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Original Article

Study on the effect of XRCC1 silencing on radiotherapeutic response of nasopharyngeal carcinoma cells and its mechanism

Hongbo Li, Chunhong Cheng

Department of Radiology, The Sixth Hospital of Wuhan, Affiliated Hospital of Jianghan University, Wuhan, Hubei Provence, China

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Abstract: Objective: To investigate the effect of X-ray repair cross-complementing gene 1 (XRCC1) silencing on radiotherapeutic response of nasopharyngeal carcinoma (NPC) cells and its underlying mechanisms. Methods: The small interfering RNA (siRNA) interference technology was adopted to inhibit XRCC1 in nasopharyngeal carcinoma CNE-2 cells, and reverse transcription polymerase chain reaction (RT-PCR) and Western blotting were adopted to verify the effect of XRCC1 silencing on expression. The whole experiment included a blank control group (added with reagent blank), negative control group (added with NC-siRNA) and siRNA interference group (added with XRCC1-siRNA), and MTT assay was adopted to examine the cell proliferation ability of those groups. After a certain dose of X-irradiation, the colony formation ability of cells in those groups was detected through the plate clone formation assay the cell cycle changes of CNE-2 cells were detected through flow cytometry and the effect of XRCC1 silencing on relevant cyclin expression of the CNE-2 cells was detected through Western blotting. The CNE-2 cells after XRCC1 silencing were implanted in nude mice and irradiated with a certain dose of X-rays to examine the volume and mass of the tumor in them. Results: The results of RT-PCR experiment and Western blotting experiment showed that the XRCC1 mRNA level and protein expression of CNE-2 cells in the siRNA interference group were significantly decreased than those in the blank control group (both $P<0.05$). The results of MTT assay showed that XRCC1 silencing could inhibit the proliferation ability of CNE-2 cells ($P<0.05$). Compared with the blank control group, the siRNA interference group showed significantly decreased number of cloning cells after 4 Gy irradiation ($P<0.05$). The results of flow cytometry showed that after 4 Gy irradiation, the percentage of cells in G0/G1 phase in the siRNA interference group was significantly lower than that in the other two groups (both $P<0.05$), and cells in G2/M phase in the siRNA interference group increased significantly ($P<0.05$). After irradiation of CNE-2 cells with XRCC1 silencing, the cyclinB1 protein expression in the XRCC1-siRNA group was significantly decreased ($P<0.05$) and the p-Cdk1 and p21 protein expression was significantly increased ($P<0.05$). The results of the animal experiment showed that the tumor growth of nude mice in the siRNA group was relatively slow and the tumor mass was significantly lower than that in the blank control group. Conclusion: XRCC1 silencing can increase the radiotherapeutic response of NPC cells, which may be due to the fact that the abnormal expression of relevant cyclin leads to induction of block of cells in G2/M phase and promotion of cells to be more sensitive to irradiation.

Keywords: X-ray repair cross-complementing gene 1, nasopharyngeal carcinoma cell, radiotherapeutic response

Introduction

Nasopharyngeal carcinoma (NPC) is a relatively common clinical head and neck neoplasm. Epidemiologic studies show that NPC occurs mainly in males and occurs easily in people of 50-60 years old [1]. The pathogenesis of NPC is relatively complicated, and virus infection, genetic factors and living habits are closely related to the incidence of NPC [2]. NPC occurs deep in a body and close to important blood vessels in the neck, so the rate of cervical lymph node metastasis is relatively high, resulting in relatively great difficulty in surgical resection [3]. Radiotherapy is commonly adopted for NPC in clinical practice, but tumor cells in mid- and late-stage NPC patients are tolerant to low-dose radiotherapy, while high-dose radiotherapy may induce complications [4]. Therefore, it is of great value to investigate the way of increas-

ing the radiosensitivity of NPC in improving the efficacy in patients.

The main principle of radiotherapy is that ionizing radiation can mutate or break the target cell's DNA and kill the target cells [5]. X-ray repair cross-complementing gene 1 (XRCC1) involves in the homologous recombination re pair, base excision repair and other DNA protection mechanism in gene regulation, so it can relive the damage caused by ionizing radiation [6]. Balla et al. found that lung cancer cells showed relatively slow proliferation and were prone to apoptosis under a low dose of ionizing radiation after the expression of XRCC1 was reduced by the siRNA interference technology [7]. XRCC1 has a certain influence on the radiosensitivity of tumor cells, but there are few studies on how XRCC1 regulates the radiosensitivity of NPC, so it is necessary to further elucidate the mechanism of XRCC1 on radiosensitivity of NPC, so as to lay a theoretical foundation for the improvement of radiotherapy effect on NPC.

In order to investigate the correlation between XRCC1 expression and radiotherapeutic response of NPC, the changes of radiotherapeutic response of NPC cells after XRCC1 silencing were examined, which provided an experimental reference for clinical treatment of NPC.

Materials and methods

Cell lines and experimental animals

Human nasopharyngeal carcinoma cell CNE-2 (Shanghai Huiying Biological Technology Co., Ltd.) was cultured in Dulbecco's modified eagle medium (DMEM) with 80 μg/mL penicillin and 10% fetal bovine serum at 37°C with 5% CO_2, which passed one time each 2 days. Twenty-four 4-6 week old BALB/c nude mice (Shanghai SLAC Laboratory Animal Co., Ltd. with production license number of SCXK (Shanghai) 2003-0003) weighing 16-18 g (half male and half female) were kept under conditions without specific pathogen at a temperature of 24 ± 2°C and humidity of 45%-55%. The feed, water and padding required during the raising were autoclaved.

Cell transfection

Relevant siRNA sequences were designed based on the human XRCC1 sequence (Accession Number: NM_006297) in the Genbank database and synthesized by Shanghai GenePharma Co., Ltd. XRCC1-siRNA sequence: 5'-CCGACTGACGTCGTCAC-3'; Nc-siRNA sequence: 5'-ACTGATCACGACTGC-3'. Before transfection, CNE-2 cells in logarithmic phase were seeded in 6-well plates with 1.5 * 10^5 cells per well. After the cell density reached 60%-80%, a CNE-2 cell culture medium was added with siRNA according to the instructions of a lipofectamine 2000 transfection reagent kit and incubated at 37°C with 5% CO_2 for 12 hours after being mixed gently.

Detection of cell proliferation ability through MTT assay

CNE-2 cells in logarithmic phase were prepared and seeded into 96-well plates with 5 * 10^5 cells per well and 3 repetitive holes were set. The cells were divided into blank control group (added with reagent blank), negative control group (added with NC-siRNA) and siRNA interference group (added with XRCC1-siRNA) and cultured at 37°C with 5% CO_2. At the 12^{th} hour, 24^{th} hour, 36^{th} hour, 48^{th} hour and 60^{th} hour, the cells were added with 30 μL of 10 μg/mL MTT solution, respectively, and then the supernatants of them was removed after they were incubated for 3 hours. Each well was added with 180 μL of dimethyl sulfoxide (DMSO), and placed on a shaker for 15 minutes. Finally, an enzyme mark instrument was used to detect the optical density value of each well at 490 nm.

Clone-forming test

CNE-2 cells in logarithmic phase were prepared and digested with trypsin (Shanghai Huiying Biological Technology Co., Ltd.) to prepare a single suspension. The single suspension was seeded into petri dishes with a diameter of 55 mm, 500 cells per dish, after being counted, and the petri dishes were divided into blank control group, negative control group, and siRNA interference group, and cultured at 37°C with 5% CO_2. Each group was set with 6 repetitive groups, of which 3 groups received 0 Gy irradiation and the other 3 groups received 4 Gy irradiation for 12 hours, respectively. The cells were cultured for additional 8 days, and the culture was stopped when there were visible cell colonies. The cells were fixed with methanol for 10 minutes after being washed with

phosphate buffered saline (PBS). Each dish was added with 4 mL of 5% crystal violet staining solution after being washed with PBS and placed on a shaker for 8 minutes, and then the cell colonies were counted under a microscope (the number of cells per cell colony must be more than 50) [5].

Quantitative real-time detection of mRNA level of XRCC1 through PCR

Cells in logarithmic phase were prepared and washed twice with pre-cooled PBS; the total RNA in those cells was extracted through Trizol (Invitrogen, USA); the RNA quality was detected using NanoDrop spectrophotometer (Thermo Fisher, USA). The ratio of OD260 to OD280 within 1.8-2.0 indicated good quality. The cDNA was synthesized through reverse transcription and stored at -20°C. GAPDH was adopted as a reference gene and PCR primer was designed using Prime6.0. XRCC1: The upstream primer was 5'-ACTGACGTGCACCTGC-3' and the downstream primer was 5'-CAGTCAGCATGCACTGC-3'. The GAPDH primer sequences: The upstream primer was 5'-ACTGCCTGACTGCACTGAC-3' and the downstream primer was 5'-GTACCGTTA-ACGACGCACTCAC-3'. The optimal system of PCR reaction was a 25 μL reaction mixture consisting of 10 μL of SYBR Premix, 2.5 μL of upstream and downstream primers of XRCC1, 8 μL of deionized water and 2 μL of cDNA template. PCR reaction was performed using a two-step method: initial denaturized at 95°C for 25 seconds, and 1 recycle, initial denaturized at 95°C for 5 seconds and at 62°C for 30 seconds, and 32 recycles. The $2^{-\Delta\Delta Ct}$ method was adopted to detect the relative expression levels of target genes.

Detection of protein level through Western blot

Cells in logarithmic phase were prepared, treated with RIPA cell lysis buffer (Beijing solarbio science & technology co., ltd.) and centrifuged at 10,000 g for 10 minutes. The protein concentration of those cells was detected through a BCA protein assay kit; 15 μg of protein was taken as a sample to mix with a loading butter and placed in a water bath at 100°C for 8 minutes. They were loaded as a sample after being cooled down, and then done with electrophoretic separation with 10% polyacrylamide gelelectrophoresis (SDS-PAGE). The separated protein was transferred to a PVDF membrane through semi-dry electroporation, blocked with tris-buffered saline Tween 20 (TBST) solution with 5% skim milk powder for 1 hour, washed three times with TBST, added with rabbit anti-human XRCC1 antibody and rabbit anti-human GAPDH antibody (Abcam plc, Cambridge, UK), and incubated overnight at 4°C, and then incubated for 1.5 hours after being washed with TBST and added with HRP-labeled secondary antibody (Beijing Bioss Biotechnology Co., Ltd.). Finally, the separated protein was developed with electochemiluminescence (ECL) (Shanghai YeSen Biotechnology Co., Ltd.) after the PVDF membrane was washed, and then the grey values of protein bands were analyzed using the Image J software.

Detection of cell cycle through flow cytometry

Nasopharyngeal carcinoma cells in logarithmic phase were prepared, adjusted to $5 * 10^5$/mL after trypsinization, seeded in a 6-well plate, $3 * 10^5$ cells per well, cultured at 37°C with 5% CO_2 for 12 hours. The cultured cells were divided into blank control group, negative control group and siRNA interference group. After transfection and incubation, each group of cells was adhered and they received irradiation for 12 hours with doses of 0 and 4 Gy, respectively. The cells were prepared after incubation at 37°C for 24 hours and trypsinization, centrifuged at 1,200 rpm/min at 4°C for 15 minutes, washed twice with pre-cooled PBS buffer, fixed with 70% pre-cooled alcohol for 1 hour, added with 400 μL of propidium iodide after being washed with PBS and stained in the dark at 37°C for 30 minutes. The cell cycle of CNE-2 cells was detected by flow cytometry (Beckman Coulter, Inc. USA) [6].

Detection of CNE-2 cell cycle-related protein after XRCC1 silencing

After cells at logarithmic phase were prepared, protein preparation, electrophoretic separation and transfer of protein membrane were performed according to Western blot experimental procedures. The PVDF membrane was blocked with TBST solution with 5% skim milk powder for 1 hour, washed for three times with TBST, added with rabbit anti-human cyclinB1, p-Cdk1, p21 and GAPDH antibody (Abcam plc, Cambridge, UK), and incubated overnight at 4°C, incubated for 1 hour after being washed with TBST and added with HRP-labeled seco-

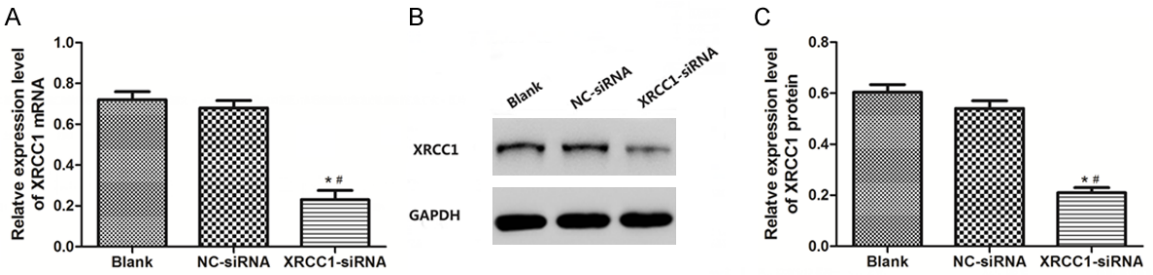

Figure 1. XRCC1 silencing effect in CNE-2 cells detected by RT-PCR and Western blot. A: Relative levels of XRCC1 mRNA in CNE-2 cells of different treatment groups; B: XRCC1 protein electrophoresis in CNE-2 cells of different treatment groups; C: Relative levels of XRCC1 protein in CNE-2 cells of different treatment groups. In comparison with the blank control group, *P<0.05; in comparison with the negative control group, #P<0.05.

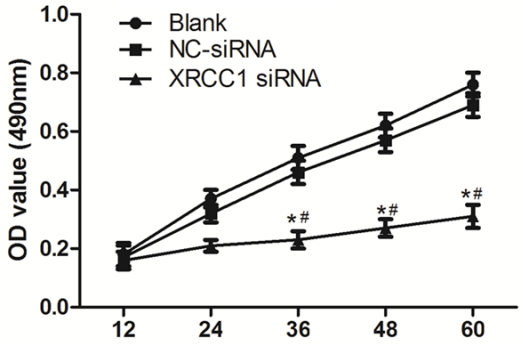

Figure 2. Proliferation ability of CNE-2 cells after XRCC1 silencing detected by MTT assay. In comparison with the blank control group, *P<0.05; in comparison with the negative control group, #P<0.05.

ndary antibody (Shanghai Yanhui Biological (b) of the tumors were measured every 5 days. The tumor volume was calculated as follows: $V = 1/6\pi(ab^2)$. After 25 days, the nude mice were killed by cervical dislocation and the tumors were detached and weighted [7, 8].

Statistical analysis

In this study, the data in this study were analyzed through SPASS21.0. The measurement data were represented as mean ± standard deviation and processed with t-test. P<0.05 is considered statistically significant.

Results

Detection of XRCC1 silencing effect in CNE-2 cells

The results of RT-PCR showed that the relative level of XRCC1 mRNA in CNE-2 cells after transfection of XRCC1-siRNA was 0.28 ± 0.06, which was significantly lower than that in the blank control group and the negative control group (both P<0.05). The results of Western blot showed that the relative level of XRCC1 protein in CNE-2 cells after transfection of XRCC1-siRNA was 0.21 ± 0.04, which was significantly lower than that in the blank control group and the negative control group (both P<0.05), suggesting that XRCC1-siRNA could effectively silence the XRCC1 expression of CNE-2 cells. See **Figure 1**.

Effect of XRCC1 silencing on CNE-2 cell proliferation in vitro

The results of MTT assay showed that there was no significant difference in cell proliferation between the blank control group and the negative control group (P>0.05). The cell proliferation level of the siRNA interference group during the 36th hour to the 60th hour was significantly lower than that of the blank control group and the negative control group (both P<0.05), suggesting that XRCC1 silencing could inhibit the proliferation ability of CNE-2 cells. See **Figure 2**.

Effect of XRCC1 silencing on radiosensitivity of CNE-2 cells

In order to investigate whether X-irradiation has an effect on the radiosensitivity of CNE-2 cells after XRCC1 silencing, each group of cells received 0 Gy irradiation after being cultured for 12 hours. The results showed the number of cell clones in each group had no difference (all P>0.05). After 4 Gy irradiation, the number of cell clones in the blank control group was 134.6 ± 14.6, that in the negative control group was 129.2 ± 11.8 and that in the siRNA interference group was 78.2 ± 12.3. The number of cell clones in the blank control group, negative

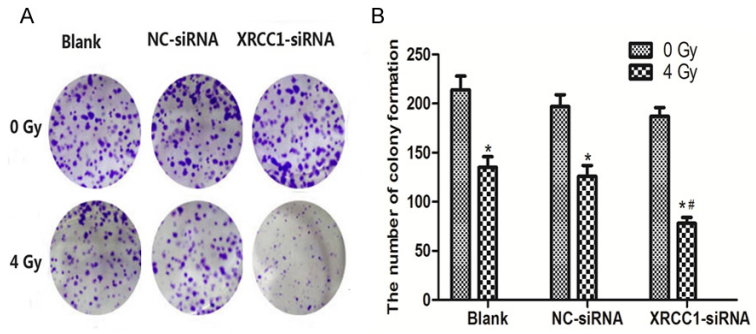

Figure 3. Radiosensitivity of CNE-2 cells after XRCC1 silencing detected through the clone-forming tes. A: Plate clone formation assay of CNE-2 cells in different treatment groups. B: The colony formation number of CNE-2 cells in different treatment groups. In comparison with that after 0 Gy irradiation, *P<0.05; in comparison with the blank control group, #P<0.05; in comparison with the negative control group, &P<0.05.

control group and siRNA interference group was significantly lower than that before irradiation (all P<0.05). After 4 Gy irradiation, the number of cell clones in the siRNA interference group was significantly lower than that in the blank control group and negative control group (both P<0.05). See **Figure 3**.

Effect of XRCC1 silencing on cell cycle of CNE-2 cells

The CNE-2 cells in each group were detected by flow cytometry in the changes of cell cycle after undergoing 0 Gy and 4 Gy irradiation, respectively, and the results showed that there was no significant difference in cell cycle distribution of cells undergoing 0 Gy irradiation (all P>0.05). After 4 Gy irradiation, the percentage of cells in G0/G1 phase in the siRNA interference group was significantly lower than that in the other two groups (both P<0.05) and the percentage of cells in G2/M phase was significantly higher than that in the other two groups (both P<0.05), suggesting that nasopharyngeal carcinoma cells after XRCC1 silencing escaped from the G0/G1 phase and remained in the G2/M phase. See **Figure 4**.

Effect of XRCC1 silencing on CNE-2 cell cycle-related protein

In this study, the results of Western blotting showed that after XRCC1 silencing, the expression of cyclinB1 protein in the siRNA interference group was 0.13 ± 0.04, which was significantly lower than that in the other two groups (both P<0.05); the relative expression level of p-Cdk1 protein in the siRNA interference group was 0.36 ± 0.05, which was significantly higher than that in the other two groups (both P<0.05); the relative expression level of p21 protein in the siRNA interference group was 0.24 ± 0.03, which was significantly higher than that in the other two groups (both P<0.05). See **Figure 5**.

Effect of XRCC1 silencing on transplanted nasopharyngeal carcinoma in nude mice

The results of an animal experiment showed that within 10 days of nude mice modeling, there was no statistically significant difference in tumor volume between the siRNA interference group and the other two groups (both P>0.05); after 15 days of nude mice modeling, the tumor volume in the siRNA interference group was significantly smaller than that in the other two groups (both P<0.05). After 25 days of nude mice modeling, the nude mice were sacrificed and the tumors were detached. The tumor mass in the siRNA interference group was 0.47 ± 0.06 g, which was significantly lower than that in the other two groups (both P<0.05). See **Figure 6**.

Discussion

Clinical practice shows that radiotherapy is effective for the treatment of most nasopharyngeal carcinomas at present, but it acts not well in about 20% patients with nasopharyngeal carcinoma. In addition, the recurrence rate and survival time of patients with nasopharyngeal carcinoma are closely linked with radiosensitivity [8]. The radiosensitivity of tumor cells is regulated by many factors, and it has been found that the DNA repair mechanism of tumor cells is closely related to radiosensitivity of the cells [9]. Kim et al. found that XRCC1 was a gene involved in DNA double-strand break repair and transfection of this gene into irradiation-sensitive tumor cells could increase the resistance of cells to radiation [10]. Liu et al. found that up-regulation of XRCC1 expression could promote the growth of hepatoma cells and nasopharyngeal carcinoma cells and enhance the in vitro invasive ability of cancer

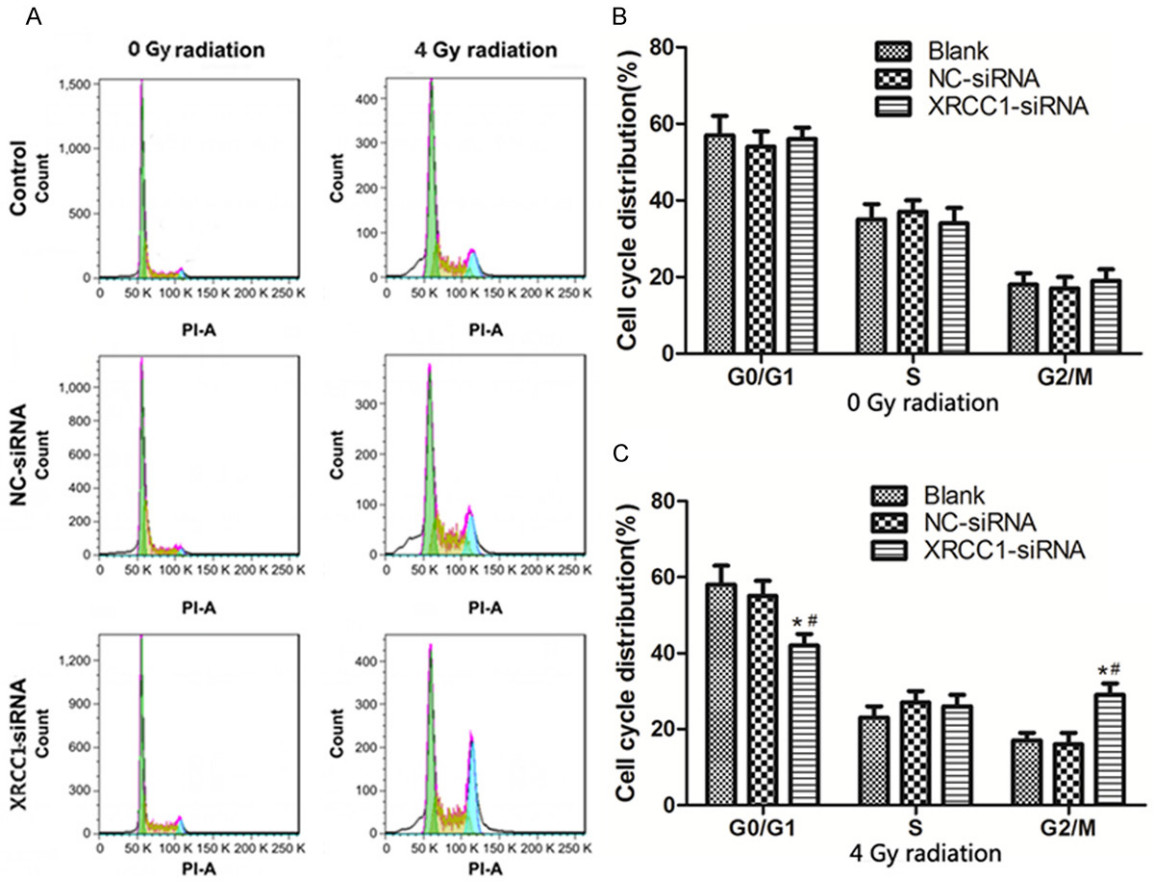

Figure 4. Cell cycles of CNE-2 cells after XRCC1 silencing detected through flow cytometry. A: Graph of flow cytometry of CNE-2 cells in different treatment groups; B: Distribution of cell cycles of CNE-2 cells in different treatment groups after 0 Gy irradiation; C: Distribution of cell cycles of CNE-2 cells in different treatment groups after 4 Gy irradiation. In comparison with the blank control group, *P<0.05; in comparison with the negative control group, #P<0.05.

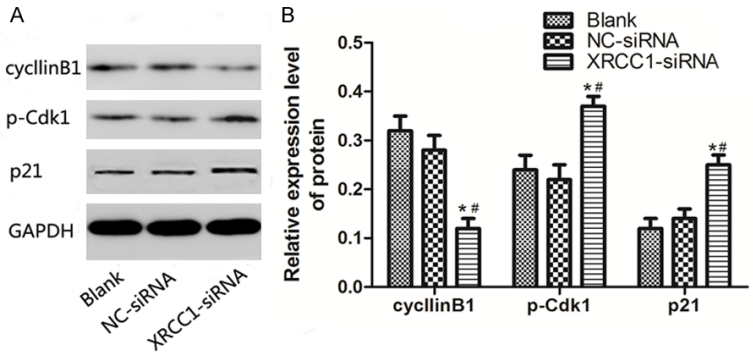

Figure 5. CNE-2 cell cycle-related protein after XRCC1 silencing detected through Western blot. A: Electropherogram of CNE-2 cell cycle-related protein in different treatment groups; B: CNE-2 cell cycle-related protein in different treatment groups. In comparison with the blank control group, *P<0.05; in comparison with the negative control group, #P<0.05.

cells [11]. Therefore, it is of great clinical value to investigate the regulatory function of XRCC1 on radiosensitivity of tumor cells in improving the treatment of nasopharyngeal carcinomas.

In this study, it was found that XRCC1 silencing significantly decreased the proliferation of CNE-2 cells, suggesting that XRCC1 silencing could inhibit the proliferation ability of CNE-2 cells. Hsin et al. found that inhibition of XRCC1 expression in human lung cancer cells could significantly inhibit cell growth, which was consistent with the results of this study [12]. Clone formation assayis an important method to detect the radiosensitivity of cells. Some studies have

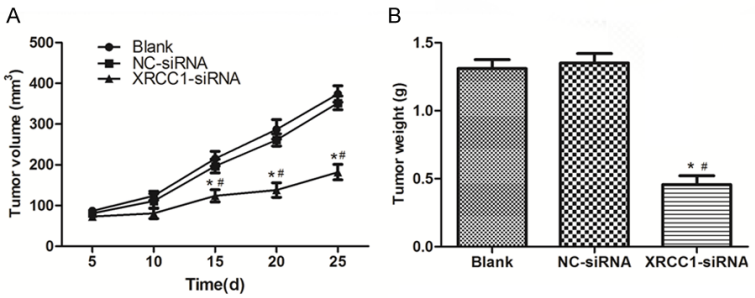

Figure 6. Effect of XRCC1 silencing on transplanted nasopharyngeal carcinoma growth of nude mice. A: Changes in tumor volume of transplanted nasopharyngeal carcinoma on nude mice in different treatment groups; B: Changes in tumor mass of transplanted nasopharyngeal carcinoma on nude mice in different treatment groups. In comparison with the blank control group, *P<0.05; in comparison with the negative control group, #P<0.05.

found that XRCC1 silencing through siRNA interference technology can enhance the radiosensitivity of many types of cells such as gastric cancer cells and lung cancer cells [13]. In this study, it was found that after XRCC1 silencing, CNE-2 cells showed a significant decrease in the number of clone formation under irradiation, suggesting that XRCC1 silencing could enhance the radiosensitivity of cancer cells. In some studies, the siRNA interference technology was adopted to suppress the XRCC1 expression in hepatoma cells and it was found that it could significantly enhance the radiosensitivity of hepatoma cells [14]. Ming et al. found that the radiosensitivity of human colorectal tumor cells was enhanced to a certain extent after XRCC1 silencing through siRNA and 4 Gy irradiation, which was consistent with the results of the study in this paper [15]. The expression product of XRCC1s is an important functional protein for DNA repair in cells, which can relieve the damage of cells due to radiation [16, 17]. The results of this study indicate that XRCC1 is involved in regulating the stress response of nasopharyngeal carcinoma cells to ionizing radiation and strong ionizing radiation can damage the genes of cells, leading to abnormal physiological functions of tumor cells and finally inducing cell death.

At present, tumors are clinically considered to be a disease with abnormal cell cycle. Studies have shown that tumor cells in different cell cycles have different sensitivities to ionizing radiation and cells in G2/M phase are prone to chromosomal abnormalities under the stimulation of ionizing radiation, thereby inducing tumor nucleus and aggravating abnormal physiological function of cell [18, 19]. Wang et al. found that the percentage of cells in G2/M phase in hepatoma cells after XRCC1 silencing increased to some extent, suggesting that XRCC1s might promote the block in G2/M phase and it was related to the radiosensitivity of tumor cells [20]. In this study, it was found that the percentage of cells in G2/M phase in CNE-2 cells after XRCC1 silencing was significantly increased under radiation stimulation and cyclinB1, p-Cdk1, and p21 were important regulator factors of checkpoints in G2/M phase. Decreased cyclinB1 level can promote the expression of p-Cdk1 and p21, and induce block of cells in G2/M phase [21]. The results of Western blotting experiment in this paper showed that the expression level of cyclinB1 protein in the siRNA interference group was significantly decreased, while those of the p-Cdk1 protein and p21 protein were significantly increased, leading to block of cells in G2/M phase, which were consistent with the existing studies [22]. The results of an animal experiment showed that the tumor volume and mass of nude mice in the XRCC1-siRNA group after radiotherapy were significantly smaller than those of the other two groups, suggesting that XRCC1 silencing could improve the radiotherapeutic response of tumors in vivo. The molecular mechanism of tumor radiation sensitivity is relatively complex. The results of this study indicated that the radiosensitivity of nasopharyngeal carcinoma cells was significantly related to the XRCC1 expression, but whether XRCC1 silencing can improve the clinical radiosensitivity of patients with nasopharyngeal carcinomas should be further studied.

In summary, XRCC1 silencing can increase the radiotherapeutic response of NPC cells and inhibit the growth of NPC cells. This may be due the fact that the changes in the expression of relevant cyclin s induce block of cells in G2/M phase and ultimately lead to the sensitivity of cells to radiation.

Disclosure of conflict of interest

None.

Address correspondence to: Chunhong Cheng, Department of Radiology, The Sixth Hospital of Wuhan, Affiliated Hospital of Jianghan University, No.168 Hongkong Road, Jiang'an District, Wuhan 430015, Hubei Provence, China. Tel: +86-027-82428332; E-mail: chengchunhong945@163.com

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