

Silibinin sensitizes CD133+ hepatocellular carcinoma cells to cisplatin treatment through suppression of OPA1

Jikang Yang

Jiaozuo People's Hospital

Zhiyuan Xing (✉ jzhiyanxing@aliyun.com)

Jiaozuo People's Hospital

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Abstract

Background: Drug resistance is still a major obstacle during the cisplatin-based chemotherapy of hepatocellular carcinoma (HCC). Recently, studies have indicated that the population of CD133⁺ cancer cells is partially responsible for the failure of cancer treatment. However, the potential mechanisms are still unclear.

Methods: CD133⁺ HepG2 and Huh7 cells were sorted via flow cytometry. CCK-8 assay was used to detect the cytotoxicity of cisplatin and silibinin against HCC cells. Western blot assay was performed to detect the protein expression, cleavage of caspases and release of cytochrome c from mitochondria into cytosol. Flow cytometry analysis was used to measure the apoptotic rate of CD133⁺ HepG2 and Huh7 cells.

Results: CD133⁺ HepG2 and Huh7 cells were observed to exhibit obvious resistance against cisplatin. However, co-treatment with silibinin significantly reduced the cisplatin resistance of CD133⁺ HepG2 and Huh7 cells. Furthermore, although CD133⁺ HepG2 and Huh7 cells were resistant to cisplatin-induced apoptosis, co-treatment with silibinin enhanced the cisplatin-induced apoptosis through promoting the release of cytochrome c from mitochondria into cytosol. In the mechanism research, we proved that silibinin inhibited the expression of OPA1 in CD133⁺ HepG2 and Huh7 cells. Under the stress of cisplatin, silibinin promoted the collapse of mitochondria and increased the release of cytochrome c. As a result, caspases-dependent apoptosis was induced in CD133⁺ HepG2 and Huh7 cells which were co-treated with cisplatin and silibinin.

Conclusion: Silibinin sensitizes CD133⁺ HCC cells to cisplatin-induced apoptosis through suppression of OPA1.

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Because of the late diagnosis and poor prognosis, HCC represents as one of the leading causes of cancer-related deaths [1, 2]. Although surgery and liver transplants are the most effective treatment strategy for the early stage of HCC, a large percentage of HCC patients have reached a stage beyond curative surgery when they are diagnosed [3, 4]. For these HCC patients in advanced stage, systematic chemotherapy is irreplaceable and valuable [5, 6]. However, HCC cells are usually resistant to anti-tumor drugs [7, 8]. It is urgent to explore the potential mechanisms and develop the strategies against the drug resistance.

CD133 is a pentaspan transmembrane glycoprotein encoded by the prominin 1 (PROM1) gene [9]. Recently, studies indicate that HCC patients with high proportion of CD133 positive cancer cells show shorter overall survival and higher recurrence rate compared to the patients with low proportion of CD133 positive cancer cells [10, 11]. More importantly, previous studies have demonstrated that CD133 positive cancer cells are resistant to conventional chemotherapy in various cancers including HCC [12–

14]. Therefore, the population of CD133 positive cancer cell is responsible for chemotherapy failure and cancer relapse. This population of cancer cell has become a novel target to overcome the drug resistance of HCC.

Silymarin is mainly extracted from milk thistle (*Silybum marianum*). Silibinin is a nature flavonoid and a major bioactive component of silymarin [15, 16]. Pharmacological studies have indicated that silibinin has favourable effects on jaundice, hepatitis and gallbladder disease [17, 18]. Furthermore, recent studies have revealed the anti-tumor effect of silibinin. For instance, silibinin can partially inhibit the proliferation of androgen-independent prostate cancer cells through increasing the insulin-like growth factor-binding protein 3 expression [19]; Silibinin suppresses bladder cancer progression through inhibiting the PI3K/AKT signaling pathway [20]. However, little is known regarding to the effect of silibinin on the CD133 positive population of HCC. The aim of this study is to explore the effect of silibinin on cisplatin treatment against CD133 positive HCC cells.

Methods

Cell lines

Human HCC cell lines HepG2 and Huh7 were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37 °C in a humidified incubator with 5% CO₂ and 95% air. To obtain the CD133⁺ and CD133⁻ HepG2 and Huh7 cells, HepG2 and Huh7 cell lines were stained with mouse anti-human CD133/1 (AC133) conjugated with VioBright FITC (Miltenyi Biotec, Germany) for 30 min at 4 °C in dark. Subsequently, cells were sorted by fluorescent-activated cell sorting equipment (Beckman Coulter, USA).

Gain-of-function and Loss-of-function of OPA1

To knockdown the gene of OPA1, OPA1 small interfering RNA (OPA1 siRNA, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. To overexpress OPA1, the OPA1 eukaryotic expression plasmid was generated by cloning the open reading frame of the OPA1 gene into the pcDNA3.1 plasmid (Life Technologies, Carlsbad, CA, USA). For transfection, OPA1 siRNA (50 pmol/ml) or OPA1 plasmid (2 µg/ml) plasmid was transfected by using the Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

Cell viability assay

Cells were inoculated onto the 96-well plate at a density of 5,000 cells/well. After overnight incubation, the medium was replaced with fresh medium containing different concentrations of cisplatin and silibinin (Sigma Aldrich, St. Louis, MO, USA) for 48 h. Subsequently, CCK-8 (10 µl) (Sigma Aldrich) was added to each well and incubated for 2 h at 37 °C. In each well, the absorbance was measured at 570 nm. Cell viability was calculated by using the following formula: Cell viability rate = OD value in drug

administration group/ OD value in control group. Half maximal inhibitory concentration (IC50) of cisplatin was calculated according to the cell viability curve.

Combination index (CI) with cisplatin and resveratrol

CI was based on the results of cell viability assay and was calculated by using the following formula: $CI = E(A + B)/(EA + EB - EA \times EB)$. EA represents as the inhibitory rate caused by cisplatin; EB represents as the inhibitory rate caused by silibinin; $E(A + B)$ represents as the inhibitory rate caused by combination treatment with cisplatin and silibinin. It was considered as simple addition of the two drugs when CI ranged from 0.85 to 1.15; It was considered as the synergistic effect of the two drugs when CI was greater than 1.15; It was considered as the antagonistic effect of the two drugs when CI was less than 0.85.

Separation of mitochondria fraction and cytosol fraction

Cells were inoculated onto the 6-well plate at a density of 5×10^5 cells/well. After overnight incubation, the medium was replaced with fresh medium containing cisplatin (5 μ M) and silibinin (5 μ M) for 48 h. Cells were then collected and washed with PBS. Subsequently, Mitochondria fraction and cytosol fraction of HCC cells were separated by using Mitochondria/Cytosol Fraction Kit (BioVision, USA) according to the manufacturer's instruction. The obtained mitochondria fraction and cytosol fraction was directly used for the western blot assay.

Western blot analysis

Total proteins from HCC cells were extracted by using lysis buffer (Cell Signaling Technology, Danvers, MA, USA). 50 μ g of the obtained proteins were separated on a 12.5% SDS-PAGE system as described [21]. Subsequently, proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA) in transfer buffer. After blocking with 5% non-fat dried milk for 2 h, the membranes were incubated with the primary antibodies. Antibodies of anti-Mfn2 and anti-OPA1 were purchased from Abcam (Cambridge, MA, USA); antibody of anti-Mfn1 was purchased from Sigma Aldrich. Antibodies of anti-cytochrome c, anti-caspase-9, anti-caspase-3 and anti-GAPDH were purchased from Cell Signaling Technologies. After incubation with primary antibodies overnight at 4 °C, the immunoreactive bands were visualized by using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection kit (Pierce, Rockford, IL, USA).

Measurement of apoptotic rate

Cells were inoculated onto the 6-well plate at a density of 5×10^5 cells/well. After overnight incubation, the medium was replaced with fresh medium containing cisplatin (5 μ M) and silibinin (5 μ M) for 48 h. Cells were then collected and washed with PBS. Subsequently, cell apoptosis was measured by using Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich) according to the manufacturer's instruction. Annexin V positive cells were calculated as the apoptotic cells.

Statistical analysis

Data (mean \pm standard deviation) were statistically analyzed by using one-way analysis of variance (ANOVA) and Bonferroni's *post hoc* test through SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant.

Results

Resistance of CD133 positive HCC cells to cisplatin

To investigate the cisplatin resistance of CD133 positive HCC cells, CD133⁺ and CD133⁻ population was sorted by flow cytometry in HepG2 and Huh7 cell lines. As shown in Fig. 1a, the sorted CD133⁺ and CD133⁻ HCC cells showed high purity and can be confirmed. We found that cisplatin sensitivity of CD133⁺ population of HepG2 and Huh7 was obviously lower than the CD133⁻ population of HepG2 and Huh7 (Fig. 1b). Specifically, IC₅₀ of cisplatin to CD133⁺ HepG2 was 7.8 fold higher than the CD133⁻ HepG2, and IC₅₀ of cisplatin to CD133⁺ Huh7 was 5.3 fold higher than the CD133⁻ Huh7 (Fig. 1c). Our data indicated that CD133 positive HCC cells were resistant to cisplatin treatment.

Silibinin increased the cytotoxicity of cisplatin against CD133⁺ HCC cells

To investigate the effect of silibinin on cisplatin-induced cytotoxicity against CD133⁺ HCC cells, we treated the CD133⁺ HepG2 and Huh7 cells with different concentrations of cisplatin and silibinin. We then found that combination index (CI) with cisplatin and silibinin was greater than 1.15 on CD133⁺ HepG2 (Table 1) and CD133⁺ Huh7 (Table 2). These data confirmed the synergistic effect of silibinin and cisplatin. As the CI with silibinin and cisplatin was highest at the concentration of 5 μ M (cisplatin) and 5 μ M (silibinin), we chose the two concentrations of cisplatin and silibinin for the following experiments. Next, we found that silibinin (5 μ M) obviously increased the sensitivity of CD133⁺ HepG2 and Huh7 cells to different concentrations of cisplatin (Fig. 2a). Specifically, silibinin decreased the IC₅₀ of cisplatin to CD133⁺ HepG2 by 87.8%. Meanwhile, silibinin decreased the IC₅₀ of cisplatin to CD133⁺ Huh7 by 81.5%. (Fig. 2b). These data indicated that silibinin increased the cytotoxicity of cisplatin against CD133⁺ HCC cells. On the other hand, we found that silibinin (5 μ M) can increase the sensitivity of CD133⁻ HepG2 and Huh7 cells to different concentrations of cisplatin (Fig. 2c). Meanwhile, silibinin reduced the IC₅₀ of cisplatin to CD133⁻ HepG2 and Huh7 cells (Fig. 2d). However, we indicated that the CD133⁺ HCC cells are more sensitive to silibinin compared to the CD133⁻ HCC cells.

Table 1
Combination index (CI) with cisplatin and silibinin in CD133⁺ HepG2

Cisplatin single treatment		Silibinin single treatment		Combination treatment	Combination index (CI)
Concentration (μM)	Inhibitory rate (%)	Concentration (μM)	Inhibitory rate (%)	Inhibitory rate (%)	
2	6.9	2	3.5	31.4	3.09
5	13.3	5	4.7	58.2	3.34
10	20.6	10	6.5	70.5	2.74
15	27.9	15	9.1	75.8	2.20
20	40.2	20	13.2	79.4	1.65
30	49.4	30	15.7	83.6	1.46
40	54.5	40	17.8	87.8	1.40

Table 2
Combination index (CI) with cisplatin and silibinin in CD133⁺ Huh7

Cisplatin single treatment		Silibinin single treatment		Combination treatment	Combination index (CI)
Concentration (μM)	Inhibitory rate (%)	Concentration (μM)	Inhibitory rate (%)	Inhibitory rate (%)	
2	4.1	2	3.8	25.4	3.28
5	10.4	5	4.9	51.5	3.48
10	18.3	10	6.3	67.2	2.87
15	25.5	15	8.8	71.8	2.24
20	35.4	20	12.6	75.6	1.74
30	44.7	30	15.5	81.4	1.53
40	49.2	40	18.1	85.2	1.46

Silibinin decreased the expression of OPA1 in CD133⁺ HCC cells

Previous research has indicated that mitochondrial dynamics determines the process of cell apoptosis. Moreover, mitochondrial fusion and fission is dependent on some specific proteins including Mfn1, Mfn2 and OPA1 [22]. Compared to the CD133⁻ HepG2 and Huh7 cells, we found that CD133⁺ HepG2 and Huh7 cells were resistant to apoptosis when they were under the stress of equal concentration of cisplatin (Fig. 3a). Next, we compared the expression of mitochondria-related proteins between CD133⁺ and CD133⁻ HCC cells. Although significant change of Mfn1 and Mfn2 was not observed between CD133⁺

and CD133⁻ HCC cells, expression level of OPA1 in the CD133⁺ HepG2 and Huh7 cells was significantly lower than that in the CD133⁻ HepG2 and Huh7 cells (Fig. 3b). To investigate the effect of silibinin and cisplatin on OPA1 expression, we treated the CD133⁺ and CD133⁻ HCC cells with cisplatin and silibinin before western blot analysis. As shown in Fig. 3c, silibinin but not cisplatin partly reduced the expression of OPA1 in CD133⁻ HepG2 and Huh7 cells. On the other hand, expression of OPA1 in CD133⁺ HepG2 and Huh7 cells became very low when they were treated with silibinin (Fig. 3d). Our data indicated that silibinin targeted OPA1 in HCC. Furthermore, decrease of OPA1 expression in the CD133⁺ HCC cells were more obvious than that in the CD133⁻ HCC cells.

Silibinin reduced the cisplatin resistance of CD133⁺ HCC cells through inhibition of OPA1

To investigate whether the synergistic effect of silibinin on cisplatin was dependent on the inhibition of OPA1, we performed gain-of-function and loss-of-function assays on OPA1 by using the OPA1 plasmid and OPA1 siRNA. Transfection efficiency of OPA1 plasmid and OPA1 siRNA in CD133⁺ HepG2 and Huh7 cells was shown in Fig. 4a. Results of CCK-8 assays showed that transfection with OPA1 plasmid abolished the synergistic effect of silibinin on cisplatin-induced cytotoxicity against CD133⁺ HepG2 and Huh7 cells (Fig. 4b). On the other hand, direct knockdown of OPA1 by using the OPA1 siRNA also can reduce the cisplatin resistance of CD133⁺ HepG2 and Huh7 cells (Fig. 4c). Taken together, we demonstrated that OPA1 expression partially determined the cisplatin resistance of CD133⁺ HCC cells. Furthermore, silibinin partially Silibinin reduced the cisplatin resistance of CD133⁺ HCC cells through inhibition of OPA1.

Silibinin enhanced cisplatin-induced apoptosis through the mitochondria pathway

As mitochondria-related proteins determine the process of cell apoptosis [22], we next investigated the role of silibinin/OPA1 axis in cisplatin-induced apoptosis in CD133⁺ HepG2 and Huh7 cells. We found that combination treatment with cisplatin and silibinin induced significant collapse of mitochondria. Cytochrome c which located at mitochondria was found to be released into the cytosol of CD133⁺ HepG2 and Huh7 cells which were co-treated with cisplatin and silibinin. However, transfection with OPA1 plasmid reduced the release of cytochrome c (Fig. 5a). Furthermore, we found that combination treatment with cisplatin and silibinin induced significant cleavage of caspase-9 and caspase-3 (Fig. 5b) and cell apoptosis (Fig. 5c). Taken together, we demonstrated that combination treatment with silibinin can enhance cisplatin-induced apoptosis in CD133⁺ HCC cells through the mitochondria pathway.

Discussion

Platinum-based anti-tumor drugs are commonly used in the treatment of HCC [23, 24]. As an important member of platinum-based chemotherapeutic drug, cisplatin cross-links with DNAs to inhibit the DNA replication and thus induces apoptosis of cancer cells [25, 26]. However, some cancer cells, especially the population of CD133 positive cancer cells, show obvious drug resistance and is responsible for the failure of chemotherapy [27]. Our study confirmed that CD133 positive HCC cells showed low sensitivity to

cisplatin. Therefore, reducing the drug resistance of CD133 positive HCC cells was a promising strategy for improving the cisplatin therapy.

Recent studies have indicated that some natural drugs exhibit good biological activity to increase the chemosensitivity of some cancers including HCC. For instance, natural drugs of resveratrol and imperatorin were found to enhance the cisplatin-induced apoptosis of HCC [7, 28]. As a natural, silibinin was also found to exhibit synergistic anti-cancer effects with conventional cytotoxic agents on breast cancer [29], ovarian cancer [30], prostate cancer [31] and HCC [32]. However, little is known regarding to the effect of silibinin on the CD133 positive population of HCC. In this study, we found that silibinin increased the chemosensitivity of HCC cells, especially the population of CD133 positive HCC cells. Our data suggested that silibinin can be used as a adjuvant drug to target the CD133 positive HCC cells which exhibited obvious cisplatin resistance.

Mitochondrial fusion and fission is dependent on some specific proteins including Mfn1, Mfn2 and OPA1 [22]. Absence of these proteins cause mitochondrial fragmentation. On the other hand, Inhibition of mitochondrial fragmentation prevents the release of cytochrome c and subsequent apoptotic steps [33]. As a major organizer of the mitochondrial inner membrane, OPA1 is involved in the sequestration of cytochrome c [34]. Therefore, OPA1 can be considered as a promising target for enhancing the apoptosis pathway of cancer cells. In this study, we found that silibinin exerted the synergistic effect with cisplatin on CD133 positive HCC cells through inhibition of OPA1. Furthermore, we observed that the effect of silibinin on CD133 positive HCC cells were more obvious than the CD133 negative HCC cells. We explained that expression of OPA1 in CD133 positive HCC cells became very low when they were treated with silibinin. Therefore, apoptotic stimuli (cisplatin) can easily elicit mitochondrial fragmentation to release the cytochrome c into the cytosol of CD133 positive HCC cells.

Conclusion

Combination treatment with silibinin significantly decreased the expression of OPA1 in CD133 positive HCC cells. Under the apoptotic signaling caused by cisplatin, silibinin promoted the fragmentation of mitochondria. As a result, cytochrome c which was a mitochondria-derived apoptotic inducers was released into the cytosol of CD133 positive HCC cells. Eventually, caspases-dependent apoptosis was induced. We concluded that combination treatment with nature drugs represented by silibinin may be considered as a promising strategy for reversing the drug resistance of CD133 positive HCC cells.

Abbreviations

HCC, hepatocellular carcinoma; CCK-8, Cell Counting Kit-8; IC50, 50% inhibiting concentration; siRNA, small interfere RNA; OPA1, optic atrophy 1; Mfn1, mitofusin 1; Mfn2, mitofusin 2

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

JKY and ZYX conducted the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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Not applicable.

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Figures

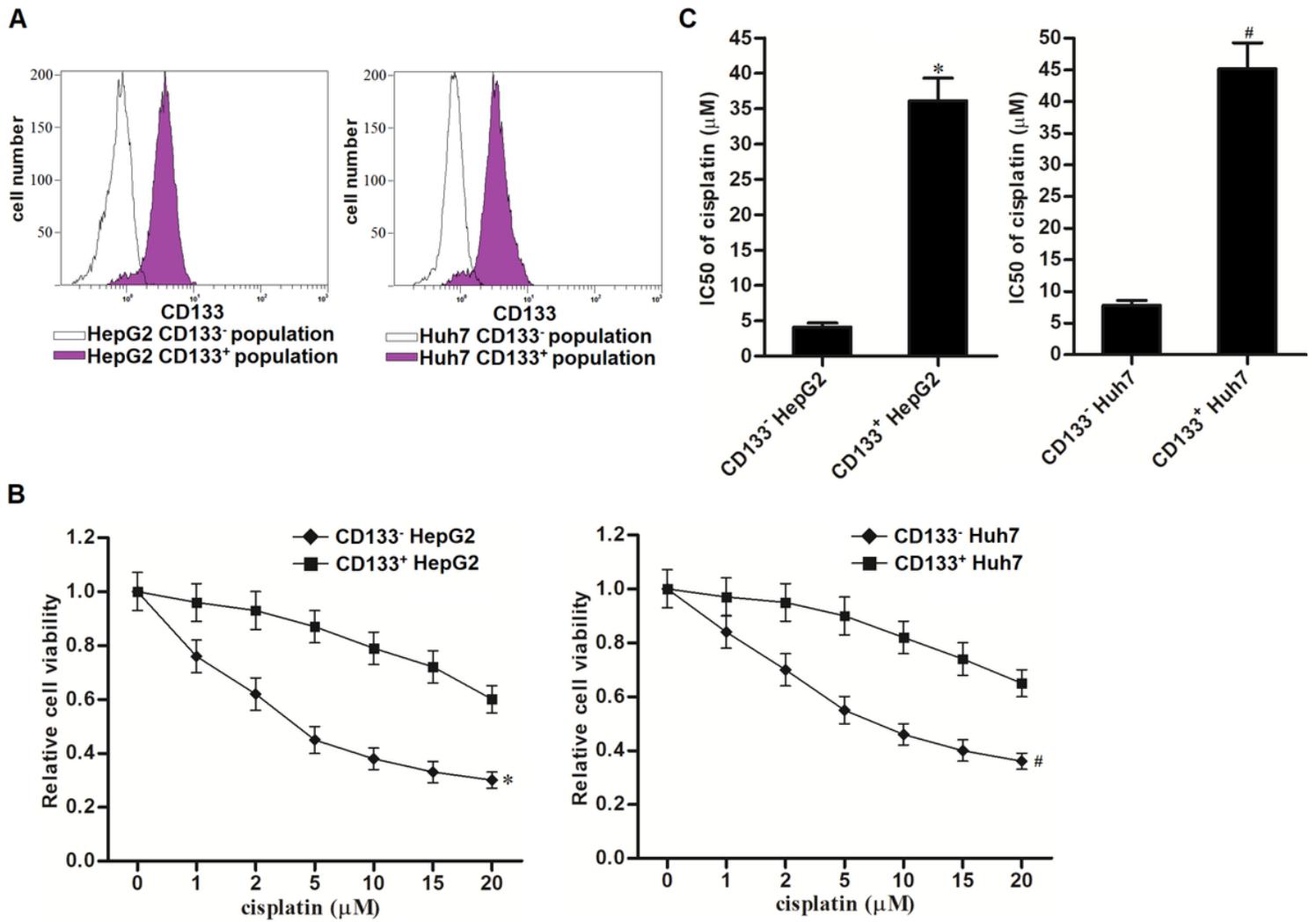


Figure 1

CD133⁺ HCC cells were resistant to cisplatin. a Purity of sorted CD133⁺ and CD133⁻ HepG2 and Huh7 cells. b Difference of cisplatin sensitivity between CD133⁺ and CD133⁻ HepG2 and Huh7 cells. c IC50 of cisplatin to CD133⁺ and CD133⁻ HepG2 and Huh7 cells. * $P < 0.05$ vs. CD133⁻ HepG2. # $P < 0.05$ vs. CD133⁻ Huh7.

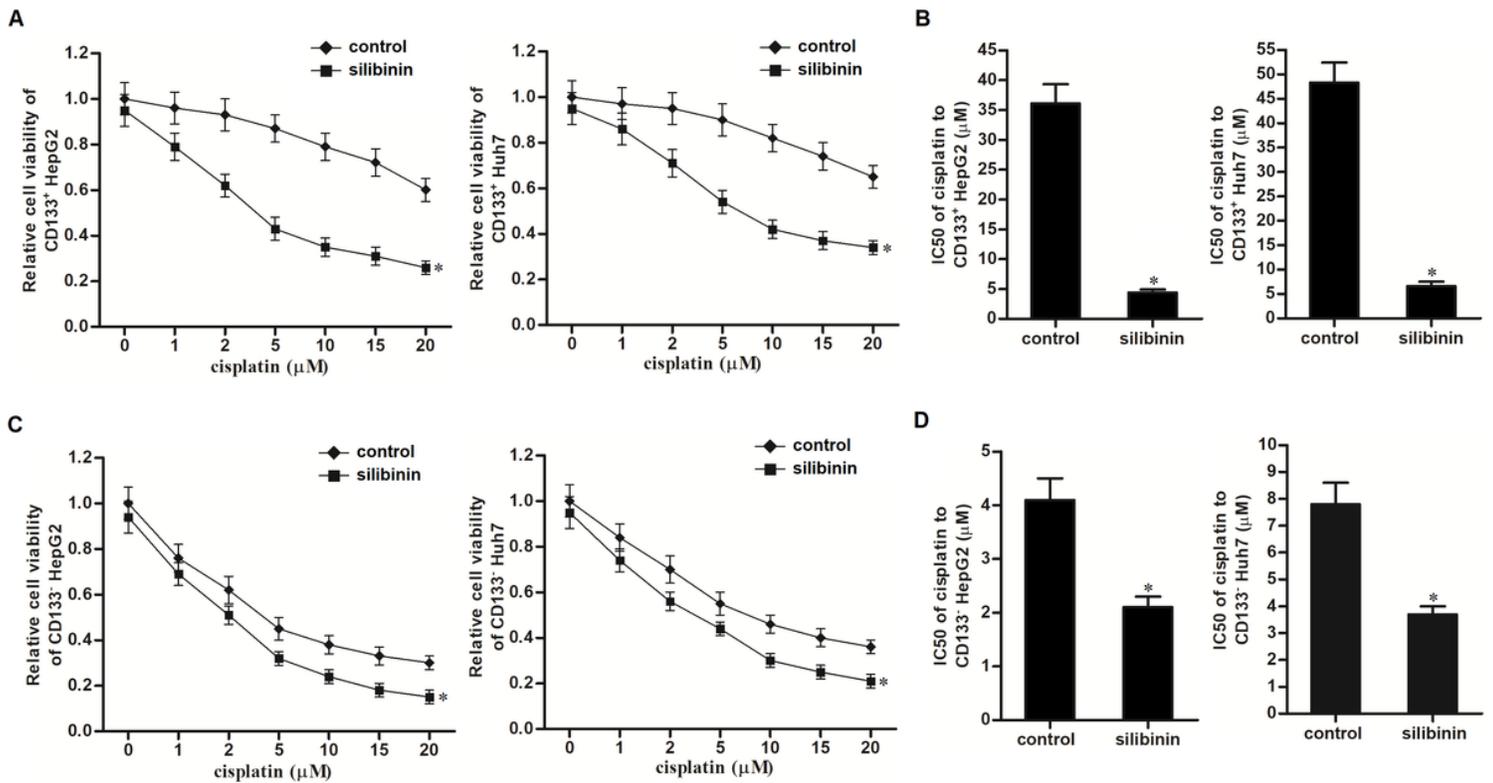


Figure 2

Silibinin reduced the cisplatin resistance of CD133⁺ HCC cells. a Silibinin (5 μM) enhanced the cytotoxicity of different concentrations of cisplatin (0~20 μM) to CD133⁺ HepG2 and Huh7 cells. b Silibinin (5 μM) decreased the IC₅₀ of cisplatin to CD133⁺ HepG2 and Huh7 cells. c Effect of Silibinin (5 μM) on cisplatin-induced cytotoxicity to CD133⁻ HepG2 and Huh7 cells. d Effect of Silibinin (5 μM) on IC₅₀ of cisplatin to CD133⁻ HepG2 and Huh7 cells. *P<0.05 vs. control group.

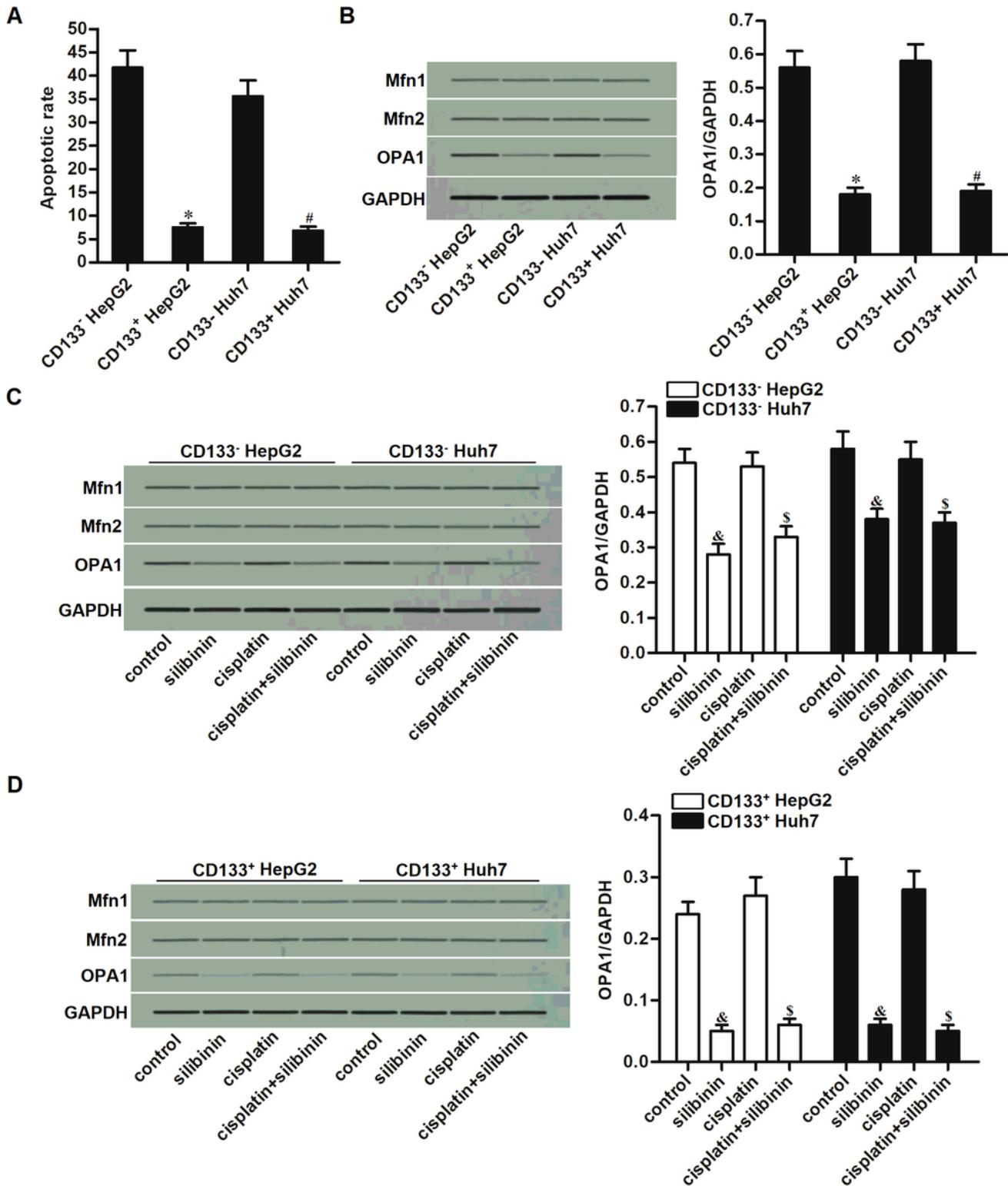


Figure 3

Silibinin decreased the expression of OPA1. a Sensitivity of CD133+ and CD133- HepG2 and Huh7 cells to cisplatin-induced (5 μ M) apoptosis. b Expression of Mfn1, Mfn2 and OPA1 in CD133+ and CD133- HepG2 and Huh7 cells. c Effect of silibinin and cisplatin on changing the expression of Mfn1, Mfn2 and OPA1 in CD133- HepG2 and Huh7 cells. d Effect of silibinin and cisplatin on changing the expression of Mfn1,

Mfn2 and OPA1 in CD133+ HepG2 and Huh7 cells. *P<0.05 vs. CD133- HepG2. #P<0.05 vs. CD133- Huh7. &P<0.05vs. control group. \$P<0.05vs. cisplatin group.

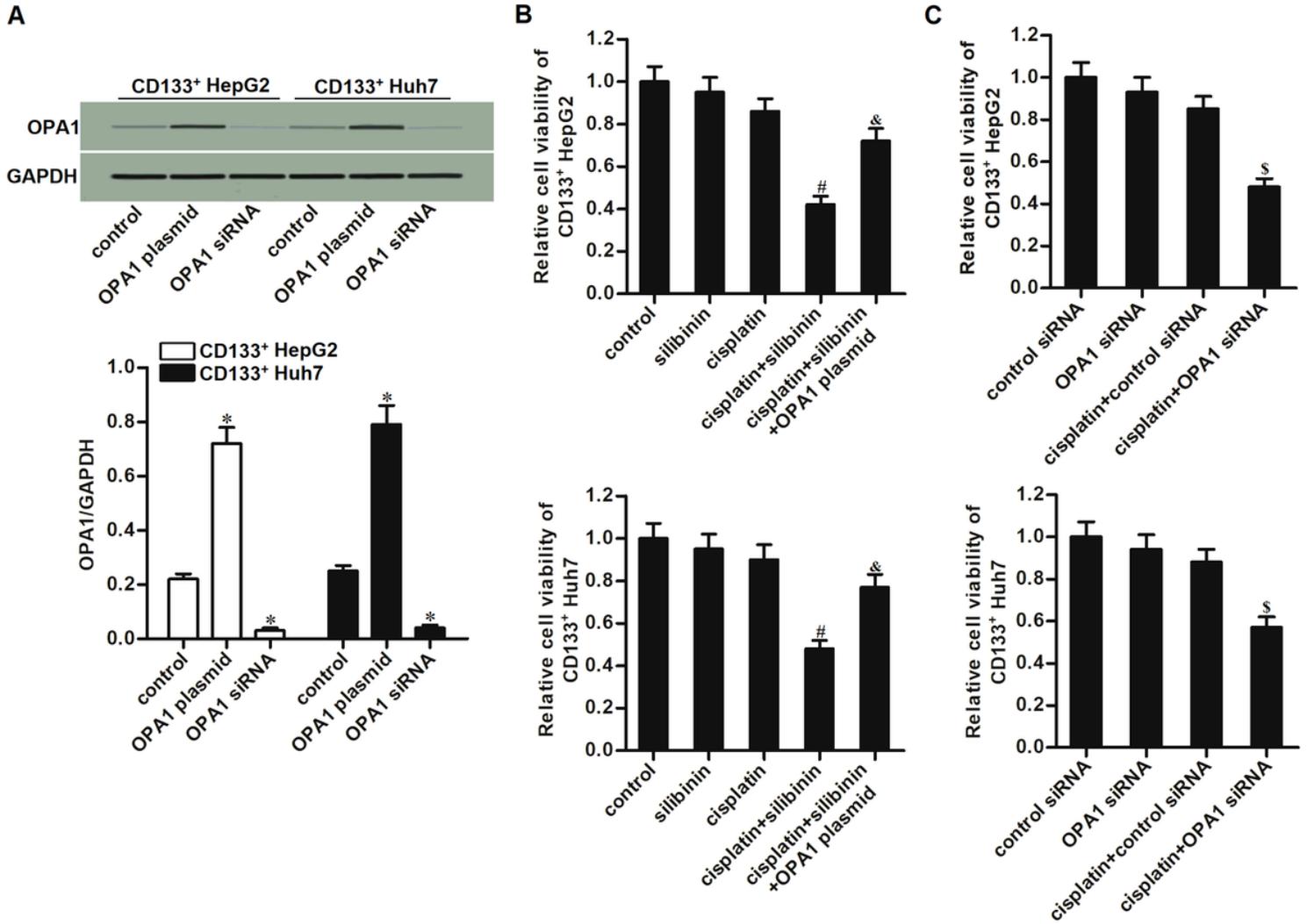


Figure 4

Silibinin reduced the cisplatin resistance of CD133+ HCC cells through inhibition of OPA1. a Transfection efficiency of OPA1 plasmid and OPA1 siRNA in CD133+ HepG2 and Huh7 cells. b Transfection with OPA1 plasmid increased the cell viability of CD133+ HepG2 and Huh7 cells which were co-treated with silibinin and cisplatin. c Transfection with OPA1 siRNA increased the sensitivity of CD133+ HepG2 and Huh7 cells to cisplatin. *P<0.05 vs. control group. #P<0.05 vs. cisplatin group. &P<0.05 vs. cisplatin+silibinin group. \$P<0.05 vs. cisplatin+control siRNA group.

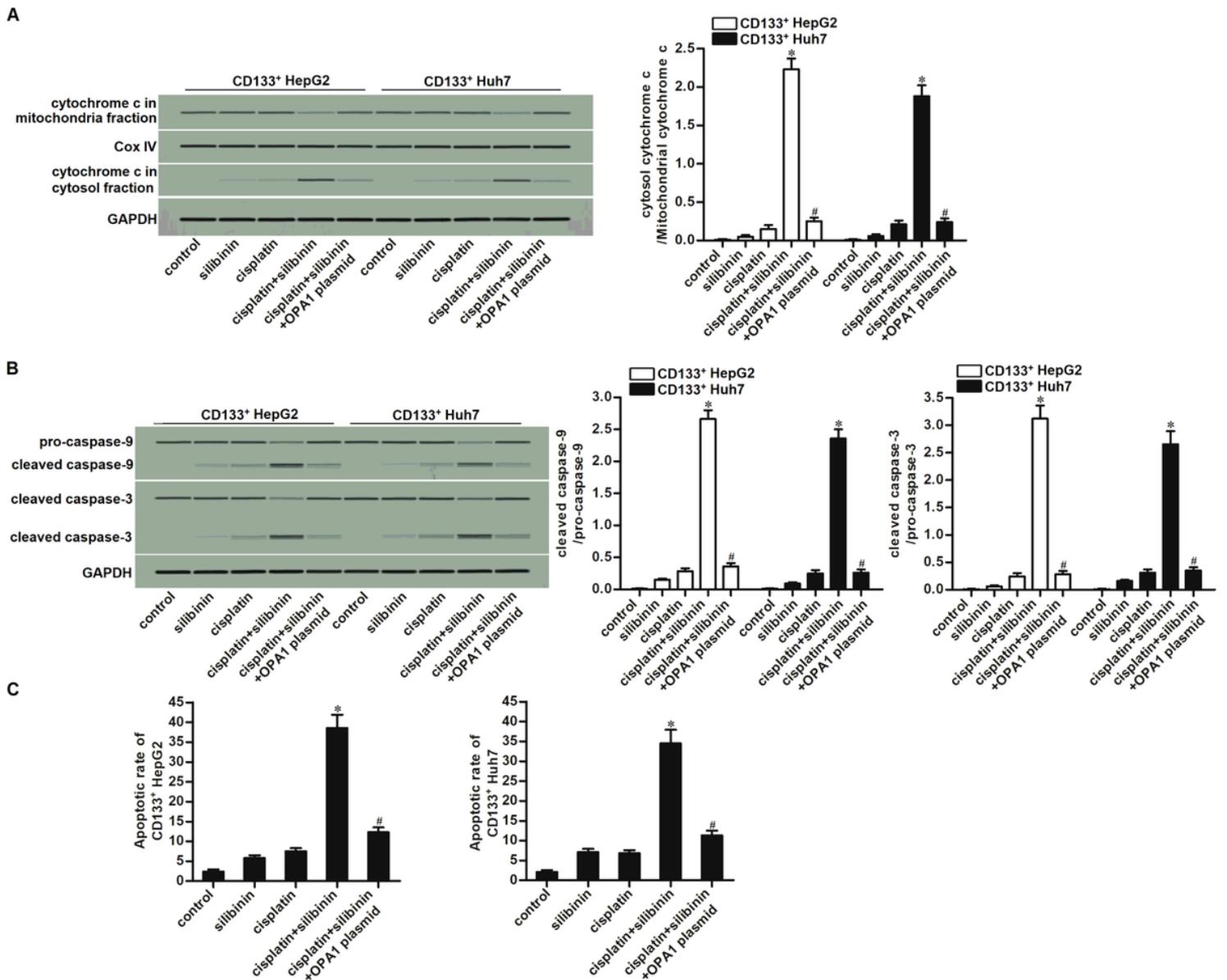


Figure 5

Silibinin enhanced cisplatin-induced apoptosis through the mitochondria pathway. a Combination treatment with cisplatin and silibinin induced significant release of cytochrome c from mitochondria into cytosol of CD133+ HepG2 and Huh7 cells. b Combination treatment with cisplatin and silibinin induced significant cleavage of caspase-9 and caspase-3 in CD133+ HepG2 and Huh7 cells. c Combination treatment with cisplatin and silibinin induced significant apoptosis of CD133+ HepG2 and Huh7 cells. * $P < 0.05$ vs. cisplatin group. # $P < 0.05$ vs. cisplatin+silibinin group.

Supplementary Files

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