

RESEARCH ARTICLE

Knocking Down Nucleolin Expression Enhances the Radiosensitivity of Non-Small Cell Lung Cancer by Influencing DNA-PKcs Activity

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Abstract

Nucleolin (C23) is an important anti-apoptotic protein that is ubiquitously expressed in exponentially growing eukaryotic cells. In order to understand the impact of C23 in radiation therapy, we attempted to investigate the relationship of C23 expression with the radiosensitivity of human non-small cell lung cancer (NSCLC) cells. We investigated the role of C23 in activating the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), which is a critical protein for DNA double-strand breaks (DSBs) repair. As a result, we found that the expression of C23 was negatively correlated with the radiosensitivity of NSCLC cell lines. *In vitro* clonogenic survival assays revealed that C23 knockdown increased the radiosensitivity of a human lung adenocarcinoma cell line, potentially through the promotion of radiation-induced apoptosis and adjusting the cell cycle to a more radiosensitive stage. Immunofluorescence data revealed an increasing quantity of γ -H2AX foci and decreasing radiation-induced DNA damage repair following knockdown of C23. To further clarify the mechanism of C23 in DNA DSBs repair, we detected the expression of DNA-PKcs and C23 proteins in NSCLC cell lines. C23 might participate in DNA DSBs repair for the reason that the expression of DNA-PKcs decreased at 30, 60, 120 and 360 minutes after irradiation in C23 knockdown cells. Especially, the activity of DNA-PKcs phosphorylation sites at the S2056 and T2609 was significantly suppressed. Therefore we concluded that C23 knockdown can inhibit DNA-PKcs phosphorylation activity at the S2056 and T2609 sites, thus reducing the radiation damage repair and increasing the radiosensitivity of NSCLC cells. Taken together, the inhibition of C23 expression was shown to increase the radiosensitivity of NSCLC cells, as implied by the relevance to the notably decreased DNA-PKcs phosphorylation activity at the S2056 and T2609 clusters. Further research on targeted C23 treatment may promote effectiveness of radiotherapy and provide new targets for NSCLC patients.

Keywords: Nucleolin/C23 - non-small cell lung cancer - radiation therapy - DNA repair - DNA-PKcs

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Introduction

Over the past twenty years, the worldwide incidence of non-small cell lung cancer (NSCLC) and associated mortality have increased steadily each year (Silvia et al., 2014). Although significant advancements in the treatment of NSCLC, only minimal increases in survival rates have been produced (Fernandes et al., 2012). Therefore, new treatment modalities that improve survival rates are urgently needed. A clearer understanding of the biology of NSCLC at a molecular level will aid materially in the development of these modalities, including ways to accurately predict the prognosis of patients with certain clinical-pathological characteristics and identification of new targets for radiation therapy.

Improving tumor radiosensitivity is an effective

way to increase the potency of radiation therapy. Tumor radiosensitivity is associated with a variety of factors, including cell apoptosis, radiation damage repair, and adjusting cell division cycle of cancer cells, etc.

Nucleolin (C23) is anti-apoptotic protein that is expressed highly in cancer cells (Andersen et al., 2005). The overexpression of C23 stabilized Bcl-2 mRNA, consequently increased the expression level of Bcl-2 and inhibited cell apoptosis (Zhang et al., 2010). Down-regulation of heat shock protein 70 in laryngeal cancer cells increased the fracture and degradation of C23, which consequently lead to increased tumor radiosensitivity (Xu et al., 2010).

As a multifunctional protein, C23 is involved in DNA damage repair (Goldstein et al., 2013). C23 represses p53 translation through binding to both the 5'- and 3'-

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UTRs of p53 mRNA. C23 binds to the 5'- and 3'-UTR interaction region that is critical for the recruitment of RPL26 to p53 mRNA after DNA damage (Chen et al., 2012). C23 interacted with Rad50 and was recruited to DNA double-strand breaks (DSBs) in an MRE11-NBS1-RAD50 complex-dependent manner (Goldstein et al., 2013). Notably, C23 knockdown exhibited repression of both ATM-dependent phosphorylation following exposure to γ -ray and activated cell cycle checkpoint subsequently. Furthermore, the knockdown of C23 gene may induce the deficiency of the catalytic subunit of autophosphorylation of DNA-dependent protein kinase (DNA-PKcs) (Kobayashi et al., 2012). The results demonstrated that C23 was involved in DNA DSBs repair. Moreover, the relationship between C23 expression and DNA-PKcs activity remains unclear in NSCLC.

In this study, we assume that the inhibition of C23 expression can improve the radiosensitivity of NSCLC. C23 may be affected by DNA-PKcs activity to inhibit DNA damage repair. We expect to provide new targets for radiation sensitivity for lung cancer.

Materials and Methods

Cell culture

Based on the mRNA expression profiles of human lung cancer cell lines used in previous studies, human lung adenocarcinoma (NSCLC) cell line A549 and human large cell lung carcinoma cell line NCI-H460 (obtained from the Institute of Cancer Prevention and Treatment, Harbin Medical University) was chosen for this analysis. Cells were cultured in a 5% CO₂ incubator at 37°C in RPMI 1640 (Hyclone, Beijing, China) supplemented with 10% bovine serum.

RNA interference

Cells were transfected with 50 nM of siRNAs targeting C23 (C23_siRNA1: UAA CAG AAC CAG GAA GCU GUU; C23_siRNA2: CCA CUA GAA CCG CCA GAG AUU; C23_siRNA3: AGA GCG AGA UGC AGA ACA TT) or nonspecial (NC) siRNA (NC_siRNA: CUA CAA CAG CCA CAA CGU CTT) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Western blotting

Cells were washed twice with ice-cold PBS and then lysed in a solution containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, sodium orthovanadate, 1 mM sodium fluoride, 1 mM EDTA and 1 μ g/mL leupeptin. Protein concentrations in the lysates were determined using Bradford reagent, and equal amounts of protein were separated by 12% SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% nonfat dried milk in TBST for 1 hour at room temperature before an incubated overnight at 4°C with rabbit monoclonal antibodies against human C23 (1:200; Santa Cruz Biotechnology), human DNA-PKcs (1:1000; Thermo Scientific), human Ku70 (1:2000; Epitomics), human phospho-specific S139 H2AX (1:1000; Cell

Signaling), human phospho-specific T2609 DNA-PKcs (1:2000; Abcam), human phospho-specific S2056 DNA-PKcs (1:2000; Epitomics) or β -actin (1:2000; Santa Cruz Biotechnology). The membranes were then washed with TBS containing 0.05% Tween 20 before incubated for 1 hour at room temperature with peroxidase-conjugated AffiniPure goat anti-rabbit or goat anti-mouse IgG (Jackson ImmunoResearch). Immune complexes were detected using an enhanced chemiluminescence detection system and autoradiography.

Clonogenic survival assay

The effectiveness of the combination of C23_siRNA and ionizing radiation was determined using clonogenic survival assays. Cells were initially transfected with C23_siRNA. After 24 hours, cells were planted on a 6-well plate in a limiting dilution assay (100, 200, 400, 1000, 5000, 10000 cells per well) and then the plate was treated with 0, 2, 4, 6, 8, 10 Gy of ionizing radiation. Briefly, the cells were irradiated with 6 MeV photons/100 cm focus-surface distance at a dose rate of 4.0 Gy/min at room temperature in T-25 flasks. After treatment, the cells were trypsinized and counted. The cells were then washed with PBS and returned to the incubator to allow for macroscopic colony development. The colonies were counted after 14 days, and the plating efficiency and surviving fraction for each treatment were calculated based on the survival of non-irradiated cells transfected with C23_siRNA alone.

Quantification of apoptosis

To quantify apoptotic cells were identified using fluorescein isothiocyanate (FITC)-labeled recombinant chicken Annexin V (ZomanBio, Beijing, China) in combination with propidium iodide (PI). Briefly, 10⁵ cells were resuspended in 500 μ L of Ringer's solution, incubated for 30 minutes at 4°C in the dark with 1 μ g Annexin V-FITC and 1 μ g PI and analyzed on a Coulter EPICS XL flow cytometer with System XL II software.

Cell proliferation assay

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) detection kit (Zomanbio, Beijing, China). Cells were seeded at a concentration of 2×10^3 cells per well in 96-well plates. After 24 hours of growth and achieving a confluence of about 70-80%, cells were treated with siRNAs. At 24, 48 and 72 hours after transfection, 10 μ L of CCK-8 solution was applied to each well followed by a 3 hour incubation at 37°C. Absorbance values for all wells were then determined at 450 nm in on a microplate reader (Elx800, BioTec).

Cell cycle analysis

Adherent cells (1×10^6 /ml) were collected by trypsinization, washed with PBS for 10 minutes and centrifuged at 120xg. The cells were resuspended in a hypertonic solution containing 50 μ g/mL PI and 10 μ g/mL RNaseA (Beyotime, Shanghai, China). Fluorescence-activated cell sorting (FACS) was performed using a Coulter EPICS XL flow cytometer (Coulter) and System XL II software.

Immunofluorescence and the quantification of

γ -H2AX foci

Cells were cultured on six-well slides and exposed to 4 Gy/min of X-ray irradiation. At 15, 30, 60, 120 and 360 minutes after X-ray irradiation, the cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. The cells were then permeabilized with 0.25% Triton in PBS for 15 minutes, blocked with 3% BSA for 1 hour at room temperature and incubated with phospho-specific H2AX (1:100; Cell Signaling) and phospho-specific S2056 DNA-PKcs (1:100; Epitomics). Immune complexes were visualized after incubation with the FITC-conjugated secondary antibody. The nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen), the coverslips were mounted in fluorescence mounting medium, and the fluorescence was visualized using a confocal laser scanning microscope equipped with the LSM5 PASCAL system (Carl Zeiss).

Real-time PCR assays

Total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with oligo dT primers (Roche, Switzerland). Real-time PCR was carried out in an Applied Biosystems 7500 System with Power SYBR Green PCR Master Mix (Roche, Switzerland). Relative levels of gene expression were determined with glyceraldehyde 3-phosphate dehydrogenase (β -actin) as the control.

Statistical analysis

All data were presented as mean \pm standard deviation and calculated using SPSS13.0 statistical software. Selection of one-way ANOVA or independent samples t-test was based on experimental setup, and $P < 0.05$ was considered statistically significant. All experiments were performed in triplicate.

Results

C23 expression was negatively correlated with the radiosensitivity of NSCLC cell lines

We detected the expression of C23 mRNA in A549 and H460 cell lines, and found that cell survival curves shifted to the left accompanied with the high grade of C23 expression (Figure 1A, 1B). As a result, we considered that the expression of C23 was negatively correlated with the radiosensitivity of NSCLC cell lines.

Down-regulation of C23 enhanced radiosensitization of NSCLC cells

The influence of C23 on NSCLC cell radiosensitivity was first examined. Transfection of A549 cells with C23_siR significantly decreased C23 expression at 24 and 48 hour time points (Figure 2A, 2B) ($P < 0.05$). Furthermore, treatment with C23_siR shifted the A549 survival curve to the left with a radiation dose enhancement factor of 1.61, as measured by clonogenic survival assays, suggesting that C23 knockdown increased the radiosensitivity of A549 cells (Figure 2C).

Further research was taken in order to investigate whether inhibition of C23 increased the radiation sensitivity. The absorbance values of the A549 cells at

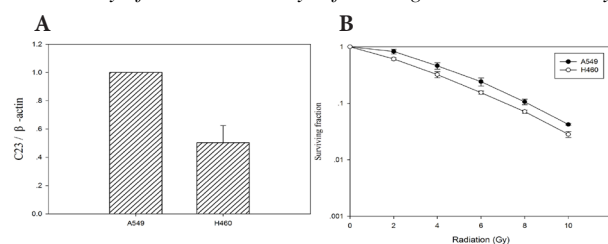


Figure 1. The High Level of C23 Along with Low Radiosensitivity. (A) C23 mRNA expression was higher in A549 cells than H460 cells. (B) The cells were irradiated with 2-10 Gy of X-rays, and drew the cell survival curves after 14 days. The cell survival curves shifted to the left in H460 cells compared with A549 cells

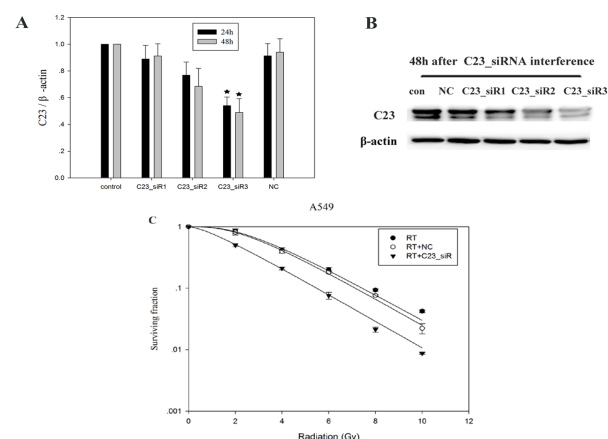


Figure 2. C23_siRNA Inhibited C23 Expression and Enhanced the Radiosensitivity of A549 Cells. (A) RT-PCR analysis of C23 mRNA expression in untreated and treated A549 cells. C23 mRNA levels were significantly lower in the C23_siR group. β -actin mRNA was used as an internal control. * $P < 0.05$ versus control or NC group. (B) Western blot analysis of C23 protein expression in untreated and treated A549 cells. C23 protein levels were notably lower in the C23_siR group. β -actin protein was used as an internal control. $P < 0.05$ versus control or NC group. (C) A549 cells were transfected with C23_siR, NC or control for 24 hours and subsequently irradiated with 2-10 Gy of X-rays. After 14 days, the cells were fixed and stained. The surviving fractions were calculated based on colony counts and plating efficiency. Treatment with C23_siR shifted the A549 survival curve to the left with a radiation dose enhancement factor of 1.61

24, 48 and 72 hours post-transfection with C23_siR were notably lower than the control or NC group, as measured by the CCK-8 assay. Moreover, the cell proliferation inhibition rate in C23_siR-treated cells showed a notable increase (i.e. proliferation decreased) compared with untreated cells (Figure 3C). Flow cytometry results showed that the amount of apoptotic cells at 24 hours post-transfection with C23_siR was greater both C23_siR and radiation given than C23_siR or radiation given alone. Collectively, these data indicated that C23 knockdown promoted radiation-induced apoptosis in A549 cells (Figure 3A).

Typically, cells are most radiosensitive in the G2/M phase and most radioresistant in the S phase. Using flow cytometry, the cell cycle was detected at 24 hours after receiving C23_siR treatment. In C23_siR-treated cells, the G2/M fraction was increased and the S phase cells was decreased compared with the control or NC cells (Figure

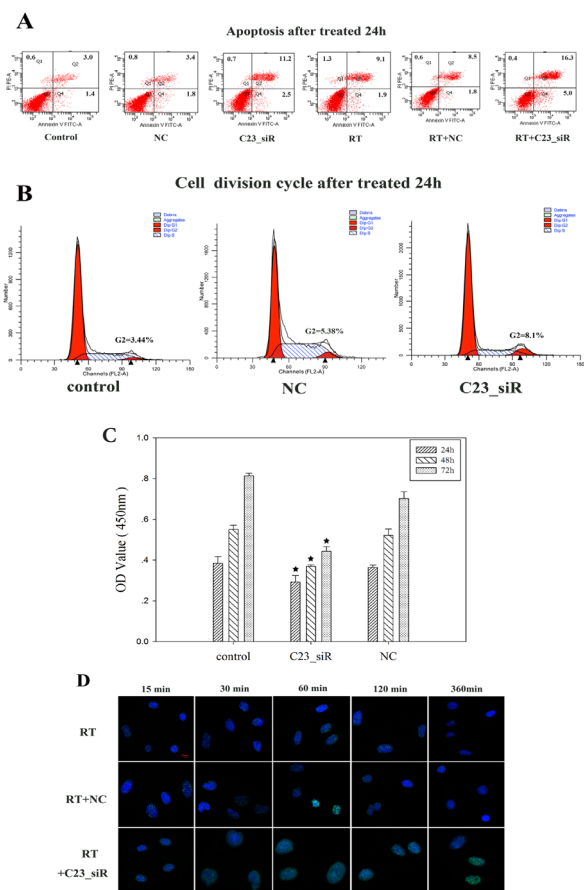


Figure 3. C23_siR Increased Radiation-induced Apoptosis, Adjusted Cell Division Cycle, Decreased Cell Proliferation and DNA Damage Repair in A549 Cells. (A) Cells were transfected with C23_siR, NC or control for 24 hours with or without 4 Gy of X-rays, then the percentage of apoptotic cells were detected by flow cytometry. Early and late apoptosis notably increased following radiation combined with C23_siR versus radiation and C23_siR alone. $P < 0.05$ versus control or NC group. (B) Cell cycle synchronization was blocked in the G1 phase. Cells were transfected by C23_siR for 24 hours and then incubated in culture medium containing serum. Cell division cycle was detected by flow cytometry. An increase in the G2/M fraction and decrease S phase were noted for C23_siR treated cells compared with control or NC cells. (C) Cells were treated with C23_siR, NC or control for 24 hours, then absorbance values were tested at 24, 48 and 72 hours using the CCK-8 assay. In cells treated with C23_siR, cell proliferation was notably lower than untreated cells. $*P < 0.05$ versus control group. (D) Cells were transfected with C23_siR or control for 24 hours with 4 Gy of X-rays. After 15-360 minutes, the cells were fixed and subjected to immunofluorescent staining for γ -H2AX (green). After treatment, the number of γ -H2AX foci increased and the extent of damage repair decreased from 15 to 360 min. Scale bar: 10 μ m

3B), suggesting that C23 knockdown may put cells in a more radiosensitive stage of the cell cycle.

DNA repair has been associated with tumor radiosensitivity. C23 has been proved to play a direct and/or indirect role in DNA repair. Therefore we assessed the effect of C23 on DNA repair using immunostaining. The foci that form at DNA DSBs were visualized in treated and untreated cells with antibodies against γ -H2AX. The induction of DNA DSBs and DNA repair was studied by detecting H2AX serine 139 phosphorylation at 15, 30, 60,

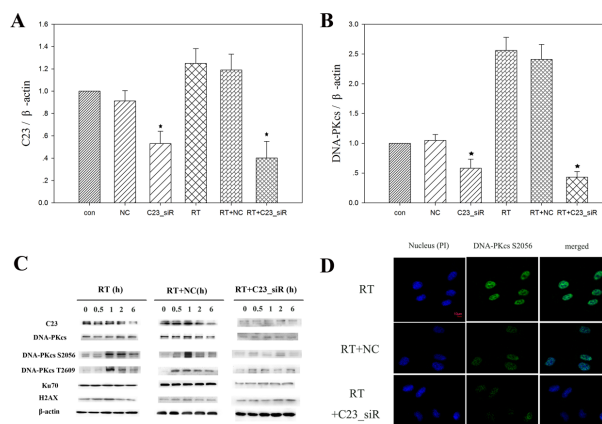


Figure 4. Down Regulation of C23 Expression Reduced the Activity of DNA-PKcs, DNA-PKcs S2056 and DNA-PKcs T2609 in A549 Cells. (A) RT-PCR analysis of C23 mRNA expression in untreated and treated A549 cells after radiation for 1 hours. C23 mRNA levels were notably lower in the RT plus C23_siR and C23_siR groups. β -actin gene was used as an internal control. $*P < 0.05$ versus control or NC group. (B) RT-PCR analysis of DNA-PKcs mRNA expression in untreated and treated A549 cells after X-ray irradiation for 1 hours. DNA-PKcs mRNA levels were notably lower in the RT plus C23_siR and C23_siR groups. β -actin gene was used as an internal control. $*P < 0.05$ versus control or NC group. (C) A549 cells were transfected with C23_siR and then exposed to 4 Gy of radiation. C23, DNA-PKcs, DNA-PKcs S2056, DNA-PKcs T2609 levels were notably decreased in the C23_siR group. (D) Cells were transfected by C23_siR, NC or control for 24 h, and treated by irradiation with 4 Gy. Immunofluorescent images of representative A549 cells stained for DNA-PKcs S2056 (Rhodamine, green) and DNA (DAPI, blue) 1 hour after irradiation. We found that the foci of phospho-specific S2056 DNA-PKcs decreased significantly in C23 knockdown cells than control and NC cells. Scale bar: 10 μ m

120 and 360 minutes after irradiation and by quantifying the formation of γ -H2AX foci at 15, 30, 60, 120 and 360 minutes. The formation of γ -H2AX foci in A549 cells was detected between 15 minutes and 360 minutes after irradiation. Compared with control and NC cells, γ -H2AX levels increased 15 minutes to 360 minutes after irradiation in C23_siR-treated cells (Figure 3D).

These results suggested that C23 knockdown increased radiosensitivity of NSCLC cells through promoting radiation-induced apoptosis, adjusting redistribution within the cell cycle, and inhibiting DNA damage repair.

C23_siR suppresses DNA damage repair of NSCLC cells through inhibiting DNA-PKcs activity

To further establish C23 activity in DNA damage repair, expression of the DNA damage gene DNA-PKcs in treated and untreated cells was examined. The results showed that the expression of C23 and DNA damage repair genes including DNA-PKcs and γ -H2AX first increased, then reduced from 30 min to 360 min after X-ray irradiation (Figure 4C). Therefore, we considered that C23 was involved in DNA damage repair after radiotherapy. RT-PCR showed that DNA-PKcs mRNA was downregulated in C23 knockdown cells (Figure 4A, 4B, 5A, 5B). Compared with control and NC cells, DNA-PKcs protein expression and phosphorylation of DNA-PKcs sites S2056 and T2609 were decreased at 30, 60, 120 and 360 minutes after irradiation (Figure 4C).

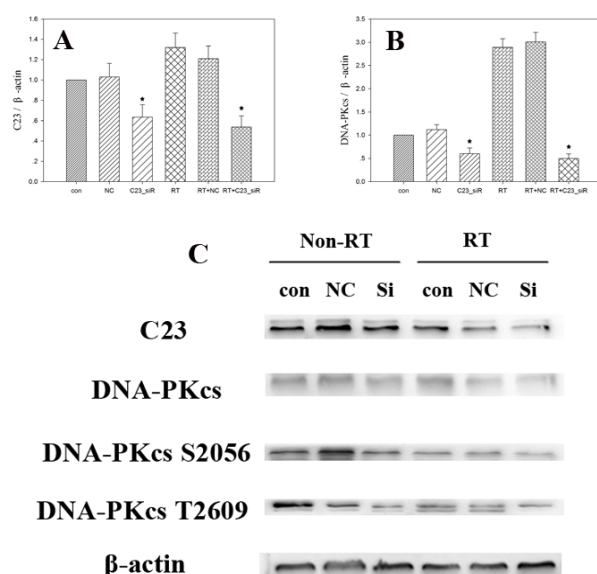


Figure 5. Knockdown C23 Expression Reduced the Activity of DNA-PKcs, DNA-PKcs S2056 and DNA-PKcs T2609 in H460 Cells. (A) RT-PCR analysis of C23 mRNA expression in H460 cells after radiation for 1 hours. C23 mRNA levels were notably lower in the RT plus C23_siR and C23_siR groups. β -actin gene was used as an internal control. * P <0.05 versus control or NC group. (B) RT-PCR analysis of DNA-PKcs mRNA expression in H460 cells after X-ray irradiation for 1 hours. DNA-PKcs mRNA levels were notably lower in the RT plus C23_siR and C23_siR groups. β -actin gene was used as an internal control. * P <0.05 versus control or NC group. (C) H460 cells were transfected with C23_siR and then exposed to 4 Gy of radiation. C23, DNA-PKcs, DNA-PKcs S2056, DNA-PKcs T2609 levels were notably decreased in the C23_siR group

When DSBs were generated by radiation, DNA-PKcs recruited by DSBs and participated in DNA damage repair. Moreover, Phosphorylation at S2056 and T2609 site clusters on DNA-PKcs was found to be critical for DNA-PKcs activation. The inhibition of C23 suppressed DNA-PK autophosphorylation and improved radiosensitivity in knockdown cells (Figure 4C, 4D, 5C).

These results suggested that C23 knockdown inhibited DNA damage repair by suppressing DNA-PKcs activity and therefore improved radiation sensitivity in NSCLC cells.

Discussion

We reported that inhibition of C23 increased the radiation sensitivity of A549 cell lines. Radiosensitivity of tumor is associated with the apoptosis, cell division cycle, radiation damage repair of tumor cells, etc. Studies have found C23 excessive expression in human various tumor cells (Guo et al., 2014) and its resistant role in apoptosis (Schokoroy et al., 2013). In this study, we found that the inhibition of C23 expression may promote radiation-induced apoptosis. C23_siR-treated cells were found to have more cells in G2/M fraction and less in S phase of the cell cycle, which indicated that C23 knock-down put cells in a more radiosensitive stage of the cell cycle.

In order to safeguard the genome against mutation or

DNA loss, cells respond to stress by transiently delaying proliferation and mobilizing repair factors (Daniely et al., 2002). Radiation-induced expression of C23 protein and mRNA were increased in our research and involved in DNA damage repair. A number of papers revealed that both heat shock and DNA-damaging agents induced a relocalization of C23 from the nucleolus to the nucleoplasm. These studies revealed that C23 subsequently bound replication protein A, which resulted in a DNA replication blockade (Daniely et al., 2000). C23 was sequestered within the nucleolus and its release which was triggered by recognition of DNA damage, functioned to help the cell regulate the complex response to DNA damage. Finally, the release of C23 might lead to cell cycle arrest and activation of DNA repair (De et al., 2006). These findings were consistent with the hypothesis that C23 was involved in DNA DSBs repair. C23 may contribute to genome stability in two ways: by modulating the activities of several DNA repair enzymes through physical interaction and by enhancing the translation of some of these protein after genotoxic stress (Yang et al., 2002).

H2AX phosphorylation is crosslinking with damaged chromatin and damage repair proteins gathering (Celeste et al., 2003). H2AX phosphorylation has a positive correlation with the accumulated number of γ -H2AX foci and DSBs (Sone et al., 2014). Our immunofluorescence results showed that down-regulation of C23 expression could increase the quantification of γ -H2AX foci and decrease radiation-induced DNA damage repair. C23 knockdown showed decrease in radiation-induced chromatin accumulation of DNA damage repair factors, which agreed with the delayed kinetics of γ -H2AX focus. We found that DNA-PKcs phosphorylation at the S2056 cluster decreased significantly more than untreated and NC cells in immunofluorescence experiments (Figure 4D). C23 knockdown may reduce radiation-induced accumulation of DNA-PKcs.

DNA-PKcs, a key protein in the NHEJ pathway, consists of a 465 kDa catalytic subunit (DNA-PKcs) and a heterodimeric regulatory complex (Ku) that comprises a 70 kDa subunit (Ku70) and an 86 kDa subunit (Ku80) (Xing et al., 2015). Studies have shown that DNA-PKcs can affect cellular radiosensitivity (Costantini et al., 2007) and recognized that. The inherent radiation sensitivity of cancer cells is in greatly influenced by the ability of DNA DSBs repair (Dikomey et al., 1998). Expression of DNA-PKcs and Ku70 decreased from 30 to 360 minutes after irradiation in C23 knockdown cells. These results suggested that DNA-PKcs protein kinase activity decreased following C23 knockdown. A large number of phosphorylation sites were clustered in different regions of DNA-PKcs (Davis et al., 2013). Two prominent phosphorylation clusters which had been identified to be phosphorylated and autophosphorylation in response to irradiation are the T2609 and S2056 (Chen et al., 2005) clusters. Both the S2056 and T2609 clusters deactivation at the same time could increase the radiosensitivity (Nagasawa et al., 2011). We found that DNA-PKcs phosphorylation sites at the S2056 and T2609 significantly suppressed after irradiation in C23 knockdown cells (Figure 5C). The activation for phosphorylation site at the

S2056 and T2609 was critical for DNA DSBs repair. If lose damage repair, DSBs can lead to chromosome fracture, loss and ultimately cause cell death; therefore, inhibiting DNA DSBs repair can increase the irradiation sensitivity of cancer cells. Our research was the first study which found that C23 could regulate the function of DNA-PKcs. C23 knockdown reduced the radiation damage repair and increased the radiosensitivity in A549 and H460 cells through inhibiting DNA-PKcs phosphorylation activity at the S2056 and T2609 cluster.

In this study, we suggested that C23 knockdown inhibited DNA damage repair by suppressing DNA-PKcs activity and therefore improved radiation sensitivity in NSCLC cells. However, we found that only a portion function of C23 in the field of DNA damage repair after irradiation. Further study in the role of C23 in DNA damage repair could provide a new target for radiosensitivity improvement in NSCLC patients (Goldshmit et al., 2014).

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