miR-520d-5p enhances radiotherapy sensitivity of hepatocellular carcinoma cells line HepG2 through targeting Ceruloplasmin

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Research

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Abstract

Background: Liver cancer is one of the most common malignant tumors in the clinic. According to statistics, there were more than 62.6 million new cases of liver cancer in the world in the past two years, with the morbidity ranking 5th in malignant tumors and the third mortality rate. The resistance of liver cancer cells to radiotherapy reduces the sensitivity of radiotherapy, limiting the widespread application of radiotherapy. The aim of this study was to investigate the effect on the radio sensitivity enhancement of hepatocellular carcinoma cells by targeting ceruloplasmin (CP) with miR-520d-5p.

Results: miR-520d-5p overexpressed can inhibit the survival of liver cancer cells and increase the sensitivity of radiotherapy. In the overexpression of miR-520d-5pgroup, the Bax and Caspase-9 protein expression levels were significantly increased, the Ki67, PCNA and Bcl-2 levels were significantly reduced. After overexpression of miR-520d-5p, the cell survival fraction decreased and the cell sensitization ratio increased. CP overexpression reversed the inhibitory effect of miR-520d-5p overexpressed on cell apoptosis and reduced cell sensitization.

Conclusion: The above results suggested that miR-520d-5p improves the sensitivity of radiotherapy and promotes the apoptosis of cells through the negative regulation of CP.

Background

Liver cancer is one of the most common malignant tumors in the clinic. According to statistics, there were more than 62.6 million new cases of liver cancer in the world in the past two years, with the morbidity ranking 5th in malignant tumors and the third mortality rate [1]. In recent years, the incidence of liver cancer in China has been increasing year by year. At present, there are about 350,000 new cases of liver cancer each year, and the mortality rate is high [2]. With the popularization and development of precision radiotherapy in recent years, radiotherapy has become an effective method for the treatment of liver cancer. Three-dimensional conformal radiotherapy is a commonly used treatment for patients with liver cancer, and its efficacy is closely related to the sensitivity of patients to radiotherapy [3]. However, the resistance of liver cancer cells to radiotherapy reduces the sensitivity of radiotherapy, limiting the widespread application of radiotherapy [4].

miR-520d-5p is a non-coding endogenous small RNA molecule. miR-520d-5p expression is reduced in hepatoma cells [5]. Overexpression of miR-520d-5p can inhibit the proliferation, migration and invasion of liver cancer cells. The mechanism may be related to its down-regulation of GAB1 and ERK1/2 expression [6]. Studies have shown that miR-520d-5p enhances the radiotherapy sensitivity of NK/T cell lymphoma by inhibiting the ATK pathway [7]. The relationship between miR-520d-5p and radiotherapy sensitivity of liver cancer cells has not been reported. Ceruloplasmin (CP) is overexpressed in hepatoma cells, and inhibiting expression of CP significantly inhibits the proliferation, apoptosis and metastasis and chemotherapy resistance of hepatoma cells [8]. CP can increase the radiation resistance of liver cancer

cells, and it is more obvious in hypoxic environment [9]. CP has been shown to be regulated by multiple small RNAs, but in liver cancer, it has not been reported whether miR-520d-5p targets CP.

In the current study, the establishment of radioresistant cell strain RR-HepG2 was induced by fractional incremental radiotherapy, we investigated the effect on the radio sensitivity enhancement of hepatocellular carcinoma cells by targeting CP with miR-520d-5p.

Materials And Methods

Cell culture

Place radiosensitive HepG2 cells (Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) in 10% fetal bovine serum (Hangzhou Siji Qing Biological Engineering Materials Co., Ltd., Hangzhou, China) and double antibodies (penicillin 100 U/mL, streptomycin 100 μg/mL) in RPMI 1640 medium (Sigma-Aldrich, USA) in 5% CO_2, 37 ℃ incubator. Establishment of radiation-resistant cell line (RR-HepG2): Induced by fractional radiotherapy radiation incremental method, using ^{60}Co at a dose of 5 Gy and a dose rate of 0.5 Gy/min to irradiate HepG2 cells to a cumulative exposure of 60 Gy.

Cell transfection

When the confluence of HepG2 cells reaches about 70%, refer to the instructions and use TM with Lipofectamine 2000 transfection reagent (Invitrogen, USA). Transfect miR-520d-5p mimics, si-CP, CP-OV into HepG2 cells respectively, continue to culture and discard the transfection medium, and add conventional medium to culture overnight to observe the transfection efficiency under an inverted fluorescence microscope.

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA from the cells and tissues were extracted by using TRIzol (Invitrogen, USA). Complementary DNA was synthesized from SuperScript first strand synthesis system (Invitrogen). Quantitative PCR analysis was performed on the Applied Biosystems 7300 using IQTMSYBR Green SuperMix (Bio-Rad, Hercules, CA, USA).

Clone formation experiment to detect radio sensitivity

Dilute the gradient multiple dilution method to dilute HepG2 and RR-HepG2 cells to 1×10^4 cells/mL. According to the increase of irradiation dose, different numbers of cells were inoculated overnight, and 24 hours after transfection, irradiation (0, 2, 4, 6, and 8 Gy) was given. The cells were changed every 3 days, cultured for 10 to 14 days, the medium was discarded, and washed twice with PBS; 400 μL of fast Giemsa stain reagent was added to each well for 2 minutes; then, 800 μL Giemsa stain reagent was added to each well to stain for 8 min, rinse the staining solution under running water, and dry naturally.

Observe the colonies of ≥ 50 cells under the light microscope, (planting efficiency, PE) = number of clones/number of inoculated cells × 100%, survival fraction (SF2) = number of colonies in the irradiation dose group/(The number of cells inoculated in this group × PE in the unirradiated group). A single-click multi-target model was used to fit the cell survival curve, SF = 1-(1-e-D/D0) N, and Dq = D0 × lnN. Where D is the irradiation dose (Gy), D0 is the average lethal dose, Dq is the quasi-threshold dose (representing the breadth of survival), and N is the extrapolated value. Radiation sensitization enhancement ratio (SER) = D0 in the simple irradiation group/D0 in the combined irradiation group. The mean analysis was carried out with the survival scores of three exposures.

CCK-8 assay

The cells were grown at 2–4 × 10^4 cells/well in 96-well microplates. CCK-8 assay (Sigma Chemical Co., St. Louis, MO) was subsequently added to medium to (0.5 mg/mL) a final concentration and incubated for 4 h at 37 °C. The absorbance was measured at 450 nm.

Dual-luciferase reporter assay

CP 3'UTR with or without mutation were subcloned into pGL3 promoter plasmid. 3 × 10^4 cells/mL were seed into 24-well culture plates in phenol red-free medium. After transfection with miRNA precursor control pre-miRNA (pre-miR-co) or pre-miR-520d-5p (Biomics Biotech) for 48 h, the luciferase activity was determined by the Dual Luciferase Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Flow cytometry to detect apoptosis

Collect cells (1 × 10^6 cells/mL) 48 h after irradiation, centrifuge at 1000 r/min for 5 min, then discard the medium, wash once with PBS, discard PBS; add 250 μL PBS to resuspend the cells, and finally add 10 μL annexin V-EGFP and 5 μL PI, mix well, incubate at room temperature in the dark for 15 min, and detect by flow cytometry.

Western blotting assay

Total proteins from cells or tissues were western-blotted using the monoclonal antibody against CP, Bcl-2, Bax and caspase-9 (all 1:500, Santa Cruz, USA). β-actin (1:5000, Sigma, USA) served as a loading control. Horseradish peroxidase (HRP)-labeled secondary antibody (1:1000, Sigma, USA) was used and incubated for 1 h at 25 °C. The band densities were quantified by the LICOR Odyssey infrared imaging system (LICOR Bio- science, Nebraska, USA).

Statistical analysis

To measure differences of groups, one-way ANOVA with a Bonferroni post hoc test, Student's *t*-test, or Wilcoxon's signed rank test was applied. All the experiences were performed at least three times and the results were expressed as means ± standard deviation (SD). $P < 0.05$ was considered statistically significant. GraphPad Prime 8.0 was used to draw the survival curve and the single-machine multi-target mode and L-Q linear model curve fitting to find the D0, Dq, N, SF2, k, SER.

Results

Expression of miR-520d-5p in radiation-sensitive and radiation-resistant hepatoma cells after different doses of radiation treatment

RR-HepG2 and HepG2 cells received different doses of radiation treatment. At the same radiation dose, the expression level of miR-520d-5p in the RR-HepG2 group was significantly lower than that in the HepG2 group ($P < 0.05$) (Fig. 1).

Overexpression of miR-520d-5p promotes radiation-induced decrease in survival fraction and apoptosis of hepatoma cell HepG2

After radiation treatment, compared with the control group and the NC-mimics group, the cell survival score of the miR-520d-5p mimics group was significantly reduced ($P < 0.05$) (Fig. 2A). According to the stand-alone multi-target model, D0, Dq, N, the calculated SF2 and k values of each group and the sensitization ratio were shown in Table 1. The results of CCK-8 assay exhibited that the cell viability was markedly reduced in the miR-520d-5p mimics group (Fig. 2B). Flow cytometry showed that the apoptosis rate in the miR-520d-5p mimics group was significantly higher than that in the control group and NC-mimics group (Fig. 2C). After 4 Gy radiation treatment, the apoptosis-related proteins Bax and Caspase-9 protein expression levels in the miR-520d-5p mimics group were significantly higher than those in the control group and NC-mimics group, and the Ki67, PCNA and Bcl-2 protein expression level was significantly lower than that in the control group and NC-mimics group ($P < 0.05$) (Fig. 2D).

Table 1
Radio sensitivity parameters of HepG2 cells in the 3 groups.

Group	D_0 (Gy)	D_q (Gy)	N	SF2	k	SER
Control	1.909	1.430	2.410	0.668	0.488	-
NC-mimics	1.564	1.233	2.128	0.450	0.599	-
miR-520d-5p mimics	1.040	1.002	1.907	0.270	0.886	1.360

miR-520d-5p targeting CP and inhibits CP protein expression

Figure 3A showed that TargetScan predicts miR-520d-5p and CP binding sites. Western blot results showed that, compared with the NC-mimics group, the CP protein level was significantly reduced ($P < 0.05$); compared with the NC-inhibitor, the CP protein level of miR-520d-5p inhibitor group was significantly increased ($P < 0.05$) (Fig. 3B). Compared with the control group, the WT of miR-520d-5p mimics luciferase activity was significantly reduced ($P < 0.05$); while the MUT of miR-520d-5p mimics luciferase activity was not changed (Fig. 3C). After radiation treatment, compared with the control group and the si-NC group, the cell survival score of the si-CP group was significantly reduced ($P < 0.05$) (Fig. 3D). According to the stand-alone multi-target model, D0, Dq, N, the calculated SF2 and k values of each group and the sensitization ratio were shown in Table 2. The results of CCK-8 assay exhibited that the cell viability was markedly reduced in the si-CP group (Fig. 3E). Flow cytometry showed that the apoptosis rate in si-CP group was significantly higher than that in the control group and si-NC group (Fig. 3F). After 4 Gy radiation treatment, the apoptosis-related proteins Bax and Caspase-9 protein expression levels in the si-CP group were significantly higher than those in the control group and si-NC group, and the Ki67, PCNA and Bcl-2 protein expression level was significantly lower than that in the control group and si-NC group ($P < 0.05$) (Fig. 3G).

Table 2
Radio sensitivity parameters of HepG2 cells in the 3 groups.

Group	D_0 (Gy)	D_q (Gy)	N	SF2	k	SER
Control	1.800	1.530	2.530	0.567	0.456	-
si-NC	1.656	1.433	2.345	0.550	0.509	-
si-CP	1.104	0.993	1.997	0.230	0.987	1.230

miR-520d-5p improves radiotherapy sensitivity of hepatoma cells by regulating CP

After radiation treatment, compared with the NC-mimics group, the cell survival score of the miR-520d-5p mimics group was markedly reduced ($P < 0.05$), compared with the miR-520d-5p + vector group, the cell survival score of miR-520d-5p + CP group increased significantly ($P < 0.05$) (Fig. 4A). According to the single-machine multi-target model, calculate the D0, Dq, N, SF2, k value and sensitization ratio of each group (Table 3). The results of CCK-8 assay exhibited that the cell viability was markedly reduced in the miR-520d-5p mimics group, while markedly increased in the miR-520d-5p + CP group (Fig. 4B). Flow cytometry also showed that the apoptosis rate in miR-520d-5p + CP group was significantly reduced (Fig. 4C). Compared with the miR-520d-5p + vector group, the Bax and caspase-9 protein expression levels were significantly reduced, and Ki67, PCNA and Bcl-2 protein expression levels were significantly increased in the miR-520d-5p + CP group (Fig. 4D).

Table 3
Radio sensitivity parameters of HepG2 cells in the 4 groups.

Group	D_0 (Gy)	D_q (Gy)	N	SF2	k	SER
NC-mimics	1.678	1.630	2.564	0.667	0.476	-
miR-520d-5p-mimics	1.040	0.987	1.887	0.245	0.886	1.030
miR-520d-5p + vector	1.104	0.979	1.987	0.235	0.900	1.130
miR-520d-5p + CP	1.788	1.543	2.455	0.547	0.365	1.112

Discussion

Liver cancer is a common malignant tumor in China. It has a high degree of malignancy, is prone to relapse, and has a high case fatality rate. The treatment methods include surgery, chemotherapy, and radiotherapy [10]. However, the resistance of liver cancer cells to radiotherapy limits its efficacy, and improving the sensitivity of liver cancer radiotherapy has important clinical significance for the treatment of liver cancer. miRNA is a type of non-coding small RNA that plays an important role in tumorigenesis [11]. Studies have shown that the survival time of triple negative breast cancer patients with low expression of miR-520d-5p is shortened, and their sensitivity to chemotherapy is reduced [12]. The results of this study showed that the expression level of miR-520d-5p in radiation-resistant RR-HepG2 cells was significantly lower than that of HepG2 cells. It has been reported that overexpression of miR-150 can increase the radiation of NK/T cell lymphoma Sensitization [13]. It has also been reported that miR-150 inhibits the proliferation, invasion and metastatic ability of liver cancer cells by negatively regulating the GAB1-ERK1/2 axis [14]. The results of this study show that miR-520d-5p overexpressed can inhibit the survival of liver cancer cells and increase the sensitivity of radiotherapy.

The Bcl-2 family plays an important role in the process of apoptosis. Bcl-2 is an anti-apoptotic protein and Bax is a pro-apoptotic protein. When excessive expression of Bax can inhibit the apoptosis of Bcl-2, the inhibitory effect promotes apoptosis [15]. Caspase-9 is a key executor of apoptosis, which can cleave substrates and promote cell apoptosis [16]. This study showed that in the overexpression of miR-520d-5p group, the Bax and Caspase-9 protein expression levels were significantly increased, the Ki67, PCNA and Bcl-2 levels were significantly reduced.

CP is involved in cell proliferation, apoptosis, metastasis, and chemotherapy resistance [17]. CP gene silencing significantly improves the radio sensitivity of DLBCL cells [18], and CP is regulated by a variety of miRNAs, miR-204-5p targeted inhibition of CP expression promotes apoptosis of hepatoma cells [19], improves drug sensitivity. miR-22 inhibits tumorigenesis and improves the Radiosensitivity of breast cancer cells by targeting CP [20]. In this study, after overexpression of miR-520d-5p, the cell survival fraction decreased and the cell sensitization ratio increased. CP overexpression reversed the inhibitory effect of miR-520d-5p overexpressed on cell apoptosis and reduced cell sensitization. The above results

suggested that miR-520d-5p improves the sensitivity of radiotherapy and promotes the apoptosis of cells through the negative regulation of CP.

Conclusion

In summary, miR-520d-5p can negatively regulate the expression of CP, which can improve the sensitivity of liver cancer cells to radiotherapy and provide a theoretical basis for molecular targeted therapy of liver cancer. However, there are still deficiencies in this study, and the cell signaling pathway is relatively complicated. The pathway that miR-520d-5p negatively regulates CP needs to be further studied.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors confirm that the manuscript, or its contents in some other form, has not been published previously by any of the authors and/or is not under consideration for publication in another journal at the time of submission.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests, and all authors confirm accuracy.

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Authors' contributions

Junling Han and Lei Wang, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization; Qiong Luo, Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition.

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Figures

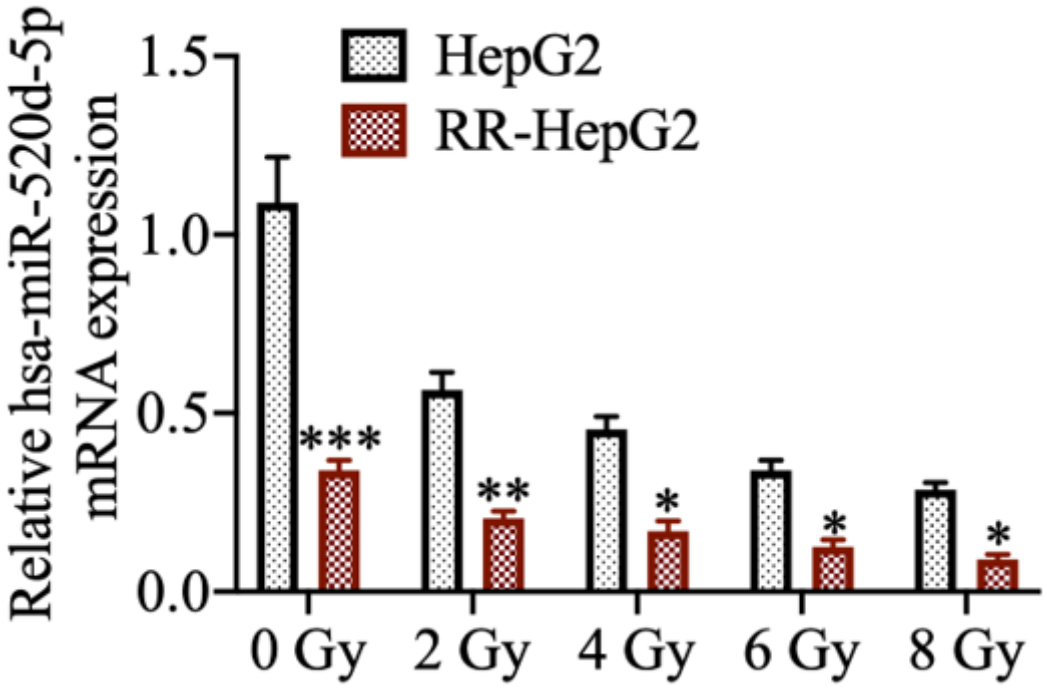

Figure 1

The miR-520d-5p mRNA expression was evaluated by qRT-PCR in HepG2 and RR-HepG2 cells treated with irradiation. *P<0.05, **P<0.01, ***P<0.001 vs. HepG2 group.

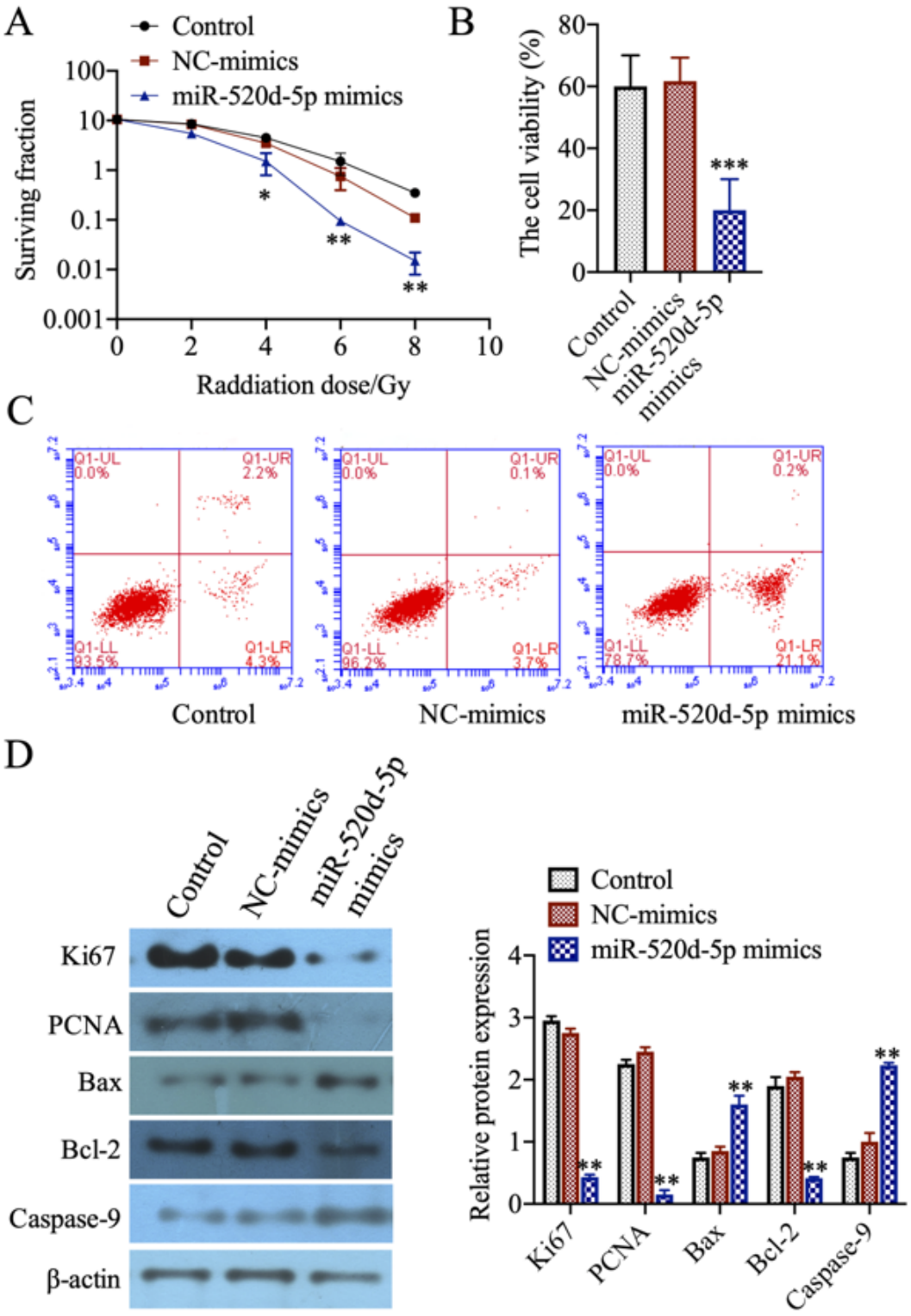

Figure 2

Overexpression of miR-520d-5p promotes radiation-induced decrease in survival fraction and apoptosis of HepG2 cell. (A) Surviving cell fractions in the HepG2 cells after radiation at different doses. (B) The cell viability was evaluated by CCK-8 assay after radiation at 4 Gy. (C) The apoptosis of HepG2 cells was evaluated by flow cytometry after radiation at 4 Gy. (D) The Ki67, PCNA, Bax, Bcl-2 and Caspase-9 protein

expressions were evaluated by western blot after radiation at 4 Gy. **P<0.01, ***P<0.001 vs. Control group.

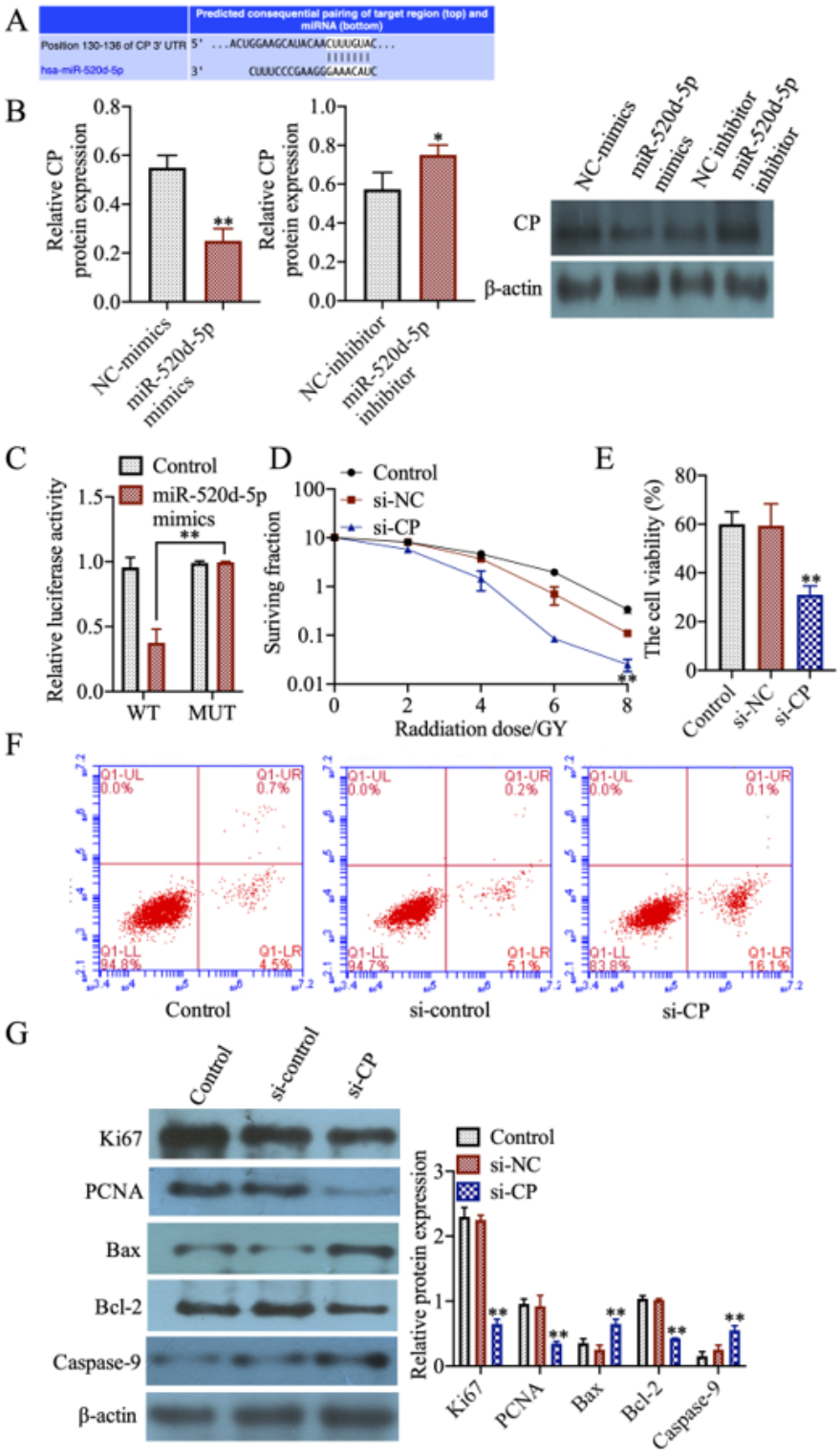

Figure 3

miR-520d-5p targeting CP inhibits the expression of CP protein. (A) TargetScan predicts miR-520d-5p and CP binding sites. (B) The CP protein expression was evaluated by western blot. (C) Luciferase activity analysis of the targeted relationship of miR-520d-5p and CP in HEK-293T cells. (D) Surviving cell

fractions in the HepG2 cells after radiation at different doses. (E) The cell viability was evaluated by CCK-8 assay after radiation at 4 Gy. (F) The apoptosis of HepG2 cells was evaluated by flow cytometry after radiation at 4 Gy. (G) The Ki67, PCNA, Bax, Bcl-2 and Caspase-9 protein expressions were evaluated by western blot after radiation at 4 Gy. *P<0.05, **P<0.01 vs. Control group.

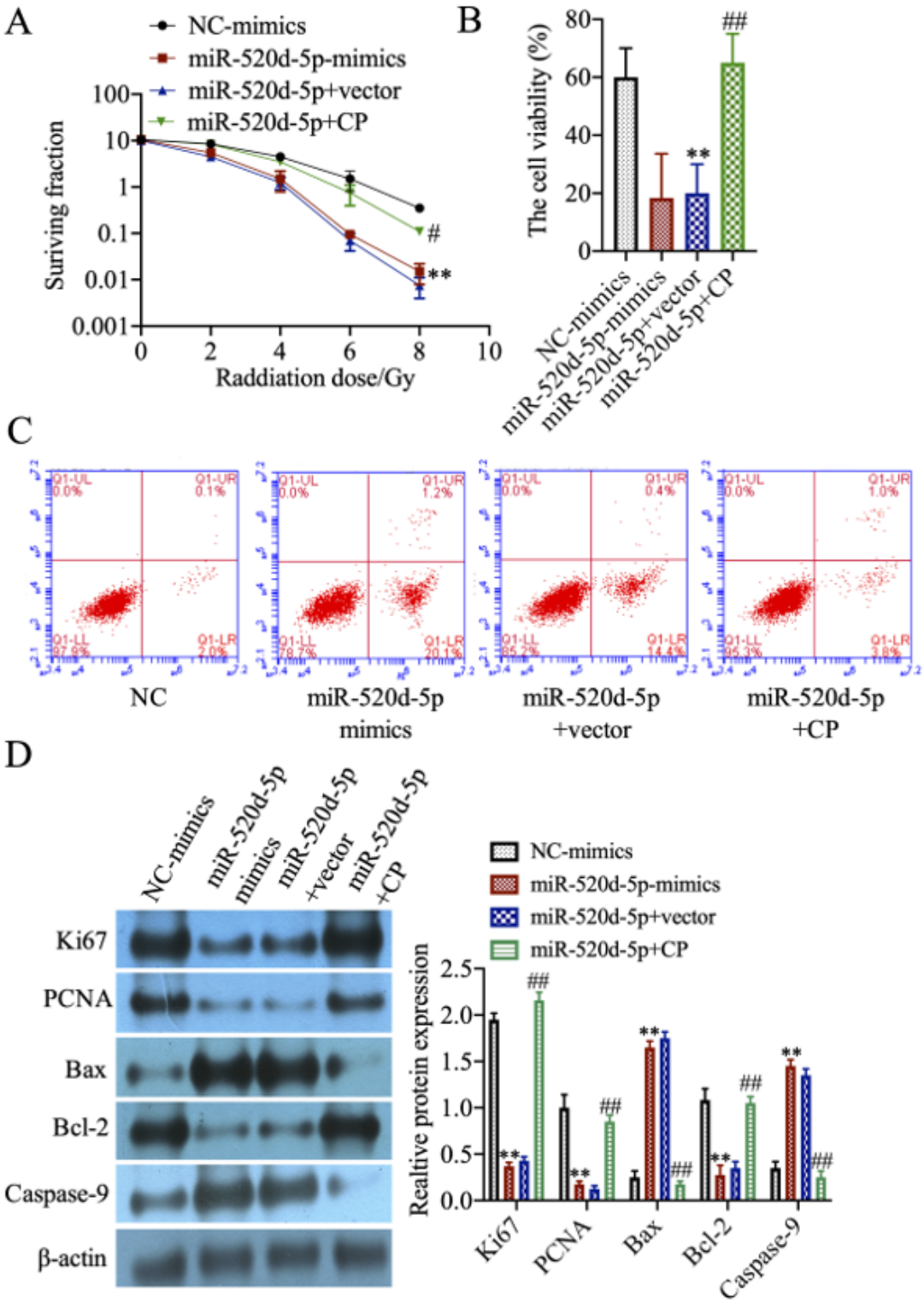

Figure 4

miR-520d-5p improves radiotherapy sensitivity of HepG2 cells by regulating CP. (A) Surviving cell fractions in the HepG2 cells after radiation at different doses. (B) The cell viability was evaluated by CCK-8 assay after radiation at 4 Gy. (C) The apoptosis of HepG2 cells was evaluated by flow cytometry after radiation at 4 Gy. (D) The Ki67, PCNA, Bax, Bcl-2 and Caspase-9 protein expressions were evaluated by western blot after radiation at 4 Gy. **P<0.01 vs. NC-mimics group, #P<0.05, ##P<0.01 vs. miR-520d-5p + vector group.