Role of YAP in lung cancer resistance to cisplatin

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Abstract. Yes-associated protein (YAP) serves a critical role in the initiation and progression of a variety of types of cancer via modulating the expression of genes involved in cell proliferation and the downregulation of apoptosis. Recent studies have suggested that YAP is responsible for the development of drug resistance and cancer metastasis and recurrence. However, the association between YAP and chemoresistance in lung cancer, particularly in lung cancer stem cells (LCSCs) remains largely unknown. In the current study, lung cancer cell spheres were established using the A549 cell line, which demonstrated stem cell properties. It was revealed that YAP was overexpressed in lung cancer spheres compared with normal A549 adherent cells and was associated with enhanced cisplatin (CDDP) resistance. Knockdown of YAP effectively sensitized the adherent A549 and tumor spheres to CDDP treatment and resulted in enhanced cell death. These results suggest that YAP serves a critical role in LCSCs drug resistance and YAP targeting could become a promising adjuvant to current the chemotherapy for lung cancer.

Introduction

Non-small cell lung cancer (NSCLC) accounts for 85% of lung cancer cases. It is one of the most common malignant tumors worldwide and is the leading cause of cancer death (1). Chemotherapy is a widely used treatment method for NSCLC in clinic, however, drug resistance usually develops during the course of therapy and limits the efficacy of chemotherapy. Cancer stem cell (CSC) is a fraction of stem-like cells in tumor, and has been considered as the root of tumor growth, relapse and metastasis. Different from other cancer cells, CSC demonstrates exclusive ability of self-renewal and tumor formation (2,3). Moreover, CSC has also been identified with impaired apoptosis, enhanced DNA damage response and overexpression of multidrug resistance protein and membrane transporter, which further drive tumor progression and recurrence after chemotherapy (4). Given these, CSC represents a promising tool for investigating cancer drug response and developing novel approaches to overcome chemo-resistance.

Yes-associated protein (YAP) is a core effector of the Hippo tumor-suppressor pathway. Recently, YAP has been implicated in chemoresistance in a variety of cancer cells. It has been shown that silencing of YAP1 increased cell sensitivity to anti-tumor drugs in multiple cancers, including ovarian CSC (5), esophageal cancer (6), pancreatic cancer (7), liver cancer (8), oral squamous cell carcinoma (9) and gastric CSC (10). YAP functions as a transcriptional coactivator. Overexpression of YAP has been associated with induction of genes involved in cell proliferation, apoptosis suppression, epithelial-mesenchymal transition and anchorage-independent growth. The activated YAP function has been found to mediate protease-activated receptor 1 (PAR1)-induced tumor initiation and spheroid colony formation in gastric cancer cells, and is also responsible for the acquired resistance to cisplatin (CDDP), 5-FU and paclitaxel in gastric CSCs (10). In addition, via upregulating the expression of glycogen synthesis kinase 3A (GSK3A) and ATP-binding cassette subfamily B member 1 (ABCB1). It has also been shown that YAP could enhance chemotherapy resistance of ovarian CSCs to CDDP, paclitaxel and (5).

All these evidences supported the role of YAP as a drug resistance mediator and implied the potential of YAP to be a novel target for cancer therapy. However, little information is available regarding the relationship between YAP and drug response in lung cancer especially in lung CSCs (LCSCs). CDDP is the first line chemotherapy drug for lung cancer. Since YAP was found to be responsible for CDDP resistance in gastric and ovarian CSCs, we hypothesized that YAP may likewise play a role in regulating cellular response to CDDP in LCSCs. Previously, we showed that A549 lung cancer cells could form tumor spheres with LCSC properties and enhanced drug resistance (11). In this study, we explored YAP expression in LCSCs, and inhibited YAP expression by siRNA to observe the proliferation inhibition and apoptosis of LCSCs. We presumed that inhibiting YAP is a new therapeutic strategy for human lung cancer though restraining the chemotherapy resistance of LCSCs.

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Materials and methods

Reagents and antibodies. Cell culture media Dulbecco's modified Eagle's medium (DMEM), DMEM/F-12 and fetal bovine serum (FBS) were obtained from Biological Industries (Cromwell, CT, USA). Trypsin and EDTA were obtained from Solarbio (Beijing, China), B27 was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA), EGF and bFGF were obtained from Proteintech (Rosemont, IL, USA). YAP rabbit monoclonal antibody was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA), β-actin mouse monoclonal antibody was purchased from Proteintech. The Cell Counting Kit-8 (CCK-8), TransZol Up Plus RNA kit, Transcript One-Step gDNA Removal and cDNA Synthesis Supermix, Goat Anti-Mouse/Rabbit IgG (H&L) were purchased from TransGen Biotech (Beijing, China). Annexin V-FITC apoptosis detection kit was obtained from BD Biosciences (San Diego, CA, USA). Western blot reagents were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA), and Pierce BCA Protein Assay kit was purchased from Applygen Technologies Inc. (Beijing, China). Small interfering RNA oligos were purchased from Invitrogen (Thermo Fisher Scientific, Inc.) and Lipofectamine® 2000 reagent purchased from TransGen (Beijing, China).

Cell line. The human NSCLC cell line A549, obtained from the Second Affiliated Hospital of Nanchang University, was cultured in DMEM supplemented with 10% FBS (v/v), streptomycin and penicillin and incubated at 37˚C under a humid atmosphere with 5% CO_2. A549 cell spheres were generated using the method as previously described (11). Briefly, A549 cells were seeded in the 6-well plates at the density of 1,000-1,500 cells per well and cultured in 2 ml 10% FBS supplemented DMEM for 8-10 days. Different single-cell derived clones formed after 8-10 days, including holoclone, meroclones and paraclones, of which holoclone consisted of tightly packed cells and demonstrated stem cell properties, such as continuous passage and unlimited proliferation. The holoclones were collected and cultured in serum-free media DMEM/F-12 supplemented with bFGF, EGF, insulin and B27. Primary A549 cell spheres were shaped after 10-14 days and were continuously grown in serum-free media to obtain secondary A549 cell spheres.

Transfection with siRNA for YAP. A549 cells were cultured in DMEM supplemented with 10% FBS, while A549 cell spheres were cultured in serum-free DMEM/F-12 supplemented with bFGF, EGF, insulin and B27. After 24 h, small interfering RNA targeting YAP (siYAP: 5'-GGUCAGAGA UACUUCUUAATT-3') and negative control (siNC: 5'-UUC UCCGAACGUGUCACGUTT-3') were introduced into cells using Lipofectamine® 2000 reagent. CDDP was added after two days at a concentration of 2.5 μg/ml.

Total RNA isolation and reverse transcription-PCR. Total RNA of A549 cells and A549 cell spheres under different treatment was extracted using TransZol Up Plus RNA kit following the manufacturer's instructions and then the RNA concentration were measured through ultraviolet spectrophotometer. cDNA synthesis was in progress with 1 μg of RNA using Transcript One-Step gDNA Removal and cDNA Synthesis Supermix according to the manufacturer's instructions. PCR was performed for 35 cycles using the following temperature profiles 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 60 sec. β-actin was served as an internal control. The sequence of primers includes: YAP_F: 5'-TGACCCTCG TTTTGCCATGA-3', YAP_R: 5'-GTTGCTGCTGGTTGG AGTTG-3'. ABCB1_F: 5'-GTCTGGACAAGCACTGAAA-3', ABCB1_R: 5'-AACAACGGTTCGGAAGTTT-3'. β-actin: F: 5'-CACGGCATCGTCACCAACT-3', R: 5'-GTCCTACGG AAAACGGCAGA-3.

Western blot analysis. Total protein of A549 cells and A549 cell spheres under different treatments was extracted using RIPA lysis buffer and protein concentration was determined by BCA method. Then proteins were denatured in 5x SDS-PAGE protein loading buffer, separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with antibody recognizing YAP (1:500) and β-actin (1:2,000), and protein bands were detected with the ECL detection system and image lab software.

Assessment of proliferation by CCK-8. 10% CCK was added into the culture media of A549 cells and A549 cell spheres under different treatments. After incubation for another 4 h, cell culture absorbance at 450 nm was read by microplate reader.

Annexin V-FITC staining. A549 cells and A549 cell spheres under different treatments were stained with Annexin V-FITC/PI following manufacturer's instructions. And apoptotic cells were analyzed determined by BD flow cytometer.

Statistical analysis. Image J software was used for data quantification and GraphPad Prism 6 software was used for the statistical analysis. Mean ± SD of three replicates are shown. An unpaired two-tailed Student's t test was used to compare the means of two groups. Two-way analysis of variance (ANOVA) was used to analyze the differences among group means, followed by a Student-Newman-Keuls (SNK) test to compare the differences between different groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Generation of A549 tumor spheres. Previously, we and others showed that adherent lung cancer A549 cells cultured in serum-free medium could form tumor spheres enriched for stem-like cells (11,12). Notably, A549 tumor spheres exhibited enhanced proliferation, cell-cycle progression as well as drug-resistant properties vs. A549 adherent cells. The morphologies of adherent A549 cells and tumor spheres are shown in Fig. 1. Specifically, A549 cells formed three morphologically different colonies: holoclone, meroclone and paraclone by using single-cell cloning culture. Only holoclones were selected, digested and further incubated with serum-free media. After three to four weeks, highly clustered tumor cell spheres were formed (Fig. 1).

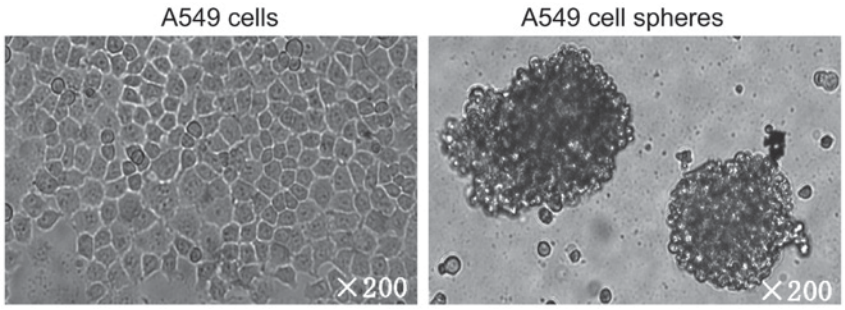

Figure 1. Morphologies of A549 adherent cells and A549 spheres. Adherent A549 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and floating A549 spheres were cultured in serum-free DMEM/F-12 medium. DMEM, Dulbecco's modified Eagle's medium.

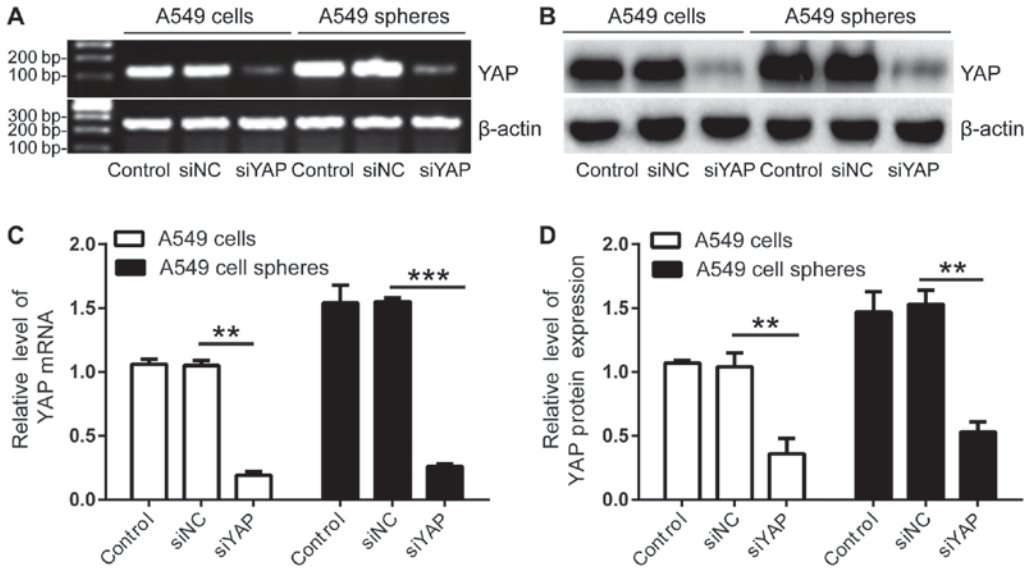

Figure 2. YAP expression in A549 cells and A549 spheres. (A) YAP expression at the mRNA level was determined by reverse transcription-polymerase chain reaction. (B) YAP expression at the protein level was determined by western blotting. The band intensity of (C) YAP mRNA and (D) YAP protein were normalized against β-actin. Data are presented as the mean ± standard deviation of three replicates. **P<0.01 and ***P<0.001. YAP, Yes-associated protein; si, short interfering; NC, negative control.

Elevated YAP expression in A549 cell spheres. Our previously studies revealed some stem cell-like properties of A549 cell spheres, including elevated expression of various stem cell markers (e.g., Sca-1, CD133, CD44s, Oct4, Sox3, Nanog) and the capability of multilineage differentiation (11). Interestingly, here our results showed that expression of YAP was likewise enhanced in A549 tumor spheres as compared with adherent A549 culture at both mRNA and protein levels (Fig. 2A and B), supporting the association of YAP with the stemness of A549 cell spheres. Notably, YAP expression was effectively knockdown using siRNA, thus allowing further investigation of YAP function in these cells (Fig. 2A-D).

Knockdown of YAP resensitized A549 cells and A549 spheres proliferation to CDDP. YAP overexpression has been implicated in the drug resistance of various CSCs. Here, we tested if YAP was associated with lung cancer cell resistance to CDDP. We treated A549 cells and A549 tumor spheres with increasing dosage of CDDP ranging from 0.001 to 50 μg/ml for 48 h. Cells were transfected with siYAP or siNC as a control. As a minor toxicity was observed by using the transfection reagent alone, here we didn't include non-transfected cells as a control. As shown in Fig. 3A, in siNC treated cells, the half maximal inhibitory concentration (IC_{50}) of CDDP was determined as 4.46 μg/ml for A549 cells and as 9.75 μg/ml for A549 spheres. The proliferation of 37.04% (±3.06%) A549 cells and 24.14% (±2.16%) A549 cell spheres were inhibited by 2.5 μg/ml CDDP (Fig. 3B), indicating the poor efficacy of CDDP on lung cancer cells. Notably, as compared with adherent cells, A549 cell spheres demonstrated higher IC_{50} of CDDP and lower inhibitory rate, suggesting increased drug resistance in tumor spheres (Fig. 3A and B). Of note, upon YAP knockdown, CDDP toxicity was significantly enhanced in these cells. The IC_{50} values of CDDP was reduced to 0.52 μg/ml A549 cells and 2.23 μg/ml for A549 spheres after YAP knockdown (Fig. 3A). Treatment with 2.5 μg/ml CDDP resulted in a significant inhibition of cancer cell proliferation in both A549 cells and A549 cell spheres by (Fig. 3B, all P<0.001). Moreover, a strong synergistic effect was observed when CDDP was combined with YAP knockdown (Fig. 3B, all P<0.001). All these evidences implied the potential of YAP silencing as an adjuvant therapy to chemotherapy.

Knockdown of YAP induced apoptosis. Since YAP is responsible for apoptosis suppression, we then investigated

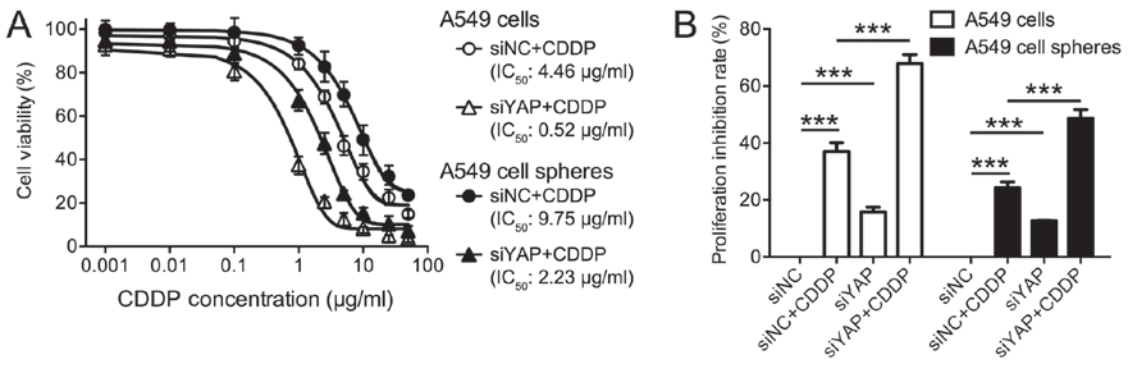

Figure 3. YAP silencing significantly enhances the efficacy of CDDP. (A) Dose response curve to determine the IC_{50} of CDDP in A549 cells and A549 tumor spheres with YAP knockdown or control knockdown. (B) The inhibitory effect of 2.5 μg/ml CDDP on A549 cells and A549 tumor spheres with YAP knockdown or control knockdown. Data are presented as the mean ± standard deviation of three replicates. ***P<0.001. CDDP, cisplatin; YAP, Yes-associated protein; si, short interfering; NC, negative control.

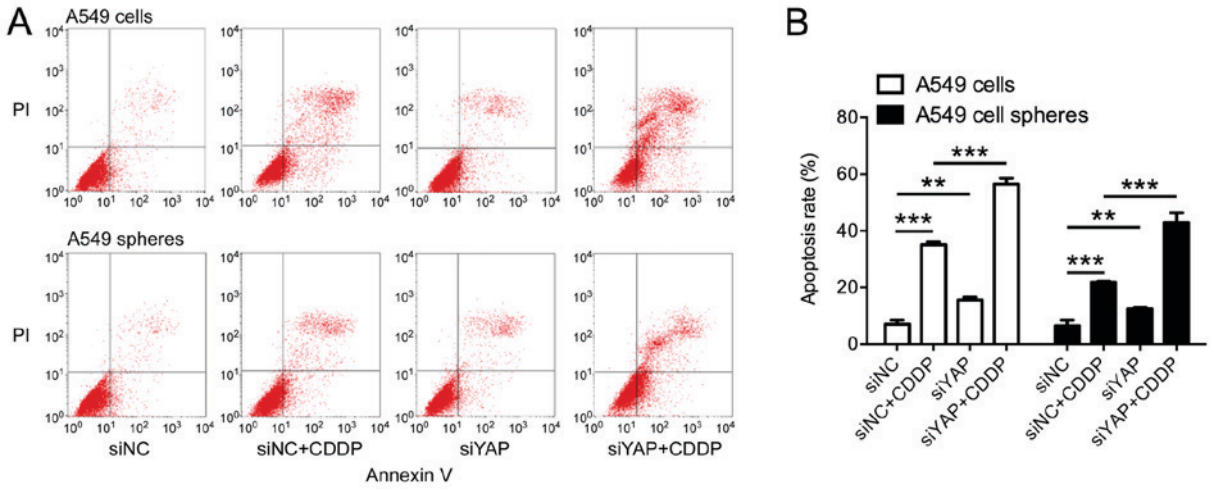

Figure 4. YAP silencing significantly induces apoptosis. (A) A549 adherent cells and A549 spheres under different treatments were collected, stained by Annexin V/propidium iodide and analyzed by flow cytometry. (B) The percentage of A549 cells and A549 sphere cells undergoing apoptosis upon different treatments. Data are presented as the mean ± standard deviation of three replicates. **P<0.01 and ***P<0.001. YAP, Yes-associated protein; CDDP, cisplatin; si, short interfering; NC, negative control.

the apoptosis of lung cancer cells on YAP depletion. As shown in Fig. 4, in both A549 cells and A549 spheres, more apoptotic cells were observed on YAP knockdown, suggesting the activation of apoptotic cascades. Moreover, we also found that CDDP induced apoptosis in 35.08% (±0.96%) A549 cells and 21.84% (±0.30%) A549 spheres, whereas much more cells were undergoing apoptosis on the co-treatment with siYAP, 56.50% (±2.07%) in A549 cells and 35.08% (±3.38%) in A549 spheres (Fig. 4B, all P<0.01), suggesting that YAP knockdown may enhance CDDP efficacy by promoting apoptosis.

Furthermore, we performed realtime PCR assay to examine the influence of YAP knockdown on ABCB1, which is a glycoprotein involved in multidrug resistance. As shown in Fig. 5, ABCB1 expression was significantly enhanced (P<0.01) in tumor spheres vs. adherent A549 cells, consistent with upregulation of YAP. Moreover, YAP silencing resulted in significant reduction of ABCB1 expression in both adherent A549 and tumor spheres (all P<0.01), indicative of a direct role of YAP in transcriptional regulation of ABCB1 expression in lung cancer cells.

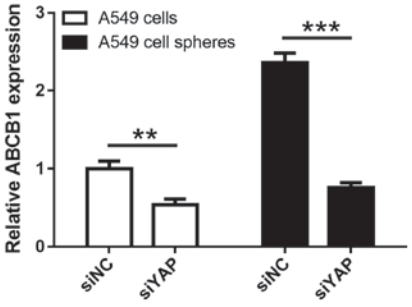

Figure 5. Dependency of ABCB1 expression on YAP. Reverse transcription polymerase chain reaction results demonstrating the relative ABCB1 expression in A549 adherent cells and A549 spheres with YAP knockdown or control knockdown. Data are presented as the mean ± standard deviation of three replicates. **P<0.01 and ***P<0.001. YAP, Yes-associated protein; si, short interfering; NC, negative control; ABCB1, ATP-binding cassette subfamily B member 1.

Discussion

According to our previous study, A549 cell spheres were

characterized with stem cell properties, including elevated expression of stem cell makers and the potential of self-renewal and multilineage differentiation (11). In the current study, we also found that A549 cell spheres demonstrated increased resistance to CDDP treatment, with enhanced cell proliferation and impaired apoptosis as compared with adherent A549 cells. This was consistent with our knowledge that CSCs are responsible for treatment failure and tumor recurrence in cancer patients and also provided A549 spheres as promising model to investigate drug response and develop novel strategies to overcome chemoresistance in NSCLC. In the present study, we found that YAP might be associated with the stemness and chemoresistance of A549 tumor spheres, while knockdown of YAP significantly enhanced sensitivity of A549 spheres to CDDP.

YAP is a major effector of Hippo tumor suppressor pathway, which is implicated in organ size control and tissue regeneration through regulating cell proliferation and apoptosis (13). YAP carries out its function by translocating into nuclear and inducing the transcription of genes involved in proliferation and anti-apoptosis (14,15), whereas the activation of Hippo pathway limits YAP function by inducing YAP phosphorylation and impairing its nuclear translocation (16,17). The major functions of the Hippo pathway have been involved in regulation of cell proliferation, differentiation, and migration in developing organs. High Hippo signaling activity has been observed in many cancer types, and functional dysregulation of Hippo signaling enhances the oncogenic properties of YAP and promotes tumorigenesis. Given these, the disruption of the balance between Hippo activity and YAP levels may disturb tissue homeostasis, and lead to a variety of disorders including cancers. Consistent with this, impaired Hippo pathway and elevated YAP expression has been frequently observed in solid tumor tissues, and YAP has been recognized as an oncogene, which is essential for cancer initiation, progression, or metastasis (18-25). Moreover, studies have also shown that overexpression of YAP is closely related to some carcinogenic properties of CSCs, such as unlimited self-renewal, the loss of cell contact inhibition (14), epithelial-mesenchymal transition (21,26) and anchorage-independent growth (27), which contribute to the development of drug resistance and result in cancer relapse (10,28,29). In the present study, we found that the expression of YAP was higher in A549 cell spheres than A549 cells, which support a role of YAP in maintaining certain characteristics of LCSCs.

It has been reported that YAP was responsible for the overexpression of anti-apoptotic Bcl-xL in hepatocellular carcinoma cells, which prevented the release of mitochondrial contents and inhibited caspase activation (30). In addition, YAP was also found to initiate chemoresistance in ovarian cancer cell via up-regulating the expression of drug resistance genes ABCB1, ABCC1 and GSK3A (5). In our study, we also provided evidences that elevated YAP expression might be associated with CDDP resistance in LCSCs, while YAP silencing increased CDDP toxicity to LCSCs by subjecting more cancer cells to apoptosis. The regulatory mechanism of YAP in lung cancer is still under investigation. Nevertheless, although YAP knockdown demonstrated the ability of restoring drug sensitivity, reduced CDDP efficacy was still observed in A549 spheres as compared with A549 cells, indicating higher basal line levels of YAP or existence of other drug resistance mechanisms.

In conclusion, our study identified increased drug resistance in LCSCs, which might be associated with the overexpression of YAP. We also provided evidences that YAP silencing could resensitize LCSCs to chemotherapy and could become a promising adjuvant therapy for NSCLC patients.

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Availability of data and materials

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

Authors' contributions

JS, LXX and XQY developed and designed the study and wrote the manuscript. JS, LXX, XYZ, PH and MFL performed the experiments. FX and JH performed the statistical analyses. 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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