RNAi-mediated knockdown of the c-jun gene sensitizes radioresistant human nasopharyngeal carcinoma cell line CNE-2R to radiation

SI-YAN GUO[1,2], XIAO-DONG ZHU[1,2], LIAN-YING GE[1,2], SONG QU[1,2], LING LI[1,2], FANG SU[1,2] and YA GUO[3]

[1]Department of Radiation Oncology, The Affiliated Cancer Hospital of Guangxi Medical University, Cancer Institute of Guangxi Zhuang Autonomous Region, Nanning, Guangxi 530021; [2]Key Laboratory of High-Incidence-Tumor Prevention and Treatment, Guangxi Medical University, Ministry of Education, Nanning, Guangxi 530021; [3]Department of Oncology, The Second Affiliated Hospital of Xian Jiaotong University, Xian, Shaanxi 710049, P.R. China

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Abstract. This study aimed to investigate the effect of RNA interference (RNAi)-mediated downregulation of the expression of the c-jun gene (a proto-oncogene) on the radiosensitivity of a radioresistant human nasopharyngeal carcinoma cell line (CNE-2R) and to validate its potential as an anticancer target. A lentiviral vector with c-jun small hairpin RNA (shRNA) was constructed and transfected into CNE-2R cells. The gene silencing efficiency of these recombinants was confirmed by RT-PCR and western blotting. Radiosensitivity, cell proliferation, cell cycle profile and apoptosis were assessed using colony formation assay, CCK-8 assay and flow cytometry, respectively. The lentiviral shRNA efficiently knocked down the expression of c-jun at both the mRNA and protein levels ($P<0.05$). c-jun-downregulated CNE-2R cells exhibited significantly decreased cell proliferation and enhanced radiosensitivity compared to the control group ($P<0.05$), and the effects were likely due to G_2/M phase arrest and enhanced cell apoptosis. These data provide evidence that c-jun may be involved in the radioresistance of nasopharyngeal carcinoma (NPC) and knockdown of the c-jun gene may be a potential strategy to enhance the radiation sensitivity of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors of the head and neck, and is endemic in Southeast Asia, particularly in Southern China (1). Local recurrence and metastasis are the main reasons that restrict the efficacy and prognosis after radiotherapy. Some studies have shown that radioresistance is a significant factor for local recurrence and metastasis of NPC (2,3). Weakening radiation resistance to increase the radiation sensitivity of tumor cells may be a promising strategy by which to improve the 5-year survival of patients with NPC.

Transcription factors play an important role in gene expression. c-jun, an important component of activating protein-1 (AP-1) transcription factor, is closely related to cell proliferation, apoptosis and malignant transformation (4). Clinically, c-jun overexpression has been associated with oral squamous cell carcinomas, breast cancer, non-small cell lung cancer and colorectal cancer (5-9). c-jun was found to promote tumor growth and progression, and c-jun binds the cyclin Dl promoter to promote its expression transcriptionally. Overexpression of c-jun was found to lead to abnormal cell proliferation and loss of apoptosis, thus it is considered to be a positive regulator of the cell cycle (9). Previous studies have shown that the expression of cyclin Dl is positively correlated with radiation resistance (10,11). Our preliminary research indicated that the expression of c-jun was significantly upregulated in CNE-2R cells compaired to its expression in CNE-2 cells, and c-jun may be associated with the radioresistance of NPC (12). However, the underlying mechanism of such outcomes remains to be elaborated by subsequent investigation.

In the present study, our data indicated that knockdown of c-jun gene expression increased the sensitivity of CNE-2R cells to radiation.

Materials and methods

Construction of the lentiviral vectors. Small interfering RNA (siRNA) targeting the c-jun sequence (CAAACCTCAGCAA CTTCAA) and a vector containing a scrambled sequence

Correspondence to: Dr Xiao-Dong Zhu, Department of Radiation Oncology, Cancer Hospital of Guangxi Medical University, Cancer Institute of Guangxi Zhuang Autonomous Region, 71 He Di Road, Nanning, Guangxi 530021, P.R. China
E-mail: zhuxdonggxmu@126.com

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(TTCTCCGAACGTGTCACGT) were transformed into short hairpin RNA (shRNA) (stem-loop-stem structure) and cloned into pLV-GV115-lentiviral vectors with *Age*I/*Eco*RI sites (Shanghai Genechem Biotechnology, Shanghai, China).

Cell culture and infection. CNE-2R, a radioresistant human NPC cell line, was constructed and maintained at the Cancer Laboratory of Guangxi Medical University. The cells were grown in RPMI-1640 culture medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA), in a humidified chamber at 37˚C in 5% CO_2. For the lentiviral infection, CNE-2R cells were cultured in 6-well plates. Then, the c-jun-shRNA-expressing lentivirus (c-jun-shRNA) or the scrambled shRNA-expressing lentivirus (NC) was added, with a multiplicity of infection (MOI) of 20 in the CNE-2R cells for 96 h. The transduction efficiency was determined by inverted fluorescence microscopy (Ix71; Olympus, Tokyo, Japan).

RNA extraction and RT-PCR analysis. Total RNA was extracted with TRIzol reagent (Invitrogen, Carslbad, CA, USA) following the manufacturer's instructions. Single-strand cDNA templates were prepared from 1 μg total RNA using the RT-PCR kit (Takara Biotechnology, Dalian, China). Primers for c-jun and β-actin were designed (13) as follows: c-jun forward, 5'-TCCCCCAGCTATCTATATGCAAT-3' and reverse, 5'-TCACAGCACATGCCACTTGA-3'; β-actin forward, 5'-ACCGAGCGCGGCTACAGC-3' and reverse, 5'-CTCATTGCCAATGGTGAT-3'. PCR amplification from cDNA was performed in a final volume of 20 μl. After 95˚C for 30 sec, the experimental reaction was subjected to 40 cycles at 95˚C for 5 sec, 60˚C for 30 sec, followed by a final elongation at 95˚C for 30 sec, and 60˚C for 1 min. The PCR products were subjected to amplification curve analysis, and fluorescence was analyzed using the Light Cycler 480 software release 1.5.0 (Roche Diagnostics, Switzerland). All samples were examined in triplicate. c-jun expression data were normalized against β-actin using the comparative threshold cycle ΔΔCt method.

Western blot analysis. Total protein was extracted from the CNE-2R cells, and each sample protein concentration was determined by Bradford assay (Beyotime, China). Proteins were separated on 10% SDS-PAGE and blotted onto PVDF membranes (Millipore, Bedford, MA, USA). After blocking in 5% non-fat dry milk in Tris-buffered saline with Tween-20 for 1 h at room temperature, the membranes were incubated with the anti-c-jun monoclonal antibody (1:1,000 dilution; Cell Signaling Technology, Boston, MA, USA) and anti-GAPDH antibody (1:3,000 dilution; Proteintech, Chicago, IL, USA) at 4˚C overnight. The membranes were washed and then incubated with a secondary antibody (1:15,000 dilution; Cell Signaling Technology) for 1 h at room temperature. Images of c-jun were obtained by an infrared fluorescence imaging system (Odyssey; Li-Cor Co., Lincoln, NE, USA). GAPDH served as a control.

CCK-8 assay. The effects on cell survival of the c-jun-shRNA-transfected cells were examined by CCK-8 assay. Cells were plated in 96-well plates at $5x10^3$ cells/well and allowed to attach overnight. The cells were then irradiated with 6 MV X-radiation at doses of 2, 4, 6 and 8 Gy. After a 24-h culture, 10 μl of 10 mg/ml CCK-8 solution was added to each well for 1 h at 37˚C. The absorbance of each well was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm. All experiments were performed in triplicate. Cell survival was calculated according to the following formula: Survival fraction % = OD treated/OD untreated x 100%, where OD is the mean absorbance.

Colony formation assay for radiosensitivity. For the colony assay, cells were plated onto 6-well plates and allowed to attach overnight. The cells were exposed to different doses of radiation (2, 4, 6 and 8 Gy), and then the cells were cultured for 14 days in a 5% CO_2 atmosphere at 37˚C until colonies appeared. The colonies were fixed with carbinol (KeLong Chemical Reagent Factory, ChengDu, China) for 20 min and then stained with 0.1% Giemsa (Solarbio Science, Beijing, China) for 15 min. The numbers of single colonies containing >50 cells were scored as survivors. All experiments were performed three times. Graphad Prism 5.0 software was used to create a fit curve. The dose responses were analyzed using multi-target single-hit mode, $SF = 1 - (1 - e^{-D/D_0})^N$, where D is the single radiation dose, D_0 is the single dose of radiation producing a 37% survival rate, SF is the survival fraction at dose D and N is the radiobiological parameter.

Cell cycle analysis. Cells were harvested and resuspended using a cell-cycle kit from Beckman Coulter (Brea, CA, USA) according to the manufacturer's instructions. The DNA content was analyzed using the FC500 flow cytometry systems (Beckman Coulter).

Apoptosis analysis. Cells were collected and resuspended in PBS, and stained using the Annexin V-PE apoptosis detection kit to detect apoptosis cells and 7-ADD to monitor dead cells according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Samples were analyzed on the FC500 flow cytometry systems.

Statistical analysis. Resulted are presented as the mean ± standard deviation (SD) of three independent experiments. Significant difference among groups were analyzed by one-way ANOVA. $P<0.05$ was considered to denote statistically significant differences. All statistical analyses were carried out with SPSS 16.0 statistical software.

Results

Effective shRNA-mediated knockdown of c-jun in the CNE-2R cells. The lentiviral shRNA was successfully constructed and transduced into the CNE-2R cells. The transduction rate was ~90% at 96 h (Fig. 1). The efficiency of the c-jun gene silencing of these recombinants was confirmed by both RT-PCR and western blotting. As shown in Fig. 2, both the mRNA and protein expression levels of c-jun were significantly decreased in the shRNA-transduced group compared to the control group ($P<0.05$).

Cell proliferation following c-jun-shRNA transduction was examined by CCK-8 assay. To further assess the role of c-jun

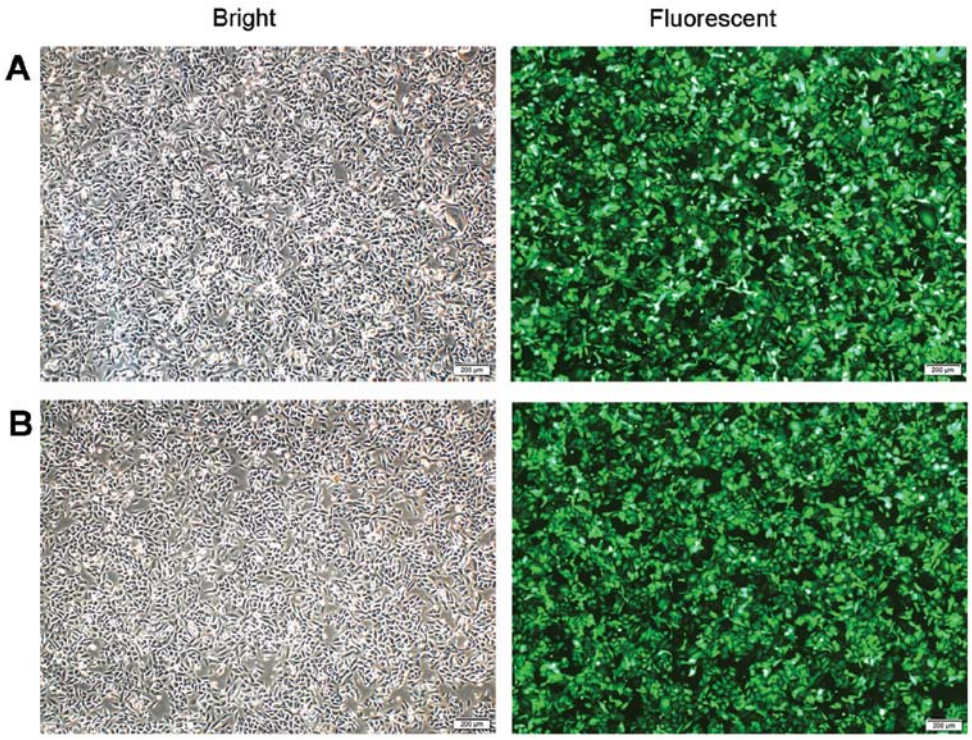

Figure 1. Evaluation of the lentivirus transduction rate, which was calculated by cellular enumeration using inverted fluorescence microscopy (x100 magnification). (A) Scrambled shRNA-transfected group (NC) and (B) c-jun-shRNA group.

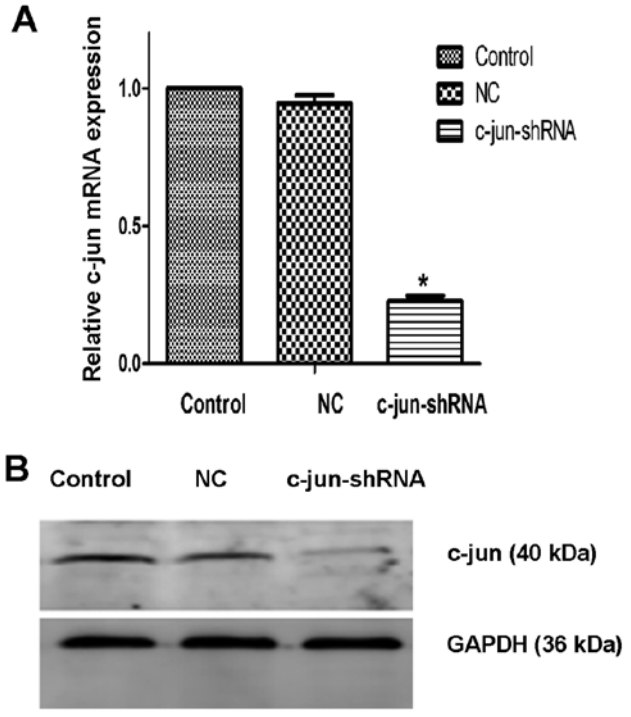

Figure 2. Expression of c-jun as suppressed by lentiviral c-jun-shRNA. (A) Quantitative analysis of c-jun mRNA expression in the different groups as assessed by RT-PCR. (B) Western blot analysis showing c-jun expression in the different groups. GAPDH was used as an internal control. Control, non-transfected group; NC, scrambled shRNA-transfected group.

in regulating cell proliferation, CCK-8 assays were performed on CNE-2R cells following lentiviral infection for 96 h. As shown in Fig. 3, there were no statistically significant differences between the scrambled shRNA-transfected cells (NC) and the non-infected cells (control), indicating that the lentiviral system itself had no cytotoxic effect on the cells, whereas the inhibition of c-jun expression significantly reduced the growth rate of the CNE-2R cells following 6 MV X-radiation compared with the control group ($P<0.05$).

Cells infected with the c-jun-shRNA lentivirus display enhanced radiosensitivity. Next, we investigated the survival curves of the colony formation assay. The curve of the c-jun-shRNA group was higher than that of the other groups. The main parameters presented in Table I and Fig. 4 are the normalized results from the clonogenic experiments. From the D_0 (dose of radiation producing a 37% survival rate), Dq (quasi-threshold dose required for cell damage) and SF2 (survival fraction at 2 Gy) values, it can be concluded that the shRNA-mediated c-jun silencing led to a greater decrease in the surviving fractions when compared with the control. When the data were analyzed by the multi-target single-hit model, $SF = 1 - (1 - e^{-D/D0})^N$, by calculating the sensitization enhancement ratio (SER), we used the D_0 of the control cells divided by the D_0 of the c-jun silenced cells, the values of $SER_{D0} = 1.41 > 1$, suggesting that silencing of c-jun in the CNE-2R cells sensitized the cells to radiation.

Inhibition of c-jun induces G_2/M cell cycle arrest. To investigate the mechanism involved in the inhibition of cell proliferation mediated by c-jun-shRNA in the CNE-2R cells, we further employed flow cytometry to study the effect of the shRNA-mediated c-jun downregulation on cell cycle progression. As shown in Fig. 5, compared with the control group, an obvious

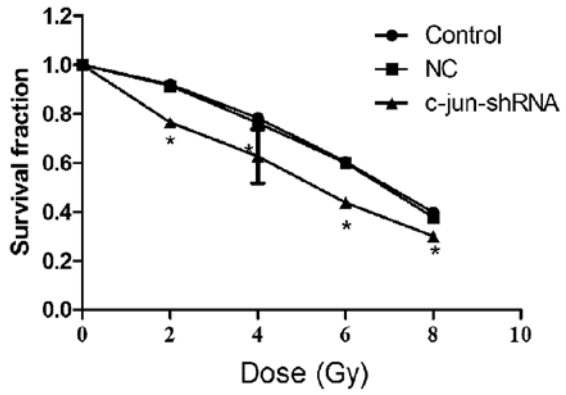

Figure 3. C-jun is important for CNE-2R cell proliferation. C-jun silencing by shRNA lentivirus resulted in growth inhibition as detected by the CCK-8 assay in CNE-2R cells. Cells infected with the c-jun-shRNA lentivirus and the scrambled shRNA (NC) for 96 h, and the non-transfected group (Control), were plated in 96-well plates and irradiated with 6 MV X-radiation at doses of 2, 4, 6 and 8Gy. Cell viability was determined at the indicated time points. *P<0.05 compared with the control.

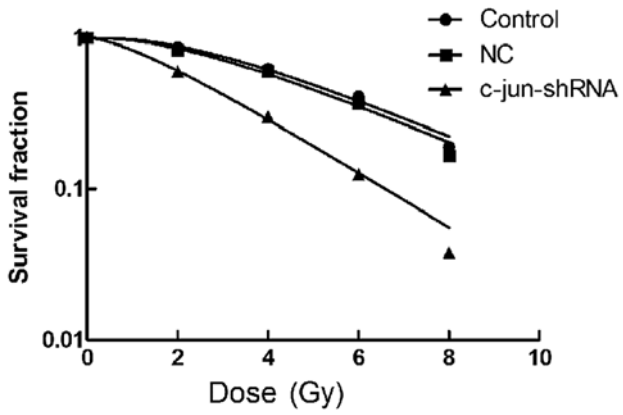

Figure 4. Survival curves of c-jun-shRNA-transfected cells, the scrambled shRNA-transfected group (NC) and the non-transfected group (Control). Cells were seeded onto 6-well plates, irradiated with various doses of X-radiation (0-8 Gy) and cultured for 14 days to allow colony formation.

increase in the G_2/M-phase cell cycle population was observed in the c-jun-shRNA group in the CNE-2R cells (P<0.05). Our results revealed that c-jun-shRNA might exert an inhibitory effect on CNE-2R cell proliferation via G_2/M cell-cycle arrest, which was related to the enhanced radiosensitivity.

c-jun-shRNA promotes the apoptosis of CNE-2R cells. FCM analysis was used to determine whether silencing c-jun had an accelerated effect on the apoptosis of CNE-2R cells. The results showed that the apoptosis rate of the CNE-2R cells following c-jun-shRNA lentiviral transduction was obviously increased, and the apoptosis rate of the control group, NC group and c-jun-shRNA group was 10.97±0.7, 10.8±1.25 and 20.93±1.99%, respectively (Fig. 6).

Discussion

NPC is a common type of cancer in Southern China. Some patients can be cured by radiotherapy which is the main therapeutic method (14), while radioresistance of NPC affects the clinical efficiency. To date, there are no effective biomarkers for predicting NPC radioresistance.

Table I. Correlation parameters in the multi-target single-hit model.

Cell lines	SF_2	D_0	Dq ($lnN*D_0$)
Control	0.88	3.32	2.18
NC	0.85	3.33	2.08
c-jun-shRNA	0.61	2.36	1.37

Control, non-transfected cells; NC, scrambled shRNA-transfected cells.

c-jun is a major constituent of activating protein-1 (AP-1) transcription factor that transduces multiple mitogen growth signals (15). Overexpression of c-jun/AP-1 has been associated with tumor invasion, metastasis and prognosis in many human cancers (5-9). In our previous study (12), we used fractionated radiation *in vitro* to construct the radioresistant NPC (CNE-2R) cell line, and with cDNA microarray, we found that the c-jun/AP-1 expression was upregulated in the radioresistant CNE-2R cells when compared with the expression in the parental CNE-2 cell line. Integrating microarray with gene ontology and protein interaction networks showed that c-jun/AP-1 can positively regulate cell proliferation. Similarly, Kajanne *et al* (16) suggested that AP-1 could mediate EGFR and PI3K signaling in prostate cancer cells, which is essential for cell proliferation, and confers protection against radiation-induced cell death. This study suggested that the transcription factor AP-1 promotes the growth and radioresistance in prostate cancer cells.

The EGFR signaling pathway has been associated with tumor radioresistance. Overexpression of EGFR is related with poor response to radiotherapy in tumors (17). In head and neck cancer cells, a high level of EGFR is correlated with radioresistance. Anti-EGFR monoclonal antibodies can improve radiosensitivity (18,19). Among the downstream signaling pathways activated by EGFR, the Ras/Raf/MEK/MAPK pathways have been well studied. Signals are transferred to the nucleus by shc, grb2, c-jun and c-fos in order, and then activate AP-1 affecting cell proliferation (20,21). c-jun may play an essential role in radioresistance through the EGFR pathway or AP-1. Therefore, specific downregulation of c-jun may be a potential strategy to enhance radiosensitivity. However, the effects of c-jun on radioresistance have not been reported in NPC cells.

In the present study, we hypothesized that c-jun may be correlated with radiation resistance in CNE-2R cells. Given the prevalence and availability of RNA interference (RNAi) technology in cancer research or cancer therapy (22), we used a lentivirus shRNA system that effectively knocked down the expression of c-jun at both the mRNA and protein levels. As shown in Fig. 2, RT-PCR and western blot analysis showed effective silencing of c-jun, thus ensuring the reliability of the subsequent assays. Our results demonstrated that inhibition of c-jun by shRNA enhanced response to radiation (Fig. 4),

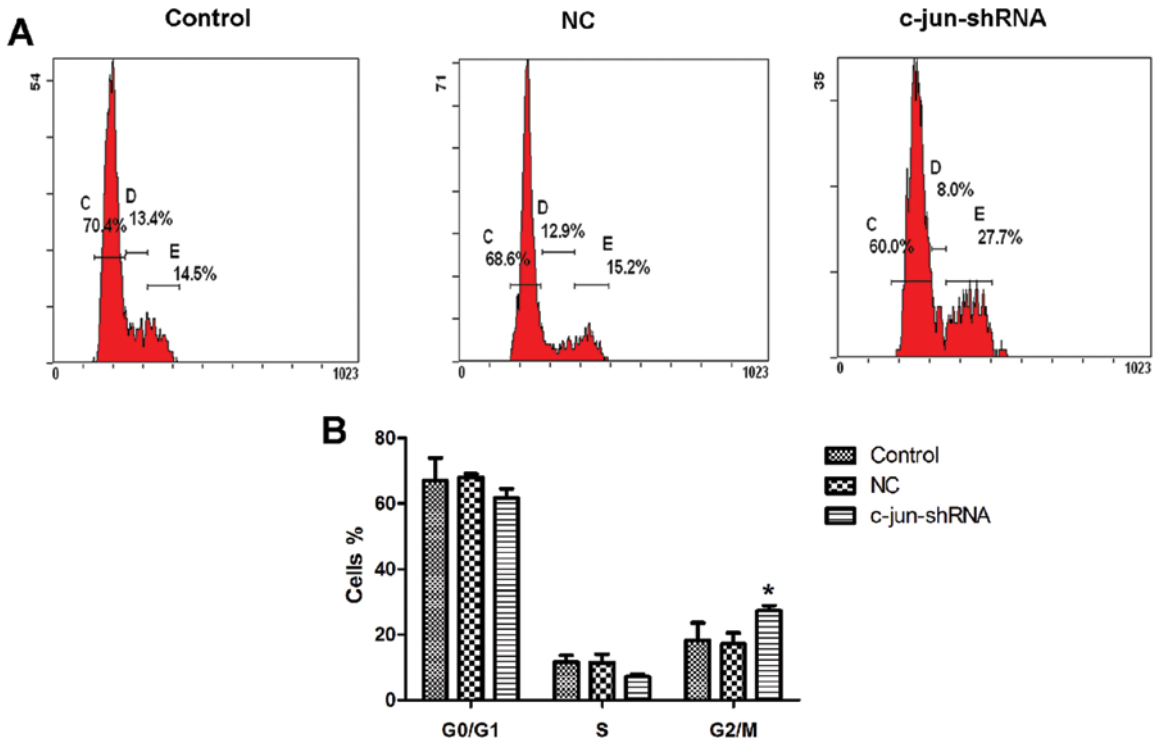

Figure 5. shRNA-mediated knockdown of c-jun increases G_2/M cell cycle arrest in CNE-2R cells compared with non-transfected cells (Control) and scrambled shRNA-transfected cells (NC). (A) Representative images showing non-transfected cells (Control), scrambled shRNA-transfected cells (NC) and c-jun-shRNA-transfected cells. (B) Quantification of A. *P<0.05 compared with the control.

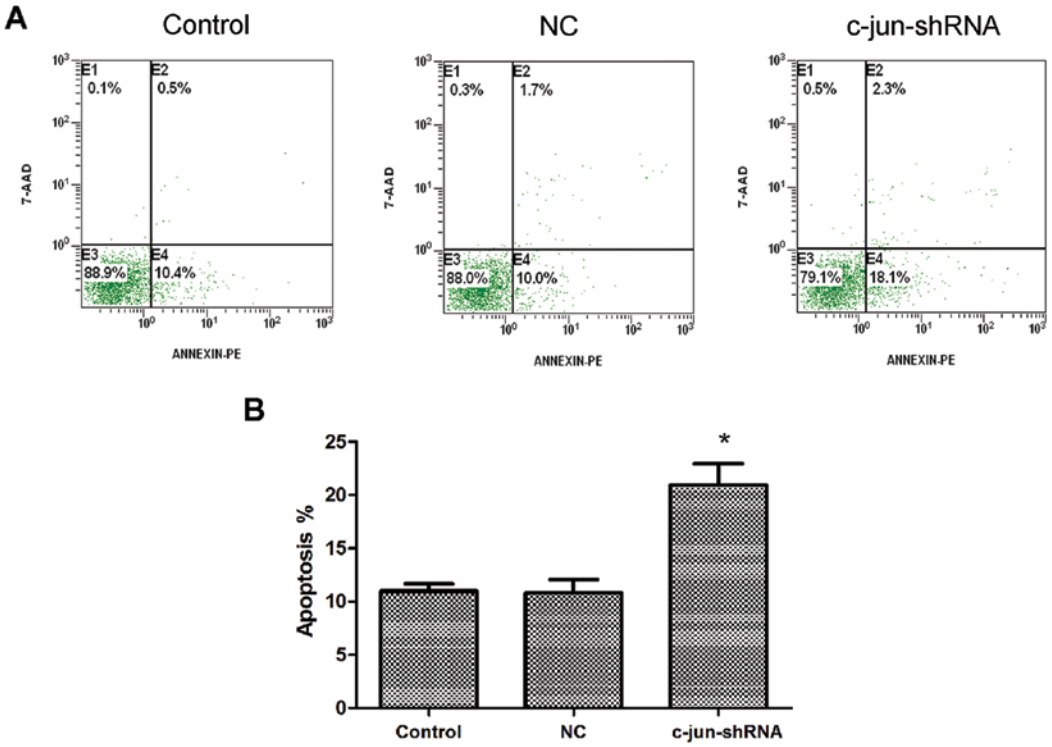

Figure 6. shRNA-mediated knockdown of c-jun enhances apoptosis in CNE-2R cells. Apoptotic cells were assessed with Annnexin V PE staining and then subjected to flow cytometry for analysis. (A) Representative images showing Annnexin V-PE staining in the non-transfected cells (Control), scrambled shRNA-transfected cells (NC) and cells infected with the c-jun-shRNA lentivirus. (B) Quantification of A. *P<0.05 compared with the control.

which significantly reduced cell proliferation (Fig. 3), altered the cell cycle (Fig. 5), and promoted cell apoptosis (Fig. 6).

The cell cycle phase determines the relative radiosensitivity of cells. Cells are the most radiosensitive in the G_2/M phase,

less sensitive in the G_1 phase and the least sensitive during the S phase (23). Our data revealed that shRNA-mediated knockdown of c-jun increased G_2/M arrest, and cells were easily killed by radiation. In other words, inhibition of c-jun in CNE-2R cells enhanced the radiosensitivity of the cells.

Apoptosis also known as programmed cell death, removes injured or disabled cells to maintain the balance of the micro-environment. The main mechanism of radiotherapy in tumors is to induce the apoptosis of cells (24,25). At present there has been some controversy regarding the effect of c-jun/AP-1 on cell apoptosis (26,27). Downregulation of c-jun expression by blocking JNK was found to inhibit the activation of AP-1 and reduce apoptosis in certain types of cells (28). However, some studies also suggest that, in some types of stress, the expression of c-jun was increased, but did not induce apoptosis. In contrast, it may promote the proliferation and differentiation of cells (29). In mice, deletion of the c-jun gene or changes in JNK phosphorylation sites were found to shrink intestinal tumors, and increase the life span of mice (30).

Our data also showed that the apoptosis rate of the c-jun-shRNA group was higher than this rate in the control groups ($P<0.05$). However, whether the mechanism involved in the promotion of apoptosis by decreased c-jun expression in CNE-2R cells is the same by inhibiting the activity of AP-1, downregulating cyclin D1 (31), arresting the cell cycle, inhibiting cancer cell proliferation or other manners needs to be further studied.

In conclusion, the levels of c-jun appear to be highly important for the radioresistance of CNE-2R cells. shRNA-mediated knockdown of c-jun in the radioresistant CNE-2R cells enhanced radiosensitivity, induced cell cycle arrest and apoptosis. Our findings imply that the overexpression of c-jun may serve as a potential target to enhance the radiation sensitivity for NPC therapy.

Acknowledgements

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