

The effect of ataxia-telangiectasia mutated kinase-dependent hyperphosphorylation of checkpoint kinase-2 on oligodeoxynucleotide 7909 containing CpG motifs-enhanced sensitivity to X-rays in human lung adenocarcinoma A549 cells

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Objective: The aim of the study reported here was to further investigate the potential effect of ataxia-telangiectasia mutated (ATM) kinase-dependent hyperphosphorylation of checkpoint kinase-2 (Chk2) on radiosensitivity enhanced by oligodeoxynucleotide 7909 containing CpG motifs (CpG ODN7909) in human lung adenocarcinoma A549 cells.

Methods: In vitro A549 cells were randomly separated into control, CpG, X-ray, CpG+X-ray, ATM kinase-small interfering RNA (siRNA)+CpG+X-ray (ATM-siRNA), and Chk2-siRNA+CpG+X-ray (Chk2-siRNA) groups. siRNAs were adopted to silence the *ATM* and *Chk2* genes. Expression and phosphorylation of ATM kinase and Chk2 were detected by Western blot assay. Cell colonies were observed under inverted phase-contrast microscopy. Cellular survival curves were fitted using a multi-target single-hitting model. Cell cycle and apoptosis were analyzed by flow cytometry.

Results: Expression of ATM kinase and Chk2 was similar among the control, CpG, X-ray, and CpG+X-ray groups. Phosphorylated ATM kinase and Chk2 were significantly increased in the CpG+X-ray group compared with in the X-ray group ($t=6.00$, $P<0.01$ and $t=3.13$, $P<0.05$, respectively), though these were hardly detected in the control and CpG groups. However, expression of ATM kinase and Chk2 was clearly downregulated in the ATM-siRNA and Chk2-siRNA groups, respectively. Similarly, their phosphorylation levels were also significantly decreased in the ATM-siRNA group ($t=14.35$, $P<0.01$ and $t=8.46$, $P<0.01$, respectively) and a significant decrease in phosphorylated Chk2 was observed in the Chk2-siRNA group ($t=7.28$, $P<0.01$) when compared with the CpG+X-ray group. Further, the number of A549 cells at Gap 2/mitotic phase and the apoptosis rate were clearly increased in the CpG+X-ray group compared with in the other groups (all $P<0.05$). The multi-target single-hitting model showed that the sensitization enhancement ratio calculated by mean death dose was 1.39 in CpG+X-ray group (vs 1.04 and 1.03 in the ATM-siRNA and Chk2-siRNA groups, respectively).

Conclusion: This study provides the first evidence, as far as we are aware, that CpG ODN7909 can potentiate A549 cell radiosensitivity via increasing ATM kinase-dependent phosphorylation of Chk2, suggesting activation of the ATM kinase/Chk2 signal pathway. However, the mechanism of ATM kinase activation is worth further exploration.

Keywords: ATM kinase, ODN, signal pathway, cell cycle, apoptosis, radiosensitivity

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Background

Worldwide, non-small cell lung cancer (NSCLC) is one of the leading cancers responsible for death in both men and women, and is still an incurable disease.¹ One of the

most vital factors that affects the survival rate of patients with NSCLC is resistance to therapeutic drugs.² Despite considerable advances in the medical and surgical treatment of patients with NSCLC, metastatic disease at presentation is common and the prognosis related to this disease remains dismal.^{3,4}

Radiotherapy is one of the most effective means of malignant tumor treatment and plays an important role in achieving local control of cancers and in the relief of symptoms resulting from metastatic lesions in patients with NSCLC. In addition, it has been well illustrated that irradiation eradicates tumor cells via inducing an array of DNA lesions, such as base damage, intra- and inter-strand cross-linking, and single- or double-strand breaks (DSBs).⁵ Among these different types of DNA damage, DSBs represent a particularly dangerous form, as they result in cell cycle arrest and/or cell death.⁶ The DNA damage sensors, such as ataxia-telangiectasia mutated (ATM) kinase and checkpoint kinase-2 (Chk2; a direct downstream substrate of ATM kinase), play central roles in response to genotoxic stress.⁷ Further, studies have shown that ATM kinase is required to sense and initiate a well-characterized response to DSBs induced by X-rays, resulting in cell cycle arrest; induction of cellular apoptosis; and, in some cases, promotion of lesion DNA repair.⁸⁻¹¹ As is well understood, tumor cells arrested at the Gap 2 (G₂)/mitotic (M) phase are the most sensitive to ionizing radiation (IR), and apoptosis could eliminate tumor cells harboring lesion DNA. Accordingly, it is also widely believed that these kinds of DNA damage responses (DDR) are responsible for the maintenance of genomic stability and preventing the development of malignant tumors.¹² However, therapeutic efficacy is compromised when tumor cells develop resistance to X-rays and long-term survival in patients with NSCLC still remains terribly low.

Previously published work on synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs (CpG ODNs) has reported that CpG ODNs might induce immune responses to tumor cells in a therapeutic adjuvant strategy through functioning as Th-1 adjuvants and activating B lymphocytes and dendritic cells.^{13,14} However, some studies have well illustrated that CpG ODNs may potentiate the sensitivity of tumor cells to chemotherapy by increasing chemotherapy-induced cellular apoptosis and inhibiting cellular proliferation.^{15,16} CpG ODN7909, one of the type-B CpG ODNs, has a fully phosphorothioate-modified backbone that can resist nuclease attack and increase the in vivo stability of ODNs by extending their half-life from a few minutes to about 2 days. Moreover, CpG ODN7909 can initiate downstream-signaling cascades that are involved in regulating transcription by interacting with Toll-like receptor 9 (TLR9), which is

also expressed in human lung adenocarcinoma A549 cells.¹⁷ Further, the results of our previous experiments have shown that there is a potential association between the increased activation of Chk2 and the enhancement of sensitivity to IR by CpG ODN7909 in A549 cells.¹⁸

Therefore, the purpose of the study reported here was to further explore whether downstream substrate Chk2 is hyperactivated depending on the increase of ATM kinase phosphorylation involved in CpG ODN7909-enhanced cell cycle arrest and apoptosis induced by X-rays, as this could provide a new theoretical basis for radiosensitivity enhanced by CpG ODN7909 in A549 cells.

Materials and methods

Antibodies, siRNAs, and reagents

A549 cells were purchased from the Chinese Academy of Science (Shanghai, People's Republic of China). Roswell Park Memorial Institute (RPMI)-1640 medium and fetal bovine serum were obtained from BioWest SAS (Nuaille, France). CpG ODN7909 (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3') was purchased from the Shanghai Sangon Biological Engineering Technology and Services Co (Shanghai, People's Republic of China) and stored at 4°C. An annexin V fluorescein isothiocyanate (FITC) apoptosis detection kit, a bicinchoninic acid protein assay kit, and secondary antibodies were obtained from the Beyotime Institute of Biotechnology (Jiangsu, People's Republic of China). Primary antibodies against beta-actin, ATM kinase, Chk2, phosphorylated ATM (pATM) kinase and phosphorylated Chk2 (pChk2) were purchased from Santa Cruz Biotechnology Inc (Dallas, TX, USA). ATM-siRNA, Chk2-siRNA, and Lipofectamine™ 2000 were bought from the Cell Signaling Technology Corporation (Danvers, MA, USA).

Cell culture

A549 cells were cultured in RPMI-1640 medium supplemented with 100 units/mL of penicillin, 100 µg/mL of streptomycin and 10% heat-inactivated fetal bovine serum at 37°C in a humidified air containing 5% carbon dioxide. The cultured cells were randomly divided into six groups: control, CpG, X-ray, CpG+X-ray, ATM kinase-siRNA+CpG+X-ray (ATM-siRNA), and Chk2-siRNA+CpG+X-ray (Chk2-siRNA). The culture medium was displaced every 2 or 3 days. A549 cells in the logarithmic growth phase were used to perform the following experiments.

Transfection of siRNAs

For siRNA transfection, A549 cells in the logarithmic growth phase were harvested and seeded in six-well culture plates.

When the cells reached 50% confluence, 100 nM of ATM-siRNA or Chk2-siRNA was transfected with 5 μ L of Lipofectamine 2000 plus 1.5 mL of serum-free RPMI-1640 medium without antibiotics following the instructions described by the manufacturer. After incubation for 6 hours, the medium was displaced with the standard culture medium already described and the transfected cells continued to be cultured for 18 hours. Then the cells were used in the following tests.

Irradiation treatment

At room temperature, A549 cells were irradiated with 6 MV X-rays using a linear accelerator (Elekta Precise; Elekta, Stockholm, Sweden) under the source-to-skin distance (the distance from the radiation source to the central surface of the six-well plate = 100 cm), and the dose rate of 2.0 Gy/minute was employed. Based on our previous studies, the cells in the CpG and CpG+X-ray groups and the cells transfected with siRNAs were all pre-treated with CpG ODN7909 (10 μ g/mL) 24 hours pre-irradiation,¹⁹ while the cells in the control and X-ray groups were treated with a corresponding volume of sterile distilled water. In colony-formation experiments, all cells except for the cells in control group and CpG group were irradiated using X-ray doses of 0, 2, 4, 6, 8, and 10 Gy. In other experiments, cells treated with X-rays were irradiated using a dose of 10 Gy.

Clonogenic survival assay

The irradiated A549 cells were immediately trypsinized and suspended post-irradiation, then seeded in triplicate in 60 mm Petri dishes at a density at which the cells would form colonies of 50–200 cells according to the results in pre-experiments. After incubation for 10 days, the cells were washed twice with phosphate-buffered saline, fixed in methanol, and then stained with Giemsa stain. A “colony” was defined as a cluster of at least 50 cells. The number of colonies was manually counted using inverted phase-contrast microscopy. Clonogenic survival fraction was calculated according to Equation 1:

$$\frac{\text{Irradiated cell colony numbers}}{\text{Unirradiated cell colony numbers}} \times 100\% \quad (1)$$

Cell cycle distribution

Cell cycle phases were analyzed by measuring the DNA fragments that were stained with propidium iodide (PI; Sigma-Aldrich Co, St Louis, MO, USA) under the conditions described by the manufacturer. The cells grown in six-well plates were harvested and centrifuged at 24 hours post-irradiation. Cell pellets were counted and washed twice

with precooled phosphate-buffered saline. Then the cells were fixed and permeabilized overnight at 4°C by adding 1 mL of 70% precooled ethanol to each tube. After centrifugation, the fixatives were decanted and the cell pellets were resuspended in 0.5 mL of staining solution containing 200 μ L of DNase-free RNase (Sigma-Aldrich Co) and PI then incubated for 30 minutes at room temperature in the dark. Finally, flow cytometry was employed to measure cell cycle using a FACScan™ system with CellQuest™ software (v 3.3, BD Biosciences, San Jose, CA, USA).

Cell apoptosis

The A549 cells grown in six-well plates were harvested and counted at 24 hours post-irradiation. The tests were performed using an annexin V FITC apoptosis detection kit. The cell pellets were resuspended in 195 μ L of binding buffer and stained with 5 μ L of annexin V FITC and PI staining solution for 10 minutes at room temperature in the dark. Flow cytometry was employed to detect cellular apoptosis. The cell apoptosis rate was calculated according to Equation 2:

$$\frac{\text{Number of apoptotic cells in each group}}{\text{Total number of cells in each group}} \times 100\% \quad (2)$$

Expression and phosphorylation of ATM kinase and Chk2s

Western blot was performed to detect the expression and phosphorylation of ATM kinase and Chk2 in the A549 cells. The treatment schedule for Western blot tests was the same as that for cell cycle assay. At 3 hours post-irradiation, A549 cells in each group were lysed in 100 μ L of radio-immunoprecipitation assay protein-lysis buffer (Beyotime Institute of Biotechnology) supplemented with 1 nM phenylmethylsulfonyl fluoride and 1 nM sodium orthovanadate. Proteins were extracted on ice for at least 30 minutes.

The concentrations of the proteins in the lysates were measured using a bicinchoninic acid protein assay kit. Lysate proteins (50 μ g) were fractionated by 12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (Thermo Fisher Scientific, Waltham, MA, USA) and blocked for 1 hour in Tris-buffered saline containing 5% bovine serum albumin and 0.05% polysorbate 20 at room temperature. Blots were probed with the appropriate primary antibodies and peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies. Specific signals were detected with an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology).

The images were analyzed using Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA, USA).

Statistical analysis

A multi-target single-hitting model was adopted to fit survival curves using SigmaPlot® software (v 10.0; Systat Software, Inc, San Jose, CA, USA). Statistics analysis and graphing were performed with SPSS (v 16.0; IBM Corporation, Armonk, NY, USA) and GraphPad Prism® 5 (GraphPad Software, Inc, La Jolla, CA, USA) software, respectively.

Measurement data are expressed as mean \pm standard deviation, and a probability <0.05 was considered statistically significant between groups, as determined by Student's *t*-test.

Results

CpG ODN7909 increased ATM kinase-dependent phosphorylation of Chk2 induced by X-ray in A549 cells

As shown in Figure 1, there was no obvious difference in expression of ATM kinase or Chk2 among the control, CpG,

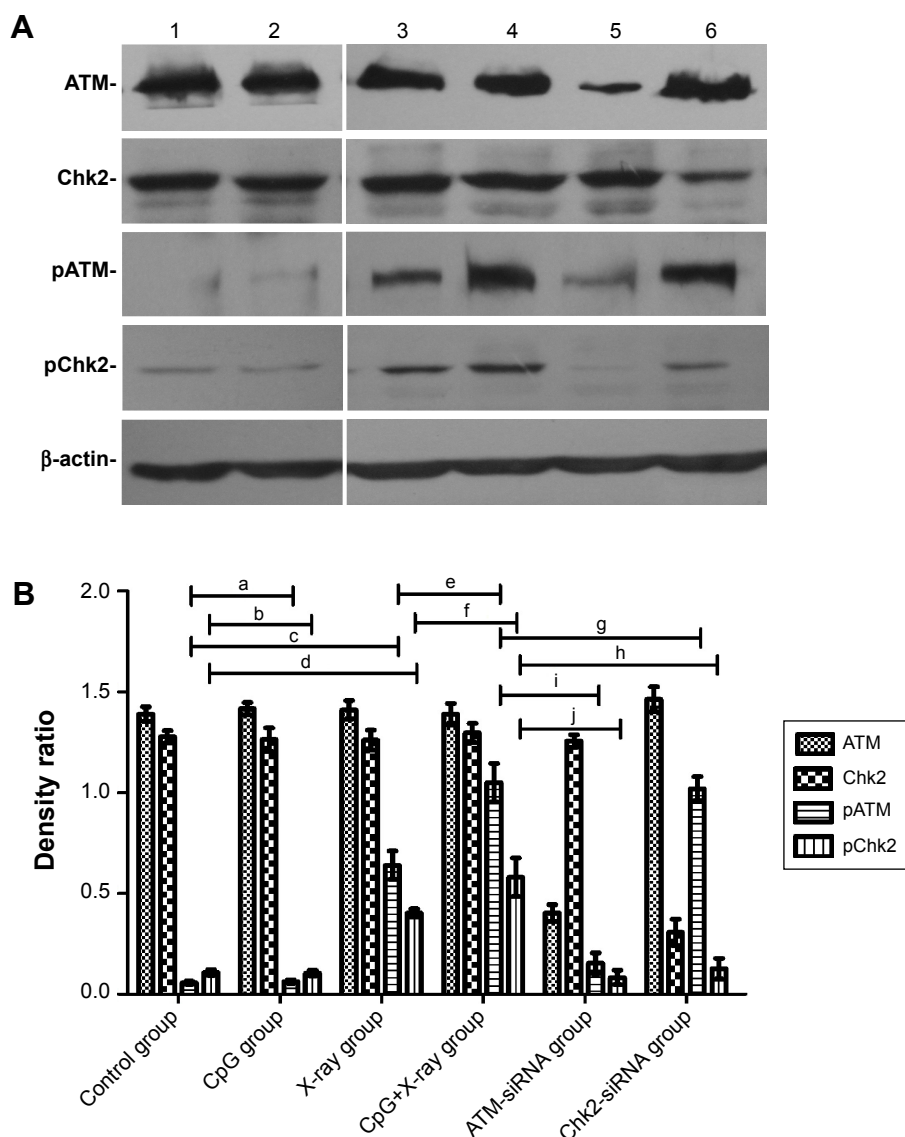


Figure 1 Effect of CpG ODN7909 on ataxia-telangiectasia mutated (ATM) kinase and checkpoint kinase-2 (Chk2) phosphorylation induced by X-rays in A549 cells. **(A)** Expression of ATM and Chk2 were detected by Western blot 3 hours post-irradiation. Columns: (1) control group, (2) CpG group, (3) X-ray group, (4) CpG+X-ray group, (5) ATM-siRNA group, (6) Chk2-siRNA group. **(B)** Expression of ATM and Chk2 were analyzed by density ratio (density ratio = ATM or Chk2 band density/ β -actin band density). Each experiment was repeated three times.

Notes: In terms of pATM and pChk2: ^a $P>0.05$ between control group and CpG group; ^c $P<0.01$ between control group and X-ray group; ^d $P<0.05$ between X-ray group and CpG+X-ray group, respectively; ^b $P>0.05$ and $P<0.01$ between CpG+X-ray group and Chk2-siRNA group, respectively; ⁱ $P<0.01$ between CpG+X-ray group and ATM-siRNA group.

Abbreviations: pATM, phosphorylated ataxia-telangiectasia mutated; pChk2, phosphorylated checkpoint kinase-2; siRNA, small interfering RNA.

X-ray, and CpG+X-ray groups. pATM kinase and Chk2 were hardly detected in the control group and CpG group, and there was no significant difference between the two groups ($t=0.93$, $P>0.05$ vs $t=0.18$, $P>0.05$). However, both ATM kinase and Chk2 were obviously phosphorylated in the X-ray group. Moreover, the level of ATM kinase and Chk2 phosphorylation was increased in the CpG+X-ray group compared with in the X-ray group ($t=6.00$, $P<0.01$ vs $t=3.13$, $P<0.05$). ATM kinase expression in the ATM-siRNA group and Chk2 expression in the Chk2-siRNA group were deregulated by specific siRNAs. Similarly, the level of pATM kinase and Chk2 was significantly decreased in the ATM-siRNA group compared with in the CpG+X-ray group ($t=14.35$, $P<0.01$ vs $t=8.46$, $P<0.01$). pChk2 was significantly decreased in the Chk2-siRNA group compared with in the CpG+X-ray group ($t=7.28$, $P<0.01$), although there was no obvious difference in the level of pATM kinase between the two groups ($t=0.46$, $P>0.05$).

CpG ODN7909 increased the number of cells arrested at G₂/M phase induced by X-ray in A549 cells

As shown in Table 1, there was no significant difference in G₂/M phase arrest between the control group and the CpG group ($t=2.67$, $P>0.05$). More cells were arrested at G₂/M phase in the X-ray group than in the control group ($t=17.2$, $P<0.01$). Further, significantly more cells were arrested at G₂/M phase in the CpG+X-ray group than in the X-ray group ($t=9.81$, $P<0.01$). However, significantly fewer cells were arrested at G₂/M phase in both siRNA groups than in the CpG+X-ray group ($t=17.73$, $P<0.01$ vs $t=18.15$, $P<0.01$).

Table 1 Effect of CpG ODN7909 on X-ray-induced Gap 2 (G₂)/mitotic (M) phase arrest in A549 cells

Group	Phase		
	G ₁	S	G ₂ /M
Control	65.37±0.23	21.70±0.18	12.93±0.19
CpG	64.79±0.21	21.36±0.20	13.85±0.13 ^a
X-ray	66.25±0.74	11.38±0.41	22.37±0.53 ^b
CpG+X-ray	57.16±0.34	10.35±0.30	32.49±0.29 ^c
ATM-siRNA	56.14±0.69	33.53±0.51	10.33±0.23 ^d
Chk2-siRNA	56.43±0.63	33.78±0.47	9.79±0.35 ^e

Notes: In terms of the number of cells arrested at G₂/M phase: ^a $P>0.05$ between control group and CpG group, ^b $P<0.01$ between control group and X-ray group, ^c $P<0.01$ between X-ray group and CpG+X-ray group, ^d $P<0.01$ between CpG+X-ray group and ATM-siRNA group, ^e $P<0.01$ between CpG+X-ray group and Chk2-siRNA group.

Abbreviations: ATM, ataxia-telangiectasia mutated; Chk2, checkpoint kinase-2; G₁, Gap 1; S, synthesis; siRNA, small interfering RNA.

CpG ODN7909 increased cellular apoptosis induced by X-ray in A549 cells

As shown in Figure 2, there was no significantly apoptotic difference between the control group and the CpG group ($t=1.18$, $P>0.05$). There was an increasing apoptosis rate in the X-ray group compared with in the control group ($t=11.24$, $P<0.01$). Moreover, the apoptosis rate was significantly increased in the CpG+X-ray group compared with in the X-ray group ($t=7.96$, $P<0.01$). However, the apoptosis rate was clearly decreased in both siRNA groups compared with in the CpG+X-ray group ($t=5.15$, $P<0.01$ vs $t=4.95$, $P<0.01$).

Increase of sensitization enhancement ratio was observed in A549 cells treated with the combination of CpG ODN7909 and X-rays

Fitted dose-survival curves using a multi-target single-hitting model are shown in Figure 3, and corresponding radiobiological parameters are shown in Table 2. Compared with those in other groups treated X-rays, the extrapolation number (N), mean death dose (D₀), and quasi field dose (D_q) values were clearly decreased in the CpG+X-ray group. Moreover, the sensitization enhancement ratio calculated by D₀ value was 1.39 in the CpG+X-ray group, while in the ATM-siRNA group and Chk2-siRNA group, the sensitization enhancement ratio was 1.04 and 1.03, respectively.

Discussion

Some in-depth research related to CpG ODNs as immunoadjuvants combined with other remedies such as immunotherapy, cryotherapy, and chemotherapy for the treatment of malignant tumors has been well performed.^{19–21} However, in recent years, there has been more focus on the direct effect of CpG ODNs on some malignant tumors. For example, Wu et al²² reported that CpG ODN2216 inhibits the invasion and migration of pancreas cancer cells in experiments, and Mason et al²³ demonstrated that CpG ODN1826 improves the therapeutic effect of docetaxel on breast cancer cells. Moreover, previous studies by our group have shown that CpG ODN1826 increases the response to X-rays in Lewis mice with lung cancer and that CpG ODN7909 might directly potentiate the radiosensitivity of A549 cells.^{18,24} However, the mechanisms involved in the direct effect of CpG ODNs on the sensitivity of lung cancer cells to IR are worth further investigation.

It is well demonstrated that cell cycle distribution is associated with tumor cell sensitivity to IR, and cells arrested at the

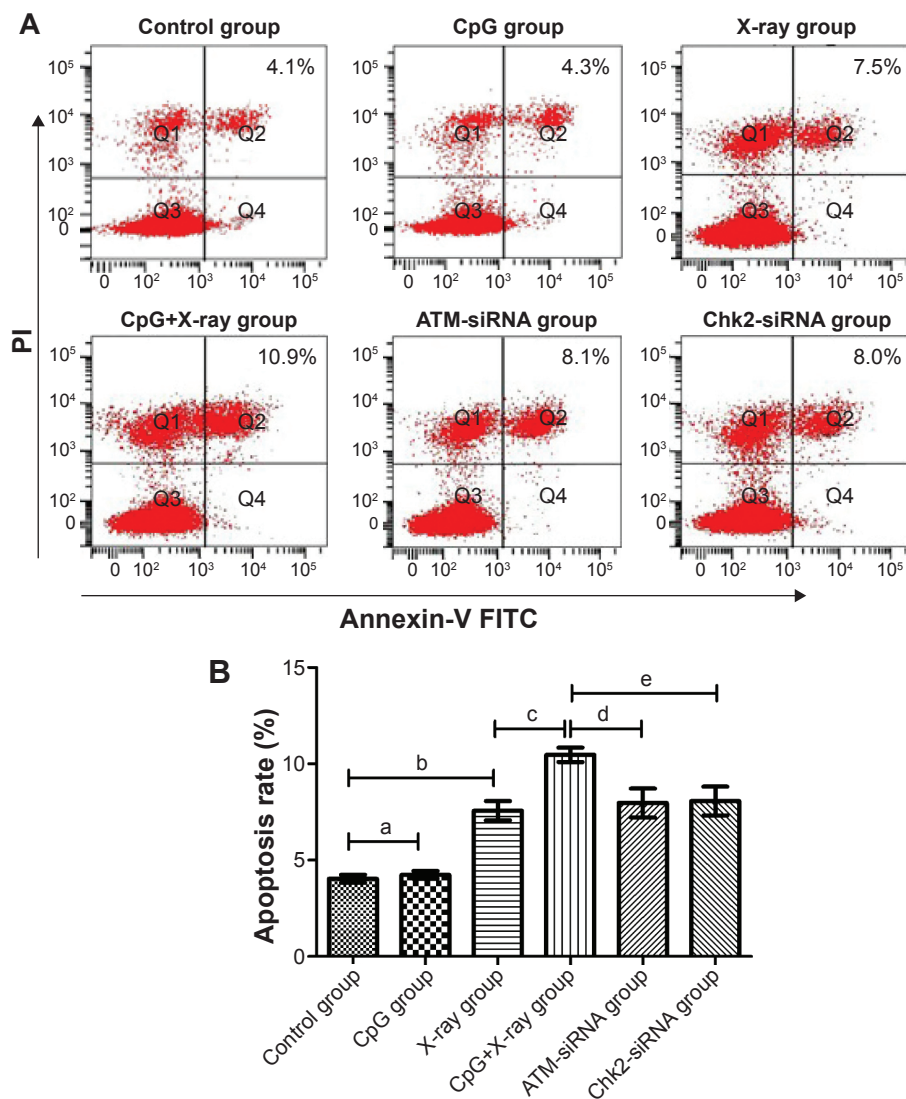


Figure 2 Effect of CpG ODN7909 on apoptosis induced by X-rays in A549 cells. **(A)** Flow cytometry showed apoptotic changes 24 hours post-irradiation. **(B)** Apoptotic fraction of cells in different groups.

Notes: ^a $P > 0.05$ between the control group and CpG group; ^b $P < 0.01$ between the control group and X-ray group; ^c $P < 0.01$ between the X-ray group and CpG+X-ray group; ^d $P < 0.01$ between the CpG+X-ray group and ATM-siRNA group; ^e $P < 0.01$ between the CpG+X-ray group and Chk2-siRNA group.

Abbreviations: ATM, ataxia-telangiectasia mutated; Chk2, checkpoint kinase-2; PI, propidium iodide; siRNA, small interfering RNA.

G_2/M phase are the most sensitive to irradiation. Therefore, an effective approach would be to increase the radiosensitivity of tumors and improve therapeutic efficacy by promoting tumor cell cycle progression then arrest at G_2/M phase. However, regulation of cell cycle is closely related to DDRs, and studies have shown that DDR signaling pathways are initiated well by phosphoinositide 3-kinase-like kinase family members, including ATM kinase and ataxia-telangiectasia and Rad3-related kinase, which recognize different types of DNA lesions.²⁵ Importantly, it is well demonstrated that ATM kinase is activated by double-stranded DNA breaks (DSBs) induced by irradiation. In response to IR-induced DNA damage, ATM kinase is recruited to the sites of DSBs and is activated by autophosphorylation at Ser-1981 and

binding to heterotrimeric Mre11/Rad50/Nbs1 adaptor complex, resulting in phosphorylation of downstream substrates such as Chk2 and p53 protein and eventually inducing cell cycle checkpoint activation to arrest cells at G_2/M phase by initiating downstream cascade reactions.²⁶

In the study presented here, our results show that CpG ODN7909 not only enhanced X-ray-induced G_2/M phase arrest but also increased X-ray-induced phosphorylation of ATM kinase and Chk2 in A549 cells. However, CpG ODN7909 did not increase Chk2 phosphorylation or the number of A549 cells arrested at G_2/M phase induced by X-rays when the expression of the *ATM* gene was silenced by specific siRNA. To further explore the relationship between ATM kinase and Chk2 phosphorylation and G_2/M phase arrest in irradiated A549 cells,

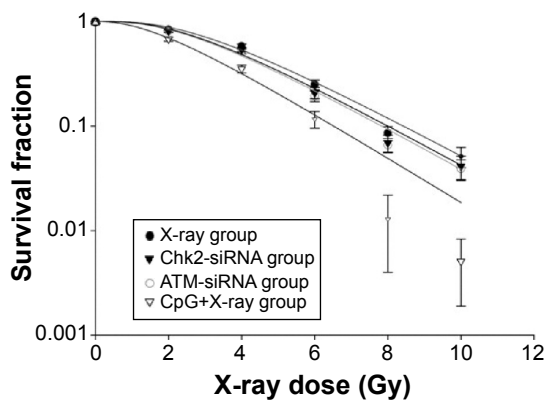


Figure 3 Dose–survival curves fitted using a multi-target single-hitting model. A549 cells were irradiated at the dose points of 0, 2, 4, 6, 8, and 10 Gy. The CpG+X-ray group showed a decrease in clonogenic survival compared with the other groups. **Abbreviations:** ATM, ataxia-telangiectasia mutated; Chk2, checkpoint kinase-2; siRNA, small interfering RNA.

we adopted the other siRNA specific targeting *Chk2* gene. Although increase of pATM kinase, CpG ODN7909 did not increase G₂/M phase arrest induced by X-rays, and pChk2 was deregulated in Chk2-silenced A549 cells. These results suggest that the increase in X-ray-induced Chk2 activation might be dependent on ATM kinase hyperphosphorylation, which played a role in enhancing X-ray-induced G₂/M phase arrest by CpG ODN7909 in A549 cells.

Irradiation-induced apoptosis is important in the use of X-rays to eradicate tumor cells, and it is now widely recognized that apoptosis induced by irradiation may be used to evaluate the sensitivity of tumor cells to IR, with an increased rate of apoptosis meaning that the cells have a higher sensitivity to irradiation.²⁷ Through in vivo retina experiments in newborn mice, Borges et al²⁸ reported that the pro-apoptotic activation of p53 protein was initiated by pChk2 in a irradiation dose-dependent manner. Moreover, Adams et al²⁹ found that the Chk2/p53 signal pathway dependent on ATM kinase plays a vital role in apoptosis induced by X-rays. The results of our present trial further suggest that CpG ODN7909 not only increases the level of ATM kinase and

Table 2 Change of radiobiological parameters in irradiated groups

Group	SF ₂	N	D ₀	D _q	SER _{D0}
X-ray	0.87	3.82	2.33	2.14	
CpG+X-ray	0.69	2.41	1.94	1.54	1.39
ATM-siRNA	0.83	3.47	2.23	2.04	1.04
Chk2-siRNA	0.84	3.50	2.27	2.07	1.03

Notes: The sensitization enhancement ratio (SER) calculated by the mean death dose (D₀) was higher in the CpG+X-ray group (1.39) than in the ATM-siRNA group (1.04) or Chk2-siRNA group (1.03).

Abbreviations: ATM, ataxia-telangiectasia mutated; D_q, quasi field dose; Chk2, checkpoint kinase-2; N, extrapolation number; SF₂, survival fraction that cells were irradiated with a dose of 2 Gy; siRNA, small interfering RNA.

Chk2 phosphorylation induced by X-rays, but also increases apoptosis in irradiated A549 cells. However, the effect of the combination of CpG ODN7909 and X-rays on apoptosis was subdued once the expression of either ATM kinase or Chk2 was inhibited by specific siRNAs in A549 cells. These results suggest that an increase in ATM kinase-mediated Chk2 phosphorylation might play a role in CpG ODN7909-enhanced apoptosis induced by X-rays in A549 cells.

However, the regulation of ATM kinase phosphorylation is complex and contentious. The precise mechanisms by which ATM kinase is phosphorylated after X-ray irradiation are yet unclear. Previously, it was thought that TLR9, described as the receptor of CpG ODNs, was only expressed in some kinds of immune cells, such as dendritic cells and B lymphocytes. However, recently, there has been increasing evidence of TLR9 expression in human tumor cells, and some studies have reported that the direct effect of CpG ODNs on tumor cells is associated with cellular TLR9 expression.¹⁶ Consistent with these results, previous studies by our group have also shown that the *TLR9* gene is overexpressed in irradiation-resistant lung adenocarcinoma A549 cells,³⁰ which is associated with an increase in irradiation sensitivity. Therefore, it is necessary to further explore whether CpG ODN7909 is involved in increasing X-ray-induced ATM kinase phosphorylation via interacting with the TLR9 signal pathway in irradiated A549 cells.

Conclusion

This study further substantiates the proposed role of CpG ODN7909 in potentiating the sensitivity of A549 cells to X-rays by assessing the effect of ATM kinase-dependent Chk2 hyperphosphorylation, which resulted in an increase in cells arrested at the G₂/M phase, an increase in cell apoptosis, and a decrease in cell survival. The present data strengthen the notion that, besides its role in immune, CpG ODN7909 is directly involved in facilitating cells sensitive to X-rays and support our earlier suggestion that an increase in Chk2 activation is involved in facilitating CpG ODN7909-enhanced radiosensitivity. We conclude that CpG ODN7909 enhances the sensitivity of A549 cells to X-rays, possibly by increasing ATM kinase-dependent Chk2 phosphorylation, leading to subsequent cascade reactions by activating the corresponding downstream substrates.

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Author contributions

Xiaoqun Liu and Xiangdong Liu contributed equally to this work. All authors read and approved the final manuscript for publication. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest in this work.

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