Radiosensitizing effects of *Cyclocarya paliurus* polysaccharide on hypoxic A549 and H520 human non-small cell lung carcinoma cells

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Abstract. *Cyclocarya paliurus* (CP) polysaccharide (CPP) is a chemical component contained in CP, which has been reported to possess significant hypoglycemic activity. The present study aimed to investigate the radiosensitizing effect and underlying mechanisms of CPP on hypoxic A549 and H520 human non-small cell lung carcinoma cells. Cell viability, apoptosis and proliferation were determined using Cell Counting kit-8 assay, flow cytometry and colony formation assay, respectively. mRNA and protein expression levels were determined by reverse transcription-quantitative PCR and western blot analysis, respectively. The results suggested that CPP markedly inhibited the viability of hypoxic A549 and H520 cells. In response to combined treatment with CPP and radiation, hypoxic A549 and H520 cells exhibited enhanced apoptosis; in addition, cell proliferation was suppressed and the expression levels of hypoxia-inducible factor-1α, survivin and cleaved caspase-3 were modified. Furthermore, CPP in combination with radiation affected the mammalian target of rapamycin (mTOR)/Akt/phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway. These findings indicated that CPP may enhance the radiosensitivity of hypoxic A549 and H520 cells; this effect may be associated with inhibition of the mTOR/Akt/PI3K pathway. The potential radiosensitizing effects of CPP on hypoxic A549 and H520 cells suggested that CPP may be an effective target for treatment of non-small cell lung carcinoma.

Introduction

Lung carcinoma is one of the most common malignant tumors worldwide; its incidence is secondary to prostate cancer among men and to breast cancer among women (1). A previous study released by the International Agency for Research on Cancer reported that the incidence rate of lung cancer was 23.1/100,000 and its mortality rate was 19.7/100,000 in 2012 (2). A previous study also suggested that lung cancer was the most common and fatal cancer in China in 2015 (3). The annual mortality rate of lung cancer is higher than that caused by colon cancer, breast cancer and prostate cancer in total (4). Lung cancer can be divided into two main types: Small cell lung cancer and non-small cell lung cancer; these types are characterized by cell size and type. Non-small cell lung cancer accounts for ~80% of lung cancer cases, leading to ~900,000 deaths worldwide on an annual basis (5). Non-small cell lung cancer is normally classified into three types: Squamous cell carcinoma, adenocarcinoma and undifferentiated large cell carcinoma. Various approaches have been adopted to treat lung cancer, including surgery, radiation therapy, chemotherapy and molecular targeted therapy (6). However, the exact pathogenesis of and mechanisms underlying non-small cell lung cancer remain unclear.

Molecular biology has revealed that the majority of tumor tissues exhibit different degrees of hypoxic cells, and that hypoxic cells are resistant to radiation, which may induce failure of tumor radiotherapy and recurrence (7). Although scientists have applied direct or indirect methods to increase oxygen content in tumors to overcome hypoxic conditions, the therapeutic effect remains unsatisfactory (8-10). Therefore, the development and exploration of tumor hypoxic cell radiosensitizers has attracted much attention from researchers in the field of tumor radiotherapy.

Cyclocarya paliurus (CP), a member of the Juglandaceae family, is a unique species and an endangered plant in China (11). CP polysaccharide (CPP) is a heteropolysaccharide and contains protein (8.44%), 17 amino acids and 18 mineral elements (12). CP has previously been reported to possess anti-oxidant effect (12). Furthermore, CPP has garnered much interest in fields of antihypertensive, hypoglycemic, anti-oxidant and anticancer research (13). Modern pharmacological studies have demonstrated that CPP possesses significant hypolipemic, hypoglycemic (14) and antitumor activity (13). However, to the best of our knowledge, only one study has been conducted regarding the radiosensitizing effect of polysaccharides on lung cancer (15).

Hypoxia-inducible factor-1α (HIF-1α) is a well-studied oxygen regulatory factor (16). Oxygen concentration is able to regulate the expression levels of relevant genes (17), and

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low oxygen concentration affects the malignant phenotype of tumors. Mammalian target of rapamycin (mTOR) and survivin are highly expressed in malignant tumors and are closely associated with tumor apoptosis (18). Furthermore, the expression levels of mTOR and survivin are associated with low oxygen status in tumors (19,20). Numerous studies have reported that the growth of tumor cells is closely related to the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt/mTOR and HIF-1α/survivin pathways (21-23). Therefore, mTOR, HIF-1α and survivin are often considered as targets for tumor therapy. However, the accurate mechanisms underlying the modulatory effects of PI3K/Akt/mTOR and HIF-1α/survivin pathways on the proliferation and apoptosis of non-small cell lung carcinoma cells remain unknown.

This study assessed the association between CPP and the radiosensitivity of hypoxic A549 and H520 non-small cell lung carcinoma cells. Furthermore, the exact roles and mechanisms of CPP in combination with radiation on the growth and apoptosis of hypoxic A549 and H520 non-small lung carcinoma cells were investigated.

Materials and methods

Cell culture and reagents. Human non-small cell lung carcinoma cell lines (A549 and H520) were acquired from the Cell Bank of Chinese Academy of Sciences. A549 and H520 cells were incubated in RPMI 1640 medium (cat. no. 61870044; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc.) in an environment containing 5% CO_2 at 37˚C. CP was obtained from Institute of Materia Medica, CAMS & PUMC. CPP was extracted from CP as previously described (24). The proportion of CPP content was ~8.11% in the CP leaves, and CPP accounted for 75.34% of the total sugar content.

Grouping
Control. In the control group, normal A549 and H520 cells were treated with PBS at 37˚C for 48 h.

Hypoxia group. After being cultured under normoxic conditions for 24 h, cells in the hypoxia group were placed in a sterile and closed hypoxia modular incubator chamber (Billups-Rothenberg, Inc.). The hypoxic conditions were controlled using a high/low oxygen control system (ProOx 110; BioSpherix, Ltd.), which can automatically inject nitrogen to reduce oxygen concentration. The cells were maintained in a humidified atmosphere containing 5% CO_2 and 1% O_2 at 37˚C for 30 min, and were then transferred to normoxic conditions. After culturing for 24 h at 37˚C, the cells were collected and used in subsequent experiments.

CPP/hypoxia group. Cells in the logarithmic growth phase were inoculated into 6-well plates (~130 cells/well). RPMI-1640 medium without serum was added to the cells, and 3 ml CPP solution was then added to the cells (A549 cells, 100 nmol/l; H520, 60 nmol/l). After 24 h, the cell medium was replaced with RPMI-1640 containing 10% FBS. The plates were placed in a hypoxic incubator and maintained under hypoxic conditions for 30 min. Subsequently, cells were incubated in a

normoxic incubator. After culturing for 24 h at 37˚C, the cells were collected and used in subsequent experiments.

X-ray/hypoxia group. Cells in the logarithmic growth phase were inoculated into 6-well plates (~130 cells/well). The plates were then placed in a hypoxic incubator and maintained under hypoxic conditions for 30 min at 37˚C. Subsequently, cells were incubated in a normoxic incubator. After culturing for 2 h at 37˚C, the cells were irradiated with 6 MV-X radiation at a dose rate of 400 cGy/min using TrueBeam (Varian Medical Systems) for 1 h. The irradiation field was 20x20 cm and the source skin distance (SSD) was 100 cm, as previously described (25). After culturing for 24 h at 37˚C, the cells were collected and used in subsequent experiments.

CPP/X-ray/hypoxia group. Cells in the logarithmic growth phase were inoculated into 6-well plates (~130 cells/well). Subsequently, 3 ml CPP solution was added to the cells (A549 cells, 100 nmol/l; H520, 60 nmol/l) and they were cultured in a normoxic incubator for 24 h at 37˚C, the cell medium was then replaced with RPMI-1640 that contained 10% FBS. The plates were then maintained under hypoxic conditions for 30 min at 37˚C. Subsequently, cells were incubated in a normoxic incubator. After culturing for 2 h at 37˚C, the cells were irradiated with 6 MV-X radiation at a dose rate of 400 cGy/min for 1 h. The irradiation field was 20x20 cm and the SSD was 100 cm. After culturing for 24 h at 37˚C, the cells were collected and used in subsequent experiments.

Cell viability analysis. Viability of hypoxic A549 and H520 cells was analyzed using the Cell Counting kit-8 (CCK-8; cat. no. C0038; Beyotime Institute of Biotechnology). Approximately $1x10^3$ cells/well cultured hypoxic A549 and H520 cells in the logarithmic phase were seeded into 96-well plates (cat. no. FPT011; Beyotime Institute of Biotechnology) and were then maintained in an environment containing 5% CO_2 at 37˚C for 12 h. After being treated with 10, 20, 50, 100, 200 and 400 nmol/l CPP, A549 and H520 cells were then maintained at 37˚C for 24 h. Subsequently, 10 µl CCK-8 reagent was added to the wells of 96-well plates and the cells were incubated for 3 h at 37˚C. A microplate reader (Bio-Rad Laboratories, Inc.) was used to record the absorbance at 450 nm. The inhibition ratio was calculated according to the following equation: Inhibition ratio (%) = (1-absorbance of experimental group/absorbance of blank control group) x 100%.

Apoptosis analysis. The apoptosis of A549 and H520 cells was evaluated using flow cytometry (FCM). After being washed with PBS, cultured cells were trypsinized in 0.25% trypsin (cat. no. C0201; Beyotime Institute of Biotechnology). Subsequently, the supernatant was discarded and A549 and H520 cells prepared for detection were suspended in 1X Annexin binding buffer (Thermo Fisher Scientific, Inc.) at a final density of $1x10^6$ cells/ml. The cells were then incubated with 1X Annexin V-FITC and 100 µg/ml propidium iodide (cat. nos. C1063, ST512; Beyotime Institute of Biotechnology) in the dark for 15 min at room temperature. A FACSCalibur flow cytometer (BD Biosciences) equipped with CellQuest software (version 3.3; BD Biosciences) was used to detect cell apoptosis.

Colony formation assay. Following irradiation, the cells in culture medium were incubated in an incubator containing 5% CO_2 at 37˚C for 14 days. Subsequently, the culture medium was discarded, and the cells were washed with PBS and fixed with ethanol (cat. no. A112719; Aladdin Shanghai Biochemical Technology Co., Ltd.) for 20 min at room temperature. The cells were then stained with 0.1% crystal violet (cat. no. C0121; Beyotime Institute of Biotechnology) for 20 min at room temperature, and washed three times with PBS. The number of colonies containing ≥5 cells was counted in a 6-well culture plate under a low magnification confocal microscope, and cell colony formation rate was calculated according to the formula: Colony formation rate = number of colonies formed following treatment/number of cells inoculated x 100%.

Western blot analysis. Proteins were extracted using NP40 lysis buffer (Beyotime Institute of Biotechnology) and the bicinchoninic acid assay was applied to determine protein concentration. Proteins (20 μg) were separated by 10% SDS-PAGE and then transferred onto a PVDF membrane (cat. no. FFP28; Beyotime Institute of Biotechnology). The membrane was blocked using 5% skimmed milk at room temperature for 60 min and was then incubated with the following rabbit anti-human primary antibodies at 4˚C for 12 h: Anti-HIF-1α (1:500; cat. no. ab51608; Abcam); anti-survivin (1:5,000; cat. no. ab76424; Abcam); anti-cleaved caspase-3 (1:500; cat. no. ab2302; Abcam); anti-phosphorylated (p)-mTOR (1:1,000; cat. no. ab109268; Abcam); anti-mTOR (1:2,000; cat. no. ab2732; Abcam); anti-p-Akt (1:500; cat. no. ab38449; Abcam); anti-Akt (1:500; cat. no. ab8805; Abcam); anti-p-PI3K (1:1,000; cat. no. ab182651; Abcam); anti-PI3K (1:1,000; cat. no. ab191606; Abcam); and anti-GAPDH (1:2,500; cat. no. ab9485, Abcam). The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:5,000; cat. no. ab205718; Abcam) at room temperature for 1 h. The bands were visualized by enhanced chemiluminescence (EMD Millipore), and densitometry was performed using the Bio-Rad ChemiDoc system with Image Lab software version 6.0 (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was isolated using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Subsequently, RNA was reverse transcribed into cDNA using a RT kit (cat. no. D7168L; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. qPCR analysis was performed using SYBR Premix Ex Taq™ Real-Time PCR kit (Takara Bio, Inc.) on an ABI 7500 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR thermocycling conditions were as follows: 4 min pretreatment at 94˚C, followed by 30 cycles at 94˚C for 30 sec and 65˚C for 30 sec, and a final extension step at 72˚C for 10 min; the samples were then maintained at 4˚C. The primers were designed by Invitrogen; Thermo Fisher Scientific, Inc., as follows: HIF-1α, forward 5'-CAGTCGACA CAGCCTGGATA-3', reverse 5'-CCACCTCTTTTGGCA AGCAT-3' (product: 210 bp); survivin, forward 5'-TAGCTG CACACCTGACAAGA-3', reverse 5'-CCGTCAGCTCAG TGAAGTCT-3' (product: 200 bp); and GAPDH, forward 5'-CCATCTTCCAGGAGCGAGAT-3' and reverse 5'-TGC TGATGATCTTGAGGCTG-3' (product: 222 bp). GAPDH was used as a control of the input RNA level. Gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method (26).

Statistical analysis. All experimental results are presented as the mean ± SD for at least three independent experiments. The CCK-8, FCM, RT-qPCR and western blotting data were analyzed by one-way ANOVA followed by Tukey's test using IBM SPSS statistical software (version 19; IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

CPP evidently inhibits the viability of hypoxic A549 and H520 cells. The present study detected the viability of hypoxic A549 and H520 cells treated with various concentrations of CPP, in order to investigate the antitumor effect of CPP. The results of the CCK-8 assay revealed that the inhibition rates of hypoxic A549 cells treated with 50, 100, 200 and 400 nmol/l CPP were significantly higher than those in the control group; the IC_{50} value was calculated as 109.4 nmol/l (Fig. 1A and B; P<0.01). Similarly, based on CCK-8 data, the inhibition rates of hypoxic H520 cells treated with 20, 50, 100, 200 and 400 nmol/l CPP were significantly higher than those in the control group; the IC_{50} value was calculated as 67.4 nmol/l (Fig. 1C and D; P<0.01). These findings indicated that CPP could significantly reduce the viability of hypoxic A549 and H520 cells in a dose-dependent manner. Furthermore, the optimal concentrations of CPP for hypoxic A549 and H520 cells were 100 and 60 nmol/l, respectively; these concentrations were used in subsequent experiments.

Apoptosis of hypoxic A549 and H520 cells is enhanced by CPP in combination with radiation. Since CPP could obviously inhibit the viability of hypoxic A549 and H520 cells, further studies were conducted to determine whether CPP also affected the apoptosis of hypoxic A549 and H520 cells. The apoptosis of hypoxic A549 and H520 cells treated with CPP and radiation was detected using FCM; the apoptotic rate was determined as the sum of the right upper quadrant and the right lower quadrant percentages. As shown in Fig. 1E and G, the results indicated that the proportion of apoptotic A549 cells in the control group was 5.23%. Following treatment with hypoxia, CPP/hypoxia, X-ray/hypoxia and CPP/X-ray/hypoxia, the apoptotic rates of A549 cells were increased to 6.00, 11.83, 16.15 and 25.16%, respectively. In addition, the proportion of apoptotic H520 cells in the control group was 4.66%. Following treatment with hypoxia, CPP/hypoxia, X-ray/hypoxia and CPP/X-ray/hypoxia, the apoptotic rates of H520 cells were increased to 5.33, 12.08, 12.77 and 23.39%, respectively (Fig. 1F and G). These findings indicated that, to some extent, CPP and radiation could enhance the apoptotic capacities of hypoxic A549 and H520 cells. In addition, compared to CPP or radiation alone, the combination of CPP and radiation significantly accelerated the apoptosis of hypoxic A549 and H520 cells.

Proliferation of hypoxic A549 and H520 cells is suppressed by CPP in combination with radiation. This study demonstrated
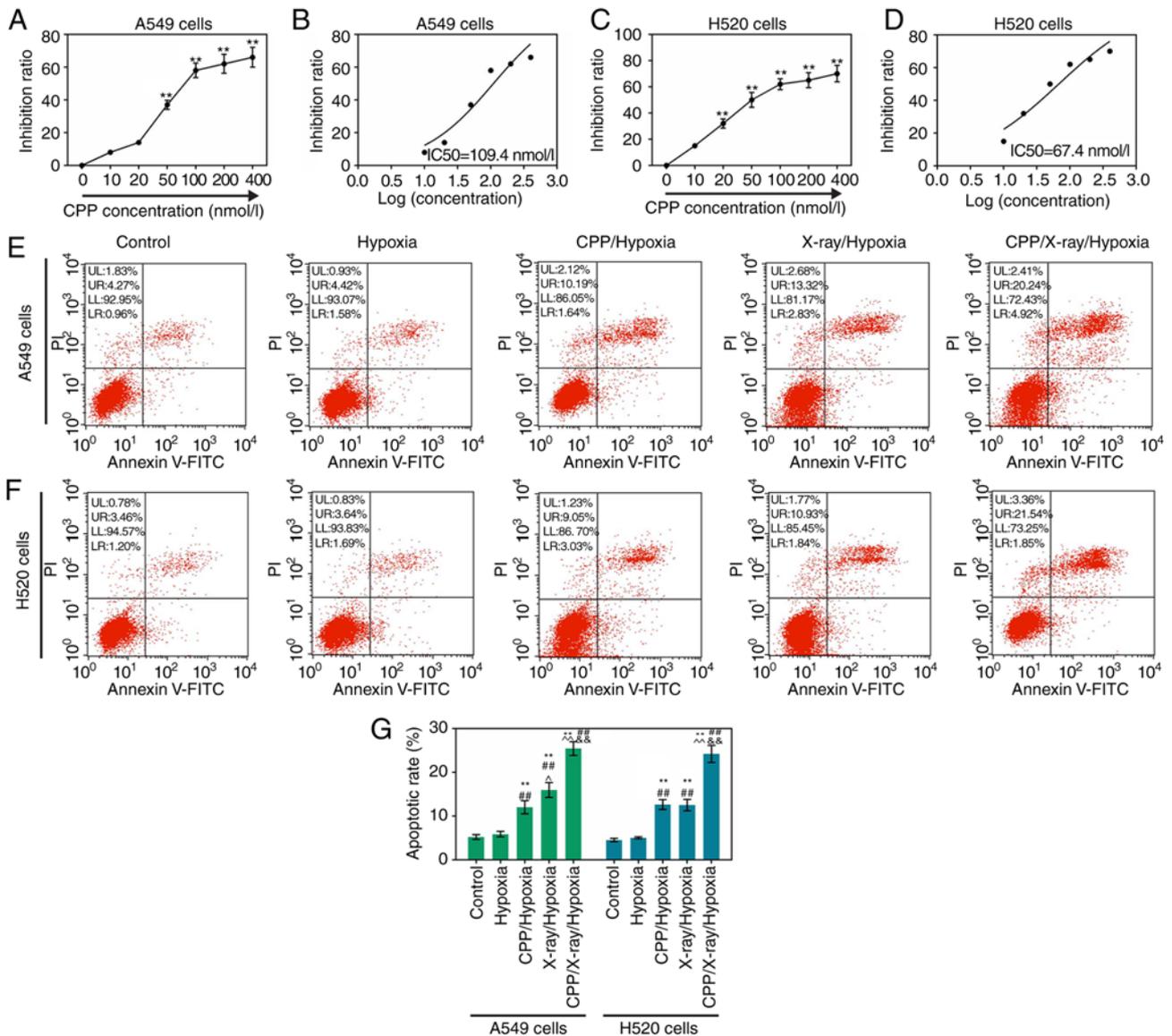
Figure 1. CPP inhibits the viability of hypoxic A549 and H520 cells, and the apoptosis of hypoxic A549 and H520 cells is enhanced by CPP in combination with radiation. Cell Counting kit-8 assays were performed on hypoxic (A and B) A549 and (C and D) H520 cells treated with PBS, 10, 20, 50, 100, 200 and 400 nmol/l CPP. (E-G) Flow cytometry was performed to detect the apoptosis of (E) A549 and (F) H520 cells in the Control, Hypoxia, CPP/Hypoxia, X-ray/Hypoxia and CPP/X-ray/Hypoxia groups. **P<0.01 vs. Control or 0 nmol/l; ##P<0.01 vs. Hypoxia; ^P<0.05 and ^^P<0.01 vs. CPP/Hypoxia; &&P<0.01 vs. X-ray/Hypoxia. CPP, *Cyclocarya paliurus* polysaccharide; PI, propidium iodide.

that the apoptotic capacities of hypoxic A549 and H520 cells were markedly enhanced by CPP in combination with radiation; therefore, the proliferative abilities of hypoxic A549 and H520 cells treated with CPP and X-ray were further detected. Colony formation assay results (Fig. 2A and B) revealed that CPP and X-ray could, to some extent, reduce the proliferation of hypoxic A549 and H520 cells. Furthermore, a noticeable reduction in the proliferative abilities of hypoxic A549 and H520 cells was determined in response to treatment with CPP and X-ray compared with CPP or radiation alone (Fig. 2A-C; P<0.05).

Expression levels of HIF-1α, survivin and cleaved caspase-3 are modulated by CPP in combination with radiation. To investigate the relevant mechanisms through which CPP in combination with radiation affected the apoptosis and

proliferation of hypoxic A549 and H520 cells, the expression levels of HIF-1α, survivin and cleaved caspase-3 were determined in A549 and H520 cells from each group. According to the RT-qPCR results, compared to CPP or radiation alone, the mRNA expression levels of HIF-1α and survivin were significantly decreased in hypoxic A549 and H520 cells following treatment with CPP in combination with X-ray (Figs. 3A and B, and 4A and B; P<0.05). According to western blot analysis, compared to CPP or radiation alone, HIF-1α and survivin protein levels were significantly decreased in hypoxic A549 and H520 cells following treatment with CPP in combination with X-ray (Figs. 3C and 4C; P<0.05). In addition, compared to CPP or radiation alone, CPP in combination with X-ray markedly promoted the activation of caspase-3 in hypoxic A549 and H520 cells (Figs. 3C and 4C; P<0.05). These findings suggested that CPP in combination with X-ray
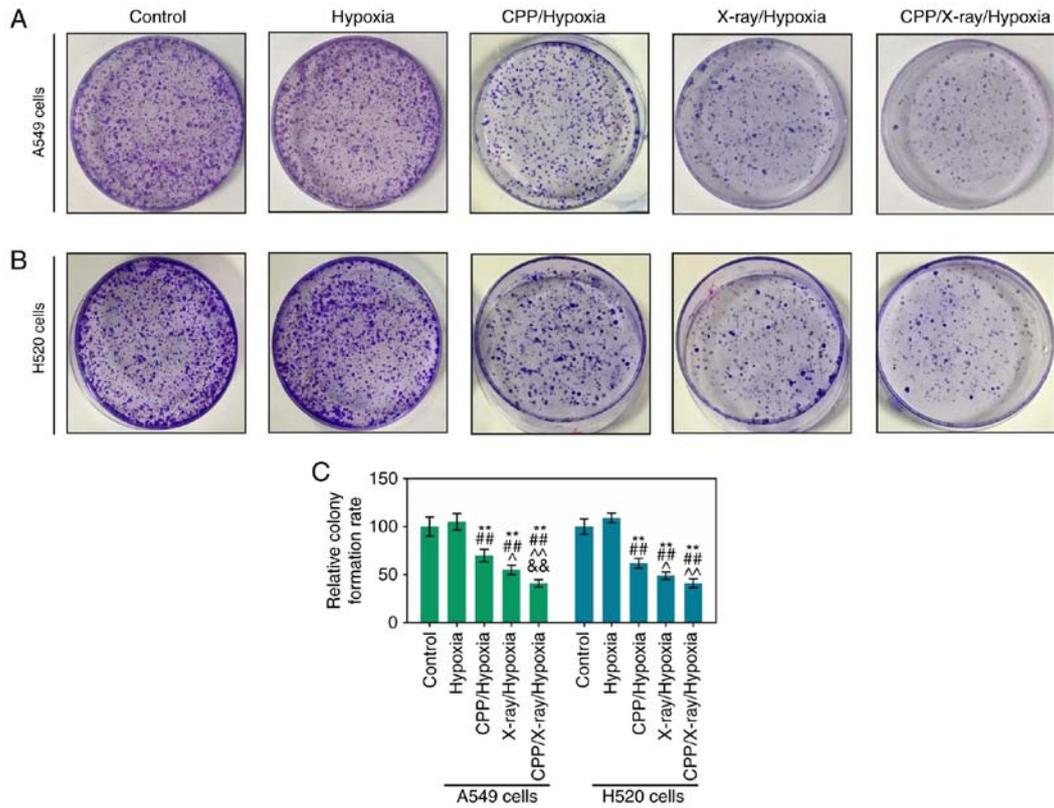
Figure 2. CPP in combination with radiation suppresses the proliferation of hypoxic A549 and H520 cells. (A-C) Colony formation assay was performed on (A) A549 and (B) H520 cells in the Control, Hypoxia, CPP/Hypoxia, X-ray/Hypoxia and CPP/X-ray/Hypoxia groups. **P<0.01 vs. Control; ##P<0.01 vs. Hypoxia; ^P<0.05 and ^^P<0.01 vs. CPP/Hypoxia; &&P<0.01 vs. X-ray/Hypoxia. CPP, *Cyclocarya paliurus* polysaccharide.
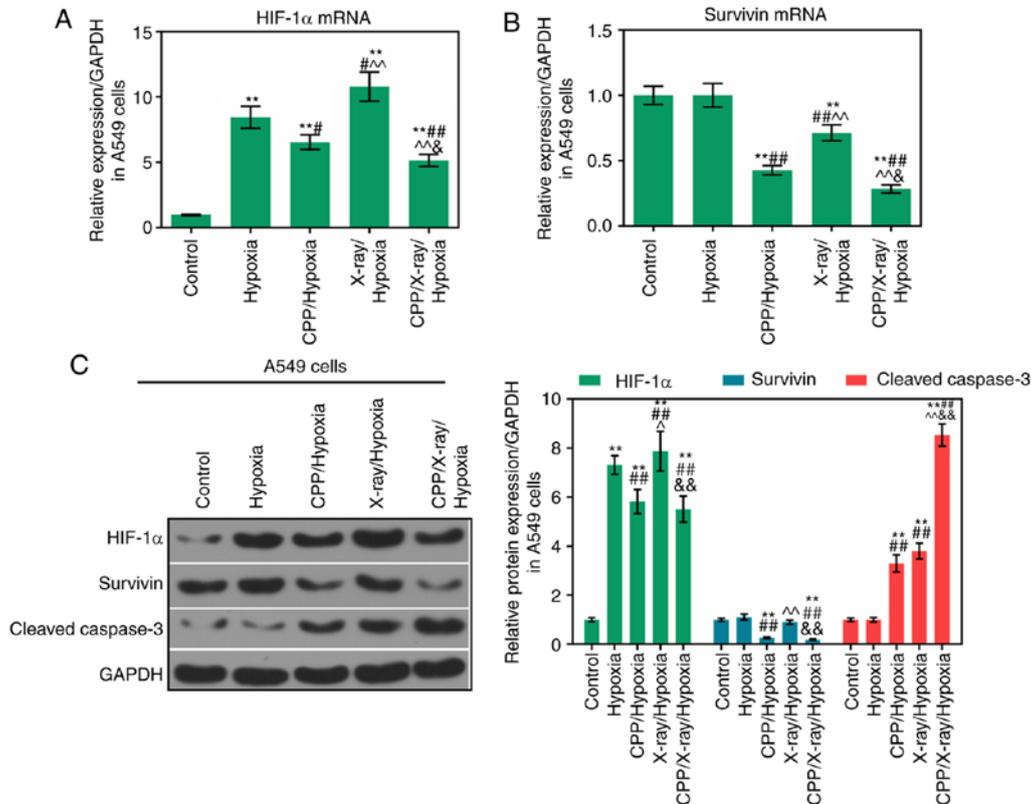
Figure 3. Expression levels of HIF-1α, survivin and cleaved caspase-3 in A549 cells are modulated by CPP in combination with radiation. (A and B) Reverse transcription-quantitative PCR and (C) western blotting were performed to detect HIF-1α, survivin and cleaved caspase-3 expression in A549 cells in the Control, Hypoxia, CPP/Hypoxia, X-ray/Hypoxia and CPP/X-ray/Hypoxia groups. **P<0.01 vs. Control; #P<0.05 and ##P<0.01 vs. Hypoxia; ^P<0.05 and ^^P<0.01 vs. CPP/Hypoxia; &P<0.05 and &&P<0.01 vs. X-ray/Hypoxia. CPP, *Cyclocarya paliurus* polysaccharide; HIF-1α, hypoxia-inducible factor-1α.
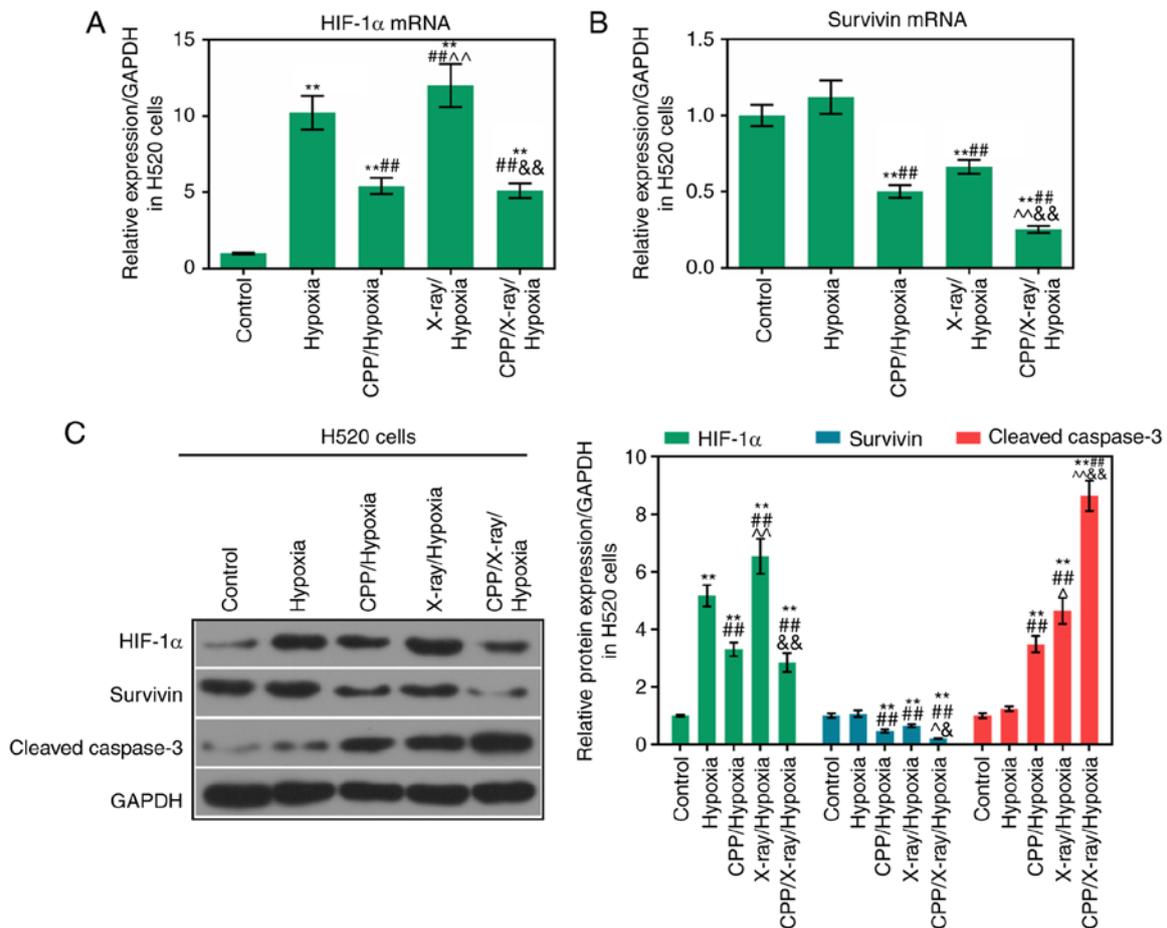
Figure 4. Expression levels of HIF-1α, survivin and cleaved caspase-3 in H520 cells are modulated by CPP in combination with radiation. (A and B) Reverse transcription-quantitative PCR and (C) western blotting were performed to detect HIF-1α, survivin and cleaved caspase-3 expression in H520 cells in the Control, Hypoxia, CPP/Hypoxia, X-ray/Hypoxia and CPP/X-ray/Hypoxia groups. [**]P<0.01 vs. Control; [##]P<0.01 vs. Hypoxia; [^]P<0.05 and [^^]P<0.01 vs. CPP/Hypoxia; [&]P<0.05 and [&&]P<0.01 vs. X-ray/Hypoxia. CPP, *Cyclocarya paliurus* polysaccharide; HIF-1α, hypoxia-inducible factor-1α.

markedly downregulated the expression levels of HIF-1α and survivin, and upregulated cleaved caspase-3 expression in hypoxic A549 and H520 cells. Therefore, CPP in combination with X-ray may affect the apoptosis and proliferation of hypoxic A549 and H520 cells via modulating the expression levels of HIF-1α, survivin and cleaved caspase-3.

CPP in combination with radiation affects the mTOR/Akt/PI3K pathway. To further investigate the mechanisms underlying the effects of the combination of CPP and radiation on hypoxic A549 and H520 cells, further studies were conducted on the mTOR/Akt/PI3K signaling pathway. Western blotting revealed that the expression levels of p-mTOR, p-Akt and p-PI3K were significantly lower in hypoxic A549 cells treated with CPP in combination with X-ray compared with in the X-ray/hypoxia group (Fig. 5A-D; P<0.05). Furthermore, the expression levels of p-mTOR, p-Akt and p-PI3K were significantly downregulated in hypoxic H520 cells following treatment with CPP in combination with X-ray compared with in the X-ray/hypoxia group (Fig. 5E-H; P<0.05). These data indicated that CPP in combination with radiation could inhibit the phosphorylation of mTOR, Akt and PI3K in hypoxic A549 and H520 cells. The schematic representation of the findings of this study is presented in Fig. 6.

Discussion

Radiotherapy is a widely adopted method used to treat non-small cell lung cancer; however, due to the resistance of tumor cells to radiation, locally recurrent or residual tumor cells hinder the efficacy of radiotherapy. Increasing the sensitivity of tumor cells to radiation has become the goal of radiotherapy (27-29). Radiosensitizers have therefore been developed and clinically applied; however, their application is not as satisfactory as expected because they are largely toxic and associated with side effects (30-32). It is of great clinical value to develop highly effective sensitizing drugs with low toxicity. Various traditional Chinese medicines and their extracts, for example *Lycium barbarum* polysaccharide and irisquinone, have been reported to possess antitumor and radiosensitizing effects (15,33). CPP is polysaccharide contained in CP, which has been shown to possess numerous bioactivities, including antioxidant, hypolipemic, hypoglycemic, antitumor and immunomodulatory effects (13). However, the radiosensitizing effects of CPP on tumor cells remain unclear. Therefore, CPP was selected as the research focus of this investigation. The IC_{50} values of CPP on A549 and H520 cells after 24 h were determined by CCK-8 assay; the results of which were 109.4 and 67.4 nmol/l, respectively.
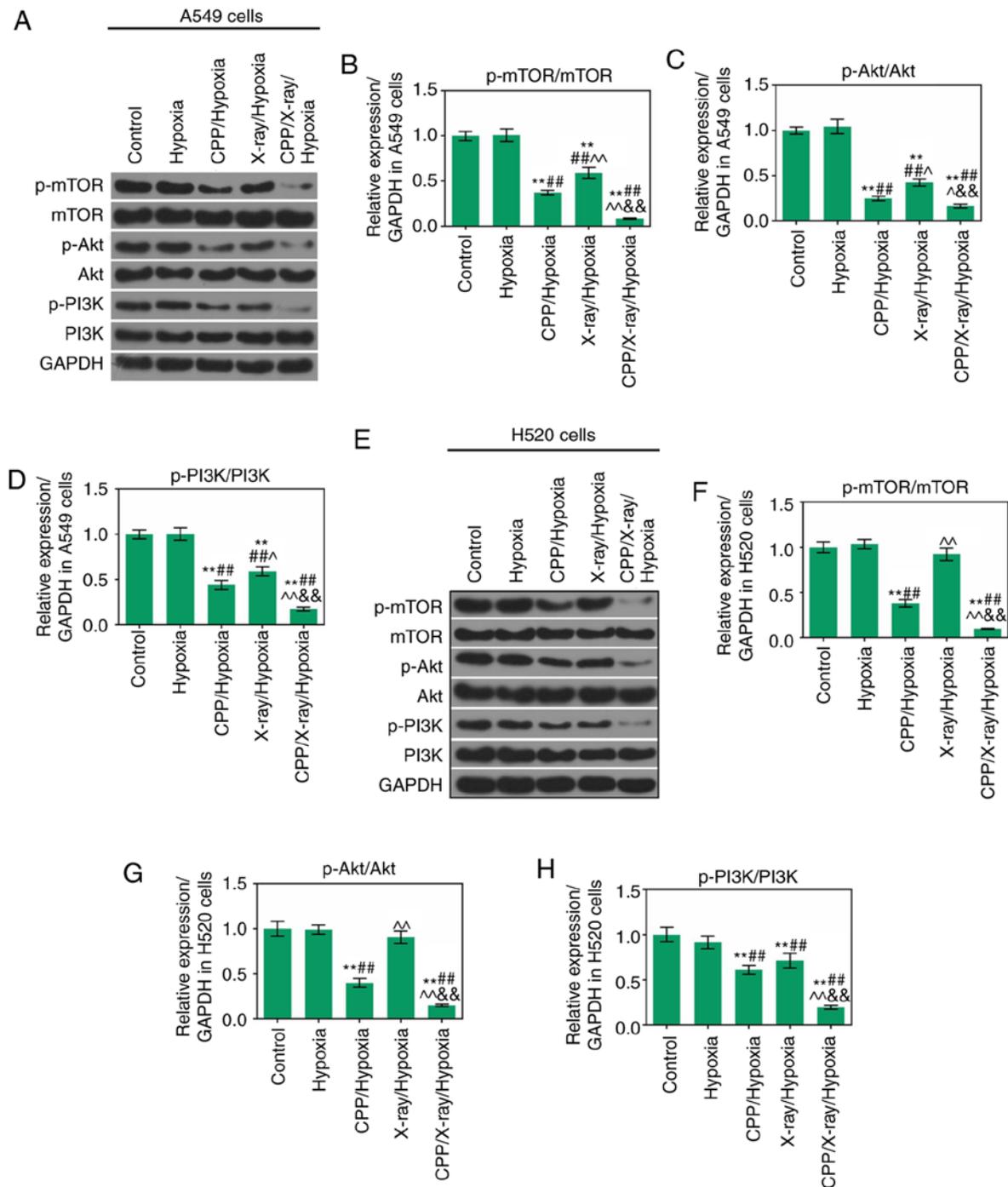
Figure 5. CPP in combination with radiation affects the mTOR/Akt/PI3K pathway. Western blotting was carried out to assess the expression levels of p-mTOR, mTOR, p-Akt, Akt, p-PI3K and PI3K in (A-D) A549 and (E-H) H520 cells in the Control, Hypoxia, CPP/Hypoxia, X-ray/Hypoxia and CPP/X-ray/Hypoxia groups. **P<0.01 vs. Control; ##P<0.01 vs. Hypoxia; ^P<0.05 and ^^P<0.01 vs. CPP/Hypoxia; &&P<0.01 vs. X-ray/Hypoxia. CPP, *Cyclocarya paliurus* polysaccharide; mTOR, mammalian target of rapamycin; p-, phosphorylated; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase.

Therefore, 100 and 60 nmol/l CPP was used to treat hypoxic A549 and H520 cells in subsequent experiments, respectively. In order to investigate the radiosensitizing effects of CPP on hypoxic A549 and H520 cells, the apoptotic capacities of hypoxic A549 and H520 cells treated with CPP and X-ray were further assessed. The FCM results revealed that in response to the same irradiation dose, the apoptotic rate of cells treated with CPP in combination with radiation was higher than that in the radiation only group. Furthermore, the proliferative abilities of hypoxic A549 and H520 cells treated

with CPP and X-ray were determined. The results of a colony formation assay demonstrated that CPP in combination with radiation markedly suppressed the proliferation of hypoxic A549 and H520 cells. These findings indicated that CPP could significantly enhance the radiosensitization of hypoxic lung cancer cells.

Hypoxia is a critical feature of solid tumors and is represented by decreased oxygen content caused by oxygen diffusion disorders, poor blood flow and blockage (34,35). Molecular biology studies have reported that the majority
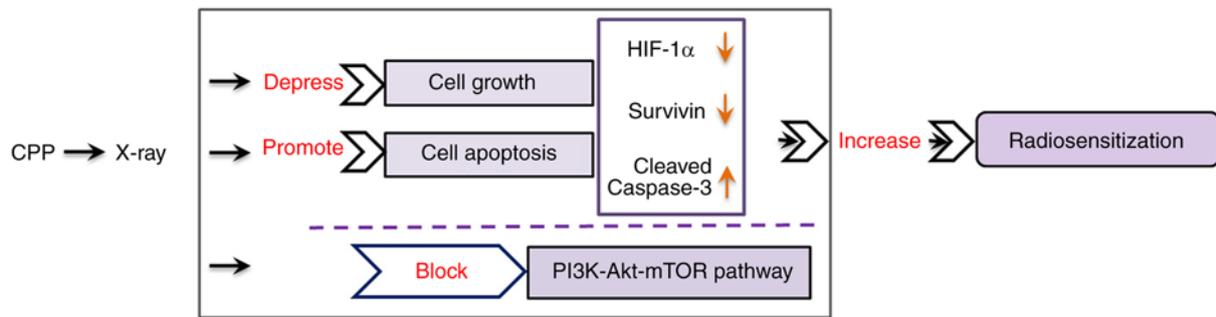
Figure 6. Schematic representation of the findings of the present study. Orange arrows indicate whether the expression levels of HIF-1α, survivin and cleaved caspase-3 were downregulated or upregulated. CPP, *Cyclocarya paliurus* polysaccharide; HIF-1α, hypoxia-inducible factor-1α; mTOR, mammalian target of rapamycin; p-, phosphorylated; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase.

of tumor tissues possess hypoxic cells. The resistance of hypoxic cells to radiation is also a direct cause of the failure of tumor radiotherapy, which may result in recurrence. HIF-1α is a well-studied gene in hypoxia. At low oxygen levels, the oxygen-dependent degradation domain of HIF-1α is unable to undergo hydroxylation and ubiquitination; therefore, the HIF-1α protein is not degraded and is more stable (36-38). Previous studies have suggested that hypoxia is associated with mTOR and survivin. Under short-term and moderate hypoxic conditions, the mechanism of mTOR upregulation may be associated with regulation of the 5'AMP-activated protein kinase pathway (39). In the present study, CPP combined with radiation treatment enhanced radiosensitivity of hypoxic A549 and H520 by blocking the PI3K/Akt/mTOR signaling pathway. In experiments conducted on human breast cancer cells and pancreatic cancer cells *in vitro*, researchers have demonstrated that the mRNA expression levels of survivin are increased under hypoxic conditions, compared with in normoxic group cells (40). In summary, mTOR, HIF-1α and survivin are oxygen-regulating molecules, and hypoxia can promote their expression. Therefore, this study evaluated the expression levels of HIF-1α, survivin, cleaved caspase-3 and the mTOR/Akt/PI3K pathway in hypoxic A549 and H520 cells treated with CPP and X-ray. RT-qPCR and western blotting demonstrated that CPP in combination with radiation significantly enhanced the expression levels of cleaved caspase-3, and reduced HIF-1α and survivin expression in hypoxic A549 and H520 cells. These outcomes suggested that CPP in combination with radiation affected the apoptosis and proliferation of hypoxic A549 and H520 cells via modulating the expression levels of HIF-1α, survivin and cleaved caspase-3. Furthermore, this study demonstrated that CPP in combination with radiation markedly suppressed the phosphorylation of mTOR, Akt and PI3K in hypoxic A549 and H520 cells. These results indicated that CPP may enhance the radiosensitivity of A549 and H520 cells via regulating the mTOR/Akt/PI3K pathway; however, the detailed mechanisms underlying the involvement of the mTOR/Akt/PI3K pathway in the radiosensitizing effect of CPP require a further investigation.

Taken together, this study demonstrated that CPP enhanced radiosensitization of hypoxic lung cancer cells through accelerating apoptosis and suppressing proliferation of hypoxic A549 and H520 cells. In addition, inactivation of the mTOR/Akt/PI3K pathway may be a possible mecha-

nism underlying the radiosensitizing effect of CPP. Previous studies (13,41) have suggested that CPP may inhibit MGC-803 cancer cell growth. Therefore, the present results were in line with the results of previous studies. Enhanced radiosensitizers in cancer cells (such as nitric oxide) may explain the increased radiosensitization in lung cancer cells treated with CPP. However, a previous study revealed that CPP is able to protect against oxidative stress in RAW264.7 cells (42). It was hypothesized that the differences may be related to the cell type and cell context. In summary, the present study provided novel information, which may improve understanding of the pathogenesis of non-small cell lung carcinoma, and may identify a potential approach for treating non-small cell lung carcinoma.

There are some limitations to this study; for example, although the hypoxic microenvironment of solid tumors in the body was simulated in tumor cells through maintenance in a humidified incubator containing 5% CO_2 and 1% O_2, whether the radiosensitizing effect of CPP can be applied to solid non-small cell lung carcinoma tumors remains to be determined. In addition, the effects of CPP on human non-small cell lung cancer under normoxic conditions must be further elucidated.

In conclusion, this study revealed that CPP enhanced the radiosensitivity of hypoxic A549 and H520 cells, and indicated that CPP may be an effective agent for the treatment of non-small cell lung carcinoma.

Authors' contributions

FZ made substantial contributions to study conception and design. LM and BF acquired, analyzed and interpreted the

data. All authors drafted the article, critically revised it for important intellectual content and gave final approval of the version to be published. BF and LM agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

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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