

## Original Article

# Vitamin D improves the sensitization of lung cancer to radiotherapy

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Received January 10, 2016; Accepted October 29, 2016; Epub March 15, 2017; Published March 30, 2017

**Abstract:** Lung cancer is the leading cause of cancer related death worldwide. Radioresistance is an obstacle in treating lung cancer. The efficacy of treatments may be improved by increasing the sensitivity of cancer cells to radiation therapy. Vitamin D has been reported to have radiosensitizing effects in cancers of the breast and the prostate. The objective of this study is to explore the effects of vitamin D on radiosensitization of human lung cancer cells. Human non-small cell lung cancer cell line A549 cells were treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> before irradiation with X-rays. The effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on cell-survival following irradiation was evaluated by colony-forming assay. DNA double-strand breaks were detected by immunostaining for γH2AX foci. Cell cycle distributions were investigated using flow cytometry. Apoptotic cells were identified on the basis of nuclear morphology. Results: 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited lung cancer cell proliferation in a dose-dependent manner. A549 cells were radiosensitized by 1,25(OH)<sub>2</sub>D<sub>3</sub>. Flow cytometric analysis for DNA content indicated that 1,25(OH)<sub>2</sub>D<sub>3</sub> increased radiation-induced G2/M arrest. Apoptotic analysis demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub> enhanced the cell apoptosis induced by irradiation. Conclusion: These findings suggest that 1,25(OH)<sub>2</sub>D<sub>3</sub> caused radiosensitization of A549 cells are associated with its enhancement effects on apoptosis and G2/M arrest induced by radiation.

**Keywords:** Vitamin D, lung cancer, radiation, radiosensitization

## Introduction

Lung cancer is one of the most frequently diagnosed cancers and the leading cause of cancer related death worldwide. Its rates have been steadily increasing in recent years. Non-small cell lung cancer (NSCLC) is accounted for more than 80% of the total lung cancers [1]. Currently, the epidemiologic survey displayed that the 5 year survival rate of patients in the Stage I NSCLC was about 55%~72%. Unfortunately, some unresectable cases such as patients with NSCLC of stage II, III B and IV only had less than 5% the 5 year survival rate [2]. Radiotherapy provides a clear survival advantage for patients with NSCLC and remains to be an effective measure in achieving the local control of the disease. However, the development of radioresistance frequently influences the efficacy of radiotherapy and thus becomes an obstacle of this technique. Therefore, it would be desirable to develop agents that could potentiate the

effects of radiation. Ideally, such agents would be non-toxic and enhance the killing effect of radiation on cancer cells.

Vitamin D is an essential fat-soluble vitamin. 1α, 25-dihydroxy-cholecalciferol (1,25(OH)<sub>2</sub>D<sub>3</sub>), also known as calcitriol, is an active form of vitamin D. It have been confirmed that vitamin D plays an important role in maintaining the calcium homeostasis and bone mineralization. Recent years, 1,25(OH)<sub>2</sub>D<sub>3</sub>, as an important bone regulator, has been suggested to have multiple functions, including anticancer effect [3]. The most of the anti-cancer actions of vitamin D have been studied in cancers of breast, prostate, and colon. Studies have provided evidence that circulating 25(OH)D levels are inversely associated with the incidence of colorectal cancer [4], prostate cancer risk [5] and breast cancer [6]. There are also investigations about vitamin D and lung cancers. Results of a meta-analysis showed an inverse associa-

tion between serum vitamin D and lung cancer risk [7]. Evidences in the studies of patients with early stage NSCLC in US and Norwegian showed that high level of vitamin D may prolong survival time [8, 9]. Studies showed Vitamin D intake was associated with a lower lung cancer risk in never-smoking, postmenopausal women [10]. An *in vitro* study showed that vitamin D inhibited the growth of lung cancer cell lines [11]. Findings of other studies suggest that vitamin D could inhibit the growth and metastasis of lung cancer in mouse models [12, 13]. These findings support the potential of vitamin D as a therapeutic agent for lung cancer. As its anti-tumor capacity, vitamin D might be also considered as a chemosensitizer or radiosensitizer. It has been found that 1,25(OH)<sub>2</sub>D<sub>3</sub> or its synthetic analogues increased the susceptibility of cancer cells to the cytotoxic action of tumor necrosis factor [14], adriamycin and paclitaxel [15]. Vitamin D<sub>3</sub> or its analog has also been shown to enhance the responses of breast cancer cells [16, 17] and prostate cancer cells [18] to radiation. Vitamin D enhances cancer cells response to radiation by decreasing cell viability, inducing cell apoptosis and cell cycle arrest [16, 19]. However, its radiosensitizing effects are not well studied in lung cancer. The aim of this study is to explore the potential of vitamin D as a radiosensitizer in lung cancer.

### Materials and methods

#### *Materials and cell cultures*

The NSCLC cell line (A549) were obtained from ATCC (Manassas, VA, USA) and maintained at 37°C in humidified incubator containing 5% CO<sub>2</sub> in RPMI-1640 (Gibco Invitrogen, Burlington, ON, USA) containing 10% fetal calf serum (Gibco Invitrogen, Burlington, ON, USA) with supplements. All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. 1,25(OH)<sub>2</sub>D<sub>3</sub> (0.1 mol/L) was dissolved in absolute ethanol and further diluted in cell culture medium as required concentrations. Control cells were always treated with the corresponding concentrations of ethanol.

#### *Cell proliferation assessment by MTT assay*

Cells were seeded at 3,000 per well in 96-well plates, allowed to attach overnight and cells were treated with different concentrations (50-

1000 nmol/L) of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Medium containing ethanol served as the controls. After 72 hour incubation, cell proliferation was assessed using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based on colorimetric assay as previously described [20]. The absorbance was measured at 490 nm using a microplate reader (BIO RAD, Hercules, CA, USA). Cellular proliferation was expressed as a percentage with vehicle-treated cells.

#### *Irradiations*

Radiation was administered with 6 MV of x-rays from a linear accelerator (Elekta Axesse, Elekta Medical Equipment Co. Ltd, Sweden) with graded doses (0, 2, 4, 6 and 8 Gy).

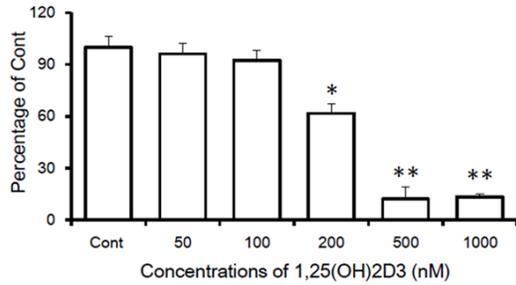
#### *Clonogenic analysis of cell survival*

Cells were seeded in 6 well plates at a density of 200 cells per well. After attachment, cells were treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (100 nmol/L) or vehicle for 24 hours. The cells were then unirradiated (control) or irradiated for total doses ranging from 0 to 8 Gy. Media containing 1,25(OH)<sub>2</sub>D<sub>3</sub> was exchanged for fresh media. Cells were subsequently cultured under standard cell culture conditions for 8 days, with media were changed every third day. Colonies were stained with Giemsa solution, and colonies containing at least 50 cells in size were counted. The surviving fraction (SF) was calculated based on the plating efficiency of nonirradiated control cultures treated with the vehicle. The survival curve was plotted using X-Y log scatter (Graph Prism 5.0). Based on the clonogenic survival assay, the dose enhancement ratio (DER) was calculated as the ratio of radiation doses required to give the same biological effect in the absence or the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub>. The DER for 10% survival was determined. A DER greater than one is indicated radiosensitization.

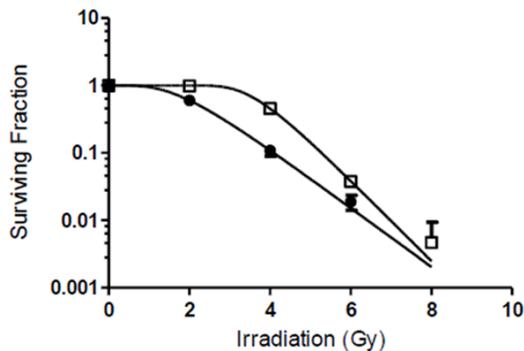
#### *γ-H2AX immunofluorescence staining of cells*

Cells were cultured onto cover slips in 24-well plates and incubated overnight. And then cells were treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (100 nmol/L) for 24 hours, then irradiated (0 or 4 Gy). At 1 hour and 24 hours post radiation, cells were fixed with 4% formaldehyde, the cells were rinsed in PBS and permeabilized with 0.2% Triton X100 in PBS for 20 minutes at room temperature and

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**Figure 1.** 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits proliferation of A549 cells. Cells were treated with increasing concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> for 72 hours. Cell proliferation assay was used to assess the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on cell growth (n = 4, mean ± SD, \*P < 0.01, \*\*P < 0.001 relative to control group).



**Figure 2.** Clonogenic survival of A549 cells treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> followed by irradiation in a range of radiation doses. Radiosensitization by 1,25(OH)<sub>2</sub>D<sub>3</sub> was assessed on the basis of clonogenic cell survival assays. A549 cells were treated with 0 (□) or 100 nmol/L (●) 1,25(OH)<sub>2</sub>D<sub>3</sub> for 24 hours then irradiated as indicated. Cells were stained after being cultured for 8 days in medium containing the agent. The survival data were normalized to those of the unirradiated control group. Values shown are the mean ± SD for three independent experiments.

blocked in PBS containing 5% normal goat serum for 30 min. Slips were incubated with Alexa Fluor 488-conjugated rabbit-anti-γ-histone-H2AX antibody (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:500 in 5% bovine serum albumin at 4°C overnight. After washing with PBS, Slips were rinsed and mounted in Gelvatol (25% polyvinyl alcohol, 30% glycerol, and 0.1% sodium azide) containing 4',6-diamidino-2-phenylindole (DAPI, 1 μg/ml). Cover slips were then mounted on glass slides, and cells were analyzed with an Olympus BX51 fluorescent microscope (Olympus Corporation, Tokyo, Japan) using 40x objective. The images

were captured using a digital camera (Olympus DP72). Cells were classified positive (containing radiation-induced H2AX foci) when more than five foci were detected. Total and γ-H2AX-positive cells were counted in at least five representative fields per cover slip using a 40x objective.

### Cell cycle analysis

Cells were seeded in 6 well plates at  $1.5 \times 10^5$  cells per well and incubated overnight. Cells were then treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> at 0 or 100 nmol/L for 24 hours. Cells were irradiated with 0 or 4 Gy of X-rays and incubated in a medium containing the drug for appropriate time (0, 6, 12, 24, 48 h). They were then fixed with 70% ethanol, treated with RNase A and incubated in propidium iodide solution after washing. Cell cycle distribution was analyzed using BD FACSCalibur (Becton, Dickinson and Company, NJ, USA).

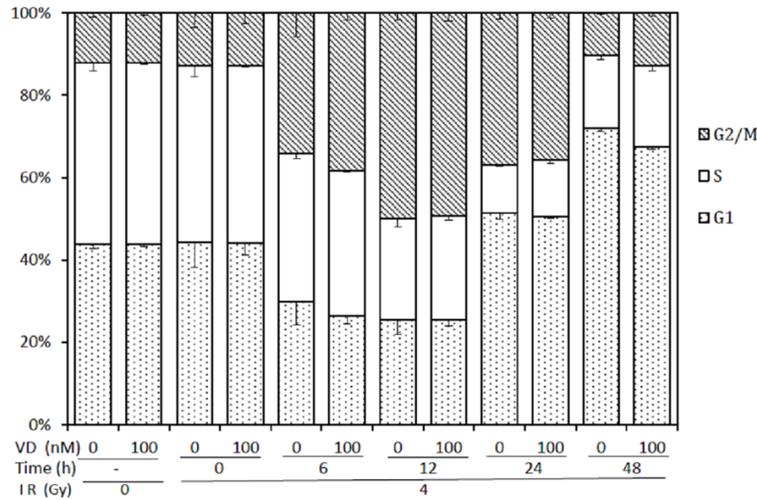
### Apoptotic assay

The apoptosis of cells were analyzed as previously described [21]. Cells were cultured onto cover slips in 24-well plates and incubated overnight. And then cells were treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (100 nmol/L) for 24 hours, then irradiated with 0 or 4 Gy of X rays. Three days after radiation, cells were fixed with 4% formaldehyde and rinsed in PBS. The cells were permeabilized with 0.2% Triton X100 in PBS for 20 minutes. The cover slips were rinsed and mounted in Gelvatol containing DAPI. Fluorescent images were captured with a digital camera linked to a fluorescent microscope. Total and apoptotic nuclei were counted at least in five representative fields per cover slip using a 40x objective. Apoptotic cells were identified on the basis of morphology (nuclear condensation and fragmentation). Values for apoptotic cells were expressed as the percentage of total cells.

### Statistical methods

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) software version 16.0 (SPSS Inc, Chicago, IL, USA). Data were presented as mean ± SD. The differences between groups were tested for significance using the one-way analysis of variance or student t test P < 0.05 was considered statistically significant.

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**Figure 3.** The influence of  $1,25(\text{OH})_2\text{D}_3$  on cell cycle phase distribution. Cells were pretreated with  $1,25(\text{OH})_2\text{D}_3$  for 24 hours and irradiated. Cells were collected and cell cycle was analyzed using flow cytometry at different time points ( $n = 3$ , mean  $\pm$  SD). VD,  $1,25(\text{OH})_2\text{D}_3$ ; IR, irradiation.

### Results

#### $1,25(\text{OH})_2\text{D}_3$ inhibits proliferation of A549 cells

The proliferation assay was performed to evaluate the effects of  $1,25(\text{OH})_2\text{D}_3$  on A549 Cell growth inhibition. It was found that  $1,25(\text{OH})_2\text{D}_3$  dose dependently inhibited A549 cell growth. Treatment with 200 nmol/L or 500 nmol/L of  $1,25(\text{OH})_2\text{D}_3$  showed approximately 40% or 90% inhibition of A549 cells growth (**Figure 1**). Concentrations greater than 500 nmol/L did not result in further growth inhibition.  $1,25(\text{OH})_2\text{D}_3$  had no inhibitory effects on cell proliferation when concentrations were at or lower than 100 nmol/L (**Figure 1**). To test its potential as a radiosensitizer,  $1,25(\text{OH})_2\text{D}_3$  at 100 nmol/L was used in the subsequent experiments.

#### Vitamin D sensitizes A549 cells to radiation

To determine whether vitamin D could radiosensitize A549 cells, cells were exposed to 0 or 100 nmol/L  $1,25(\text{OH})_2\text{D}_3$  for 24 hour, and then irradiated with 0, 2, 4, 6 or 8 Gy of X-rays. As shown in **Figure 2**, A549 cells treated only with 100 nmol/L  $1,25(\text{OH})_2\text{D}_3$  showed no effect on cell survival. However, A549 cells pretreated with  $1,25(\text{OH})_2\text{D}_3$  plus X-ray irradiation exhibited significantly lower clonogenic survival fractions than cells treated with radiation alone. Survival fraction at 4 Gy was reduced

from 45.5% in the irradiated-only controls to 10.8% in  $1,25(\text{OH})_2\text{D}_3$  treated and irradiated cells. The DER for 10% cell survival was 1.33 (**Figure 2**).  $1,25(\text{OH})_2\text{D}_3$  treatment significantly sensitized the cells to irradiation.

*1,25(OH)<sub>2</sub>D<sub>3</sub> does not enhance the radiation-induced DNA damage or inhibit the DNA damage repair*

$\gamma$ -H2AX has been identified as a marker of DNA double-strand break. In order to determine the effects of  $1,25(\text{OH})_2\text{D}_3$  on DNA damage, immunofluorescent staining using the anti- $\gamma$ -H2AX antibodies was conducted.  $\gamma$ -H2AX foci could be clearly distinguished 1

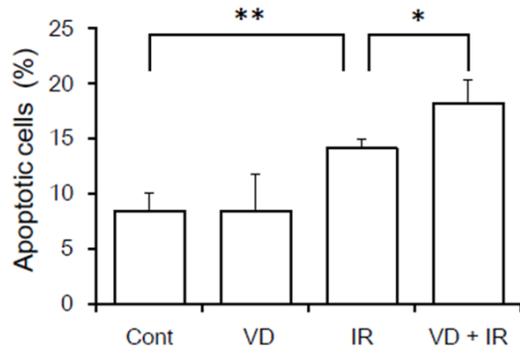
hour post 4 Gy radiation.  $\gamma$ -H2AX positive cells were shown to be reduced dramatically at 24 hour time point. The percentages of  $\gamma$ -H2AX positive cells were no difference between cell treated with a combination of  $1,25(\text{OH})_2\text{D}_3$  and radiation and treated with radiation alone at either 1 hour or 24 hour time point. These results implicate that the DNA damage or damage repair is not influenced by the presence of vitamin D.

#### $1,25(\text{OH})_2\text{D}_3$ pretreatment enhances radiation-induced G2/M arrest

To test the radiosensitization nature of  $1,25(\text{OH})_2\text{D}_3$ , cell cycle was evaluated by flow cytometry. As shown in **Figure 3**, radiation induced cell cycle arrest in G2/M phase at 6, 12 and 24 hour time points and the altered cell cycle recovered 48 hours after irradiation. However, cells pretreated with  $1,25(\text{OH})_2\text{D}_3$  showed more cells arrested in G2/M phase at 48 time point compared with cells treated with radiation alone ( $13.7 \pm 1.0\%$  vs  $10.3 \pm 0.3\%$ ,  $P < 0.05$ ; **Figure 3**).  $1,25(\text{OH})_2\text{D}_3$  alone had no effect on cell cycle phase distribution.

#### The treatment with $1,25(\text{OH})_2\text{D}_3$ increases radiation-induced A549 cell apoptosis

Apoptosis assay performed after 72 hour irradiation and demonstrated that 4 Gy irradiated cells pretreated with VD had more apoptotic



**Figure 4.** 1,25(OH)<sub>2</sub>D<sub>3</sub> pretreatment increases IR-induced A549 cell apoptosis. A549 cells were pretreated with 1,25(OH)<sub>2</sub>D<sub>3</sub> for 24 hours and then irradiated (0 Gy or 4 Gy). Three days post irradiation apoptotic cells were analyzed and expressed as the percentage of total cells (n = 4, mean ± SD, \*P < 0.05, \*\*P < 0.01). VD, 1,25(OH)<sub>2</sub>D<sub>3</sub>; IR, irradiation.

cells (18.2 ± 2.2%) than cells treated with irradiation alone (14.1 ± 0.9%, P < 0.05; **Figure 4**). 1,25(OH)<sub>2</sub>D<sub>3</sub> (100 nmol/L) alone had no effects on A549 cell apoptosis.

### Discussion

Recent years, vitamin D has been suggested to have multiple functions, including anticancer effect [22, 23]. Vitamin D may prevent some type of cancers such as breast cancer, colon cancer and prostatic cancer. Inhibition of cancer cell growth is suggested to be one of the mechanisms of its anticancer effects [23]. In the present study we demonstrate that vitamin D dose-dependently inhibit the growth of A549 cells. These findings add to the evidence that vitamin D may have anti-cancer effects.

Given the ability of 1,25(OH)<sub>2</sub>D<sub>3</sub> to inhibits the growth of some type of cancer cells, we sought to investigate its potential as a radiosensitizer in lung cancer cells. Radiosensitizing effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> have been reported in breast cancer cells [16, 17] and prostate cancer cells [18]. In the present study using clonogenic assays, we assessed the effects of vitamin D on lung cancer cell survival. Our results showed that vitamin D pretreatment followed by radiation exposure dramatically reduced colony formation compared to radiation alone. The present results indicate that 1,25(OH)<sub>2</sub>D<sub>3</sub> pretreatment enhance the cell killing and showed a significant radiosensitizing effect on the lung cancer cell line.

The mechanism of vitamin D-induced radiosensitization remains largely unknown at the cellular level. Increasing radiation-induced DNA damage and decreasing DNA damage repair have been demonstrated to play roles in tumor radiosensitivity. γ-H2AX, as a marker of DNA double-strand break, has shown to be positively associated with tumor radiosensitivity [24]. In the present study, the DNA damage was assessed by measuring the expression levels of γ-H2AX in response to radiation and radiation combined with 1,25(OH)<sub>2</sub>D<sub>3</sub>. Our results demonstrate that 1,25(OH)<sub>2</sub>D<sub>3</sub> fails to augment DNA damage induced by radiation or inhibit DNA damage repair in A549 cells, which is consistent with findings in a previous study [25]. These findings suggest that vitamin D promoted lung cancer cell killing induced by radiation may not correlate to the alteration of DNA damage and DNA damage repair. Similar finding was also observed in breast cancer cells [26, 27].

The use of different agents to arrest cells in the radiosensitive phases of the cell cycle is a strategy for radiosensitization. Cells are most sensitive to the effects of radiation in G2/M phase and most resistant in G0/G1 [28]. Studies have shown that the radiosensitizing effect of some anticancer drugs is due to cell cycle alteration, such as Paclitaxel and Cucurbitacin B. These drugs Potentiate radiosensitivity by mainly blocking cells in G2/M phase of the cell cycle [29, 30]. To investigate the mechanism by which vitamin D enhance lung cancer cell sensitivity to radiation, we examined the effects of combined treatment or radiation alone on cell cycle regulation by flow cytometry. Our results demonstrated a slight but statistically significant increase in the proportion of cells in G2/M phase for cells receiving combined treatment of radiation and 1,25(OH)<sub>2</sub>D<sub>3</sub> than cells receiving radiation alone. The results suggest that the enhanced radiation effects by vitamin D seem to be associated with cell cycle arrest. Therefore, cell cycle regulation may be one of the mechanisms of the effects of vitamin D on the radiosensitization. However, in the present study the cell cycle regulating effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> is modest. Therefore, cell cycle redistribution does not appear to play a major role in vitamin D-induced radiosensitization. Other mechanisms, such as apoptosis, might contribute to the increased cell killing observed in the clonogenic assays.

Apoptosis is a programmed cell death. It is important defense mechanisms of the system to either repair the damage or eliminate the defective cells (containing damages) [31]. Apoptosis has previously been regarded as a potential mechanism of radiosensitization. Different studies have reported the role of apoptosis in radiosensitization [32, 33]. Apoptosis enhancement in vitamin D induced radiosensitization has been observed in prostate cancer cells [18] and breast cancer cells [16]. It has been suggested that pretreatment with  $1,25(\text{OH})_2\text{D}_3$  lowers the apoptotic threshold of cancer cells to the subsequent stress of radiation [18]. Increased apoptosis of cancer cells may result in reoxygenation for hypoxic cancer cells, and thus contribute to cytotoxicity of subsequent radiation treatments [34, 35]. In the present study we showed that pretreatment with  $1,25(\text{OH})_2\text{D}_3$  could significantly enhance the radiation-induced apoptosis in A549 cells, which could be also one of the mechanisms underlying the sensitization of lung cancer cells to the effects of radiation. However, a previous study showed that apoptosis enhancement was not observed in A549 cells co-treated with  $1,25(\text{OH})_2\text{D}_3$  and radiation [25]. The inconsistent results may attribute to different experimental conditions. In their experiment, they used  $^{137}\text{Cs}$  gamma rays, but X-rays were used in our experiments. The duration of total exposure time to  $1,25(\text{OH})_2\text{D}_3$  was also different in these two studies. The results in our study suggest that vitamin D may increase the efficacy of irradiation-induced lung cancer cell death. However, further investigations and characterization are need for evaluating radiosensitizing effects of vitamin D.

In conclusion, vitamin D radiosensitized A549 cells by significantly decreasing the cell colony formation ability. Enhanced G2/M cell cycle arrest and apoptosis could be possible mechanisms underlying the effects of vitamin D on radiosensitization. The finding implicates that vitamin D may be a useful radiation sensitizer in human lung cancer cells.

### Acknowledgements

This work was supported by the Medical Research Grant of 306 Hospital of PLA. The authors wish to thank Yaya Qin, Xiaoqing Tan and the staff in the Department of Therapeutic Radiology and Oncology in 306 Hospital of PLA for their technical assistance.

### Disclosure of conflict of interest

None.

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