. To investigate whether YAP was involved in the resistance to CDDP in LC cells, the CDDP-resistant LC cells were transfected with si-YAP-1 and si-YAP-2 (Fig. 3A). si-YAP-1 was used for subsequent experiments since it displayed increased efficiency. The results revealed that si-YAP-1 markedly increased the CDDP sensitivity of HepG2/CDDP (Fig. 3B), Huh-6/CDDP (Fig. 3C)

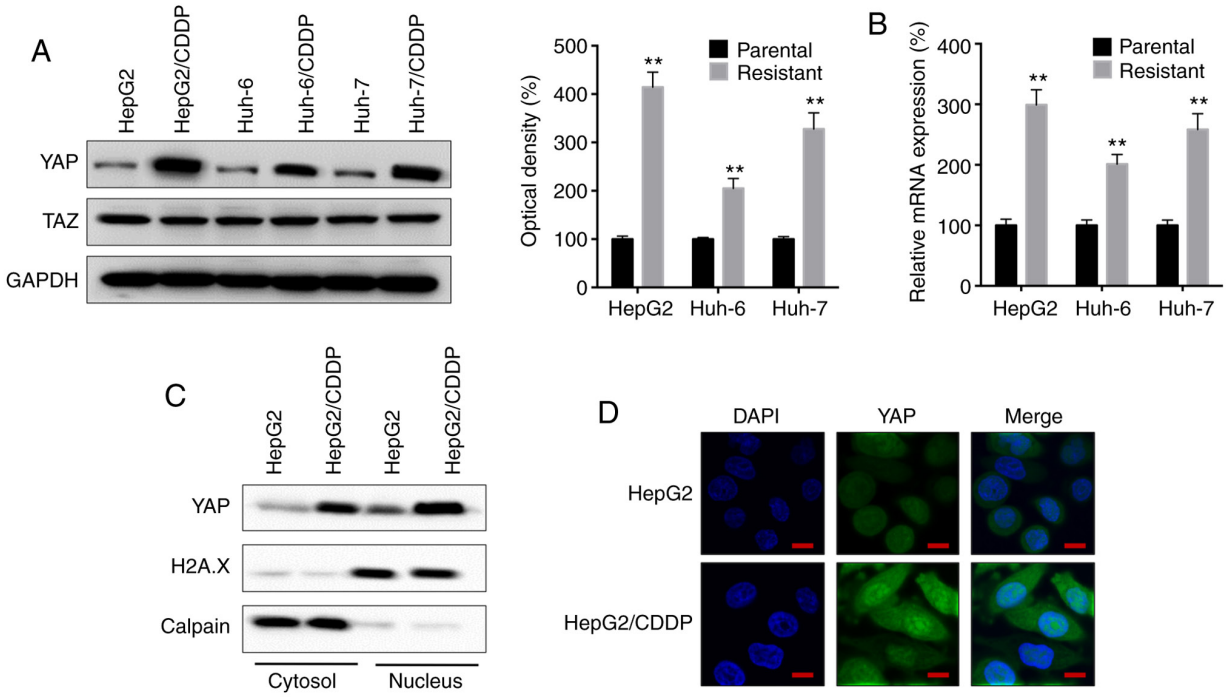

Figure 2. YAP expression is upregulated in CDDP-resistant LC cells. (A) YAP and TAZ protein expression in LC/CDDP or parental cells was analyzed by western blot analysis (left) and YAP expression was quantitatively analyzed (right). (B) YAP mRNA expression in LC/CDDP or parental cells was analyzed by reverse transcription-quantitative PCR. The subcellular localization of YAP in HepG2/CDDP or HepG2 cells was checked by (C) western blot analysis and (D) confocal microscopy (scale bar, 20 μm). Data are presented as the mean ± SD of three independent experiments. **P<0.01 vs. parental. LC, liver cancer; CDDP, cisplatin; LC/CDDP cells, CDDP-resistant LC cells; YAP, Yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif.

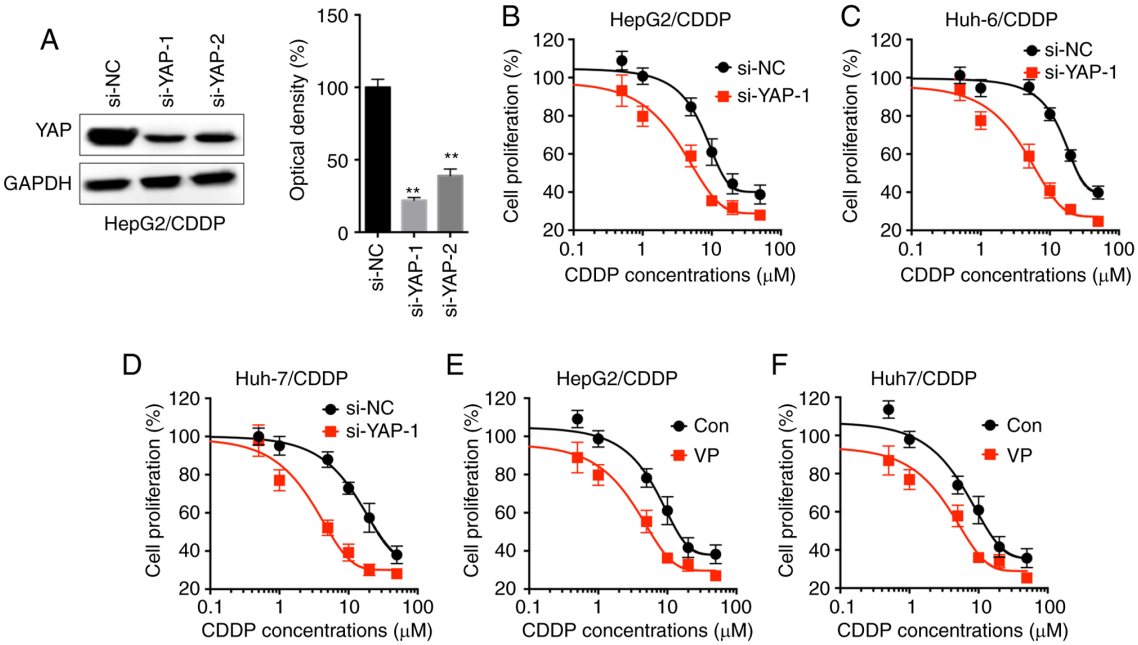

Figure 3. YAP is involved in the CDDP resistance of LC cells. (A) HepG2/CDDP cells were treated with si-NC or si-YAP-1/2 for 24 h, and YAP expression was analyzed by western blot analysis. Cell proliferation of (B) HepG2/CDDP, (C) Huh-6/CDDP and (D) Huh-7/CDDP cells transfected with si-NC or si-YAP-1 for 12 h and then further treated with increasing concentrations of CDDP (0, 0.5, 1, 5, 10, 20 and 50 μM) for 48 h. Cell proliferation of (E) HepG2/CDDP and (F) Huh-7/CDDP cells pre-treated with or without 4 μM VP for 90 min and then further treated with increasing concentrations of CDDP (0, 0.5, 1, 5, 10, 20 and 50 μM) for 48 h. Data are presented as the mean ± SD of three independent experiments. **P<0.01 vs. si-NC. LC, liver cancer; CDDP, cisplatin; LC/CDDP cells, CDDP-resistant LC cells; YAP, Yes-associated protein; si-NC, siRNA negative control; VP, verteporfin; Con, control.

and Huh-7/CDDP (Fig. 3D) cells. Since the results revealed that YAP expression was markedly increased in HepG2/CDDP and Huh-7/CDDP cells, these cell lines were further treated with VP, a suppressor of the YAP-TEAD complex (21). VP increased the sensitivity of CDDP in HepG2/CDDP (Fig. 3E) and Huh-7/CDDP (Fig. 3F) cells.

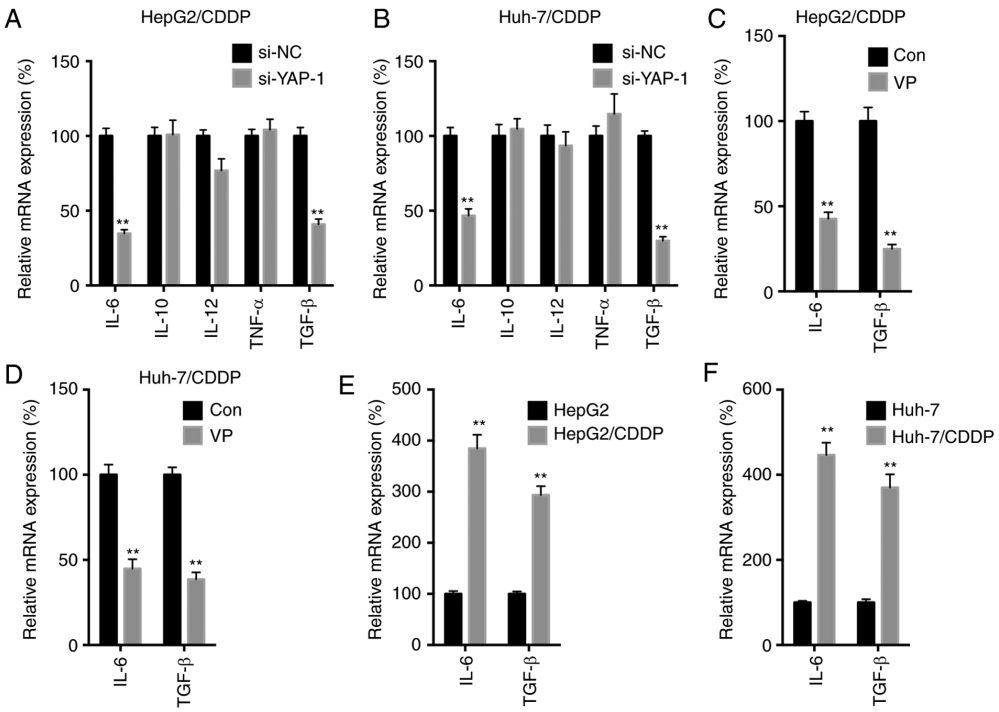

Figure 4. YAP regulates the expression levels of IL-6 and TGF-β in LC/CDDP cells. (A) HepG2/CDDP or (B) Huh-7/CDDP cells were transfected with si-NC or si-YAP-1 for 24 h, and the mRNA expression levels of different cytokines were measured by RT-qPCR. (C) HepG2/CDDP or (D) Huh-7/CDDP cells were treated with or without 4 μM VP for 24 h, and the mRNA expression levels of IL-6 and TGF-β were measured by RT-qPCR. IL-6 and TGF-β expression in (E) HepG2/CDDP and (F) Huh-7/CDDP cells and their corresponding parental cells were measured by RT-qPCR. Data are presented as the mean ± SD of three independent experiments. **P<0.01 vs. si-NC, Con or parental cells. LC, liver cancer; CDDP, cisplatin; LC/CDDP cells, CDDP-resistant LC cells; YAP, Yes-associated protein; si-NC, siRNA negative control; VP, verteporfin; Con, control; RT-qPCR, reverse transcription-quantitative PCR.

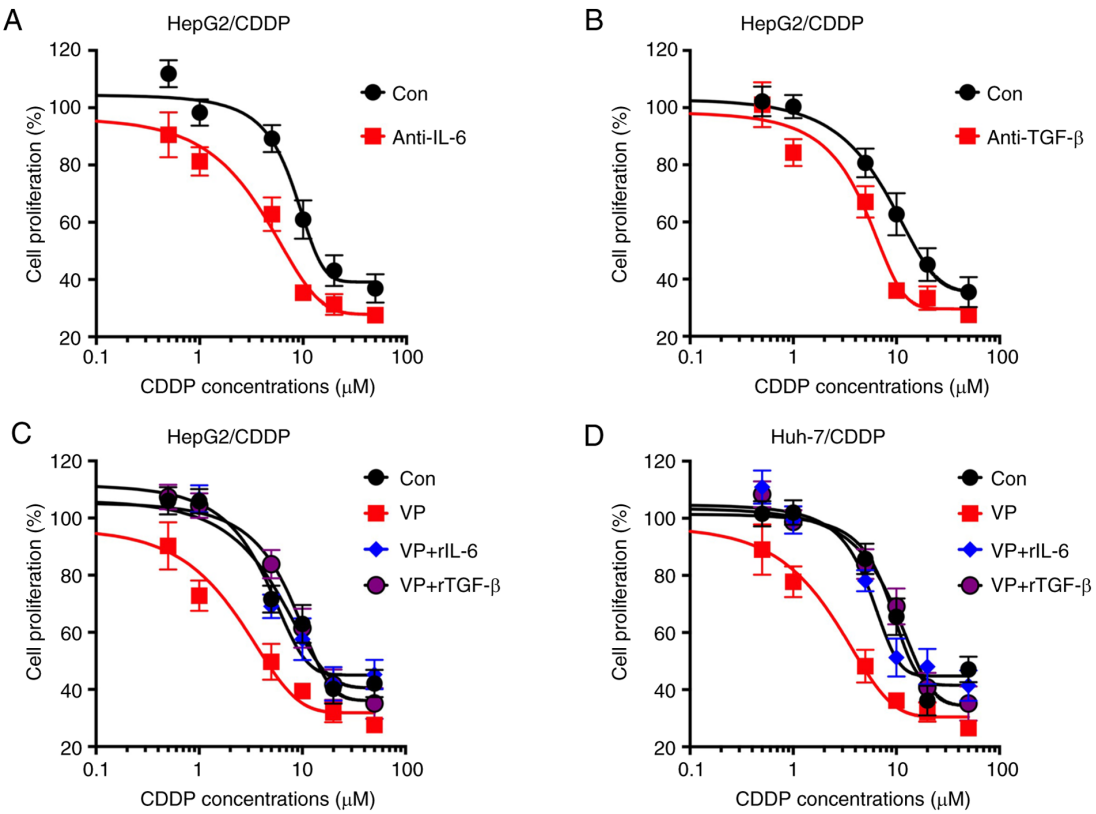

Figure 5. IL-6 and TGF-β are involved in YAP-regulated chemoresistance of LC cells. Cell proliferation of HepG2/CDDP cells pre-treated with 100 ng/ml (A) anti-IL-6 or (B) anti-TGF-β for 2 h and then further treated with increasing concentrations of CDDP (0, 0.5, 1, 5, 10, 20 and 50 μM) for 48 h. Cell proliferation of (C) HepG2/CDDP or (D) Huh-7/CDDP cells pre-treated VP (4 μM) combined with rIL-6 (100 ng/ml) or rTGF-β (100 ng/ml) for 2 h, and then further treated with increasing concentrations of CDDP (0, 0.5, 1, 5, 10, 20 and 50 μM) for 48 h. Data are presented as the mean ± SD of three independent experiments. LC, liver cancer; CDDP, cisplatin; LC/CDDP cells, CDDP-resistant LC cells; YAP, Yes-associated protein; VP, verteporfin; Con, control; r, recombinant.

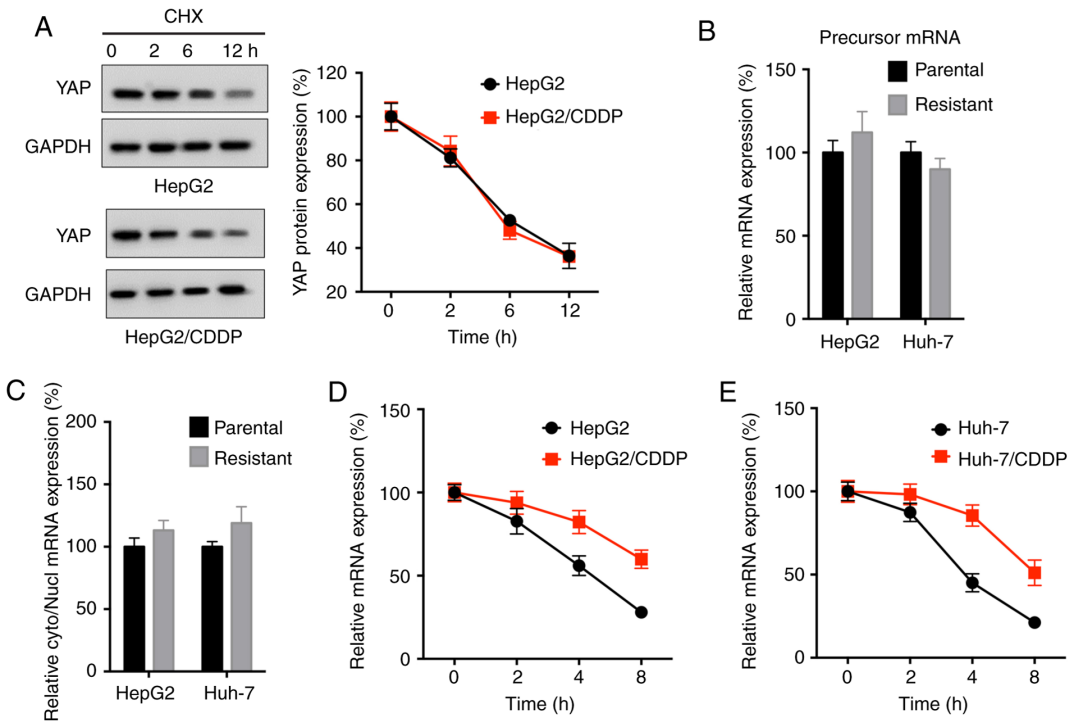

Figure 6. mRNA stability is responsible for the upregulation of YAP expression in LC/CDDP cells. (A) HepG2 and HepG2/CDDP cells were treated with CHX (100 μg/ml) for the indicated time periods, and YAP protein expression was analyzed by western blot analysis (left) and quantitatively analyzed (right). (B) Expression levels of the precursor mRNA of YAP in LC and LC/CDDP cells were measured via RT-qPCR. (C) Relative cyto/nucl levels of YAP mRNA expression in LC and LC/CDDP cells were measured via RT-qPCR. (D) HepG2/CDDP or (E) Huh-7/CDDP cells and their corresponding parental cells were treated with actinomycin D for the indicated time periods, and YAP mRNA expression was analyzed via RT-qPCR. Data are presented as the mean ± SD of three independent experiments. LC, liver cancer; CDDP, cisplatin; LC/CDDP cells, CDDP-resistant LC cells; YAP, Yes-associated protein; RT-qPCR, reverse transcription-quantitative PCR; CHX, cycloheximide; cyto, cytoplasm; nucl, nucleus.

YAP regulates the expression levels of IL-6 and TGF-β in LC/CDDP cells. It has been previously reported that YAP regulates the expression levels of various cytokines to regulate cancer progression (12-14). In the present study, an array of cytokines was analyzed, including IL-6, IL-10, IL-12, TNF-α and TGF-β, in si-YAP-1-transfected LC/CDDP cells. si-YAP-1 significantly downregulated the expression levels of IL-6 and TGF-β in both HepG2/CDDP (Fig. 4A) and Huh-7/CDDP (Fig. 4B) cells. In addition, VP treatment significantly downregulated the expression levels of IL-6 and TGF-β in both HepG2/CDDP (Fig. 4C) and Huh-7/CDDP (Fig. 4D) cells. On the other hand, the expression levels of IL-6 and TGF-β in both HepG2/CDDP (Fig. 4E) and Huh-7/CDDP (Fig. 4F) cells were significantly upregulated compared with in their corresponding control cells. The current results suggested that YAP may regulate the expression levels of IL-6 and TGF-β in LC/CDDP cells.

IL-6 and TGF-β are involved in the YAP-mediated chemoresistance of LC cells. The current study further analyzed whether IL-6 and TGF-β were involved in the YAP-mediated chemoresistance of LC cells. The data demonstrated that neutralization antibodies anti-IL-6 (Fig. 5A) and anti-TGF-β (Fig. 5B) significantly increased the CDDP sensitivity of HepG2/CDDP cells. In addition, rIL-6 (Fig. 5C) and rTGF-β (Fig. 5D) significantly attenuated the VP-induced CDDP sensitivity of HepG2/CDDP cells. All these data indicated that IL-6 and TGF-β may be involved in the YAP-mediated chemoresistance of LC cells.

mRNA stability is responsible for the upregulation of YAP expression in LC/CDDP cells. The potential mechanisms responsible for the upregulation of YAP expression in LC/CDDP cells were subsequently investigated. The protein stability of YAP in HepG2 and HepG2/CDDP cells following CHX treatment was similar to each other (Fig. 6A). Additionally, the expression levels of the precursor mRNA of YAP, analyzed by RT-qPCR, were not significantly different between HepG2 and HepG2/CDDP cells or between Huh-7 and Huh-7/CDDP cells (Fig. 6B). In addition, the nuclear turnover rate of YAP was not significantly different between HepG2 and HepG2/CDDP cells, as analyzed by RT-qPCR (Fig. 6C). However, the data revealed that the mRNA stability of YAP in HepG2/CDDP cells following Act-D treatment was markedly increased compared with in HepG2 cells (Fig. 6D). Consistently, the mRNA stability of YAP in Huh-7/CDDP cells was also increased compared with in Huh-7 cells (Fig. 6E). These results indicated that increased mRNA stability may be responsible for the upregulation of YAP expression in LC/CDDP cells.

miR-375 decreases the mRNA stability of YAP in LC/CDDP cells. miRNAs can decrease mRNA stability via binding to the 3'-untranslated regions of mRNA (22). It has been revealed that miR-375 (23), miR-506 (24), miR-132 (25) and miR-129 (26) directly target YAP mRNA to downregulate its expression. Thus, the expression levels of these miRNAs in both LC/CDDP and LC cells were subsequently analyzed. The data revealed that, among all miRNAs, only the expression levels of miR-375

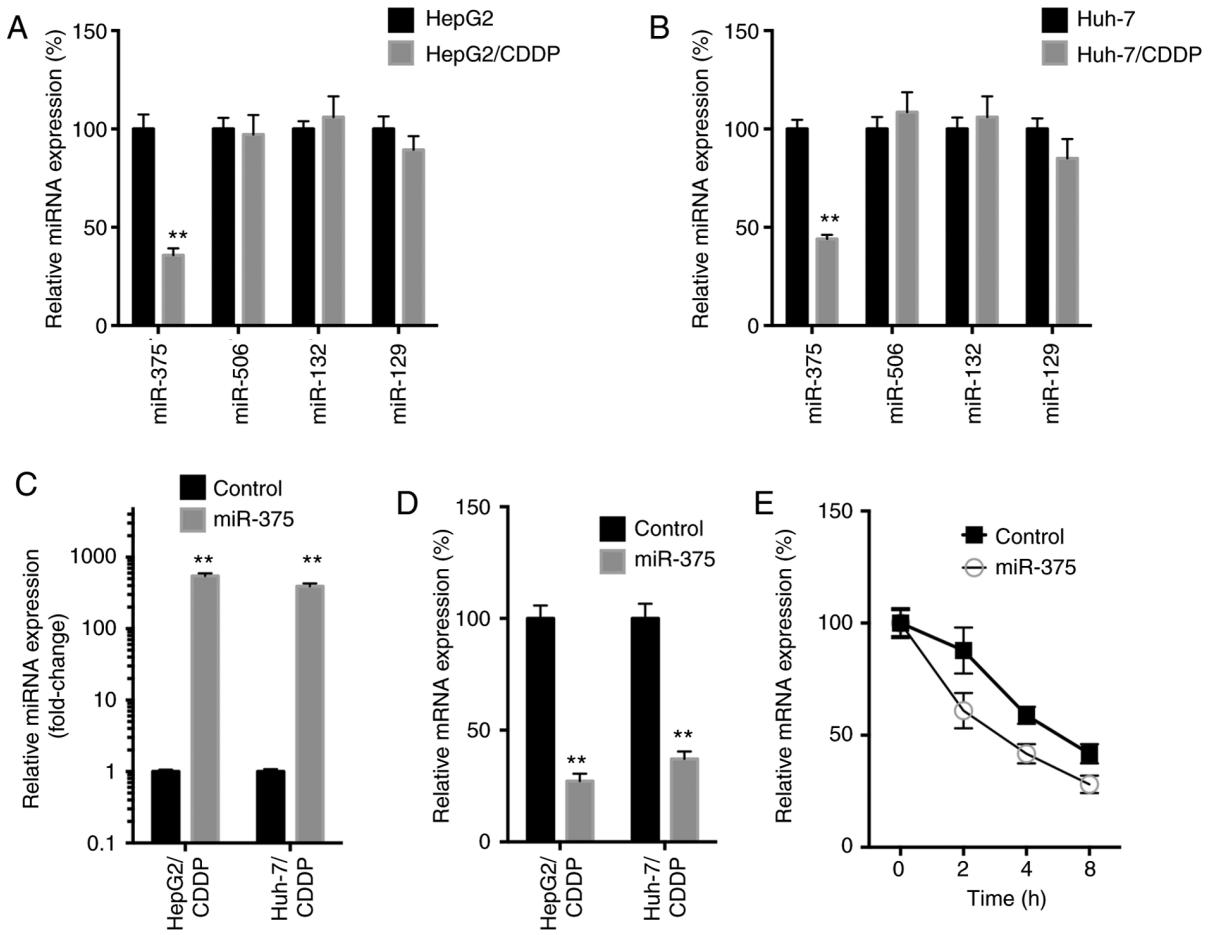

Figure 7. miR-375 decreases the mRNA stability of YAP in LC/CDDP cells. Expression levels of miRNAs in (A) HepG2/CDDP or (B) Huh-7/CDDP cells and their corresponding parental cells were measured via RT-qPCR. Cells were transfected with miR control or miR-375 mimics for 24 h, and the expression levels of (C) miR-375 and (D) YAP were measured via RT-qPCR. (E) HepG2/CDDP cells were transfected with miR control or miR-375 mimics for 24 h and further treated with actinomycin D for the indicated time periods, after which YAP mRNA expression was analyzed via RT-qPCR. Data are presented as the mean ± SD of three independent experiments. $^{**}P<0.01$ vs. parental cells or control. LC, liver cancer; CDDP, cisplatin; LC/CDDP cells, CDDP-resistant LC cells; YAP, Yes-associated protein; RT-qPCR, reverse transcription-quantitative PCR; miR/miRNA, microRNA.

were significantly downregulated in both HepG2/CDDP (Fig. 7A) and Huh-7/CDDP (Fig. 7B) cells. Furthermore, the overexpression of miR-375 (Fig. 7C) using miR-375 mimics significantly downregulated the mRNA expression levels of YAP in both HepG2/CDDP and Huh-7/CDDP cells (Fig. 7D). This was due to the fact that miR-375 decreased the mRNA stability of YAP (Fig. 7E).

Discussion

Chemotherapy is an important treatment for patients with LC, especially for those with advanced LC (27). Cisplatin has been widely used as a therapeutic agent for patients with LC; however, its application has been significantly limited due to the development of chemoresistance (28). To the best of our knowledge, the molecular mechanisms involved in LC chemoresistance to CDDP are not fully understood. The results of the present study suggested that YAP, an important downstream signaling protein of the Hippo signaling pathway, may mediate the CDDP resistance of LC cells via upregulating IL-6 and TGF-β expression. In addition, the downregulation of miR-375 expression in LC/CDDP cells was responsible for the upregulation of YAP expression. Collectively, these results suggested that the miR-375/YAP axis-induced expression of IL-6 and TGF-β may be critical for the CDDP resistance of LC cells.

The present study discovered that YAP was involved in the CDDP resistance of LC cells. It has been previously revealed that YAP upregulation is strongly associated with the carcinogenesis of LC (29,30). The activation of YAP suppresses the sensitivity of cancer cells to various drugs, such as anti-tubulin drugs and DNA-damaging agents (31-34). In LC cells, it has been reported that YAP upregulation confers resistance to doxorubicin (35) and the topoisomerase I inhibitor SN38 (36). The data of the present study illustrated that the expression levels and nuclear localization of YAP were increased in LC/CDDP cells. In addition, the targeted inhibition of YAP via siRNA or an inhibitor restored the CDDP sensitivity of LC cells, which indicated that YAP may be involved in the chemoresistance of LC cells.

The data of the current study also demonstrated that IL-6 and TGF-β were involved in the YAP-mediated chemoresistance of LC cells. It has been previously reported that the activation of YAP stimulates IL-6 gene transcription during colonic tumorigenesis (37). In LC cells, YAP induces IL-6 expression to recruit tumor-associated macrophages (38). Additionally, a recent study has confirmed that YAP can directly bind to the

promoter of IL-6 to regulate its transcription (39). As to TGF-β, it has been reported that YAP promotes the TGF-β-induced tumorigenic phenotype in breast cancer cells (40). In addition, YAP/TAZ regulate TGF-β/Smad3 signaling through the induction of Smad7 via activator protein 1 in human skin dermal fibroblasts (41). However, whether YAP can directly activate the transcription of TGF-β requires further investigation.

Furthermore, the present study indicated that the downregulation of miR-375 expression may be responsible for the upregulation of YAP expression in LC/CDDP cells, indicated by the fact that YAP mRNA stability was increased, while miR-375 expression was downregulated, in LC/CDDP cells compared with in LC cells. In gastric cancer cells, the upregulation of miR-375 expression increases the CDDP sensitivity via the regulation of ERBB2 (42). miR-375 is induced in CDDP nephrotoxicity to repress hepatocyte nuclear factor-1β (43). Furthermore, miR-375 can target YAP in LC to inhibit cancer cell viability (23,44). Similarly, miR-375 suppresses YAP expression in lung cancer (45) and mouse pancreatic progenitor (46) cells. All these data suggested that miR-375 may be involved in the CDDP resistance and progression of LC.

In conclusion, the results of the present study revealed that the miR-375/YAP axis may regulate the CDDP resistance of LC via the regulation of IL-6 and TGF-β. Therefore, the targeted inhibition of this axis and signaling pathway may be useful in overcoming the CDDP resistance and enhancing the clinical treatment of patients with LC. Whether the miR-375/YAP axis-induced expression of IL-6 and TGF-β is involved in the TKI resistance of LC requires further investigation in future studies.

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Availability of data and material

All data generated or analyzed during this study are included in this published article.

Authors' contributions

KY, KW and HL conceived and designed the study. ZJ, HJH, HCH, YZ and KW acquired the data. KY, KW, HL, ZJ and YZ analyzed and interpreted the data. KY, HCH, YZ and KW wrote and revised the manuscript. The authenticity of the raw data has been assessed by all authors. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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