

## Original Article

# Interference of Ape1/Ref-1 enhances neutron radiosensitivity in non-small cell lung cancer 95D cells

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**Abstract:** Non-small Cell Lung Cancer (NSCLC) is the major pathological type of lung cancer that accounts for more than 80%. Surgery, radiotherapy, and chemotherapy are the main treatment method. Following the continuous development of radiation oncology, radiotherapy has become one of the methods for NSCLC with significant curative effect. However, it also has certain limitation such as radioresistance. Investigation of the mechanism of radioresistance and the corresponding measures becomes the hotspot. This study explores the role of Ape1/Ref-1 in NSCLC radiotherapy via RNAi technology to knockdown Ape1/Ref-1 expression in NSCLC cell line 95D. RNAi technology was applied to downregulate Ape1/Ref-1 expression in 95D cells. MTT assay and colony formation assay were used to analyze 95D cell survival and colony formation ability after radiotherapy. Alkaline comet assay and  $\gamma$ H2AX expression were adopted to investigate DNA injury, while flow cytometry was performed to detect cell apoptosis to explore the killing effect of radioactive rays on 95D cells. Under the same radiation dose, 95D cell survival rate and fraction were significantly lower, while radiosensitivity was obviously higher after pSilence APE1 transfection compared with pSilence Control ( $P < 0.05$ ). Moreover, 95D cells exhibited markedly enhanced comet tail moment, increased apoptosis, and upregulated  $\gamma$ H2AX mRNA and protein ( $P < 0.05$ ). Downregulation of Ape1/Ref-1 expression elevated the radiosensitivity of 95D cells, which may be related to the inhibition of DNA repair involved by Ape1/Ref-1.

**Keywords:** Ape1/Ref-1, NSCLC, 95D cells, neutron, radioresistance

## Introduction

Lung cancer is one of the most common malignant tumors worldwide. It is the leading cause of death in all types of malignant tumors [1]. As the aggravation of environmental pollution and the change of lifestyle in recent years, the morbidity and mortality of lung cancer exhibit the increasing trend year by year. In addition, it shows a younger trend [2]. Non-small Cell Lung Cancer (NSCLC) is the major pathological type of lung cancer that accounts for more than 80% of all types. Surgery, radiotherapy, and chemotherapy are the main therapeutic method [3]. With the continuous development of radiation oncology, radiation therapy has become an important method for the local treatment of malignant tumor. It also exhibits obvious therapeutic effect on NSCLC [4]. However, radiotherapy also has certain limitation, as the radiotherapy resistance often leads to treatment failure, especially in the middle-late and rela-

ted malignant tumor patients. Radiosensitivity is affected by multiple factors, including DNA damage repair, cell cycle, and cell apoptosis [5]. Further studies find that regulation of gene expression can influence the radiosensitivity. Nagel discovered that FOXM1 and MASTL participated in cell cycle can decrease the radiosensitivity of NSCLC [6]. It is found that DNA damage caused by radiotherapy can lead to cell growth arrest or apoptosis, while DNA damage repair system within the tumor cells is one of the important mechanisms of the radioresistance [7].

Apurinic/apyrimidinic endonuclease (APE), also named redox effector factor 1 (Ref-1), is one of the important rate-limiting enzymes involved in DNA base excision repair (BER). Meanwhile, it also has the function of redox and regulates the DNA binding activity of various transcription factors, such as NF- $\kappa$ B, HIF-1, and p53, which are closely related to tumor radioresistance [8].

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Current study shows that APE1/Ref-1 abnormally expressed in multiple tumor tissues and other diseases, such as ovarian cancer, osteosarcoma, and liver cancer [9, 10]. The expression of APE1/Ref-1 in lung cancer can significantly influence the patient's survival. Higher APE1/Ref-1 level is related to shorter survival time and worse prognosis [11].

RNA interference (RNAi) is gene silencing induced by double-stranded RNA (dsRNA). dsRNA complementarily combines with homologous mRNA to specifically degrade mRNA. RNAi is widely applied in gene function exploration and malignant tumor treatment because of its stability, efficiency, and specificity. This study knocked down APE1/Ref-1 in NSCLC cell line 95D by RNAi to investigate the role of APE1/Ref-1 in NSCLC radiotherapy.

### Materials and methods

#### *Main reagents and materials*

NSCLC cell line 95D was provided by the Chinese Academy of Sciences in Shanghai. RPMI1640 medium (Cat No: 11875119), FBS (Cat No: 16000044), and penicillin-streptomycin (Cat No: F15140122) were bought from Gibco (New York, NY). Trypsin (Cat No: SH30-042.01B) was got from Hyclone (Logan City, UT). MTT (Cat No: M5655) and DMSO (Cat No: D2650) were purchased from Sigma (St Louis, MO). RNA extraction reagent Trizol (Cat No: 15596018) was acquired from Invitrogen (Carlsbad, CA). ReverTra Ace qPCR RT Kit (Cat No: FSQ-101) was got from TOYOBO (Osaka, Japan). SuperFect Transfection (Cat No: 301305) was from QIAGEN (Dusseldorf, Germany). Rabbit anti human APE1 monoclonal antibody (Cat No: ab92744), rabbit anti human  $\gamma$ H2AX polyclonal antibody (Cat No: ab11175), and rabbit anti human  $\beta$ -actin polyclonal antibody (Cat No: ab8227) were purchased from Abcam (Cambridge, UK).  $^{252}\text{Cf}$  neutron therapy equipment was from Shenzhen double loop arlington technology development co., Ltd.

#### *APE1 siRNA expression vector pSilence APE1 construction*

According to the APE1 siRNA cDNA sequence designed by Wang D [12], pSilence2.0-U6 was selected as plasmid vector to construct APE1 siRNA expression vector and control plasmid

pSilence Control. DNA sequencing was adopted to identify the target fragment. Its effect was verified in 95D cells.

#### *Cell culture and plasmid transfection*

95D cells were cultured in 1640 medium containing 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO<sub>2</sub>. The cells in logarithmic phase were chosen for experiment. A total of  $2 \times 10^5$  cells were seeded in six-well plate and transfected after 24 h according to SuperFect Transfection kit. APE1 siRNA and control plasmid pSilence Control were transfected to cells using liposome, respectively. The medium was changed to normal complete medium after 3 h cultivation in serum free medium.

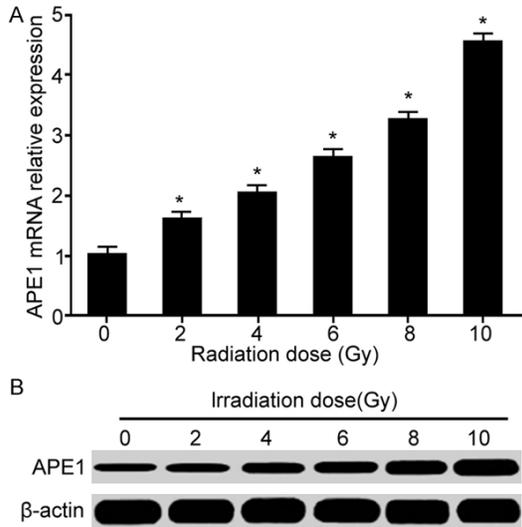
#### *RNA extraction and real time PCR*

Trizol was used to extract RNA from cells. Briefly, a total of 1 ml Trizol was added to the cells together with 200  $\mu$ l chloroform. After vibrated for 15 s, the cells were put for 15 min standing. The supernatant was moved to a new 1.5 ml EP tube and added with 1 ml isopropanol. After centrifuged at 10000 g for 10 min, the RNA was washed by 1 ml 70% ethanol for twice and solved in DEPC water. Its concentration and purification was evaluated by spectrophotometer. RNA was reverse transcribed to cDNA using TOYOBO ReverTra Ace qPCR RT Kit. The reverse transcription system in 20  $\mu$ l was composed of 2  $\mu$ g RNA, 4  $\mu$ l RT Buffer (5 $\times$ ), 1  $\mu$ l oligo dT, 1  $\mu$ l RT Enzyme Mix, 1  $\mu$ l RNase inhibitor, and ddH<sub>2</sub>O. The reaction condition contained 37°C for 50 min and 98°C for 5 min. The cDNA was adopted for PCR reaction. The PCR reaction system in 10  $\mu$ l contained 2 $\times$  SYBR Green Mixture at 4.5  $\mu$ l, 2.5  $\mu$ M/L primers at 1.0  $\mu$ l, cDNA at 1  $\mu$ l, and ddH<sub>2</sub>O at 3.0  $\mu$ l. The PCR reaction was composed of 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 74°C for 30 sec. The PCR reaction was performed on ABI7500.

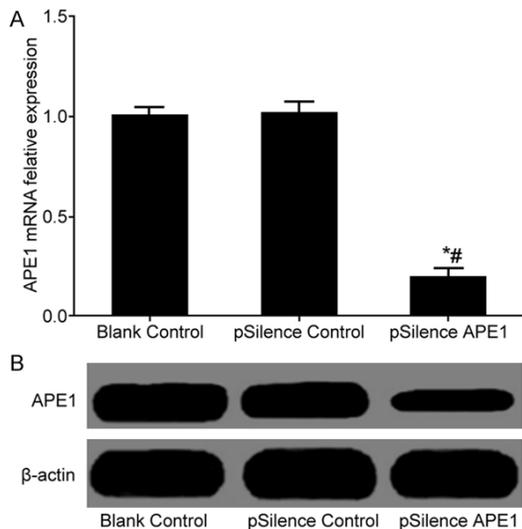
#### *Protein extraction and Western blot*

The cells were digested by trypsin and added with RIPA on ice for 30 min. After centrifuged at 10000 g for 30 min, the supernatant was collected and quantified by BCA kit. A total of 50  $\mu$ g protein was separated by 8% SDS-PAGE and transferred to PVDF membrane. After blocked by 5% skim milk at room temperature for 60

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**Figure 1.** The impact of  $^{252}\text{Cf}$  neutron irradiation on APE1 expression in 95D cells. A. qRT-PCR detection of APE1 mRNA. B. Western blot detection of APE1 protein expression. \* $P < 0.05$ , compared with control.



**Figure 2.** Plasmid construction. A. qRT-PCR detection of APE1 mRNA expression. B. Western blot detection of APE1 protein expression. \* $P < 0.05$ , compared with Blank Control. # $P < 0.05$ , compared with pSilence Control.

min, the membrane was incubated in primary antibody at 4°C overnight. After washed by PBS for three times, the membrane was further incubated in HRP labeled secondary antibody at room temperature for 60 min. After washed by PBS for three times, the membrane was treated by ECL reagent for 2-3 min and devel-

oped. Quantity One software was applied for gray intensity analysis.

### $^{252}\text{Cf}$ neutron irradiation

$^{252}\text{Cf}$  neutron therapy equipment was used for radiotherapy. The average energy of  $^{252}\text{Cf}$  neutron source was 2.34 MeV. The half-life period was 2.645 year. The effective range of  $^{252}\text{Cf}$  source was 1.4 mm × 9.0 mm. The volume intensity was 500 μg. The emissivity of neutron was  $2.3 \times 10^6/\text{s}/\mu\text{g}$ . The γ-ray emissivity was  $1.3 \times 10^7/\text{s}/\mu\text{g}$ . The neutron dose equivalent rate at 1 cm from radioactive source in the air was 1.6 mGy/h/mg. The biologic equivalent dose ( $D_{\text{Gy-eq}}$ ) was calculated by neutron RBE × neutron dose + γ-ray RBE × γ-ray dose. The exposure dose was verified at 0, 2, 4, 6, 8, and 10 Gy.

### MTT assay

95D cells were washed by PBS for twice after radiation and digested to single cell suspension. Next, the cells were seeded in 96-well plate at 5000/well with three replicates in each group. After incubated for 48 h, the cells were added with 20 μl MTT at 5 mg/ml and further incubated for 4 h. Then 100 μl DMSO was added to each well for 15 min, and the plate was read on microplate reader at 490 nm.

### Colony formation assay

95D cells were washed by PBS for twice after radiation and digested to single cell suspension. Then the cells were seeded on 6 cm dish at 100, 400,  $1 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$  cells/well. After 10 day incubation, the cells were fixed by absolute ethyl alcohol and stained by crystal violet. Cell clone was counted to draw the dose-effect curve and calculate survival fraction, mean lethal dose ( $D_0$ ) and quasi-field dose (Dq). Survival fraction = cell clone number/seeded cell number/non-irradiated cell clone formation rate.  $D_0$  refers to the dose needed to decrease survival rate from 0.1 to 0.037. Dq refers to the dose intersect with survival rate at 1.

### Alkaline comet assay

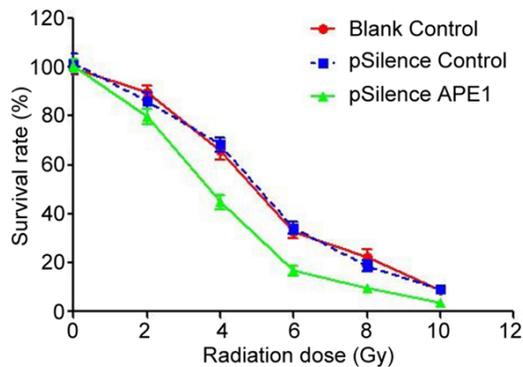
Comet slab rubber was prepared using 0.65% agarose gel at normal melting point. 95D cells were washed by PBS for twice after radiation

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**Table 1.** 95D cell survival rate after  $^{252}\text{Cf}$  neutron irradiation (%)

Groups	Irradiation dose (Gy)					
	0	2	4	6	8	10
Blank Control	100	89.25±1.86	63.82±3.27	35.18±2.04	13.96±2.45	7.38±1.85
pSilence Control	100	88.45±2.05	62.76±3.08	34.69±2.23	13.26±2.61	7.16±1.90
pSilence APE1	100	82.74±2.21*	53.19±2.85*	22.84±1.83*	7.34±2.93*	3.27±1.74*

\*P < 0.05, compared with pSilence Control.



**Figure 3.** 95D cell survival rate analysis under  $^{252}\text{Cf}$  neutron irradiation.

**Table 2.** 95D cell radiosensitivity comparison under  $^{252}\text{Cf}$  neutron irradiation

	$D_0$ (Gy)	$Dq$ (Gy)
Blank Control	2.85	2.64
pSilence Control	2.80	2.69
pSilence APE1	1.83*	2.05*

\*P < 0.05, compared with pSilence Control.

and digested to single cell suspension. A total of 20  $\mu\text{l}$  cells at  $1 \times 10^5/\text{mL}$  were mixed with 180  $\mu\text{l}$  0.5% low melting-point agarose and then seeded on the rubber. After dried at  $4^\circ\text{C}$  for 20 min, the rubber was put into the alkaline lysate at  $4^\circ\text{C}$  for 3 h. Next, the rubber was washed by ice water for twice and then unwinded in the electrophoresis buffer at room temperature avoid of light for 20 min. After electrophoresis for 30 min and neutralized for twice, the rubber was fixed by absolute ethyl alcohol and stained by PI. At last, the rubber was observed under the inverted microscope to calculate tail moment and tail DNA content to evaluate DNA damage.

### *$\gamma\text{H2AX}$ expression detection*

95D cells in logarithmic phase were seeded in 6 cm dish and cultured for 24 h. After irradiated

at 0, 2, 5, and 10 Gy, the cells were further cultured for 24 h. RNA and protein were extracted. qRT-PCR and Western blot were adopted to detect  $\gamma\text{H2AX}$  mRNA and protein expression.

### *Flow cytometry*

95D cells in logarithmic phase were seeded in 6 cm dish and cultured for 24 h. After irradiated at 0, 2, 5, and 10 Gy, the cells were further cultured for 24 h. The cells were further digested by trypsin and resuspended in 195  $\mu\text{l}$  binding buffer. Next, the cells were added with 5  $\mu\text{l}$  Annexin V-FITC and 10  $\mu\text{l}$  PI and incubated avoid of light for 15 min. At last, the cells were incubated on ice for 5 min and tested by flow cytometry.

### *Statistical analysis*

All data analysis was performed on SPSS 18.0 software. Measurement data was depicted as mean  $\pm$  standard deviation and compared by t test. P < 0.05 was considered as statistical significance.

## Results

### *APE1 expression in 95D cells received different doses of $^{252}\text{Cf}$ neutron irradiation*

qRT-PCR detection showed that APE1 mRNA gradually increased in 95D cells following irradiation dose elevation (Figure 1A). Western blot revealed that compared with non-irradiation group, APE1 protein level obviously upregulated in 95D cells treated by different doses of  $^{252}\text{Cf}$  neutron irradiation.

### *Plasmid construction*

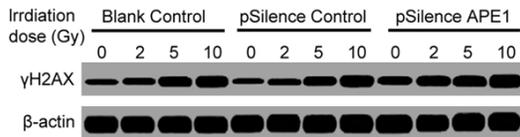
APE1 mRNA and protein levels in pSilence Control showed no statistical difference compared with blank control (Figure 2A and 2B). APE1 mRNA and protein expression in pSilence APE1 group was markedly lower than that in

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**Table 3.** DNA damage degree analysis in 95D cells under  $^{252}\text{Cf}$  neutron irradiation

Irradiation dose (Gy)	n	Tail moment ( $\mu\text{m}$ )		
		Blank Control	pSilence Control	pSilence APE1
0	20	3.246 $\pm$ 0.417	3.428 $\pm$ 0.453	3.681 $\pm$ 0.502
2	20	6.485 $\pm$ 0.708	6.782 $\pm$ 0.735	8.364 $\pm$ 0.617*
5	20	18.947 $\pm$ 1.472	19.728 $\pm$ 1.538	26.317 $\pm$ 1.194*
10	20	30.841 $\pm$ 1.218	31.274 $\pm$ 1.195	39.486 $\pm$ 1.108*

\*P < 0.05, compared with pSilence Control.



**Figure 4.** The impact of different doses of  $^{252}\text{Cf}$  neutron irradiation on  $\gamma\text{H2AX}$  expression in 95D cells.

pSilence Control (**Figure 2A** and **2B**). It indicated the successful plasmid construction.

### MTT assay analysis

95D cell survival rate significantly reduced following the enhancement of the dose of  $^{252}\text{Cf}$  neutron irradiation. Compared with pSilence Control, the survival rate of 95D cells after pSilence APE1 transfection was obviously lower under the same irradiation dose (P < 0.05) (**Table 1**).

### Colony formation assay analysis

95D cell survival fraction markedly declined following the enhancement of the dose of  $^{252}\text{Cf}$  neutron irradiation. Compared with pSilence Control, the survival rate of 95D cells after pSilence APE1 transfection was apparently lower under the same irradiation dose (P < 0.05) (**Figure 3**; **Table 2**).

### Alkaline comet assay analysis

The half lethal dose of  $^{252}\text{Cf}$  neutron irradiation on 95D cells was 4.8 Gy. Thus, we selected 2, 5, and 10 Gy for irradiation analysis. The tail moment was significantly upregulated following irradiation dose elevation. Compared with pSilence Control, the tail moment of 95D cells after pSilence APE1 transfection was apparently lower under the same irradiation dose (P < 0.05) (**Table 3**).

### $\gamma\text{H2AX}$ expression in 95D cells under different doses of $^{252}\text{Cf}$ neutron irradiation

The half lethal dose of  $^{252}\text{Cf}$  neutron irradiation on 95D cells was 4.8 Gy. Thus, we selected 2, 5, and 10 Gy for irradiation analysis.  $\gamma\text{H2AX}$  expression was obviously increased following irradiation dose elevation. Compared with pSilence Control,  $\gamma\text{H2AX}$  expression in

95D cells after pSilence APE1 transfection was apparently lower under the same irradiation dose (P < 0.05) (**Figure 4**).

### Cell apoptosis comparison under different doses of $^{252}\text{Cf}$ neutron irradiation

The half lethal dose of  $^{252}\text{Cf}$  neutron irradiation on 95D cells was 4.8 Gy. Thus, we selected 2, 5, and 10 Gy for irradiation analysis. Cell apoptosis obviously enhanced following irradiation dose elevation. Compared with pSilence Control, cell apoptosis in 95D cells after pSilence APE1 transfection was significantly lower under the same irradiation dose (P < 0.05) (**Figure 5**).

## Discussion

Malignant tumor is one of the major problems affecting human health. In recent years, following the exacerbation of environmental pollution and the change of lifestyle, the incidence of malignant tumor increased year by year, greatly threatening the human's life. Lung cancer is one of the most common malignant tumors. There are more than 1.2 million cases worldwide every year. It exhibits the leading morbidity and mortality in all kinds of cancer, seriously affecting human life and health [13, 14]. NSCLC accounts for more than 85% of all lung cancers. Surgery, chemotherapy, and radiotherapy are the common treatment methods [3, 15]. Radiotherapy plays an important role in the comprehensive treatment of NSCLC. Following the continuous development of radiation oncology in recent years, radiotherapy has gradually become the important and effective method for the local treatment of malignant tumor [4, 16]. In addition to postoperative adjuvant therapy for patients in early stage, radiotherapy is also widely applied in local palliative treatment for patients in advanced stage. However, the

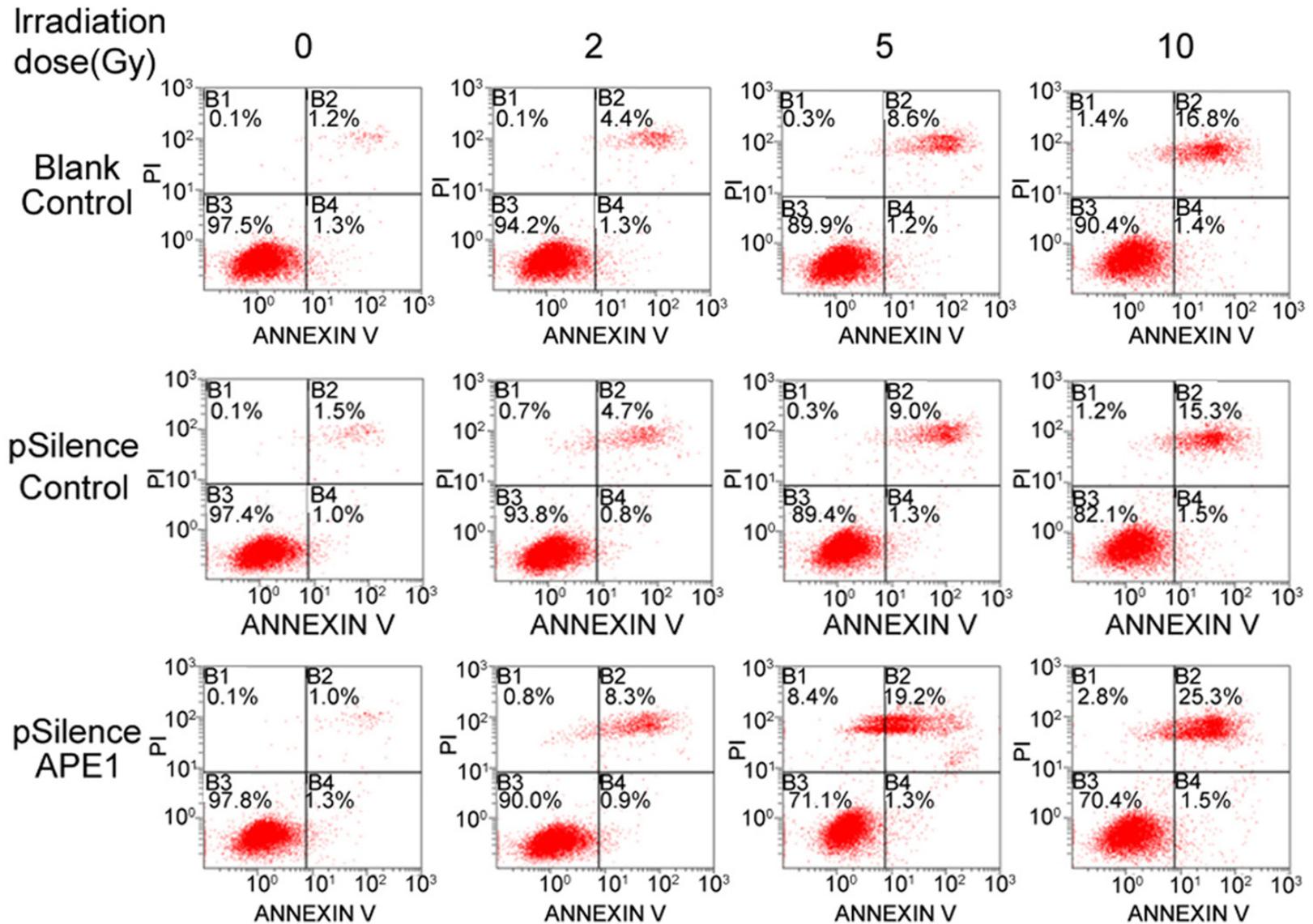


Figure 5. Cell apoptosis comparison under different doses of <sup>252</sup>Cf neutron irradiation.

curative effect of single radiotherapy is not so satisfactory on lung cancer, while some treatment may be failed because of radiotherapy resistance. Radiation causes tumor cell DNA rupture or other types of damage through direct ray effect or indirect reactive oxygen species (ROS). Tumor cells may appear apoptosis, mutation, and cell cycle stagnation under the condition that DNA damage cannot be repaired. A variety of studies suggested that the generation of radiotherapy resistance may be related to DNA damage repair system within tumor cells [4].

APE/Ref-1 is not only an important repairase in the process of DNA base excision repair, but also an important intermediary molecule in cell oxidative stress response pathway. It can participate in the regulation of a variety of transcription factors, such as p53, AP-1, NF- $\kappa$ B, Myb, and HIF-1, which are closely associated with tumor radiotherapy resistance [8, 10]. The unique dual function of APE/Ref-1 makes it play a critical role in tumor cell growth and chemoradiotherapy sensitivity. On the one hand, tumor cells may undergo apoptosis upon the lack of DNA repair ability of APE/Ref-1. On the other side, the deletion of Redox activity of APE/Ref-1 may lead to the lack of DNA binding ability of transcription factors, thus to block the tumor cell growth signaling pathway and neovascularization, reducing tumor invasion and metastasis. This study found that following the increase of radiation dose, tail moment significantly enhanced,  $\gamma$ H2AX mRNA and protein expression obviously upregulated, and APE1 mRNA and protein level markedly elevated. It indicated that DNA damage aggravated, whereas APE1 expression participated in damage repair also elevated following the increase of radiation dose. It was found that APE/Ref-1 is an important rate-limiting enzyme in DNA base excision repair pathway [17]. It is involved in DNA damage repair in purine pyrimidine deletion site [18], thus is an important repair factor of DNA damage caused by radioactive and chemical damage [19]. Our results also confirmed that APE1 may participate in DNA damage repair induced by radiation.

Current study found that APE/Ref-1 was abnormally expressed in ovarian cancer, osteosarcoma, liver cancer, and other tumor tissues [9]. The aberrant expression of APE/Ref-1 in lung

cancer significantly affects the patient's survival. Higher APE/Ref-1 expression indicates the worse prognosis [11]. This study investigated the impact of APE1 on NSCLC radiosensitivity by constructing interference plasmid to reduce APE1 level in 95D cells. MTT assay and colony formation assay revealed that 95D cell survival rate and survival index significantly declined under the same radiation dose after APE1 interference, indicating that APE1 downregulation may enhance radiotherapy sensitivity. Wang found that APE/Ref-1 upregulation may result in NSCLC radioresistance, while the suppression of APE1 expression using isoflavones or methoxyamine can elevate the radiosensitivity of cancer cells [12]. In addition, Kim also reported that the radiotherapy effect on colorectal cancer patients with APE1 downregulation was markedly better than that in high expression patients [20]. Alkaline comet assay demonstrated that the tail moment of 95D cells after pSilence APE1 transfection was apparently lower compared with pSilence control, suggesting that DNA damage aggravation may be related to the inhibition of APE1 repair. Flow cytometry confirmed that cell apoptosis in 95D cells after pSilence APE1 transfection was significantly lower compared with pSilence control. Singh-Gupta V found that isoflavone can enhance the radiosensitivity of A549 cells via suppressing APE1 and may be related to DNA DSBs [21]. Our results indicated that downregulation of Ape1/Ref-1 can increase the radiosensitivity in 95D cells. We speculated that targeting Ape1/Ref-1 expression may enhance the curative effect of radiotherapy in clinic, while further study is needed to confirm the conclusion.

### Conclusion

Downregulation of Ape1/Ref-1 elevated the radiosensitivity of 95D cells, which may be associated with the suppression of DNA damage repair.

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### Disclosure of conflict of interest

None.

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