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Original Research

Effect of HIF-1αsiRNA-linked AuNRs on radiotherapy of nasopharyngeal carcinoma

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Abstract: The radiation sensitivity of tumor cells is closely related to tumor cell hypoxia. Hypoxia-inducible factor-1α (HIF-1α) is considered a key transcription factor which regulates the sensitivity of hypoxic tumor cells to radiotherapy. On the other hand, some studies have shown that gold nanomaterials improve radiation sensitivity. However, studies on the effect of gold nanomaterials carrying HIF-1αsiRNA on tumor radiotherapy, and the underlying mechanisms are limited. Thus, the present study was aimed at investigating the effect of gold nanocomposites (AuNRs) carrying HIF-1αsiRNA (AuNRs-HIF-1αsiRNA) on the radiation sensitivity of nasopharyngeal carcinoma (NPC) hypoxia cells. The effect of AuNRs-HIF-1αsiRNA on radiation sensitivity of hypoxic NPC cells was determined under X-ray irradiation. The results showed that Au-HIF-1αsiRNA improved the radio-sensitivity of NPC tumor. Thus, this study has demonstrated that gold nanomaterials carrying HIF-1αsiRNA effectively increased the radio-sensitivity of hypoxic tumor, thereby improving the effect of radiotherapy on NPC cells.

Key words: AuNRs; HIF-1α; siRNA; Radiotherapy; Sensitivity; NPC; Hypoxias.

Introduction

The incidence of nasopharyngeal carcinoma (NPC) in Asian countries is generally high, and most NPC patients are subjected to irradiation therapy. Studies have shown that the 5-year survival of NPC patients after receiving concurrent chemotherapy and radiotherapy is about 94% (1). However, the efficacy of radiotherapy in patients with NPC depends on the radio-sensitivity of NPC cells, which affects the local recurrence rate and prognosis. The radio-sensitivity of tumor cells is related to the hypoxic micro-environment in which the tumor cells are located. It has been reported that the hypoxic environment of cells in a solid tumor reduces radio-sensitivity and inhibits tumor cell apoptosis, thereby reducing the therapeutic effect of radiation (2). Previous studies have shown that HIF-1 is a key transcription factor involved in tumor hypoxia adaptation (3). It is composed of HIF-1α subunit and HIF-1β subunit, but its function is determined mainly by the stability and activity of the HIF-1α subunit. Under hypoxic micro-environments, HIF-1α is overexpressed, which induces the expression of related downstream genes, thereby enhancing the adaptation of tumor cells to hypoxia, and increasing their resistance to damaging effects of radiotherapy (4, 5). Thus, a knockdown of HIF-1α expression may be effective in reversing the radiotherapy resistance of hypoxic NPC cells. The technique of RNA interference (RNAi), an emerging gene knockout technology with high specificity and efficiency, has been widely used in many fields. It is a small interfering RNA (siRNA) which promotes silencing of targeted HIF-1α mRNA, thereby down-regulating the expression of the target gene (6). However, due to the negative charge on siRNA, its membrane permeability is very poor, resulting in impaired delivery into the cell (7). Therefore, the present research was focused on how to safely and effectively carry out targeted delivery of siRNA molecules into the cell.

Rapid advances in research on gold nanomaterials have provided new ideas for improving the radio-sensitization of hypoxic tumor cells. Gold nanomaterials, ranging in size from 1 to 100 nm, have chemical inertness and good bio-compatibilities. Gold nanoparticles (AuNRs) combine with nucleic acids (DNA and RNA) (8). Upon irradiation, AuNRs generate secondary electrons and reactive oxygen species (ROS) which damage cellular DNA and lead to cell death, thereby increasing the sensitivity of hypoxia tumor cells to irradiation therapy (9, 10). Hainfeld, Slatkin (11) were the first to report (in 2004) that nanogold improved radiation sensitivity in animals. In 2017, Chang and collaborators designed core-shell gold nanocomposites for tumor radiotherapy (12).

The present study was aimed at investigating whether the gold nanorod attached to HIF-1αsiRNA (AuNRs-HIF-1αsiRNA) could effectively increase the sensitivity of hypoxic NPC cells to radiation. This was with a view to enhancing the effect of radiotherapy on NPC cells.

Materials and Methods

Preparation of AuNRs-HIF-1αsiRNA

Crystal growth method was used to synthesize AuNRs-CTAB at the Shenzhen Institute of Advanced Technology of Chinese Academy of Sciences. Subsequently, the cetyltrimethylammonium bromide (CTAB) on the surface of AuNRs was replaced with mercaptoundecanoic acid (MUA), resulting in generation of AuNRs-MUA. The MUA made it easy to combine AuNRs with DNA and RNA. Twenty micromolar (20 μM) HIF-1αsiRNA solution (i.e. approximately 0.246mg/mL dissolved in diethylpyrocarbonate, DEPC) was mixed with AuNRs (5mg/mL). The mixture was allowed to stand at room temperature for 30 min in the dark, resulting in formation of AuNRs-HIF-1αsiRNA at a concentration of approximately 5.2mg/mL.

Effect of AuNRs- HIF-1αsiRNA on the radio-sensitivity of tumor cells

Three groups of tumor cells were used in this experiment: control group (normal saline group), simple AuNRs group (5mg/mL), and Au-HIF-1αsiRNA group (5.2mg/mL). All tumor cells were treated with $CoCl_2$ to simulate hypoxic environment. The cells (CNE-2) were cultured for 48 h after irradiation, and a single cell suspension was prepared and seeded in a 6-well plate at a density of 600 cells/well, following counting with a cell counting plate, and dilution. Three radiotherapy doses (0, 2 and 4 Gy) were used, with three duplicates for each dose. High-energy photons (MV) were delivered using a linear accelerator (Siemens in Germany) with a 20×20 cm radiation field at a rate of 500 MU/min. After the irradiation, the 6-well plate was placed in an incubator for about 1 to 2 weeks until colony formation was visible. Thereafter, the cells were fixed with 4% paraformaldehyde, and stained with crystal violet for 10 min. Under the microscope, inoculation efficiency and survival fraction (SF) were calculated for cell colonies with cell number > 50. The correlation coefficients of equations for cell viability curve were calculated using SPSS13.0 software. The dose-survival curve for each group was drawn.

In vivo NPC xenograft growth in nude mice after irradiation

Animal experiments in this study have been approved by the local ethics committee. Twenty-four female SPF BALB/c nude mice weighing 16–18 g were obtained from SLAC Laboratory Animals (Shanghai, China). The xenograft nude mice tumor model was established via ventral subcutaneous injection of CNE-2 cells at logarithmic growth phase at a dose of 1×106 cells per mouse. After 10 days, the diameter of transplanted tumors reached 7-9 mm, indicating successful establishment of the NPC xenograft model. Then, 24 nude mice were divided into 4 groups: control group (normal saline group), simple AuNRs (5mg/mL) group, AuNRs-NCsiRNA group (negative control), and AuNRs-HIF-1αsiRNA group (5.2mg/mL). The nude mice were intravenously administered their respective treatments via the tail vein on days 1, 3, and 5. Using Western blotting , the expression of HIF-1α was determined in 5 nude mice from control group, simple HIF-1αsiRNA group, simple AuNRs group, AuNRs-NCsiRNA group, and AuNRs-HIF-1αsiRNA group. The tumor tissue was added to liquid nitrogen for full grinding, and 1 ml lysate was added to fully mix, 4°C, 1200r/min centrifuged for 20 min, to take the supernatant.The loading buffer and supernatant were added in a 95°C water bath for 20 min and then centrifuged to obtain the extracted total protein. After the electrophoresis gel is placed in an electrophoresis tank, each group of protein samples is separately added to the sample well for electrophoresis using a micropipette. After the electrophoresis was completed, the electrophoretic transfer was set to a constant current of 300 mA, and the membrane was rotated for 90 min. The transferred PVDF membrane was immersed in the blocking solution and shaken at room temperature for 1 h at room temperature, and then washed with 1×PBST for 3 times to add primary antibody (HIF-1α 1:1000, β-actin 1:1000), and incubated at 4°C shaker overnight. After rinsing 3 times with 1×PBST, secondary antibody (1:1000) was added and followed by incubation for 80 min at room temperature on a shaker. The front side of the PVDF membrane was plated in a kit, and the chemiluminescent substrate was added to SuperSignal for 1 min. The PVDF membrane was taken out to remove the residual liquid and placed in a fluorescence imager for imaging.

It were divided into 4 groups: simple HIF-1αsiRNA group, simple AuNRs group, AuNRs-NCsiRNA group, and AuNRs-HIF-1αsiRNA group. Each group was sub-divided into two treatment types: non-irradiation and 4-Gy irradiation. Each sub-group contained 3nude mice. Forty-eight hours (48 h) after the last treatment, the irradiation group of nude mice was subjected to a single irradiation dose of 4 Gy on the tumor site for 3 consecutive days (total dose was 12Gy, depending on tumor size and tolerance of nude mice).

The source-surface distance (SSD) was set at 100 cm, and the relevant irradiation parameters were identical to those in the cell experiment. At the end of the irradiation, the tumor volume (V) was calculated using the formula:

$$V\,(cm^3) = \frac{(length \times width^2)}{2}$$

Tumor volume was calculated and recorded for 3 weeks, after which the nude mice were sacrificed and tumor tissues were collected and weighed.

Safety evaluation

After the model was successfully established, the mice were treated with normal saline (control group), AuNRs (5mg/mL), AuNRs-NCsiRNA (5.2mg/mL), or Au-HIF-1αsiRNA (5.2mg/mL) once every 3 days for 3 times, with or without irradiation treatment. At 24 h after the final injection, the nude mice were sacrificed, and the relevant tissues were taken and processed for light microscopy. The tissue sections were stained with hematoxylin and eosin (H&E) and observed under a microscope. Blood samples were collected for determination of indices of liver and kidney functions i.e. 0.5 ml of serum from nude mice are used by ELISA kit (ZIKER,Shenzhen,China) to detect ALT and Urea .alanine transaminase (ALT) and urea , prior to sacrificing the animals.

Statistical analysis

The results obtained are expressed as mean ± standard deviation (SD). Statistical analyses were carried out using *t*-test and analysis of variance (ANOVA). All analyses were done with the SPSS software 13.0. Values of $p < 0.05$ were considered statistically significant.

Results

AuNRs-HIF-1α siRNA improved the radio-sensitivity of tumor cells

The results of colony formation showed that values of survival fraction at 2 Gy (SF_2) in the control group, simple AuNRs group and AuNRs-HIF-1α siRNA group were 0.65, 0.46, and 0.26, respectively, while the corresponding values of cell survival fraction at 4 Gy (SF_4) were 0.29, 0.166, and 0.018, respectively. The sensitizing enhancement ratio (SER) of the AuNRs group was 1.12, while the SER of the Au-HIF-1α siRNA group was 2.89 (Figures 1 and 2).

AuNRs-HIF-1αsiRNA enhanced radio-sensitivity in nude mouse xenografts

In the western blot experiments, it was observed that HIF-1α protein expression was significantly reduced in the AuNRs-HIF-1αsiRNA group (Figure 3). The tumor volume of radiotherapy group was smaller than that of the absence of irradiationgroup. The tumor volume of simple HIF-1αsiRNA group,simple AuNRs group,AuNRs-NCsiRNA group, and AuNRs-HIF-1αsiRNA group decreased gradually in radiotherapy group(Figure 4). In the absence of irradiation, the tumor volume was measured for each group of nude mice. As shown in Figure 5, the tumor volume of simple AuNRs group (1.92±0.60 cm^3), AuNRs-NC siRNA group (2.13±1.48 cm^3) and AuNRs-HIF-1αsiRNA group (1.33±0.40 cm^3) were significantly lower than that of control group (3.05±0.58, $p<0.05$). However, there were no statistically significant differences among the three groups ($p>0.05$). Moreover, there were no statistically significant differences ($p>0.05$) in tumor weight among the control group (4.27±0.28 g), simple AuNRs group (3.29±1.15g) and the AuNRs-NCsiRNA group

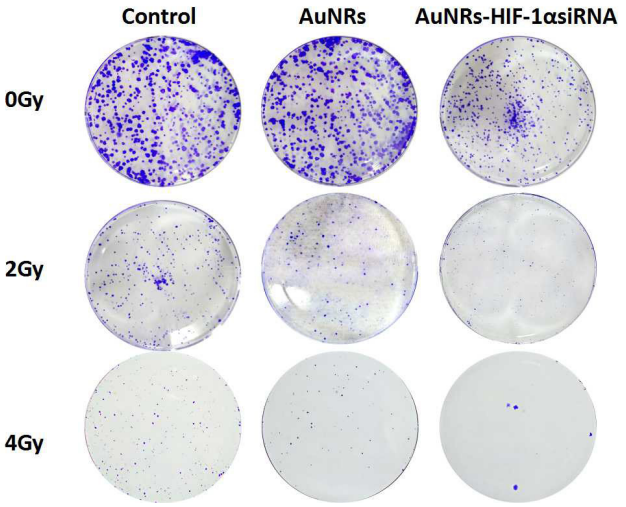

Figure 1. AuNRs-HIF-1αsiRNA improves the radiosensitivity of tumor cells (2 weeks).

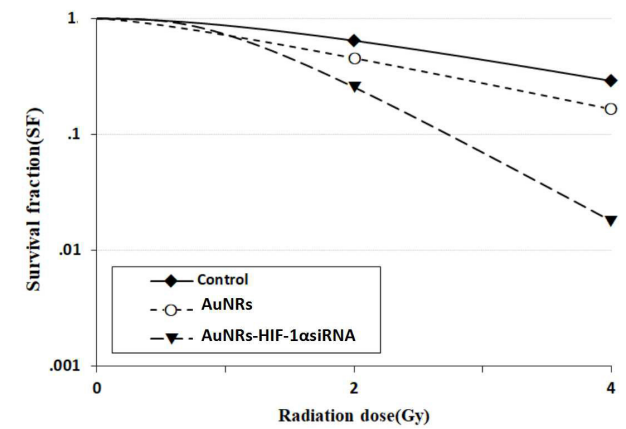

Figure 2. Calculation of sensitizing enhancement ratio (SER). Dose-survival curves after exposure to radiotherapy in each group (click-on multi-target model). Dose-survival curves of each group after irradiation with 0, 2 and 4 Gy. SF: survival fraction; Control: saline control group.

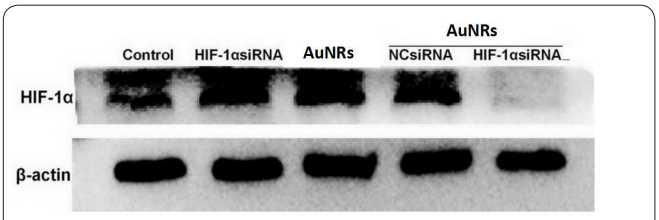

Figure 3. Western blot showing the effect of AuNRs-HIF-1αsiRNA on HIF-1 protein expression.

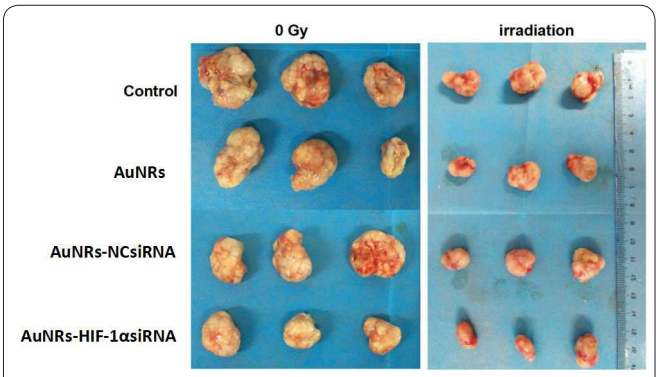

Figure 4. Nude mouse xenografts.

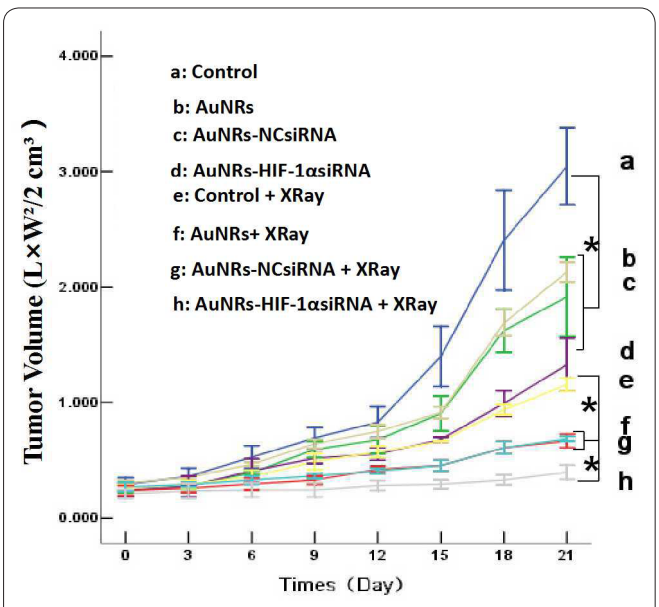

Figure 5. The nude mouse xenografts (volume growth curve). "*"P < 0.05.

(3.65±0.27 g), while the AuNRs-HIF-1αsiRNA group (3.04±0.33 g) had a slightly reduced tumor weight, relative to the other 3 groups (p<0.05; Figure 6).

On the other hand, tumor volume and tumor weight were decreased in three different groups after irradiation in nude mice. Compared with the irradiation control group (1.16±0.10 cm3), the tumor volume was decreased in thesimple AuNRs-irradiation group (0.67±0.10 cm3), the AuNRs-NCsiRNA-irradiation group (0.69±0.035 cm3) and the AuNRs-HIF-1αsiRNA-irradiation group (0.40±0.11 cm3; Figure 5). However, there was no significant difference between the simple AuNRs-irradiation and the AuNRs-NCsiRNA-irradiation groups. Tumor volume in the AuNRs-HIF-1αsiRNA irradiation group was significantly lower than tumor volume in the AuNRs-irradiation group or the AuNRs-NC siRNA-irradiation group (p<0.05). As shown in Figure 6, the weights of nude mice in the simple AuNRs-irradiation group (2.20±0.34 g), AuNRs-NCsiRNA-irradiation group (2.30±0.12g), and AuNRs-HIF-1α siRNA-irradiation group (1.20±0.26 g) were lower than that of mice in the control group (2.87±0.66 g; p<0.05), while weight of nude mice was significantly lower in the AuNRs-HIF-1αsiRNA-irradiation group than in simple AuNRs-irradiation group and the AuNRs-NCsiRNA-irradiation group (p<0.05). However, there was no significant difference in nude mice weight between the simple AuNRs-irradiation group and the AuNRs-NC-siRNA-irradiation group.

Results of H&E staining in the non-irradiation group showed that only the tumor cells of the AuNRs-HIF-1αsiRNA group showed slight apoptosis. Sections from each irradiation group showed different degrees of tissue necrosis, which was most evident in the AuNRs-HIF-1αsiRNA-irradiation group, and cell disruption was also observed (Figure 7). In all groups, examination of liver and kidney tissues under the microscope revealed no obvious apoptosis and necrosis (Figure 7). In addition, there were no significant increases in blood levels of ALT and urea in nude mice (Figure 8).

Discussion

In the animal experiments, nude mice received the drug (nanorod complex) via the tail veins. After 21 days, histology results showed no evidence of obvious apoptosis in liver and kidney tissues. In addition, there were no significant increases in blood levels of liver function biomarker (ALT) and renal function index (urea). These results indicate that the gold nanorod complex (AuNRs/AuNRs-HIF-1αsiRNA) did not exhibit significant toxic accumulation in organs such as the liver and kidney. The cetyltrimethylammonium bromide (CTAB) on the surface of AuNRs was replaced with mercaptoundecanoic acid (MUA) to obtain AuNRs-MUA. Mercaptoundecanoic acid (MUA) decreases the toxicity of AuNRs. Research has shown that gold nanomaterials exert radio-sensitization effects (11, 12). Gold nanomaterials generate large amounts of secondary electrons and free radicals through photo-absorption under photon irradiation. The secondary electrons and free radicals damage DNA and lead to programmed cell death, there by increasing the radiotherapy sensitivity of tumors (13, 14). In this study, the results of cell experiments demonstrated that simple AuNRs also increased the radiotherapy sensitivity of tumor cells to varying degrees. The tumor volume of non-irradiated simple AuNRs group was also lower than that of the normal control group. However, the tumor weight of the non-irradiated AuNRs group was comparable to that of the normal control group. It is not unlikely that the apoptosis of tumor cells was due to the cytotoxicity of the gold nanomaterials. In general, simple AuNRs did not signi-

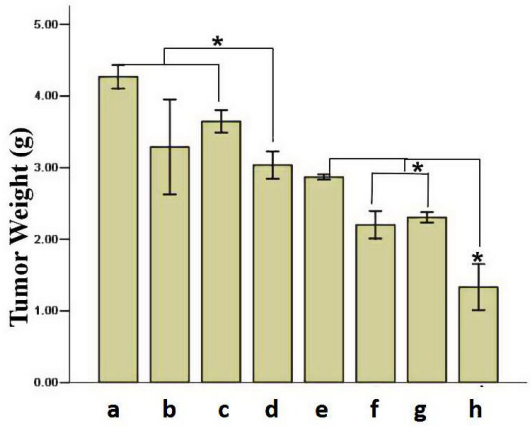

Figure 6. Weights of nude mouse xenografts . **a:**Control ; **b:** AuNRs ; **c:**AuNRs-NCsiRNA ; **d:**AuNRs-HIF-1αsiRNA ; **e:**Control+X Ray ; **f:** AuNRs+X Ray ; **g:**AuNRs-NCsiRNA+X Ray ; **h:**AuNRs-HIF-1αsiRNA +X Ray, *"P<0.05.

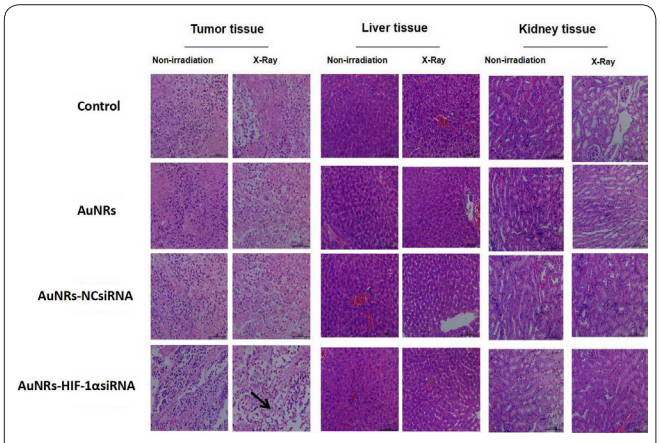

Figure 7. Photomicrographs of tumor tissues and sections of hepatic and kidney tissues of non-irradiated and irradiated nude mice in the various groups.

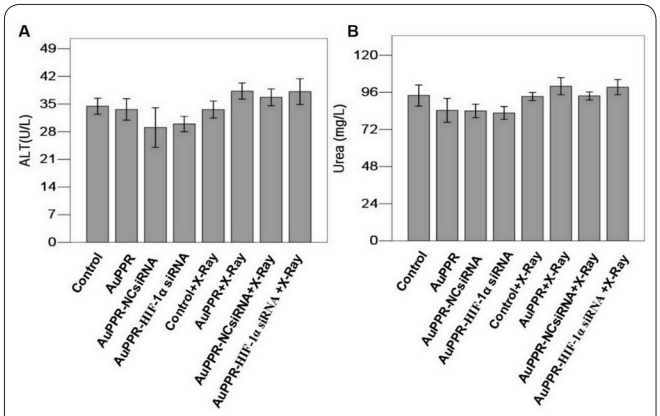

Figure 8. Effect of AuPPR-HIF-1αsiRNA, AuPPR and AuNRs/AuNPs on liver and kidney function indices (ALT and urea) in nude mice (irradiated and non-irradiated).

ficantly inhibit the growth of hypoxic tumor cells in the absence of irradiation. The tumor volume of the irradiated simple AuNRs group was lower than that of the irradiated control group. The tumor weight of the irradiated simple AuNRs group was slightly lower than that of the control irradiation group, but there was still statistical difference between the two groups. Some studies have reported that gold nanoparticles produce less secondary electrons and ROS through high-energy photon radiation (MeV) than through low-energy grades (KeV) (15, 16). The X-ray intensity of the linear accelerator used in this study was in the range of mega volts (MeV). This may be one of the reasons for the low radiation-sensitization effect of simple AuNRs on the transplanted tumor. In addition, in this study, the drug was injected intravenously through the tails of the nude mice. Thus, the local concentration of AuNRs reaching the tumor may be lower than expected. Therefore, the distribution and targeting of gold nanoparticles in intracorporeal circulation need to be further studied. Furthermore, the hypoxic microenvironment of the tumor cells in solid tumors is a major cause of decreases in the sensitivity of tumor to radiotherapy. However, the mechanism involved may be more complex than that of in vitro cell experiments, and further studies are needed to elucidate it. Based on these assumptions, Au-HIF-1αsiRNA group was incorporated into this study, and it was demonstrated it was more effective in enhancing the radiosensitivity of hypoxic NPC cells. Radiotherapy is one of the most effective treatments for most patients with NPC, but about 20% of patients are still insensitive to irradiation. The tumor hypoxia and tumor cell DNA repair systems are considered to be the main mechanisms underlying tumor resistance to radiotherapy. During the growth of solid tumors, the tumor cells located far from blood vessels are in a hypoxic microenvironment. The expression of HIF-1α is up-regulated in tumor cells located in a hypoxic microenvironment. Studies have shown that HIF-1α activates the expressions of downstream genes which adapt tumor cells to hypoxic environment, promote cellular DNA repair, and reduce apoptosis, thereby weakening the radio-sensitivity of hypoxic tumor cells (17, 18). In this study, AuNRs-HIF-1αsiRNA effectively reduced HIF-1 protein expression. Tumor volume and tumor weight of the non-irradiated AuNRs-HIF-1αsiRNA group were lower than those of the normal control group and simple AuNRs group. Sections of H&E-stained tumor tissue from the non-irradiated AuNRs-HIF-1αsiRNA group revealed slight apoptosis. These results demonstrate that HIF-1αsiRNA can effectively silence the protein expression of HIF-1α, increase apoptosis of hypoxic tumor cells and inhibit their growth. In addition, in the cell clone formation experiment, the SER of the simple AuNRs group was about 1.12, while the SER of the AuNRs-HIF-1αsiRNA group was 2.89. These results demonstrate that AuNRs-HIF-1αsiRNA can significantly enhance the killing effect of X-rays on tumor hypoxia cells, since it inhibited tumor cell growth and significantly increased their sensitivity to radiotherapy. Therefore, it can be concluded that AuNRs-HIF-1αsiRNA significantly increases

the sensitivity of NPC cells to radiotherapy, when compared with simple gold nanoparticles. Although both simple AuNRs and AuNRs-HIF-1αsiRNA exerted radio-sensitizing effects on hypoxic NPC cells, the effect of AuNRs-HIF-1αsiRNA was more evident, due to the combination of AuNRs with siRNA. In the hypoxic tumor cells, siRNA down-regulated the expressions of HIF-1α and its downstream components, weakened the ability of tumor cells to adapt to hypoxia, and increased the sensitivity of tumor hypoxia cells to radiotherapy. At the same time, gold nanoparticles entered the cells and produced a large amount of ROS via irradiation, thereby resulting in DNA damage. The combined effect of the two mechanisms greatly increased the sensitivity of the NPC tumor cells to radiotherapy. However, the in vitro

metabolic processes and targeting mechanism of gold nanoparticles need to be investigated in subsequent studies. Moreover, there is need to investigate whether the ROS produced by gold nanoparticles are related to the apoptosis signal pathway regulated by HIF-1α.

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Conflicts of interest
There are no conflicts of interest in this study.

Authors' contributions
Xianming Li designed the study and revised the manuscript. Kun Mao and Yang Sheng aided the animal experiment. Zhibin Li, Xuefeng Yu, Shihai Wu and Rencui Quan performed the experiments and analyzed the data. Gang Xu and Haosheng Zhang wrote the first draft of the manuscript. All authors read and approved the final manuscript. Gang Xu and Haosheng Zhang contributed equally to this work and should be considered as co-first authors.

Declaration
Ethical approval and consent to participate
The animal studies were performed in accordance with the declaration of Helsinki, and were approved by the institutional Ethics Committee of Shenzhen People's Hospital, China.
Consent for publication
All authors read and approved that the manuscript be submitted for publication.
Availability of data and material
The datasets supporting the conclusions in this article are included within the article.

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