Radix Angelicae Sinensis and *Radix Hedysari* enhance radiosensitivity of $^{12}C^{6+}$ radiation in human liver cancer cells by modulating apoptosis protein

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ABSTRACT

الأهداف: دراسة آثار عشبة حشيشة الملاك الصينية وآلية عملها في خلايا الكبد السرطانية.

الطريقة: أجريت هذه الدراسة خلال الفترة من سبتمبر 2010م حتى أغسطس 2012م في جامعة قانسو للطب الصيني التقليدي، لانتشو، الصين. تم تقسيم المجموعات إلى مجموعة التحكم، ومجموعة الدواء، ومجموعة الإشعاع، ومجموعة مشتركة. أجري تحليل كلا من مقايسة CCK-8، وتحليل التشكيل للمستعمر، وتغيرات الدورة الخلوية، وتحليل الإستماته، وتحليل كاسباس 9، وسرفيفين بواسطة تفاعل السلسلة المبلمرة ولطخة ويسترن في المجموعات الأربع.

النتائج: أن الأثر المثبط لعشبة حشيشة الملوك الصينية على خلايا H22 تعتمد على كلا من الوقت والتركيز، كما أن عشبة حشيشة الملوك الصينية قادرة على زيادة حساسية الإشعاع لخلايا H22 بزيادة مؤشرات الإشعاع التحسسي وجزء حياة الخلية. أن استماتة الخلايا والمرحلة الانتقالية G_2/M ظهرت نتيجة الإشعاع الأيوني الثقيل الناتج عن طريق علاج حشيشة الملوك الصينية. يقلل كلا من الإشعاع بالتعاون مع عشبة حشيشة الملوك الصينية من تعبير سرفيفين ويرفع تعبير كاسباس 9 في خلايا H22.

الخاتمة: ترفع عشبة حشيشة الملوك الصينية إحصائياً حساسية الإشعاع لخلايا H22 من الإشعاع الأيوني الثقيل وآلية عملها المحتملة لحساسية الإشعاع كما أنها تساعد على موت الخلايا المبرمج كاسباس عن طريق سرفيفين الذي يعمل كمحس للاشعاع فعال.

Objectives: To investigate the radiosensitizing effects of *Radix Angelicae Sinensis-Radix Hedysari (*RAS-RH [an ultra-filtration extract]) and its underlying mechanisms in human liver cancer cells H22.

Methods: This study was conducted between September 2010 and August 2012 in the Gansu University of Traditional Chinese Medicine, Lanzhou, China. The groups were assigned as the control group, drug (RAS-RH) group, $^{12}C^{6+}$ radiation group, and combination group. The cell counting kit-8 assay, colony formation assay, cell cycle changes, and apoptosis analysis were carried out, and survivin and casepase-9 were evaluated by reverse transcription polymerase chain reaction and Western blot analyses in the 4 groups.

Results: The inhibitory effect of RAS-RH on H22 cells was dependent on both concentration and time, RAS-RH was able to enhance the radiosensitivity of H22 cells by increasing cell survival fraction and radiosensitization parameters. Apoptosis and the gap2/mitosis (G2/M) phase transition induced by $^{12}C^{6+}$ heavy ion radiation was enhanced by RAS-RH treatment. Irradiation, combined with RAS-RH, decreased survivin expression while increasing casepase-9 expression in H22 cells.

Conclusion: The RAS-RH increased the radiosensitivity of H22 cells of $^{12}C^{6+}$ heavy ion radiation significantly, and its possible mechanism of radiosensitization is to enhance caspase-dependent apoptosis through the down-regulation of survivin, thus, it can be used as an effective radiosensitizer.

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Primary liver cancer, in which 85-90% are hepatocellular carcinoma (HCC), is the most lethal of all cancers worldwide with a dismal prognosis. Radiotherapy is one of the most effective treatments of HCC. It is reported that approximately 50% of HCC patients receive radiation therapy, and $^{12}C^{6+}$ heavy ion is recognized, as the ideal radiation rays for HCC radiotherapy. Compared with the conventional low-linear-energy-transfer (LET) irradiation like x-rays, $^{12}C^{6+}$ heavy ions have more potential therapeutic advantages, such as, better spatial dose distribution, higher relative biological effectiveness (RBE), and lower oxygen enhancement ratio.[1-3] However, radio resistance, the main reason leading to the failure of HCC treatment as other radiotherapies do have gained increasing attention nowadays. *Radix Angelicae Sinensis-Radix Hedysari (*RAS-RH) is the ultra-filtration extract mixture from *Radix Angelicae Sinensis* and *Radix*. Its main components are *Radix Hedysari* polysaccharide and *Angelica Ferulic* acid, Formononetin, astragaloside, and astragalus polysaccharides.[4,5] A previous study[5,6] found that RAS-RH has certain anti-tumor effects, improving immune functions, removing free radicals, and inducing apoptosis. However, the radiosensitivity induced by RAS-RH in primary liver cancer cells has not been reported. Radiotherapy works by damaging the DNA of cancer cells, which can block the progression of cells through gap2 (G2) phases by breaking the checkpoint pathways, which provide the cells time to repair damaged DNA prior to resuming cell cycle progression, or to induce apoptosis.[7-9] Apoptosis are triggered by different signals, they merge at a common cytoplasmic regulator of this multistep process. According to the mechanisms of apoptosis, activation of caspases is the likely candidate for being the regulator. Caspases are responsible for initiating the hallmarks of the degradation phase of apoptosis, including DNA fragmentation.[10,11] Caspase-9 is the essential initiator caspase required by apoptosis signalling.[12] Survivin occupies an outstanding position among the inhibitor of apoptosis protein (IAP) family due to its complex mode of anti-apoptotic action and its additional function in the control of cell division.[13] Survivin shows a cell-cycle dependent expression with a marked increase in the G2/mitosis (M) phase. Caspase-9 is responsible for the progression of cells in the apoptotic process and survivin is able to inhibit the pro-apoptotic activity of caspase-9.[12,14] This study aims to investigate the radiosensitizing effects of RAS-RH and its underlying mechanisms. Human liver cells H22 were utilized for this study.

Methods. This study conducted in Gansu University of Traditional Chinese Medicine, Lanzhou, China between September 2010 and August 2012, was designed to investigate the role of RAS-RH in the radiosensitivity of human liver cancer cells. Samples were divided into 4 groups with different treatments: 1) control group: 0 mg/L RAS-RH and 0 Grey (Gy); 2) drug group: cells were treated with 100 mg/L RAS-RH; 3) radiation group: cells were treated only with $^{12}C^{6+}$ heavy ion radiation; and 4) combination group: the cells were treated with $^{12}C^{6+}$ heavy ion radiation exposure, and then treated with 100 mg/L RAS-RH for 24 h. The effect of RAS-RH on the survival rates of H22 cell, and the radiosensitivity for the H22 cell line was determined separately by a cell counting kit (CCK)-8 assay and clonogenic assay. Cell cycle progression and apoptosis were analyzed by flow cytometry, while survivin and casepase-9 were evaluated by reverse transcription polymerase chain reaction (RT-PCR) and Western blot analyses. This study was approved by the ethical board of the School of Basic Medical Sciences of Lanzhou University, Lanzhou, China.

Materials and reagents. The RAS-RH from KaiRui Company was purchased in the Minxing County of Gansu Province, China. *Radix Angelicae Sinensis* is an umbelliferae dry root, and *Radix Hedysari* is a pod-bearing plant of *Hedysarum polybotrys Hand.-Mazz* dry root identified by the Department of Pharmacy, Gansu University of Traditional Chinese Medicine, China. The RAS-RH was prepared by the Gansu Academy of Membrane and Technology, which has obtained the patent right. The RAS-RH was dissolved in distilled water, filter-sterilized with 0.22 µm filter disc, and stored at 4ºC before use. Human liver cancer cells H22 were provided by the Department of Pathology, Medical College of Lanzhou University, China. The Dulbecco's Modification of Eagle's Medium (DMEM) was purchased from Sigma Corporation (St. Louis, MO, USA). The CCK-8, propidium iodide (PI), fetal calf serum (FCS), agarose, RNA primer, and all routine chemicals were purchased from Shanghai Biological Engineering Limited, Shanghai, China.

Cell culture. Human liver cancer cells (H22) were cultured in DMEM medium supplemented with 10% FCS, 100 µg/mL streptomycin and 100 µg/mL penicillin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed every 48-72 hours.

Irradiation. High-LET irradiation was carried out with $^{12}C^{6+}$ heavy ion radiation at the Institute of

Modern Physics (IMP) of Chinese Academy of Sciences in Lanzhou, China. Cells were exposed to different doses of $^{12}C^{6+}$ heavy ion (160-350 MeV/u, original energy). The dose rate for $^{12}C^{6+}$ heavy-ion irradiation was set at approximately 0.5 Gy/min.

The CCK-8 colorimetric assay. The CCK-8 test is based on the enzymatic reduction of the tetrazolium salt WST-8 in metabolically active cells. Cells at approximately 85-90% confluency were harvested, and seeded into a 96-well plate at a density of 4×10^3 cells/well. Cells were incubated with various concentrations (5, 10, 25, 50, 100, 200 mg/L) of RAS-RH for an indicated time (12-72 hours). Cells in the control group were treated in the same way, except RAS-RH, was replaced by sterile phosphate buffered saline (PBS). After treatment, the medium was changed to a fresh one, and cells were incubated with 0.01 mL of CCK-8 for 2 hours. The optical densities (Å) in the 96-well plates were determined using a Microplate Reader at 450 nm. Cell growth inhibition was estimated using the following formula: growth inhibition (%)=1-Å (treated cells)/Å (control cells) ×100%.

Clonogenic survival assay. Cell survival rate was determined by the clonogenic survival assay as previously mentioned.[15] In brief, H22 cells at log phase were placed into a 6-well plate with the density of 10^3-10^5 cells in each well, and then the plate was shaken gently, ensuring that the cells are well-distributed. The cells were incubated at the temperature of 37°C in a saturated humidity of 5% CO_2 for 24 hours. After that, culture solution was removed and the cells were divided into the 4 groups, while only the radiation group and combination group cells were exposed to $^{12}C^{6+}$ heavy ion from 1, 2, 4, 6, 8, 10 Gy, then all of the cells in the 4 groups were cultured for 14 days in the incubator at a temperature of 37°C with 5% CO_2 humidified atmosphere. After rinsing with PBS, colonies were fixed with absolute alcohol for at least 30 minutes, stained with crystal violet for 20 minutes, and then counted under the microscope. Only colonies containing more than 50 cells were seen as survivors. The experiment was repeated 3 times. The plating efficiency (PE) was calculated according to the following formula: PE = (clone count/cell count) × 100%, while surviving fraction (SF) was estimated by the following formula: SF = number of colonies formed/(number of cells seeded × plating efficiency of the control), the survival fractions at various dose points were calculated. Plotting was carried out through the curve fitting performed with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA) according to the multi-target single-hit model ($SF = 1 - [1 - e^{-Dq/D0}]^N$) where D_0 is the mean lethal dose, Dq is the quasi-threshold, and N is the extrapolation number, the survival fraction at irradiation dose of 2 Gy (SF2), and sensitization enhancement ratio (SER) in each group was calculated accordingly.[16,17]

Cell cycle and apoptosis analysis with flow cytometer. Cell cycle distribution was analyzed as described previously.[8] In brief, H22 cells at log phase were harvested. Both the combination group and radiation group were exposed to $^{12}C^{6+}$ heavy ion radiation, and then treated with 100 mg/L RAS-RH for 24 hours. Cells in the 4 groups were collected after 24 hours, and fixed in 70% ethanol after being washed once with ice-cold PBS. Fixed cells were washed in PBS, then incubated with 100 g/ml RNase A for 20 min at 37°C, washed in PBS, and incubated with 50 mg/ml PI for 20 minutes on ice. Intensities of fluorescence signals were measured on Becton Dickinson FACS Flow Cytometer (Becton Dickinson & Co., San Jose, CA, USA). The apoptosis was analyzed by the Apo Alert annexin V apoptosis kit (Clontech Laboratories Inc., Takara Bio Co., Mountain View, CA, USA). At least 10,000 or more cells were measured for each sample. All of the samples were assayed in triplicate, and the fraction of every cell cycle phase was calculated. Cell cycle distribution and apoptosis were assessed by flow cytometric analysis of cells with DNA content.

The RT-PCR for survivin and caspase-9. The expression of survivin and caspase-9 mRNA were analyzed by RT-PCR amplification,[18,19] and the RT-PCR test was carried out according to instructions of the reagent company. The total RNA of the 4 groups was isolated using Triazol according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA. The synthesized cDNA was used for PCR amplification. The primers used for survivin reactions were 5'-AGCCCTTTCTCAAGGACCAC-3,' and 5'-TGGCTCGTTCTCAGTGGGGCAGT-3' (198 bp), 5'-ATGCTGTCCCATACCAGG-3,' and 5'-CAGGAACCGCTCTTCTTGT-3' (212 bp) were used as primers for casepase-9 reactions. The primers used for the β-actin (513 bp) reactions 5'-GACCTGACTGACTACCTCATGAAGAT-3,' and 5'-GTCACACTTCATGATGGAGTTGAAGG-3' were used as internal control. The thermal cycles were: 1 cycle of 94° for 3 minutes; 30 cycles of 94° for 40 seconds, 56° for 40 seconds, 72° for 90 seconds followed by a final elongation step of 72° for 10 minutes. The

PCR products were separated by electrophoresis on a 2% agarose gel. The gels were stained by ethidium bromide and photographed using the image analysis system.

Western blot for survivin and caspase-9. The Western blot performed as previously described,[20] was carried out by following the instructions of the reagent company. For Western blotting, equal amounts of protein were loaded per lane and resolved on a 10% (for survivin), or 8% low-bis (for casepase-9) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) gel (Millipore Billerica, MA, USA) and transferred onto 0.22 mm polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for one hour with 5% skim milk in 0.05% Tris-buffered saline/Tween (TBST), and then incubated with the primary antibody (diluted 1:1000 in blocking buffer for survivin, 1:1000 for casepase-9 (Cell Signaling Technology, Boston, USA), and 1:1000 for actin at room temperature for 4ºC overnight. The membranes were washed, incubated with horseradish peroxidase (HPR)-conjugated anti-mouse immunoglobulin (Ig)G (1:2500), and then detected by an enhanced chemiluminescence system the next day. The protein image was recorded by the BIO-RAD ChemiDoc XRS, and analyzed by the Quantity One 1-D Analysis software (Bio-Rad Laboratories Inc., Hercules CA, USA).

Statistical analysis. All analyses represent experiments that were performed in at least triplicate. The results were evaluated by one- or 2-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences version 13 (SPSS Inc., Chicago, IL, USA) to determine the significance. The sensitizer enhancement ratios (SER) were measured using GraphPad Prism 5.0 software according to the multi-target single-hit model, and $p<0.05$ was considered statistically significant.

Results. *The cyto-inhibition of RAS-RH on human liver cancer cells H22.* To evaluate the inhibition effects of RAS-RH on cultured H22 cells, the H22 cells were treated with different concentrations of RAS-RH for different treatment times, while cell viability was measured by CCK-8 assay. The inhibitory effects elicited by RAS-RH on the H22 cells were dependent on both concentration and time (**Figure 1**). To evaluate the ability of RAS-RH on sensitize tumor cells to $^{12}C^{6+}$ radiation, moderate inhibitory doses were applied. We applied 117.6±2.15 mg/L inhibiting concentration 20% (IC_{20}), so the 100 mg/L of RAS-RH was used for subsequent experiments.

The effect of RAS-RH on the radiosensitivity of human liver cancer cells (H22). To explore the effects of RAS-RH on the radiosensitivity of H22 cells, we performed an in vitro clonogenic cell survival assay using RAS-RH treatment plus $^{12}C^{6+}$ radiation. The H22 cells treated with 100 mg/L RAS-RH plus $^{12}C^{6+}$ irradiation exhibited significantly lower clonogenic survival rates than the cells treated with radiation alone. The SER of the combination group (1.86±0.08) was higher compared with the radiation group (1.0)(**Figure 2 & Table 1**). The values of D_0 and D_q were 1.17 Gy,

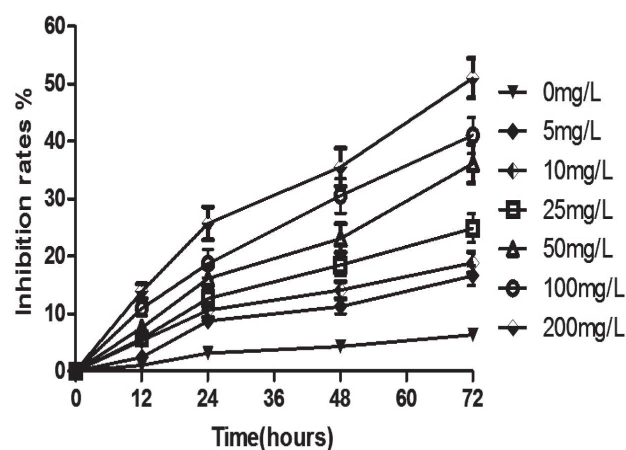

Figure 1 - A graph showing the *Radix Angelicae Sinensis-Radix Hedysari* (RAS-RH)-induced proliferation inhibition rate in H22 cells. The H22 cells were exposed to the indicated concentrations of RAS-RH for different time points. Cell survival was assessed using CCK-8 assay. The RAS-RH induced cyto-inhibition in H22 cells. The data are shown as the mean values ± standard error of the mean (±SEM) for 3 independent experiments.

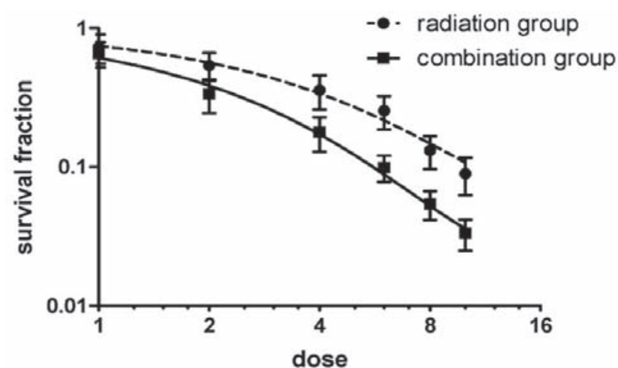

Figure 2 - The *Radix Angelicae Sinensis-Radix Hedysari* (RAS-RH)-induced radiosensitivity in H22 cells. Clonogenic cell survival curves were generated for H22 cells that were treated with the indicated concentrations of RAS-RH for 24 hours, and then were exposed to 1, 2, 4, 8 or 10 Gy irradiation. The survival data were normalized to that of the unirradiated control group. The sensitizer enhancement ratios was calculated for H22 cells that were treated with 100 mg/L RAS-RH prior to $^{12}C^{6+}$ irradiation. The values shown are the mean values ± standard error for 3 independent experiments.

and 1.53 Gy in the combination group, while 1.93 Gy and 2.48 Gy in the radiation group ($p<0.01$) (**Table 1**), 2-way ANOVA were used to test the interactive effects between the combination and radiation group, the results indicated that RAS-RH and radiation have significant interactive effects ($p<0.01$) for H22 cells, suggesting that RAS-RH treatment could sensitize H22 cells to $^{12}C^{6+}$ irradiation. The above results demonstrated that treatment with RAS-RH could increase the radiosensitivity of human H22 cells.

The effect of RAS-RH plus $^{12}C^{6+}$irradiation on cell cycle progression and cell apoptosis. To determine whether the observed RAS-RH-induced $^{12}C^{6+}$ radio sensitization was associated with changes in cell cycle progression, H22 cells were cultured for 24 hours prior to adding RAS-RH or RAS-RH with 4 Gy $^{12}C^{6+}$ irradiation. As shown in **Table 2**, $^{12}C^{6+}$ radiation induced a G2 arrest in the H22 cells. Combined treatment with RAS-RH and $^{12}C^{6+}$ irradiation increased the population of H22 cells. This result indicated that RAS-RH is able to block the repair of damaged DNA. The apoptosis rates were: 4.82±0.06% in the control group, 12.7±0.31% in the drug group, 21.9±0.23% in the radiation group, and 32.5±0.26% in the combination group, the differences among drugs group, radiation group, and combination group were statistically significant ($p<0.05$) (**Table 2**).

Table 1 - Parameters of human liver cancer cells (H22) in a single-hit multitarget model under different conditions.

Parameters	Radiation group	Combination group	*P*-value
D_0 (Gy)	1.93 ± 0.12	1.17 ± 0.11*	0.00456
D_q (Gy)	2.85 ± 0.13	1.53 ± 0.10*	0.00271
N	2.64 ± 0.46	1.34 ± 0.26*	0.00514
SF2	0.71 ± 0.09	0.43 ± 0.06*	0.00428
SERDq	1	1.86 ± 0.08	

D_0- mean lethal dose, D_q- quasi-threshold, N - extrapolation number, SF2 - survival fraction at irradiation dose of 2 Gy, SERDq - sensitization enhancement ratio. *$p<0.01$ compared with the radiation group

The RAS-RH can down-regulate survivin expression and up-regulate casepase-9 expression. To explore the role of survivin and casepase-9 genes in cellular sensitivity to radiation, the mRNA and protein expression intensities of survivin and casepase-9 gene were analyzed by RT-PCR and Western blotting. The mRNA and protein levels of an internal control gene, and β-actin were performed in the same run. As shown in **Figure 3**, the level of survivin mRNA in the combination group was significantly decreased compared with the control group, drug group, and radiation group ($p<0.05$). Compared with the other groups, casepase-9 mRNA has higher expression in the combination group ($p<0.05$) (**Figure 3**). In addition, as shown in **Figure 4**, Western blot analysis showed survivin protein was down-regulation expressed in the combination group compared with the other 3 groups, however, the result of casepase-9 protein was contrary to the other groups. The above results indicated that the down-regulation expression of survivin gene could be the up-regulated expression of casepase-9.

Discussion. The primary liver cancers, in which 85-90% are HCC, are the most deadly cancers due to poor prognosis (5 year survival rate <15%) worldwide. Management of HCC continues to be challenging because of high recurrence rate after surgical resection and resistance to chemotherapy and radiotherapy.[21] Radiotherapy is one of the major and effective treatments for tumors. However, it was reported that the recurrence (20-30%), and treatment failure rate (30-40%) is still very high.[22,23] The RAS-RH are the prevalent-regional drugs of Gansu province. The RAS-RH was identified by our research team and collaborators.[6,24] It was reported[25,26] that RAS-RH has the function of improving immunity, anti-tumor properties, and removing free radicals, in addition, one of the compounds of RH shows anti-tumor activities on liver hepatocellular cells G2 with IC_{50} values of 10.69

Table 2 - The distribution of H22 cell cycle and cell apoptosis in the 4 groups (mean ± standard deviation).

Groups	G_0/G_1	S	G_2/M	*P*-value	Apoptotic rate (%)	*P*-value
Control group	49.37 ± 3.24	34.23 ± 1.78	11.23 ± 1.26	0.0247	4.82±0.06	0.0153
Drug group	30.53 ± 2.13	30.53 ± 2.13	24.42 ± 1.45*	0.0185	12.7 ± 0.31*	0.0114
Radiation group	20.21 ± 1.62	28.19 ± 1.75	29.19 ± 1.89*	0.00724	21.9 ± 0.23*	0.00318
Combination group	9.43 ± 1.48	11.01 ± 1.69	47.23 ± 2.71†		32.5 ± 0.26†	

*indicates $p<0.05$ compared with control group; † indicates $p<0.01$ compared with the radiation and drug group. G - gap, S - S phase, M - mitosis

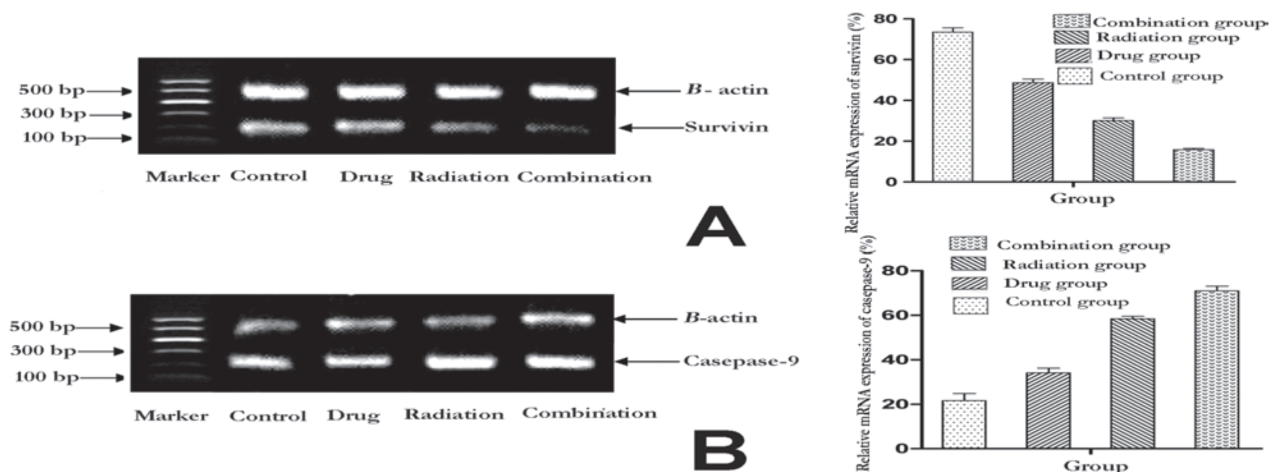

Figure 3 - Detection of survivin and casepase-9 expression by reverse transcription polymerase chain reaction (RT-PCR) analysis: **A**) mRNA expression of survivin detected by RT-PCR; and **B**) mRNA expression of casepase-9 detected by RT-PCR. Total RNA of survivin and casepase-9 from the control group, drug group, radiation group, and combination group were reverse transcribed and amplified by the primers from survivin, casepase-9, and β-actin. The products of PCR were separated on a 2% agarose gel, the survivin mRNA expression was observed in the control group (73.5%), drug group (48.6%), radiation group (20.4%), and combination group (10.7%) (*$p<0.05$). The casepase-9 mRNA expression was also observed in the control group (21.6%), drug group (34.1%), radiation group (58.4%), and combination group (71.3%) ($p<0.05$).

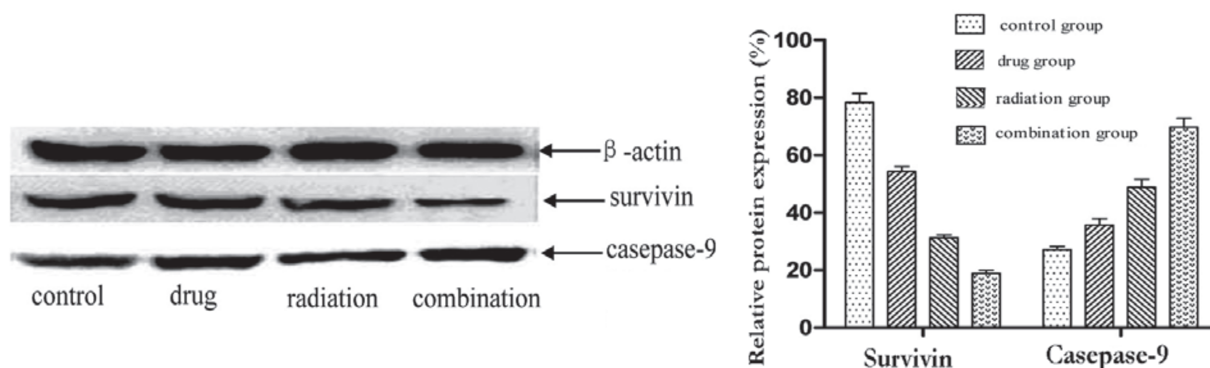

Figure 4 - Detection of casepase-9 and survivin expression by Western blot analysis. Membranes were probed with antibodies for target protein, and expression levels were normalized for loading by probing for β-actin, the survivin protein expression in the control group was 78.3%, 54.4% in the drug group, 31.2% in the radiation group, and 18.9% in the combination group ($p<0.05$), while the casepase-9 protein expression in the control group was 27.1%, 39.7% in the drug group, 50.1% in the radiation group, and 73.7% in the combination group ($p<0.05$).

µmol/L.[27] Other studies[7,8] indicate that RAS-RH has certain effects that induce apoptosis.

In the current study, we found that RAS-RH elicits inhibitory effects on human liver cancer H22 cells in a concentration- and time-dependent manner. Moreover, RAS-RH enhanced radiosensitivity and promoted the apoptosis of H22 cells. We also found that the combination treatment (RAS-RH plus $^{12}C^{6+}$ radiation treatment) can decrease survivin, while increasing casepase-9 expression level. In summary, these data demonstrate the effect of combination treatment, and provide a potential mechanism of RAS-RH-mediated radiosensitization.

Radiosensitivity of tumors is a complex interaction, which is affected by many factors. The mechanism of radioresistance remains unclear, although some studies[28-31] have shown that tumor radiosensitivity can effectively be controlled by affecting DNA damage repair, cell cycle checkpoint, apoptosis, radio-related signal transduction pathways, and tumor microenvironment. One possible mechanism common to radiosensitivity is killing tumor cells via apoptosis. It was reported that distinct pathways leading to apoptosis are triggered by different signals.[27] Studies of the mechanisms of apoptosis have yielded compelling evidence that activation of caspases is the likely candidate for being

this regulator. This provides a common biochemical pathway to explain the irreversible commitment point for this type of cell death. In the past 10 years, the possible involvement of caspases in radiation-induced apoptosis has been explored, caspases are intracellular proteases that function as initiators and effectors of apoptosis.[32] Caspases function as a component in cell signaling pathways, which are involved in events such as apoptosis, cell growth, and differentiation. As the essential initiator, caspase is required for apoptosis signalling, caspase-9 is activated on the apoptosome complex, and failure to activate caspase-9 has profound pathophysiological consequences. Intrinsic apoptosis results in apoptosome formation, activation of caspase-9, and subsequent activation of effector caspases.[12]

Survivin, a new member of the IAP family, is one of the most prominent cancer-associated genes that have been studied extensively during the recent years. Some research indicated that it is a requisite factor in the cell cycle regulators assisting completion of cytokinesis.[33] Survivin shows a cell-cycle dependent expression with a marked increase in the G2/M phase. In metaphase, survivin localizes to kinetochores, and then transfers to the central spindle midzone in anaphase. In most cancer cell lines, survivin is upregulated in the G2/M phase.[34,35] Caspase-9 is responsible for the progression of the cell in the apoptotic process, while survivin works as an inhibitor of pro-apoptotic activity of caspase-9.[12,14] Survivin is able to block the activation of caspase-9, ribozyme-mediated inhibition of survivin induced caspase-9-dependent apoptosis in prostate cancer cells.[36] Likewise, it is one of the most important tumor-specific molecules as it plays a special role in molecular mechanisms, such as, apoptosis inhibition promoter, tumor-associated angiogenesis, and a resistance factor of anticancer therapies.[37] Mori et al[38] found that apoptosis induced by high LET radiation was suppressed by inhibitors of caspase-9. These observations suggest that caspase-9 may contribute to caspase dependent apoptosis after exposure to high LET radiation. Therefore, the findings of this study provide valuable information such as; RAS-RH can result in enhanced radiosensitizing effect of $^{12}C^{6+}$ radiation, restrain survivin expression, and upregulating casepase-9 expression.

The limitation of this study is that the RAS-RH were only studied in human liver cancer H22 cells in vitro, therefore, in vivo animal experiments should be conducted in the future. We expect that RAS-RH is able to restrain tumor by enhancing the radiosensitivity of $^{12}C^{6+}$ radiation, induce the apoptosis by blocking survivin expression, and up-regulation of casepase-9 expression. However, the mechanism of radioresistance is still complicated, in our further studies we will investigate the relationships between the presence of DNA damage and radiosensitivity of $^{12}C^{6+}$ radiation induced by RAS-RH treatment.

In conclusion, this study shows that RAS-RH treatment can enhance the radiosensitivity of H22 cells by suppressing survivin, and increasing casepase-9 levels after $^{12}C^{6+}$ irradiation. The RAS-RH can increase the apoptosis rate and G2/M cell cycle arrest, which probably drives more irradiation-damaged cells into mitosis. Clinical application of this anti-malarial drug can be expanded to complement treatment of radiation therapy for cancer.

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