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Aurora-A/NF-κB Signaling Is Associated With Radio-resistance in Human Lung Adenocarcinoma

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Abstract. *Background/Aim: This study aimed to discuss the effect and possible molecular mechanisms of Aurora-A/NF-κB signaling on the radiotherapy resistance of human docetaxel-resistant lung adenocarcinoma cells. Materials and Methods: The human lung adenocarcinoma SPC-A1 and SPC-A1/DTX cell lines were utilized in the present study. The MTT assay measured the sensitivity of cells to radiotherapy. The tumor-initiating ability of the cells was detected in vitro by cloning assays. Apoptosis was quantified by flow cytometry. Real-time quantitative PCR and western blotting were used to detect the mRNA and protein expression of the Aurora-A/NF-κB, respectively. Tumors transplanted subcutaneously into nude mice were used to test the effect of Aurora-A on the in vivo sensitivity of the tumors to radiotherapy. Results: The SPC-A1/DTX docetaxel-resistant lung adenocarcinoma cells were radio-resistant compared with the parental SPC-A1 cells. Up-regulated aurora-A was responsible for the in vitro radio-resistance of docetaxel-resistant SPC-A1/DTX cells. Nuclear transcription factor NF-κB was identified as a downstream target gene of Aurora-A in SPC-A1/DTX cells, and NF-κB also participated in the radio-resistance of SPC-A1/DTX cells regulated by Aurora-A. Conclusion: The Aurora-A/NF-κB pathway is association with radio-resistance of human lung adenocarcinoma docetaxel-resistant cells.*

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Lung cancer is one of the leading causes of cancer-related death (1). Lung adenocarcinoma accounts for the main pathological types of lung cancer. Many patients are diagnosed at a late stage, losing the opportunity for surgery (2). Chemotherapy and radiotherapy are often used to improve the survival rate and prognosis of patients with advanced lung adenocarcinoma (3, 4). However, many patients with lung adenocarcinoma, especially those resistant to chemotherapy, are also resistant to radiotherapy (5). Therefore, it is important to understand the potential mechanisms of radio-resistant lung adenocarcinoma. In the present study, we generated a multidrug resistant strain of human lung adenocarcinoma, SPC-A1/DTX. The resistant cells also showed radio-resistance compared with the parental SPC-A1 cells. The present study aimed to further explore the potential molecular mechanisms of radio-resistance of human lung adenocarcinoma SPC-A1/DTX cells.

Materials and Methods

Cell line and chemical reagents. The following materials were used: NF-κB inhibitor (Selleck, Houston, TX, USA); Aurora-A interference plasmid (6) and docetaxel (Qi Lu Pharmaceutical, Jinan, PR China); antibodies against Aurora, NF-κB, IκBa, and GAPDH (Abcam, Cambridge, UK); fetal bovine serum (Sijiqing Co. Ltd., Hangzhou, PR China); RPMI-1640 culture medium (HyClone, Logan, UT, USA); a SYBR PrimeScript™ RT-PCR Kit (TaKaRa, Kusatsu, Japan); PVDF membrane (Millipore Co. Ltd, Billerica, MA, USA); human lung adenocarcinoma SPC-A1 cells (Shanghai Cell Research Institute, Shanghai, PR China).

Cell culture and transfection. Human lung adenocarcinoma SPC-A1 cells were cultured in RPMI-1640 medium containing 10% FBS. Human lung adenocarcinoma SPC-A1/DTX cells were cultured in docetaxel at a concentration of 50 μg/l. The culture conditions were 37˚C, 5% CO_2, saturated humidity, with subculture every 48 h. The Aurora-A interference plasmid was transfected into the cells using Lipofectamine 2000 according to the manufacturer's instructions.

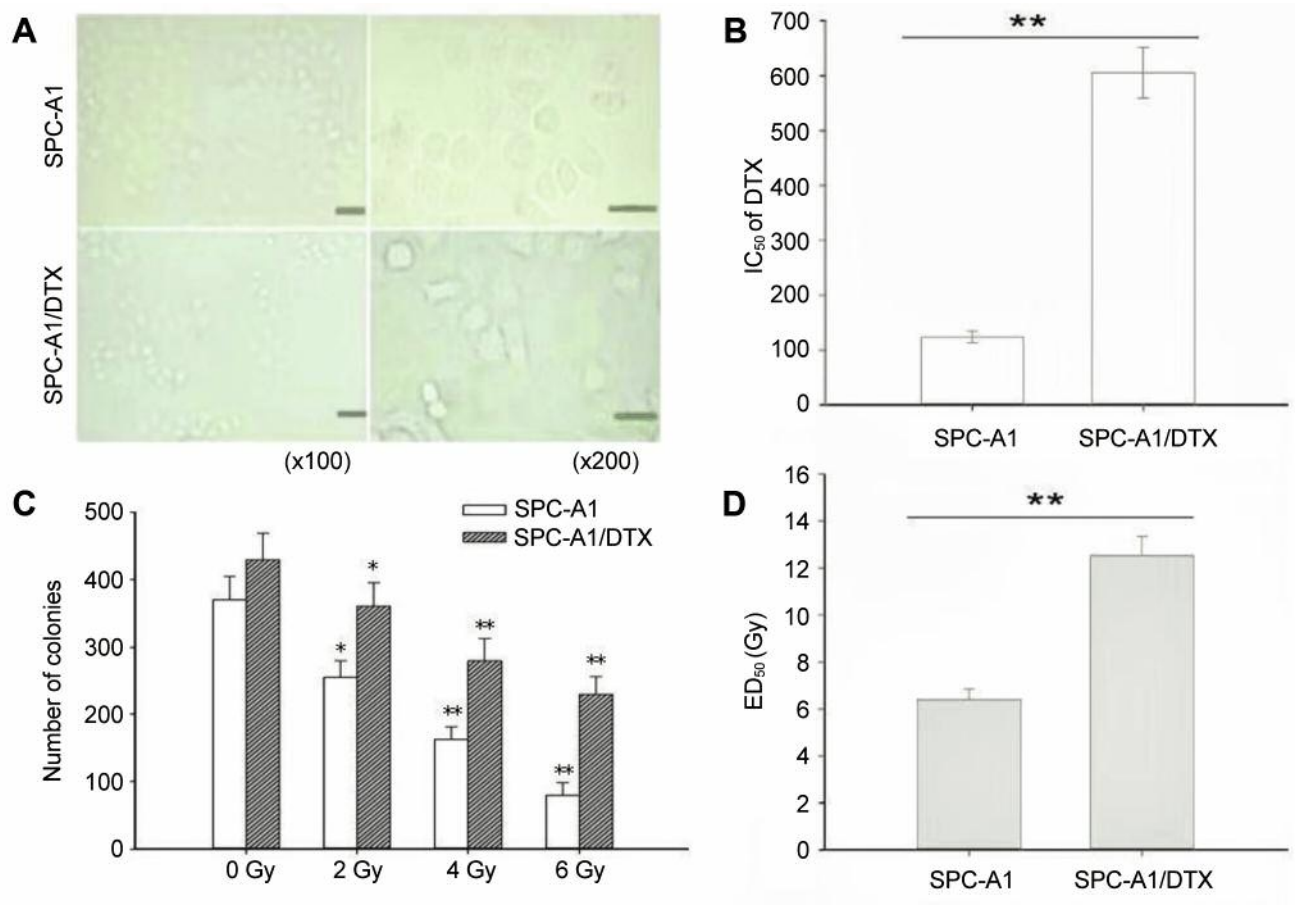

Figure 1. *Multidrug-resistant human lung adenocarcinoma cells (SPC-A1/DTX) are radio-resistance. A. Morphology of human lung adenocarcinoma (LAD) parental cells (SPC-A1) and multidrug-resistant cells (SPC-A1/DTX) under a light microscopy. Upper: parental cells (SPC-A1); Lower: multidrug-resistant cells (SPC-A1/DTX). B. The MTT assay was used to calculate the IC_{50} value of docetaxel (DTX) for human LAD cells. C. The colony formation assay was conducted to estimate the proliferation rate of human LAD cells treated with different doses of irradiation. D. The MTT assay was used to calculate the ED_{50} value of irradiation for human LAD cells. *p<0.05, **p<0.01.*

In vivo radiotherapy assay. Five-six weeks old BALB/c athymic nude mice were provided by the Department of Comparative Medicine of the Jing Ling hospital. A total of 2.0×10^6 SPC-A1/DTX/ sh-control or SPC-A1/DTX/ sh-Aurora-A cells were suspended in 100 μl PBS and transplanted subcutaneously into the right side of the posterior flank of male BALB/c athymic nude mice. Tumor volumes were calculated by using the equation: $V=A\times B2/2$ (mm^3), where A is the largest diameter and B is the perpendicular diameter. The tumor-bearing nude mice were exposed to X-ray of 2.0 Gy alone when the average tumor size reached about 50 mm^3. The treatment was repeated three times (the interval time was 5 days). After six weeks, all mice were sacrificed, and the primary tumors were excised, paraffin-embedded, formalin-fixed, stained with hematoxylin and eosin (H&E) and immunostained for Ki-67 and PCNA protein expression according to the manufacturer's instructions.

In vitro radio sensitivity assay. The *in vitro* radio sensitivity assay was determined with the MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in accordance with the manufacturer's instructions. Briefly, 2000 cells were plated in 96-well plates 24 h after transfection. Next, the cells were treated with different doses of radiation and cultured for 48 h. Then, MTT was added and incubated at 37°C for 4 h, followed by the addition of DMSO to dissolve the resulting formazan crystal. The absorbance was detected at 490 nm with a microplate reader.

Cloning assay. One thousand cells were inoculated in 6-well culture plates. After 10-14 days of incubation, the cells were washed with PBS, fixed with pure methanol and stained with crystal violet.

Flow cytometric analysis. The early apoptosis rate of the cells was evaluated with the Annexin V/FITC-PI double-staining method. Briefly, digested cells were washed with precooled PBS. Then, 100 μl of a working solution of propidium iodide (PI) and Annexin V was added to the cells. The cells were incubated in the dark for 15 min, and stained cells were detected using flow cytometry (Becton Dickinson, NJ, USA).

Real-time quantitative RT-PCR. Total RNA was extracted using the Trizol reagent and reverse transcribed into cDNA with a SYBR Prime Script Kit (Takara Company, Shiga, Japan). Aurora-A primer was then added, and amplification was performed using the following reaction

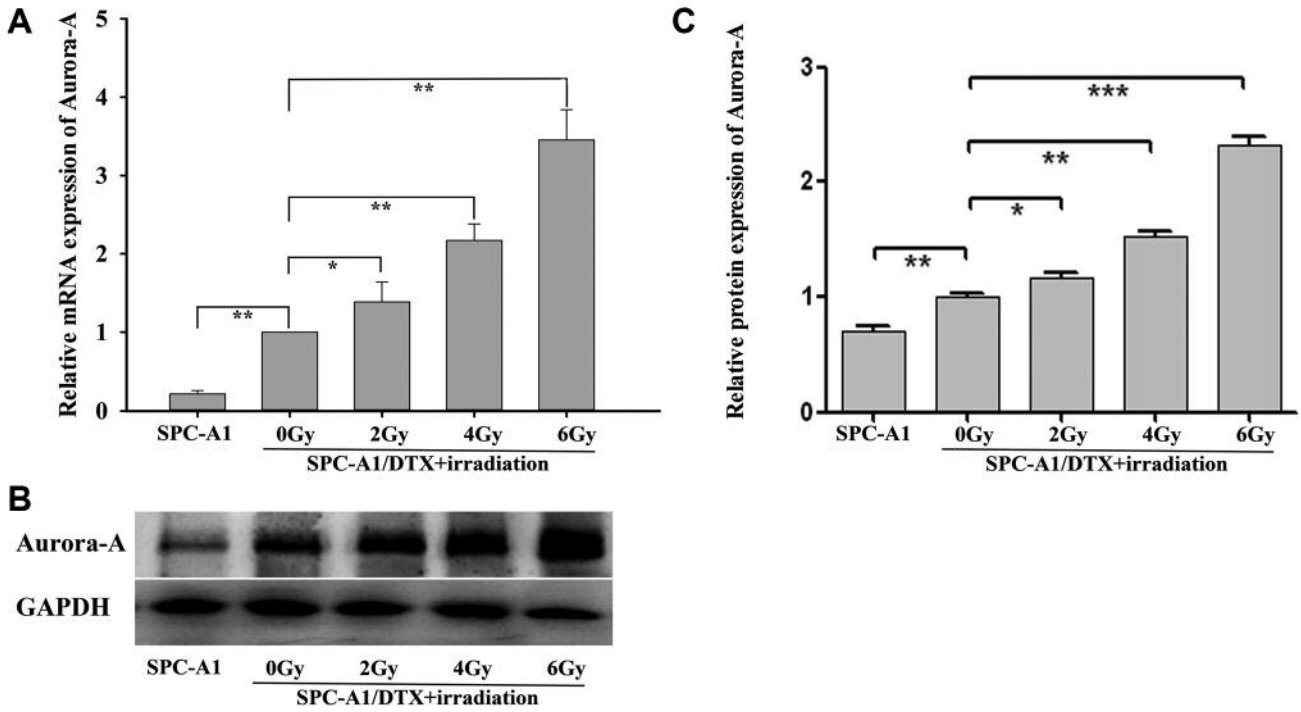

Figure 2. *Irradiation up-regulates Aurora-A expression. A. The mRNA levels of Aurora-A were detected by real-time quantitative PCR in human LAD parental cells (SPC-A1) and multidrug-resistant LAD cells (SPC-A1/DTX) treated with different intensities of irradiation. GAPDH was used as a reference. B-C. The expression of Aurora-A protein was detected by western blotting in human LAD parental cells (SPC-A1) and multidrug-resistant cells (SPC-A1/DTX) treated with different intensities of irradiation. GAPDH was used as a reference. The histogram represents the relative expression. *p<0.05, **p<0.01.*

conditions: 95˚C pre-denaturation for 3 min, followed by 35 cycles of 94˚C denaturation for 30 sec, 55˚C annealing for 30 sec, and 72˚C extension for 60 sec. The expression of the target gene was detected using the Prism ABI 7000 Sequence Detection System. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting. Total protein was extracted and separated by twelve percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to a membrane, blocked with 5% skim milk, and incubated with primary antibody at 4˚C overnight. The membrane was then washed 3 times for 5 min each, incubated with secondary antibody, followed by 4 washes for 10 min each. Electrochemiluminescence was performed, and the Image J scanning gray value was evaluated.

Statistical methods. SPSS software (version 17.0) was used for the statistical analysis. The data were from three or more independent experiments. The data are shown as the mean±standard deviation. Two-group comparisons of quantitative data were analyzed by Student's *t*-test. *p*-Value <0.05 was considered statistically significant.

Results

Multidrug-resistant human lung adenocarcinoma SPC-A1/DTX cells show radiation resistance compared with parent SPC-A1 cells. The human lung adenocarcinoma cell line SPC-A1/DTX, previously generated from parental human lung adenocarcinoma SPC-A1 cells, has multidrug resistance characteristics (7). Compared with the parental SPC-A1 cells, drug-resistant SPC-A1/DTX cells were larger and multinucleated, and some cells had pseudopodia (Figure 1A). The IC_{50} value of docetaxel (DTX) in drug-resistant SPC-A1/DTX cells was significantly higher than that of parental cells (Figure 1B, $p<0.01$). In addition, we found that the proliferation rate of SPC-A1 cells was significantly decreased *in vitro* (Figure 1C). Furthermore, SPC-A1/DTX cells were more resistant to radiation than SPC-A1 cells (Figure 1D, $p<0.01$). The above results suggested that the drug-resistant human lung adenocarcinoma SPC-A1/DTX cells also acquired radiation resistant.

Aurora-A is involved in the radiotherapy resistance of human lung adenocarcinoma drug-resistant SPC-A1/DTX cells. Aurora-A is highly expressed in many kinds of malignant tumors. In the present study, we observed that the levels of Aurora-A mRNA and protein in the resistant strain were significantly higher than those in the parental strain (Figure 2A-C, $p<0.01$). Treatment with increased intensity of radiation, led to a further increase in the expression of Aurora-A in the resistant strain (Figure 2A-C). To further verify whether Aurora-A is involved in radiation

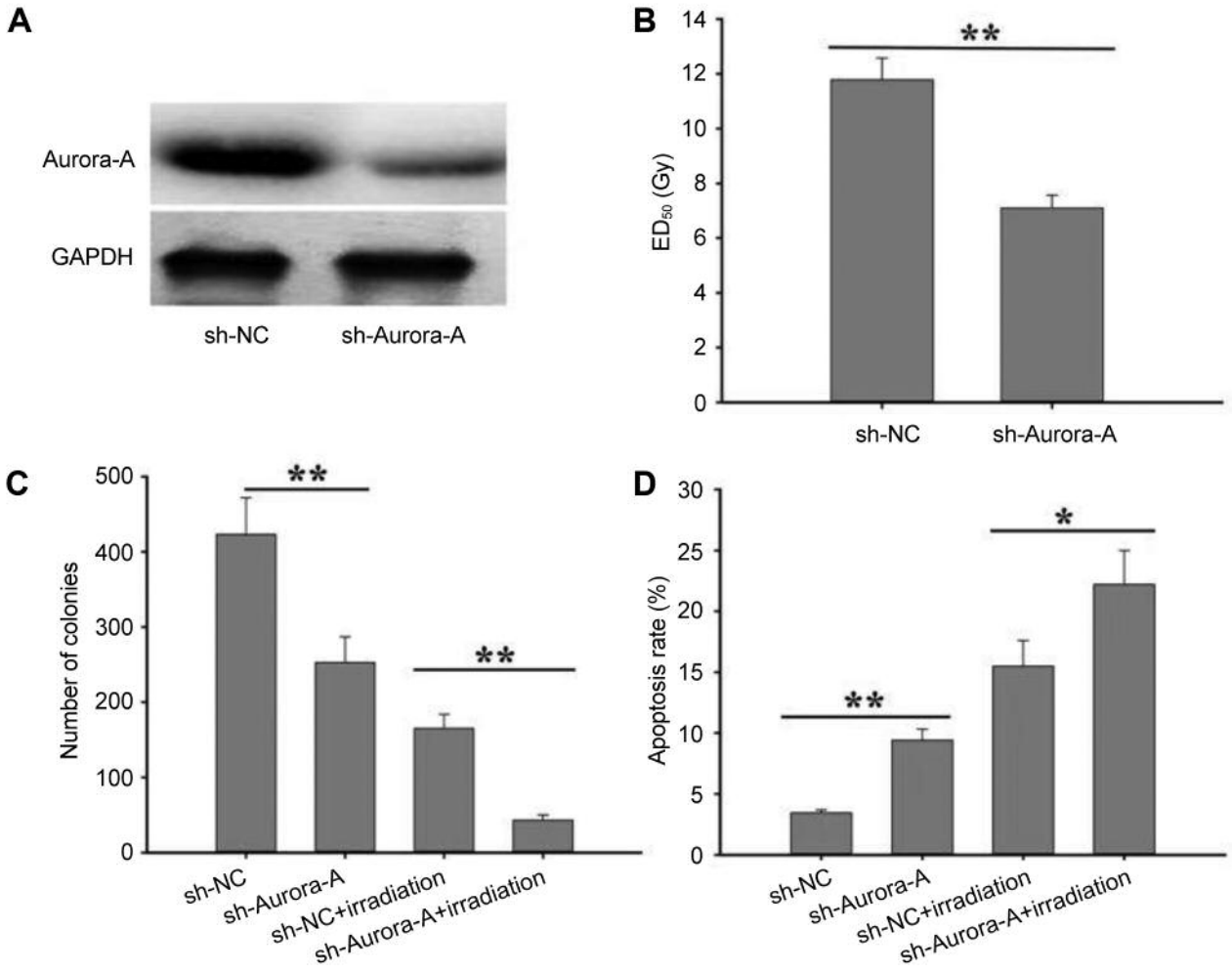

Figure 3. *Down-regulation of Aurora-A expression can enhance the in vitro sensitivity of human LAD multidrug-resistant cells. A. Changes in Aurora-A protein expression in SPC-A1/DTX cells after transfection with small interfering RNA against Aurora-A. The expression levels of Aurora-A protein were detected by western blotting, and GAPDH was used as a reference. B. The ED_{50} of radiation of SPC-A1/DTX cells after transfection with small interfering RNA of Aurora-A. The ED_{50} of each cell line was calculated with the MTT assay. C. The proliferation of SPC-A1/DTX cells in vitro following different treatments was determined by a colony-forming assay. Each cell type was plated in a 6-well plate with 1000 cells/well and was subjected to the corresponding treatment, crystal violet staining, image acquisition and calculation of the number of colonies after 2 weeks. D. The levels of early apoptosis of SPC-A1/DTX cells following different treatments was determined by flow cytometry. Each cell type was plated in a 6-well plate at 200,000 cells/well and was subjected to the corresponding treatment, and then, the rate of early apoptosis was determined by flow cytometry after 48 h. *$p<0.05$, **$p<0.01$.*

resistance of human lung adenocarcinoma drug-resistant SPC-A1/DTX cells, we constructed a small interfering RNA that targets Aurora-A, and the expression of Aurora-A protein was decreased after transfection (Figure 3A). Thereafter, we found that the effective dose $(ED)_{50}$ of radiotherapy for SPC-A1/DTX cells was significantly decreased following down-regulation of Aurora-A (Figure 3B, $p<0.01$). Irrespective of radiation treatment, down-regulation of Aurora-A expression inhibited the proliferation (Figure 3C, $p<0.01$) and rate of early apoptosis (Figure 3D, $p<0.05$) of SPC-A1/DTX. The above results suggested that Aurora-A is involved in the radiation resistance of human lung adenocarcinoma.

Nuclear transcription factor NF-κB is a downstream target gene of Aurora-A. Previous studies have suggested that Aurora-A can inhibit the expression of IκBa and cause the activation of NF-κB. However, whether this regulatory mechanism plays a role in human lung adenocarcinoma has not been reported. The present study found that down-regulation of Aurora-A resulted in increased expression of IκBa and inhibition of the expression of NF-κB (Figure 4A, $p<0.01$). Additionally, we found that the expression level of IκBa was decreased in the drug-resistant strain compared with the parental strain (Figure 4B). The expression level of NF-κB was increased in the drug-resistant strain as the intensity

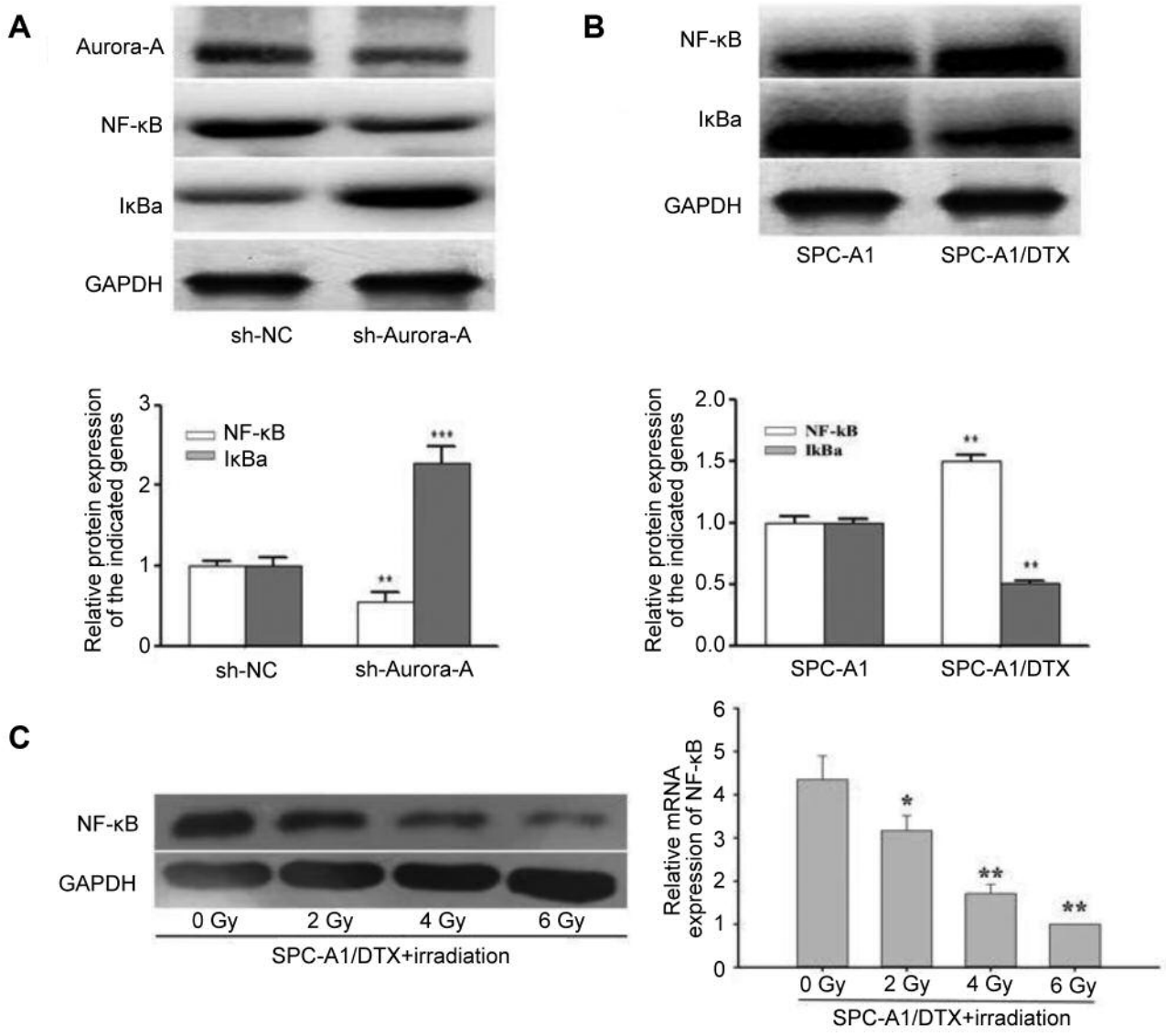

Figure 4. *In drug-resistant SPC-A1/DTX cells, NF-κB is the downstream target gene of Aurora-A. A. The expression levels of Aurora-A, NF-κB and IκBα in SPC-A1/DTX cells after transfection with different small interfering RNAs. After 72 h, the expression levels of Aurora-A, NF-κB and IκBα in each group was detected by western blotting, and GAPDH was used as a reference. The upper panel depicts representative western blots, and the lower panel represents the relative expression in a histogram. B. Expression levels of NF-κB and IκBα proteins in SPC-A1 and SPC-A1/DTX cells. The expression levels of NF-κB and IκBα proteins in each group were detected by western blotting, and GAPDH was used as a reference. The upper panel depicts representative western blots, and the lower panel represents the relative expression in a histogram. C. The expression levels of NF-κB protein in lung adenocarcinoma drug-resistant SPC-A1/DTX cells following treatment with different intensities of radiation. SPC-A1/DTX cells were treated with 0, 2, 4 and 6 Gy of radiation. After 48 h, the expression levels of NF-κB protein were detected by western blotting, and GAPDH was used as a reference. The left panel depicts representative western blots, and the right panel represents the relative expression in a histogram. *p<0.05, **p<0.01.*

of the radiation dose increased (Figure 4C). These results indicated that nuclear transcription factor NF-κB is a downstream effector gene of Aurora-A in the human lung adenocarcinoma cell line SPC-A1/DTX.

NF-κB participates in the radiotherapy resistance of the human lung adenocarcinoma drug-resistant strain SPC-A1/DTX. To further verify that the downstream factor of Aurora-A, NF-κB, participates in radiation resistance of the human lung adenocarcinoma-resistant SPC-A1/DTX cells, we treated the cells with NF-κB inhibitor (5 μmol/l). We found that treatment of cells with the NF-κB inhibitor resulted in decreased expression of NF-κB protein (Figure 5A). The median ED_{50} was decreased after inhibition of NF-κB (Figure 5B, $p<0.01$).

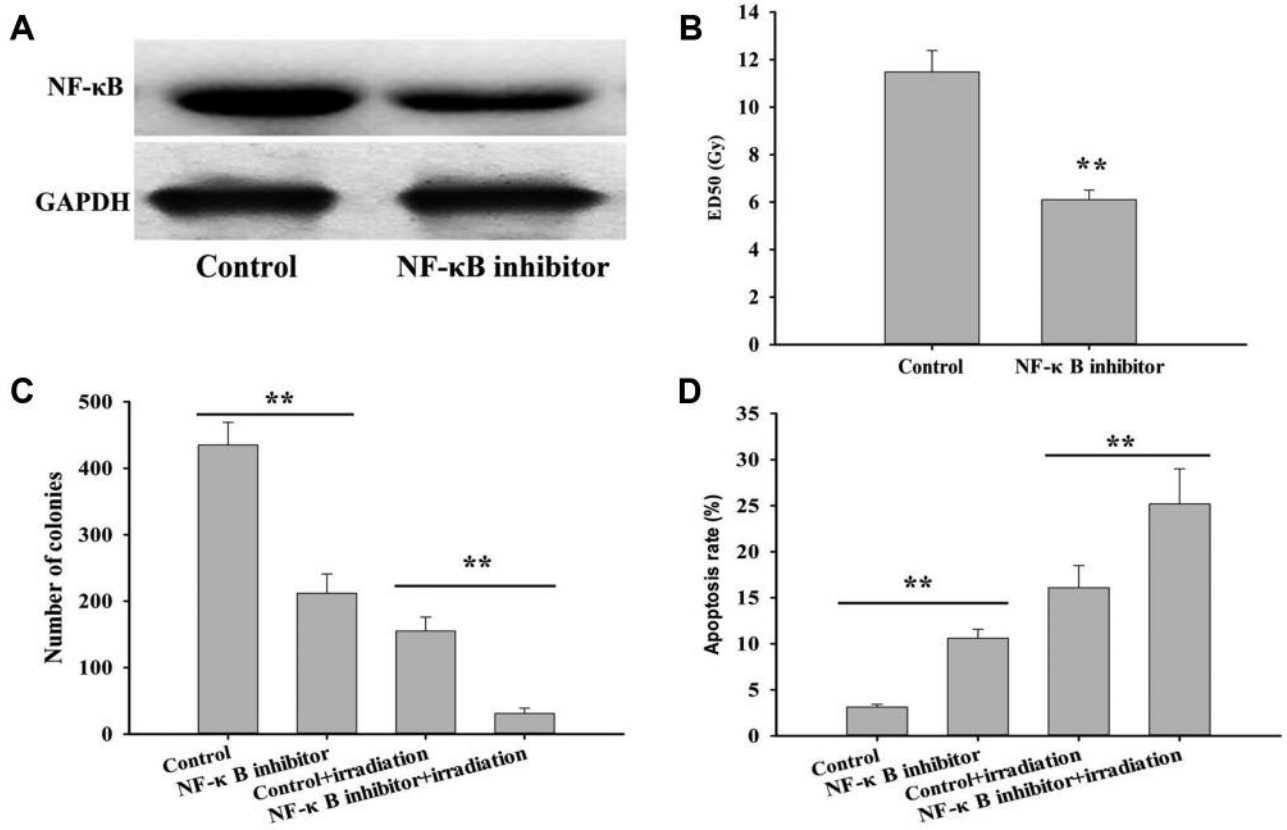

Figure 5. *Inhibition of NF-κB can reverse the sensitivity to radiation of human lung adenocarcinoma drug-resistant SPC-A1/DTX cells. A. Changes in the NF-κB protein expression in SPC-A1/DTX cells after inhibiting NF-κB. The expression levels of NF-κB protein was detected by western blotting, and GAPDH was used as a reference. B. The ED_{50} of radiation in SPC-A1/DTX cells after inhibiting NF-κB. The ED_{50} in each cell line was calculated by applying the MTT assay. C. The proliferation of SPC-A1/DTX cells in vitro was determined by the colony-forming assay. Each cell line was plated in a 6-well plate at 1000 cells/well and subjected to the corresponding treatment, followed by crystal violet staining, image acquisition and calculation of the number of colonies after 2 weeks. D. The levels of early apoptosis of SPC-A1/DTX cells were determined by flow cytometry. Each cell type was plated in a 6-well plate with 200,000 cells/well and subjected to the corresponding treatment, and then the rate of early apoptosis was determined by flow cytometry after 48 h. *$p<0.05$, **$p<0.01$.*

Additionally, inhibition of NF-κB expression inhibited the proliferation of SPC-A1/DTX (Figure 5C, $p<0.01$) and increased the rate of early apoptosis with or without radiotherapy (Figure 5D, $p<0.01$). These results suggested that NF-kB is a downstream factor of Aurora-A in human lung adenocarcinoma.

Aurora-A is involved in the radiation resistance of human lung adenocarcinoma drug-resistant SPC-A1/DTX cells in vivo. To demonstrate the role of Aurora-A in radiation resistance of human lung adenocarcinoma drug-resistant SPC-A1/DTX cells *in vivo*, we subcutaneously implanted SPC-A1/DTX cells in nude mice. Compared with the control group, down-regulation of Aurora-A inhibited the growth of the subcutaneous tumors (Figure 6A, $p<0.05$). Following down-regulation of Aurora-A, the expression of Aurora-A and NF-κB in tumor tissue was decreased (Figure 6B). Furthermore, down-regulation of Aurora-A also inhibited the proliferation of subcutaneously implanted tumor cells in nude mice (Figure 6C and D, $p<0.05$).

Discussion

Aurora-A is a subtype of serine/threonine kinases that plays an important role in the normal process of cell mitosis and the occurrence and development of tumors (8). Aurora-A kinase is often over-expressed in various malignant tumors, such as lung cancer, esophageal cancer, liver cancer, breast cancer, and colon cancer, and it is related to the patient prognosis (9-13). It has also been reported that Aurora-A is involved in chemoresistance (14). Aurora-A is involved in chemotherapy sensitivity of hepatocellular carcinoma *via* the NF-κB/miR-21/PTEN/Akt signaling pathways. Overexpression of Aurora-A is closely related to the recurrence-free survival rate of patients. Aurora-A is an independent prognostic factor in patients with

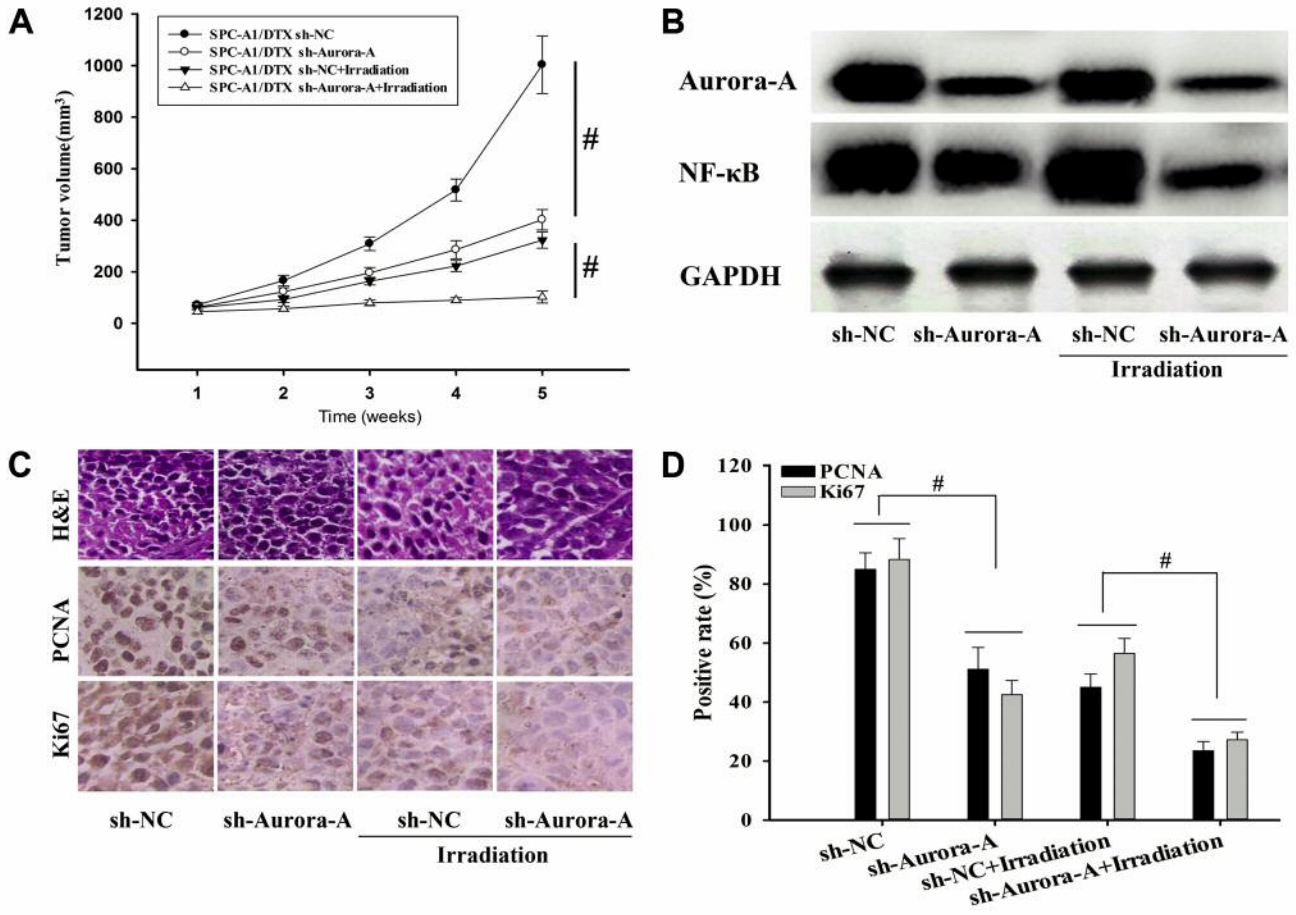

Figure 6. *Down-regulation of Aurora-A expression can reverse the sensitivity to radiation of human lung adenocarcinoma drug-resistant SPC-A1/DTX cells in vivo. A. Growth curves of the subcutaneously growingof SPC-A1/DTX cells tumors treated. The SPC-A1/DTX cells were transfected with Aurora-A small interfering RNA or the corresponding control. A tumor cells were subcutaneously implanted in nude mice (2×10^6/each mouse, 4 mice/each group). The tumor size was measured every week, and all the mice were sacrificed after 5 weeks. Tumors were then removed, and growth curves were generated. B. The expression levels of Aurora-A and NF-κB proteins were changed in each group. The expression levels of Aurora-A and NF-κB proteins in each group of tumors were detected by western blotting. GAPDH was used as a reference. C. H&E staining, and PCNA and Ki67 expression. The expression levels of PCNA and Ki67 were determined by immunohistochemistry. D. The expression levels of PCNA and Ki67 were calculated in each experimental group. *$p<0.05$.*

hepatocellular carcinoma (6). In non-small cell lung cancer, overexpression of Aurora-A is closely related to cisplatin resistance, lymph node metastasis and patient prognosis. The present study found that the expression of Aurora-A in drug-resistant cells was significantly increased and down-regulation of Aurora-A could significantly reverse radio-resistance of human lung adenocarcinoma drug-resistant strain, suggesting that high levels of Aurora-A were involved in the radiation resistance of human lung adenocarcinoma drug-resistant SPC-A1/DTX cells *in vivo* and *in vitro*. However, the underlying mechanism of the involvement of Aurora-A in radiation resistance has not yet been fully elucidated.

Previous studies have shown that Aurora-A decreases the stability of IκBα in p53-knockout lung cancer, increasing the transcriptional activity of NF-κB, which is involved in resistance to gefitinib (15). miR-15a/16 increased radiation sensitivity of non-small cell lung cancer by targeting the TLR1/NF-κB signaling pathway (16). NF-κB is not only an important factor regulating cell growth, differentiation, and inflammation but also an endogenous tumor-promoting factor. NF-κB binds to the pathway inhibitor protein IκB in the cytoplasm at resting state. Activation of NF-κB occurs primarily *via* the dissociation of IκB or an increase in NF-κB expression. Radiotherapy can reduce the levels of NF-κB in tumor cells, and excessive activation of NF-κB can promote production of apoptosis-inhibiting factors and enhance tumor cell growth, resulting in radiation resistance (17-19).

In the present study, we found that the expression of NF-κB in the resistant strain was significantly higher than that in the parental strain, while the expression of IκB was decreased, and the expression of NF-κB was increased following down-regulation of Aurora-A. Radiation resistance can cause the expression of NF-κB to be further decreased. These results suggested that high levels of Aurora-A expression induce the activation of NF-κB that participates in the radiation resistance of human lung adenocarcinoma. In conclusion, the Aurora-A/NF-κB pathway is associated with radio-resistance of human lung adenocarcinoma docetaxel-resistant cells.

Conflicts of Interest

None of the Authors has a conflict of interest with regard to this study.

Authors' Contributions

Zhang Yi designed the study. Junbao Liu and Liang Hu performed the *in vitro* study. Yu Sun performed the *in vivo* study. Zhijian Yang and Robert M. Hoffman revised the manuscript.

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