Atorvastatin potentiates the chemosensitivity of human liver cancer cells to cisplatin via downregulating YAP1

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Abstract. Atorvastatin is a competitive inhibitor of β-hydroxy β-methylglutaryl-CoA reductase, which is involved in anticancer effects in numerous types of cancer, including in human liver cancer. However, its functions and underlying mechanisms of chemosensitivity in liver cancer remain to be elucidated. The present study investigated the effect of atorvastatin on cisplatin chemosensitivity and its molecular mechanisms, with a focus on the Yes1-associated transcriptional regulator (YAP1) protein. The present study demonstrated that atorvastatin significantly potentiated chemosensitivity to cisplatin in the liver cancer HepG2 and Huh-7 cell lines. Furthermore, cell survival and apoptosis in liver cancer cell lines were analyzed using MTT assay and flow cytometry, respectively. Atorvastatin suppressed HepG2 and Huh-7 cell viability in a dose-dependent manner, similar to cisplatin and paclitaxel. Subtoxic levels of atorvastatin significantly increased cisplatin-induced apoptosis in Huh-7 cells. Atorvastatin-promoted chemosensitivity was predominantly mediated by caspase 3, caspase 9 and poly-(ADP ribose)-polymerase activation, and YAP1 downregulation. Finally, YAP1 overexpression significantly reversed the susceptibility of Huh-7 cells to cisplatin. Overall, the results of the present study suggested the underlying mechanisms of atorvastatin chemosensitivity in inducing liver cancer cell apoptosis via downregulating YAP1 and implicated the potential application of atorvastatin-potentiated chemosensitivity in liver cancer therapy.

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Introduction

Liver cancer is the third main cause of cancer-associated mortalities worldwide, with 782,500 new diagnosed cases and 745,500 deaths estimated each year (1). Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most critical known risk factors of liver cancer (1). Although surgery and chemotherapy have improved the survival time of patients with liver cancer, a considerable number of patients still undergo recurrence due to the resistance of cancer cells to chemotherapeutic drugs (2). However, the chemoresistance mechanisms of liver cancer remain unknown. Therefore, the identification of drugs that increase sensitivity to liver cancer chemotherapy is essential for the development of effective therapies, which will be beneficial for patients.

Atorvastatin, a competitive inhibitor of β-hydroxy β-methylglutaryl-CoA reductase, exerts beneficial effects on circulating lipid levels and is used for the treatment and prevention of coronary heart disease and stroke (3-5). Additionally, atorvastatin has been proposed as an anticancer drug candidate, since previous studies have demonstrated that atorvastatin exerts antiproliferative, pro-apoptotic and immunoregulatory effects (6-9). However, the underlying mechanisms of atorvastatin-induced sensitization to chemotherapy in liver cancer has not been elucidated.

The present study investigated the synergistic effect of atorvastatin on cisplatin chemosensitivity and its associated molecular mechanisms. Additionally, the role of the Yes1-associated transcriptional regulator (YAP1) in liver cancer cells was evaluated. Furthermore, cell survival and apoptosis in liver cancer cell lines were analyzed using MTT assay and flow cytometry, respectively.

Materials and methods

Cell culture. The human liver cancer HepG2 and Huh-7 cell lines were purchased from the American Type Culture Collection. All cells were grown in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; Cytiva), 2 mM L-glutamine (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin (100 U/ml) and streptomycin (100 μg/ml; Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated at 37˚C with 5% CO_2 in a humidified incubator

and passaged at ≥80% confluence using trypsin (Gibco; Thermo Fisher Scientific, Inc.).

Drug treatment. Firstly, HepG2 and Huh-7 cells were incubated with different concentrations of atorvastatin (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μM; Selleck Chemicals) at 37˚C for 24 h. Secondly, 0, 10 and 100 μM atorvastatin combined with different concentrations of cisplatin (Selleck Chemicals) or paclitaxel (Selleck Chemicals) were incubated with HepG2 and Huh-7 cells for 24 h at 37˚C. Since HpG2 and Huh-7 cells had different sensitivities to cisplatin, the concentrations of cisplatin incubated with HepG2 or Huh-7 cells were 0, 0.25, 0.5, 1 and 10 μg/ml, and 0, 1, 5, 10 and 20 μg/ml, respectively, while the concentrations of paclitaxel incubated with HepG2 or Huh-7 cells were 0, 100, 500, 800 and 1,000 μM. Finally, 4 μg/ml cisplatin alone, 40 μM atorvastatin alone and 4 μg/ml cisplatin plus 40 μM atorvastatin were incubated with HepG2 cells for 24 h at 37˚C, while 5 μg/ml cisplatin alone, 100 μM atorvastatin alone and 5 μg/ml cisplatin plus 100 μM atorvastatin were incubated with Huh-7 cells for 24 h at 37˚C. Untreated cells were used as the control check (CK). After treatment, the cell viability assay was performed. Additionally, after treatment of Huh-7 cells with 5 μg/ml cisplatin alone, 100 μM atorvastatin alone, 300 μM paclitaxel alone, 5 μg/ml cisplatin plus 100 μM atorvastatin and 5 μg/ml cisplatin plus 300 μM paclitaxel for 24 h at 37˚C, the flow cytometric analysis of apoptosis was performed.

Cell viability assay. Cell viability of HepG2 and Huh-7 was tested *in vitro* using MTT assays. A total of $1x10^4$ cells were seeded in 96-well plates. Following treatment, cells were incubated with MTT solution (Sigma-Aldrich; Merck KGaA) in PBS for 3 h at 37˚C according to the manufacturer's protocol. The purple formazan was solubilized using DMSO. The absorbance was read on a microplate reader at a wavelength of 490 nm (Molecular Devices, LLC). The combination index (CI) values between 100 μM atorvastatin and cisplatin in treating HepG2 and Huh-7 cells were calculated using the following formula: Cell viability of cisplatin + atorvastatin group / (cell viability of cisplatin group x cell viability of atorvastatin group). The cut-off of CI value to determine whether a synergistic effect was observed was 1.

Flow cytometric analysis of apoptosis. Apoptosis was assessed using FITC-labeled Annexin-V (BD Biosciences) and propidium iodide (PI; Sigma-Aldrich; Merck KGaA) via flow cytometry. Briefly, following treatment for 24 h, Huh-7 cells were collected and stained with 500 μl solution containing Annexin V-FITC in the dark at room temperature for 30 min. This was followed by addition of PI for 5 min in the dark at room temperature. Flow cytometry (FACSCanto; Becton, Dickinson and Company) was used to detect fluorescent signals in the cells. Both early and late apoptotic cells were calculated using FlowJo 7.6 (FlowJo LLC).

Western blotting. Huh-7 cells were lysed in RIPA lysis buffer (Sigma-Aldrich; Merck KGaA), and protein concentration was quantified using a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Following protein separation (20 μg/lane) via 12 or 15% SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membranes. Subsequently, membranes were blocked in 5% skimmed milk for 90 min at 37˚C and incubated with primary antibodies against caspase 3, caspase 9, poly-(ADP ribose)-polymerase (PARP), YAP1 and β-actin overnight at 4˚C. Subsequently, membranes were incubated with an HRP-conjugated secondary antibody for 2 h at 37˚C.

The primary antibodies used for immunoblotting included anti-caspase3 (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc.), anti-caspase9 (1:1,000; cat. no. 9508; Cell Signaling Technology, Inc.), anti-PARP (1:1,000; cat. no. 9532; Cell Signaling Technology, Inc.), YAP1 (1:1,000; cat. no. 14074; Cell Signaling Technology, Inc.) and anti-β-actin (1:5,000; cat. no. A5316; Sigma-Aldrich; Merck KGaA). The secondary antibodies were HRP-conjugated anti-rabbit (1:3,000; cat. no. 7074; Cell Signaling Technology, Inc.) and anti-mouse IgG antibodies (1:3,000; cat. no. 7076; Cell Signaling Technology, Inc.). Protein bands were detected using an ECL chemiluminescence reaction kit (EMD Millipore).

Quantification of western blotting data, which was performed using ImageJ 2.0 (National Institutes of Health) was calculated as follows: i) Quantification of each protein density in triplicate; ii) quantification of β-actin density in triplicate; iii) dividing each protein density by the β-actin density to obtain the relative band density in triplicate; and iv) setting each replicate of relative density in the CK group as the control (as one), and the relative density in other groups was calculated based on the control.

Plasmid construction and transfection. The human YAP1 coding sequence was synthesized and subcloned into pcDNA3.1 (Addgene, Inc.). The integrity of the respective plasmid constructs was confirmed by DNA sequencing (data not shown). After $2x10^5$ Huh-7 cells were seeded in 6-well plates overnight at 37˚C, cells were transfected with 0.8 μg plasmid using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Additionally, control pcDNA3.1 was synthesized and served as a negative control. Following incubation for 24 and 48 h at 37˚C, the overexpression efficiency of the plasmid was determined using western blotting, as aforementioned. At 24 h after transfection, Huh-7 cells transfected with the empty vector pcDNA3.1 were treated with 5 μg/ml cisplatin alone or 5 μg/ml cisplatin plus 100 μM atorvastatin and Huh-7 cells transfected with the pcDNA3.1-YAP1 were treated with 5 μg/ml cisplatin alone or 5 μg/ml cisplatin plus 100 μM atorvastatin for another 24 h at 37˚C. Subsequently, the flow cytometric analysis of apoptosis was performed.

Statistical analysis. Data are presented as the mean ± SD from ≥3 separate experiments. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc.) and SPSS 13.0 (SPSS, Inc.) software packages. Statistical significance was determined using a two-sided unpaired Student's t-test or one-way ANOVA followed by Dunnett's or Tukey's multiple comparison test as appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. CI values between 100 μM atorvastatin and cisplatin in treating liver cancer cells.

Cisplatin doses	CI values
HepG2 cells	
0.25 μg/ml	1.034±0.028
0.5 μg/ml	0.950±0.020
1 μg/ml	0.978±0.103
10 μg/ml	1.229±0.167
Huh-7 cells	
1 μg/ml	1.081±0.024
5 μg/ml	0.834±0.028
10 μg/ml	0.837±0.080
20 μg/ml	0.613±0.081

CI, combination index.

Results

Cytotoxicity of atorvastatin alone or in combination with cisplatin or paclitaxel on liver cancer cells. To determine whether atorvastatin could inhibit liver cancer cell viability, two liver cancer cell lines, as well as cisplatin and paclitaxel, were used for experiments. Cytotoxicity was evaluated using an MTT assay. The results revealed that atorvastatin significantly suppressed HepG2 cell viability at 10, 30, 60, 70, 80, 90 and 100 μM, while Huh-7 cell viability was only inhibited at 80, 90 and 100 μM (Fig. 1A). Since 10 μM was the lowest concentration to inhibit cell viability and 100 μM was the highest (Fig. 1A), these concentrations were chosen for further experimentation. Additionally, the present study examined whether combined treatment of atorvastatin with cisplatin or paclitaxel exerted enhanced lethality on liver cancer cell lines. As shown in Fig. 1B-D, following co-treatment with the indicated concentrations of atorvastatin and cisplatin or paclitaxel for 24 h, cells were subjected to an MTT assay. The combination of atorvastatin and cisplatin significantly inhibited cell viability in HepG2 and Huh-7 cells, but only slightly with paclitaxel. Using 100 μM atorvastatin combined with 0-10 μg/ml or 0-20 μg/ml cisplatin significantly inhibited HepG2 or Huh-7 cell viability compared with 0 μM atorvastatin, respectively; additionally, 10 μM atorvastatin alone and 10 μM atorvastatin combined with 10 μg/ml cisplatin significantly inhibited HepG2 cell viability compared with 0 μM atorvastatin (Fig. 1B). However, only 100 μM paclitaxel combined with 100 μM atorvastatin or 100 μM atorvastatin alone significantly inhibited cell viability in both HepG2 and Huh-7 cells compared with 0 μM atorvastatin; additionally, 10 μM atorvastatin with 100 or 500 μM paclitaxel significantly inhibited HepG2 cell viability compared with 0 μM atorvastatin (Fig. 1C). Further experiments indicated that 4 μg cisplatin combined with 40 μM atorvastatin significantly inhibited HepG2 cell viability and 5 μg cisplatin combined with 100 μM atorvastatin significantly inhibited Huh-2 cell viability compared with the control Fig. 1D). These results indicated that atorvastatin may potentiate the chemosensitivity of liver cancer cells to cisplatin. Furthermore, CI values were calculated based on relative cell viability data, revealing that atorvastatin synergized with 5-20 μg/ml cisplatin in killing Huh-7 cells (CI values <1), but not HepG2 cells (CI values near or >1) (Table I).

Atorvastatin potentiates the chemosensitivity of liver cancer cells by inducing apoptosis. Subsequently, whether the sensitization effect of atorvastatin to cisplatin and paclitaxel involved the induction of apoptosis was examined. Huh-7 cells were subjected to flow cytometry analysis following treatment with 100 μM atorvastatin alone or in combination with 5 μg/ml cisplatin or 300 μM paclitaxel. The drug concentrations used for these experiments were determined due to the following: i) 100 μM atorvastatin, but not 10 μM atorvastatin, significantly enhanced the effect of cisplatin in suppressing relative Huh-7 cell viability (Fig. 1B) and 100 μM atorvastatin was therefore chosen for subsequent experiments; ii) 5 μg/ml cisplatin plus 100 μM atorvastatin achieved ~50% of Huh-7 cell inhibition rate (Fig. 1B), therefore 5 μg/ml cisplatin was chosen for subsequent experiments; and iii) paclitaxel at various concentrations plus 100 μM atorvastatin did not achieve 50% of Huh-7 cell inhibition rate (Fig. 1C), but 100 μM atorvastatin enhanced the effect of paclitaxel on Huh-7 cell inhibition at 100 μM, but not 500 μM paclitaxel, therefore 300 μM paclitaxel (the median between 100 and 500) was chosen for subsequent experiments.

As shown in Fig. 2A and B, atorvastatin significantly enhanced cisplatin-induced apoptosis in Huh-7 cells. The percentage of Annexin-V$^+$ cells increased from 16.37% (atorvastatin alone) and 23.12% (cisplatin alone) to 54.62% (atorvastatin combined with cisplatin). However, atorvastatin slightly enhanced paclitaxel-induced apoptosis in Huh-7 cells. The percentage of Annexin-V$^+$ cells increased from 16.37% (atorvastatin alone) and 14.35% (paclitaxel alone) to 32.35% (atorvastatin combined with paclitaxel) (Fig. 2A and C). The present results suggested that atorvastatin significantly potentiated cisplatin sensitivity in Huh-7 cells via inducing apoptosis, while atorvastatin only slightly potentiated paclitaxel sensitivity in Huh-7 cells.

Apoptosis is involved in the synergistic effect of atorvastatin on cisplatin sensitivity in liver cancer cells. There are two fundamental pathways of apoptosis, which are the extrinsic and intrinsic apoptosis pathways (10,11). Cleavage of caspases and PARP are hallmarks of intrinsic and extrinsic apoptosis pathways activation (12). As shown in Fig. 3A and B, co-treatment with atorvastatin and cisplatin in Huh-7 cells significantly increased the cleavage of caspases 3 and 9, and PARP compared with CK. Additionally, increasing evidence has demonstrated that increased YAP1 expression is involved in liver cancer progression and chemoresistance (13,14). To evaluate the effect of atorvastatin treatment on the expression of YAP1 in liver cancer, the Huh-7 cells treated with atorvastatin. As shown in Fig. 3C and D, atorvastatin treatment significantly inhibited YAP1 protein levels. The current observations indicated that the intrinsic and extrinsic apoptotic pathways and YAP1 may be involved in the synergistic effect of atorvastatin on cisplatin sensitivity in liver cancer cells.

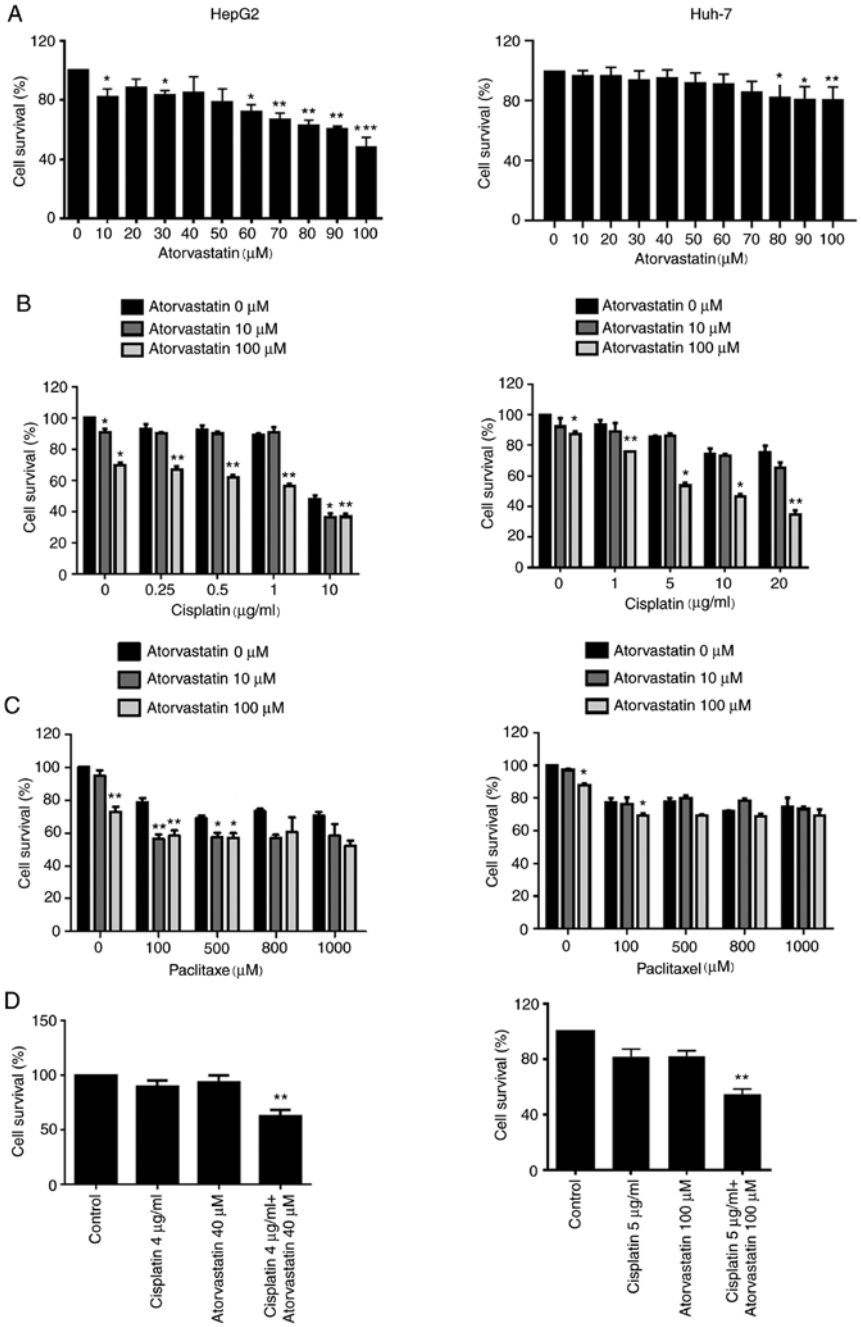

Figure 1. Cytotoxicity of atorvastatin alone or in combination with cisplatin or paclitaxel in human liver cancer cells. (A) Atorvastatin alone inhibited human liver cancer cell viability *in vitro*. The cancer cells were incubated in the presence of various concentrations of atorvastatin for 24 h. Cell viability was determined using an MTT assay. Atorvastatin at the indicated concentrations combined with various concentrations of (B) cisplatin and (C) paclitaxel inhibited human liver cancer cell viability *in vitro*. Cell viability was determined using an MTT assay. (D) Atorvastatin (40 or 100 μM) combined with 4 or 5 μg/ml cisplatin, respectively, in HepG2 and Huh-7 inhibited human liver cancer cell viability *in vitro*. Data are presented as the mean ± SD of three separate experiments. One-way ANOVA followed by Dunnett's multiple comparison test was used. *P<0.05, **P<0.01 and ***P<0.001 vs. control or 0 μM.

Atorvastatin enhances the effect of cisplatin on treating liver cancer cells via regulating YAP1 expression. To further confirm whether atorvastatin enhanced cisplatin chemosensitivity via YAP1, Huh-7 cells were transfected with a YAP1 overexpression plasmid or an empty vector pcDNA3.1 The transfection efficiency was verified using western blotting, revealing that YAP1 levels were significantly increased in Huh-7 cells transfected with YAP1 expression plasmid after both 24 and 48 h of transfection (Fig. 4A and B).

Huh-7 cells were transfected with empty vector pcDNA3.1 or pcDNA3.1-YAP1 plasmid and incubated for 24 h. Subsequently, cells were treated with 5 μg/ml cisplatin and 100 μM atorvastatin for another 24 h. Following treatment, flow cytometry was performed to determine the apoptotic cell percentage by co-staining with Annexin V-FITC and PI (Fig. 4C). YAP1 overexpression significantly attenuated the apoptosis mediated by the combination of atorvastatin and cisplatin (Fig. 4D). Overall, the present results indicated that atorvastatin may sensitize liver cancer cells to cisplatin, at least partially via inhibiting YAP1.

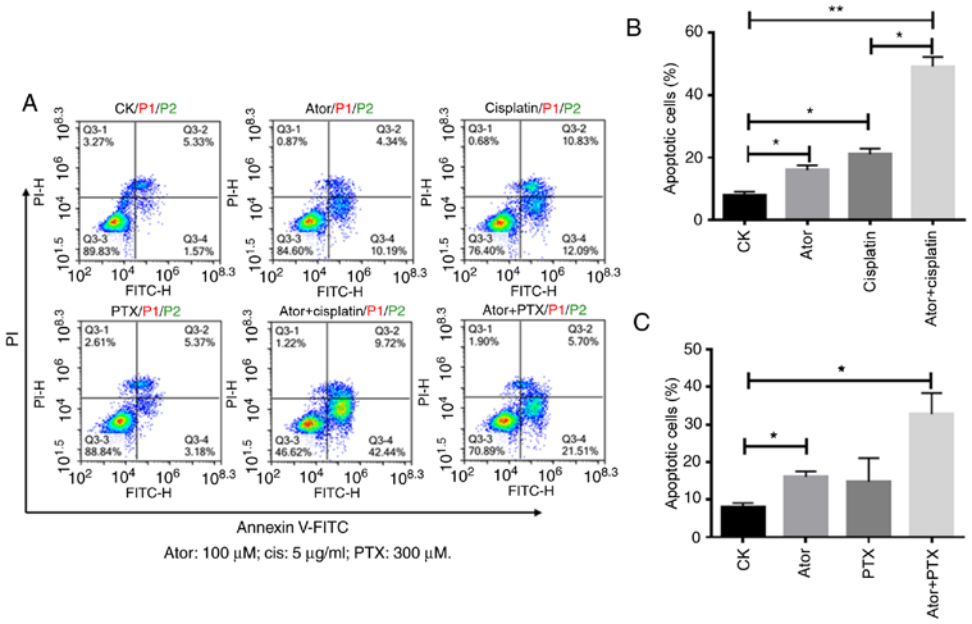

Figure 2. Atorvastatin potentiates chemosensitivity to cisplatin or paclitaxel in human liver cancer cells by modulating apoptosis. (A) Huh-7 cells were treated with the indicated concentrations of atorvastatin alone and/or combined with indicated concentrations of cisplatin or paclitaxel for 24 h. Cells were subjected to Annexin V-FITC and PI staining, and flow cytometry was performed to detect the percentage of apoptotic cells. Quantification of apoptosis in cells treated with (B) cisplatin or (C) paclitaxel. Data are presented as the mean ± SD of three separate experiments. One-way ANOVA followed by Tukey's multiple comparison test was used. *P<0.05 and **P<0.01. PI, propidium iodide; Ator, atorvastatin; PTX, paclitaxel; cis, cisplatin; CK, control check.

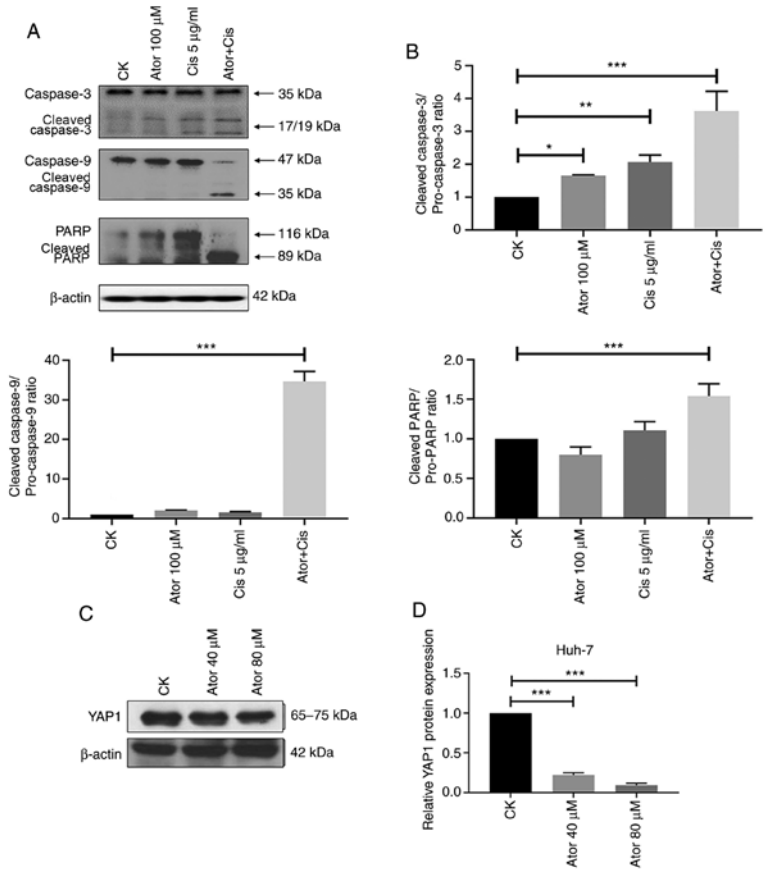

Figure 3. Apoptotic pathway is initiated in the sensitization effect of atorvastatin on cisplatin in human liver cancer cells. (A) Huh-7 cells were treated with 100 μM atorvastatin alone or combined with 5 μg/ml cisplatin. Proteins were extracted and subjected to western blotting to evaluate the levels of cleaved caspase 3 (17/19 kDa) and 9 (35 kDa) and PARP (89 kDa). (B) Quantitative analysis of the protein bands. Data are presented as the mean ± SD of three separate experiments. (C) Huh-7 cells were treated with the indicated concentrations of atorvastatin alone. Proteins were extracted and subjected to western blotting to evaluate YAP1 expression. (D) Quantitative analysis of YAP1 protein expression. Data are presented as the mean ± SD of three separate experiments. One-way ANOVA followed by Dunnett's multiple comparison test was used. *P<0.05, **P<0.01 and ***P<0.001. PARP, poly-(ADP ribose)-polymerase; YAP1, Yes1-associated transcriptional regulator; Ator, atorvastatin; Cis, cisplatin; CK, control.

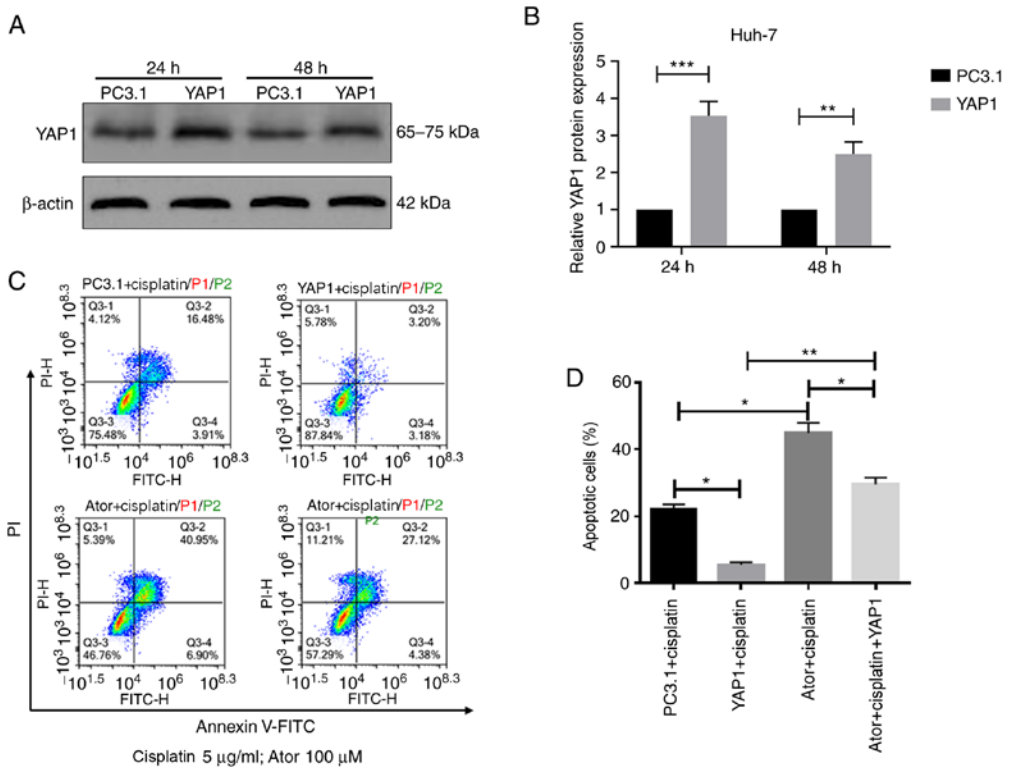

Figure 4. Atorvastatin enhances the effect of cisplatin in treating liver cancer cells via regulating YAP1 expression. (A) Huh-7 cells were transfected with empty vector pcDNA3.1 or pcDNA3.1-YAP1 and incubated for 24 h. The transfection efficiency was confirmed by western blotting. (B) Quantitative analysis of YAP1 protein expression levels. A two-sided unpaired Student's t-test was used. (C) For flow cytometry analysis, Huh-7 cells were transfected with empty vector pcDNA3.1 or pcDNA3.1-YAP1 and incubated for 24 h. Cells were treated with 5 μg/ml cisplatin and 100 μM atorvastatin for another 24 h. Following treatment, flow cytometry was performed to determine the apoptotic cell percentage by co-staining with Annexin V-FITC and PI. (D) Quantification of apoptosis levels. Data are presented as the mean ± SD of three separate experiments. One-way ANOVA followed by Tukey's multiple comparison test was used. *P<0.05, **P<0.01 and ***P<0.001. YAP1, Yes1-associated transcriptional regulator; PI, propidium iodide; Ator, atorvastatin.

Discussion

To date, increasing evidence has associated the YAP1 oncogene to tumorigenesis of several types of cancer, including pancreatic ductal adenocarcinoma, lung cancer, colon cancer, prostate cancer and liver cancer (15-19). YAP1 is the downstream effector of the Hippo signaling pathway, and in cooperation with the TEA domain transcription factor 1, increased YAP1 expression stimulates a number of target genes responsible for cell viability and apoptosis (20,21). Several studies have demonstrated that increased YAP1 expression is associated with elevated drug resistance in numerous cancer cells, such as neuroblastoma, esophageal cancer and colorectal cancer cells (22-25). The present study investigated the mechanism of the synergistic effects of YAP1 with cisplatin. Firstly, the present study revealed that atorvastatin inhibited liver cancer cell viability in a dose-dependent manner. Secondly, the present study demonstrated that sub-cytotoxic levels of atorvastatin sensitized HepG2 and Huh-7 cells to different concentrations of cisplatin and paclitaxel using an MTT assay. Subsequently, the synergistic effect of atorvastatin on cisplatin or paclitaxel sensitivity was analyzed, revealing that this mechanism involved apoptosis induction in Huh-7 cells subjected to flow cytometry analysis following treatment with atorvastatin alone or in combination with cisplatin or paclitaxel. The present results suggested that atorvastatin may regulate the intrinsic and extrinsic apoptotic pathways to increase cell sensitivity to cisplatin and paclitaxel. In addition, western blotting was performed to evaluate the protein expression levels of cleaved caspase 3 and 9, and PARP, which were all upregulated in Huh-7 cells co-treated with atorvastatin and cisplatin compared with CK.

Finally, the YAP1 protein was further investigated, since increased YAP1 expression is best known as a regulator of cell viability, survival and chemoresistance (26-29). The present study demonstrated that YAP1 levels were decreased by atorvastatin treatment in Huh-7 cells. Furthermore, transfecting Huh-7 cells with pcDNA3.1-YAP1 expression plasmid significantly reversed the apoptosis mediated by the combination of atorvastatin with cisplatin. Therefore, the current data revealed that atorvastatin may potentiate the chemosensitivity of liver cancer cells to cisplatin by regulating YAP1 expression.

Despite the findings of the present study, there are still some limitations. First, the combined effect of atorvastatin plus cisplatin or paclitaxel on cell apoptosis, and protein expression levels, such as YAP1, were detected in a single cell line; therefore, further validation in multiple cell lines is required in future studies. Second, the deeper molecular mechanism of atorvastatin plus cisplatin treatment via YAP1 requires further exploration. Third, due to lack of funding, *in vivo* validation was not performed in the present study and should therefore be performed in future studies.

In conclusion, the current results demonstrated that elevated levels of YAP1 in liver cancer may serve a role in cancer cell chemoresistance. Although other downstream target genes may also be involved in regulating apoptosis following atorvastatin treatment, the present data illustrated that atorvastatin may potentiate chemosensitivity to cisplatin in liver cancer cells by regulating YAP1, which may serve a role as an apoptosis suppressor. Therefore, the results of the present study indicated that atorvastatin plus cisplatin therapy may be a potential strategy for the treatment of chemoresistant liver cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GS designed the experiments. LG, JZ, HZ and ZZ performed the experiments. LG and JZ analyzed the data. LG wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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