DOI: 10.55522/jmpas.V12I6.5821 www.jmpas.com ISSN NO. 2320 – 7418

International peer reviewed open access journal

Journal of Medical Pharmaceutical and Allied Sciences

Journal homepage: www.jmpas.com CODEN: JMPACO

Review article

Effects and mechanism of anlotinib on radiosensitivity of non-small cell lung cancer cell lines a549

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Received - 21-10-2023**, Revised** - 27-11-2023, **Accepted** - 09-12-2023 **(DD-MM-YYYY)**

Refer this article

Zhang Ming, Tan Suyono, Ali Napiah Nasution, 2023. Effects and Mechanism of Anlotinib on Radiosensitivity of Non-small Cell Lung Cancer Cell Lines A549. Journal of medical pharmaceutical and allied sciences, V 12 - I 6, Pages - 6223 – 6229. Doi: 10.55522/jmpas.V12I6.5821

ABSTRACT

To investigate the effect and mechanism of Anlotinib on radio sensitization of human lung adenocarcinoma cell line A549. Human lung adeno carcinoma cell line A549 was teated with anlotinib and/or radiotherapy, then divided four groups, control group (Ctrl), Anlotinib treatment group (A), irradiation group (RT) and Anlotinib combined with irradiation group (A+RT) . CCK8 method was used to determine cell proliferation; the clone formation experiment was used to determine the inhibitory effect on cell growth; flow cytometry was used to determine cell cycle and apoptosis; immunofluorescence of γ-H2AX was used to determine DNA damage; expression of DNA-PKcs were detected by Western blot. Anlotinib inhibited proliferation and clonogenic survival following irradiation. The dose (Dq), the average lethal dose (D0) and the survival score (SF2) in the anlotinib combined radiotherapy group was significantly lower than those in the radiotherapy group. Anlotinib decreased G2/M phase arrest and promoted the cells apoptosis induced by in irradiation. The confocal microscopy results showed the average number of γ-H2AX foci in the A+RT group was more than that in RT group. The protein levels of DNA-PKcs were higher in A+RT group than that in RT group. Anlotinib enhances the radio sensitivity of A549 cells, which may be attributed to the delay DNA damage repair. It provides a rationale strategy by Anlotinib combined with irradiation for NSCLC.

Keywords: Anlotinib, Non-small cell lung cancer, Radio-sensitization, DNA damage repair.

INTRODUCTION

Lung cancer is the most common of all malignant tumors with the highest incidence and mortality, and the five-year survival rate of patients with advanced lung cancer is still less than 15% [1]. Non-small cell lung cancer (NSCLC) accounts for 80% of lung cancers [2]. Due to no specific clinical signs and symptoms in the early stages, many patients suffer from the advanced stage at the initial diagnosis [3]. Therefore, surgery may not be suitable for patients with advanced lung cancer, and the prognosis or clinical outcome is inferior [4]. Thus, the clinical strategy for the treatment of advanced lung cancer is a great challenge.

Anti-angiogenesis drugs can target vascular endothelial cells in a proliferating state, have a significant inhibitory effect on tumor angiogenesis, and effectively reduce tumor micro vessel density, thereby achieving the goal of "starving" the tumor [5]. Recent studies have shown that small molecule multi-target tyrosine kinase inhibitors (tyrosine kinase inhibitors, TKIs) can inhibit the binding of EGFR and DNA-PK, thereby inhibiting the repair of tumor cell DNA damage, thereby promoting the killing effect of radiotherapy on tumors [6]. Anlotinib is a new type of TKI drug, which has now become a treatment drug for various cancers, including non-small cell lung cancer [7]. Compared with other TKI drugs, Anlotinib can inhibit more targets, which provides a potential basis for the strategy of Anlotinib combined with radiotherapy [8].

This study investigates the therapeutic effect of anlotinib and radiotherapy combination strategy on NSCLC in the non-small cell lung cancer (NSCLC) cell line A549 and studies the effect of anlotinib on DNA damage repair, and clarifies the molecular mechanisms.

MATERIALS AND METHODS

Cell culture and irradiation

Human lung adenocarcinoma cell line A549 (purchased from Kunming Institute of Zoology, Chinese Academy of Sciences), cultured in essential medium (RPMI1640 + 10% FBS) and incubated at 37°C and 5% CO_2. The experimental groups were set as blank control group (Ctrl group), anlotinib treatment group (group A), radiotherapy group (RT group), and radiotherapy + anlotinib group (RT + A group). The radiotherapy group and combined group were irradiated by 60Coγ linear accelerator, source to surface distance = 100 cm, radiation field 40 cm × 40 cm, irradiation dose 2 Gy, and the irradiated cells were placed in a 37°C with 5% CO_2 incubator for further culture.

CCK8 test to detect cell survival and proliferation

A549 cells in the logarithmic growth phase were prepared a single-cell suspension and seed in a 96-well plate at a density of 5000 cells/well per well, with 5 replicate wells in each group. After being cultured in groups for 24, 48, 72, 96, and 120 h, 20 μL of 0.5 mg/mL CCK8 solution was added for proliferation detection. Read the absorbance value (A value) at the wavelength of 492 nm on the microplate reader, take the culture time as the abscissa and the absorbance as the ordinate, draw the cell growth curve, and repeat the experiment three times.

Clone formation experiment

Cells were seeded in a 6-well plate at a density of 1000 cells/well, cultured for 24 hours to adhere to the wall, and then processed in groups. Radiation therapy was performed 2 hours after the drug was added, and the radiation doses were 0, 2, 4, 6, and 8 Gy, respectively. There are 5 multiple holes for each dose. After treatment for 5 minutes, the culture was continued for 48 hours, replaced with a drug-free complete medium, and cultured for 14 days. When there are macroscopically visible clones in the 6-well plate, discard the culture medium, wash twice with PBS, and fix methanol for 15 min. Abandon the fixative solution, add an appropriate amount of Giemsa staining solution to stain for 15 minutes, and count the number of cells in each well with more than 50 cells. Calculate the survival score and draw the dose-effect curve. Surviving fraction (SF) = number of clones per well/number of planted cells x adhesion rate. The single-click multi-target mathematical model was used to fit the cell survival curve, and the survival fraction (SF), mean lethal does (D0), quasi threshould does (Dq), and radiation of each group were calculated. Sensitivity enhancement ratio (sensitive enhancement ratio, SER).

Flow cytometry detection of cell cycle and apoptosis

The cells in the logarithmic growth phase were digested and seeded in a 6-well plate, and treated in groups after the cells adhered to the wall. After 2h of drug action, radiotherapy treatment (4Gy, 5min) was performed, the complete medium was replaced and the culture was continued for 48h, the cells were washed with PBS and collected, and the Annexin V-FITC/PI kit was used for detection operation, and flow cytometry was used to determine each group Apoptosis rate. After the same treatment, the cells were collected, stained with 400ul PI, and mixed, incubated at 4°C for 30 minutes in the dark, and then the cell cycle changes were measured by flow cytometry.

Detection of DNA damage by immunofluorescence

5000/well cells were plated in 6-well plates with cell slides and treated according to the experimental groups. After 48 hours, the cells were fixed with 4% paraformaldehyde, and the sealing solution (0.5%) was added at room temperature. TritonX-100+3%BSA+5% goat serum) were treated for 1.5 h. Discard the blocking solution, add FITC-labeled primary antibody (H2AX Rabbit Monoclonal Antibody, 1:200), incubate for 2 h at room temperature, stain with DAPI solution for 10 min; take out the slides, mount the slides with gum, and observe under a fluorescent microscope.

Western blot assay

The total protein of each group of cells were extracted by using RIPA lysate. An equal amount of total cellular protein per sample was run on a SDS–PAGE and transferred to a PVDF membrane. After blockage with 5% skimmed milk at room temperature for 1h. DNA-PKcs primary antibody was (diluted 1:300) incubate overnight at 4°C. The HRP-labeled secondary antibody was incubated for 1.5h at room temperature, washed with TBST, and then detected with the ECL chemiluminescence reaction kit (Amersham Pharmacia Biotech) in accordance to the manufacturer's instructions. Quantity One software 2.64 was used to analyze the gray value of the image. GAPDH was selected as the internal control.

Statistical analysis

All experiments were repeated 3 times. SPSS 22.0 software was used for data processing, and the data were expressed as mean ± standard deviation (x±s). The independent sample t test was used to compare the differences between the two groups, and the one-way analysis of variance was used to compare the three groups and above in parallel between the groups (SNK method). $P < 0.05$ is considered statistically significant.

RESULTS AND DISCUSSION

The effect of Anlotinib on the proliferation of human lung adenocarcinoma cells

The growth curve of A549 cells treated with different concentrations of Anlotinib is shown in Figure 1. The cells of the control group and the Anlotinib group both showed a proliferation trend. At 0h and 24h, the proliferation rates of the two groups were similar, and there was no statistical difference. At 48h, 72h and 96h, the cell proliferation rate of anlotinib group was significantly lower than that of the control group (all $p<0.05$). Moreover, the inhibitory effect of Anlotinib on lung adenocarcinoma cells was dose-dependent (Figure 1B).

The effect of Anlotinib on radiosensitivity of human lung adenocarcinoma cells

The cell SF value and colony formation rate in the RT group, A group and A+RT group decreased by increasing radiation dose (Figure 2). The single-click multi-target model is used to curve-fit SF and radiation dose to obtain D0 and Dq values. Compared with the RT group (4,8, 16 Gy radiation), different does of anlotinib significantly reduced Dq and SF values in the RT+A group. However, there was no statistical difference between 0.28nM and 0.56nM anlotinib on radiotherapy sensitization (P=0.875). It suggests that Anlotinib can enhance the radio sensitivity of A549 cells.

The effect of anlotinib combined with radiotherapy on the cell cycle and apoptosis of lung adenocarcinoma cells induced by radiotherapy

In order to explore the mechanism for sensitization, we evaluated the effect of Anlotinib on the cell cycle and apoptosis of lung adenocarcinoma cells induced by radiotherapy. Compared with the Ctrl group (7.55%±1.17%), anlotinib and radiotherapy both induced A549 cell apoptosis (23.84%±1.52%, p<0.001 and 31.94%±2.25%, p <0.001), while the apoptosis rate of lung adenocarcinoma cells in the A+RT group was significantly higher than that in the RT group (44.44%±2.30%, p<0.001) (Figure 3). Thus, it suggests that anlotinib promoting radio-sensitization may be related to cell apoptosis.

Flow cytometry showed that compared with the control group, the ratio of cells in G1/G0 phase of group A and RT increased significantly. In contrast, the proportion of cells in the S phase and G2/M phase was significantly decreased (Figure 4). Compared with the RT group, the ratio of cells in the G1/G0 phase of the A+RT group increased, while the proportion of cells in the S phase and G2/M phase significantly reduced. It indicated that Anlotinib can block cells in G1/G0 phase; after combined radiotherapy, S phase cells reduced considerably.

Anlotinib delayed radiotherapy for DNA damage repair of lung adenocarcinoma cells

The mechanism of radiotherapy is to induce cell DNA double-strand breaks to form γH2AX spots and recruit DNA-pK factors to the break location for DNA damage repair. Thus, we examined the changes in the DNA damage marker γ-H2AX focus (Figure 5A-B). Immunofluorescence staining showed that the proportion of positive cells with γ-H2AX foci in the nucleus of A549 cells increased (to 65.67%±6.11%) at 2h after 4 Gy radiotherapy, and then decreased to 25.67%±2.52% at 24h. Anlotinib treatment alone did not cause significant changes in γ-H2AX foci. However, compared with the RT group, anlotinib combined with radiotherapy can increase the formation of γ-H2AX foci in the cells after 2 hours (83.33%±4.16%, p=0.014). But at 24h, the proportion of γ-H2AX-positive cells in the combined group was significantly higher than in the radiotherapy group (54.67% ± 3.79%, p<0.05). It suggests that Anlotinib can delay DNA damage repair in lung adenocarcinoma cells under radiotherapy.

Several studies reported that tinib drugs could regulate the binding activity of EGFR and DNA-PKcs, which leads to the inhibition of the function of DNA-PKcs and affects DNA damage repair. Immunoblot assay demonstrated that compared with the Ctrl group, the DNA-pKcs levels in the A group and the RT group increased significantly, while the level of DNA-pKcs expression reduced significantly in the RT+A group compared with the RT group (Figure 5C). It indicates that anlotinib treatment could reverse the radiotherapy-induced accumulation of DNA-pKcs expression in A549 cells.

Recent studies have shown that receptor tyrosine kinases (RTKs) are involved in various stages of tumor genesis, such as angiogenesis, invasion and metastasis, immune escape and metabolic adaptation [9]. Because radiation can promote RTK activity, it helps tumor cells survive and proliferate after irradiation. In addition, activated RTK promotes cellular DNA repair and makes tumor cells resistant to radiotherapy [10]. Therefore, combining radiotherapy with RTK inhibitors provides a potential tumor treatment strategy. Existing studies have shown that compared with DNA damage or anti-mitotic agents, small molecule targeted tyrosine kinase inhibitors (TKI) or monoclonal antibodies (mAb) inhibit tumor-specific receptor tyrosine kinases, may be a reasonable method combined Radiotherapy for effective tumor control, with fewer side effects and patients can get a better quality of life [11,12].

Anlotinib is a new type of oral TKI independently developed by China, which could anti-tumor angiogenesis and inhibit tumor growth [13]. This study investigated the effect of Anlotinib on the radio sensitivity of NSCLC cells. Our data shows that Anlotinib can induce synergistic radio sensitization in A549 cells, including promoting radiation-induced cell apoptosis and S2 phase block in A549 cells. It is the first study to show the combined effect of Anlotinib and radiotherapy on lung adenocarcinoma cells. Currently, several FDA-approved RTK antagonists (TKI: imatinib, gefitinib, erlotinib, afatinib, etc. or mAb: cetuximab, bevacizumab, etc.) has been verified to enhance the radio-sensitization in lung cancer [13-19]. Compared with other RTK inhibitors (including sorafenib, sunitinib and pazopanib), Anlotinib can inhibit more targets, thereby inhibiting tumor angiogenesis and tumor cell proliferation, which provides potential feasibility for anlotinib combined with radiotherapy for lung cancer.

Radiotherapy is a critical clinical strategy for the treatment of lung cancer. Although the molecular mechanism of radiotherapy is complex and multifactorial, most tumor cells killed by radiotherapy are mainly through DNA damage response. Many events in the DNA damage response are the key to cellular radiosensitivity, including the activation and repair of checkpoints [20]. γ-H2AX is considered to be a key checkpoint for radiation-induced DNA double-strand break (DSB). In addition, G2/M phase block allows cells to repair damaged DNA and cause radiation resistance. In this study, pretreatment with anlotinib resulted in the increase of γ-H2AX foci in the irradiated A549 cells and persisted for a long time, and reduced the G2/M and S phases, allowing the cells to enter the G1/G0 phase, shorten the time for cells to repair DNA damage and promote cell apoptosis.

Existing evidence shows that after tumor cells are exposed to radiation, active EGFR directly enters the nucleus from the cytoplasm, binds to DNA-PK, activates the catalytic subunit DNA-PKcs of DNA-PK, repairs damaged DNA, and appears radiation resistance [21]. Therefore, this study also detected the expression level of DNA-PKcs, and the results showed that Anlotinib could significantly attenuate the increase in the level of DNA-PKcs in the nucleus induced by radiation. It implies that anlotinib can inhibit the EGFR of lung adenocarcinoma cells from entering the nucleus and binding to DNA-PK after radiotherapy, thereby preventing DNA damage repaired and increasing the radio-sensitivity. However, it should be noted that different TKI drugs have different effects on DNA damage repair. Cao et al. found that gefitinib can prevent nucleus entry and promote sensitization [22], which is not observed in Tanaka et al.'s study [15]. The present study is similar to most TKIs—preventing EGFR from binding to DNA-PK affects DNA damage repair. However, we cannot rule out that Anlotinib affects the radiotherapy sensitivity of A549 cells through other means. The molecular mechanism of Anlotinib for sensitization still needs to be further explored.

Figure 1: Effect of anlotinib on the proliferation of A549 cell. (A) Inhibition rate of anlotinib in different concentrations; (B) Growth curve of A549 cells in control group and anlotinib treatment group

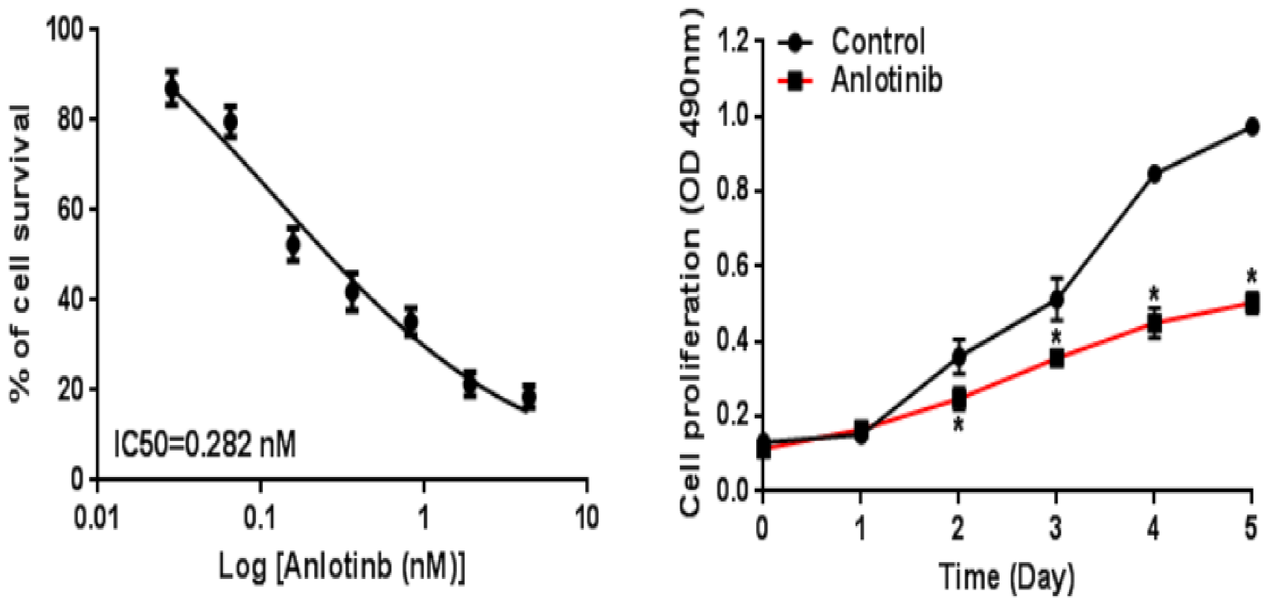
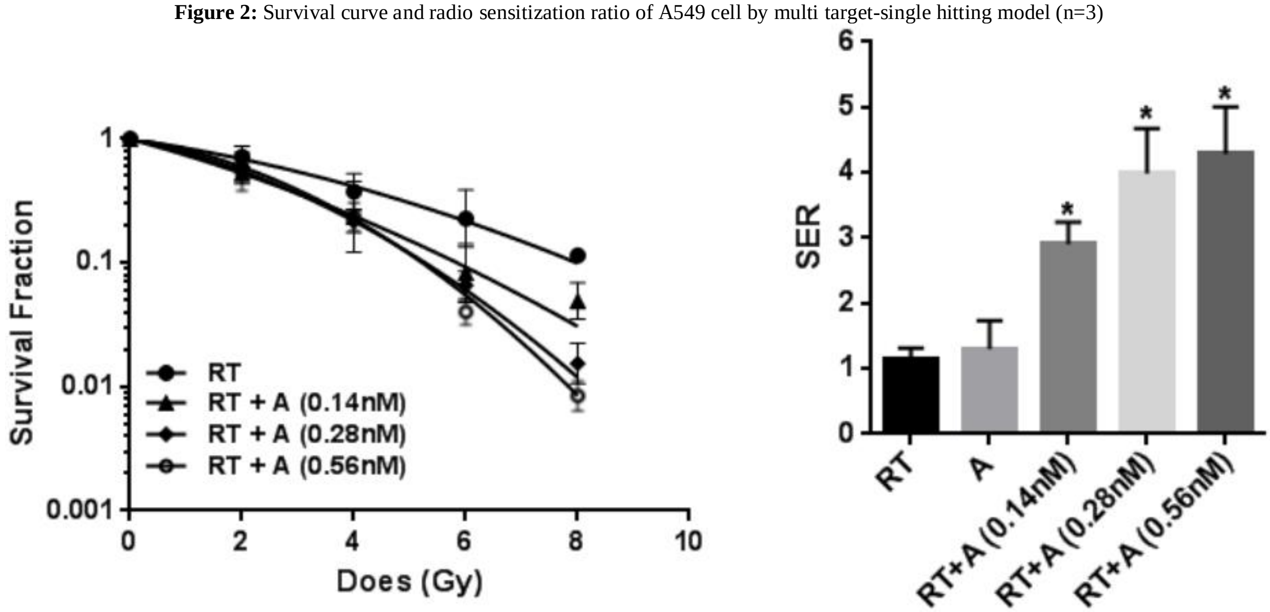


Figure 2: Survival curve and radio sensitization ratio of A549 cell by multi target-single hitting model (n=3)



DOI: 10.55522/jmpas.V12I6.5821 ISSN NO. 2320 – 7418

Figure 3: Effects of anlotinib and/or combined with radiation on the apoptosis of A549 cells

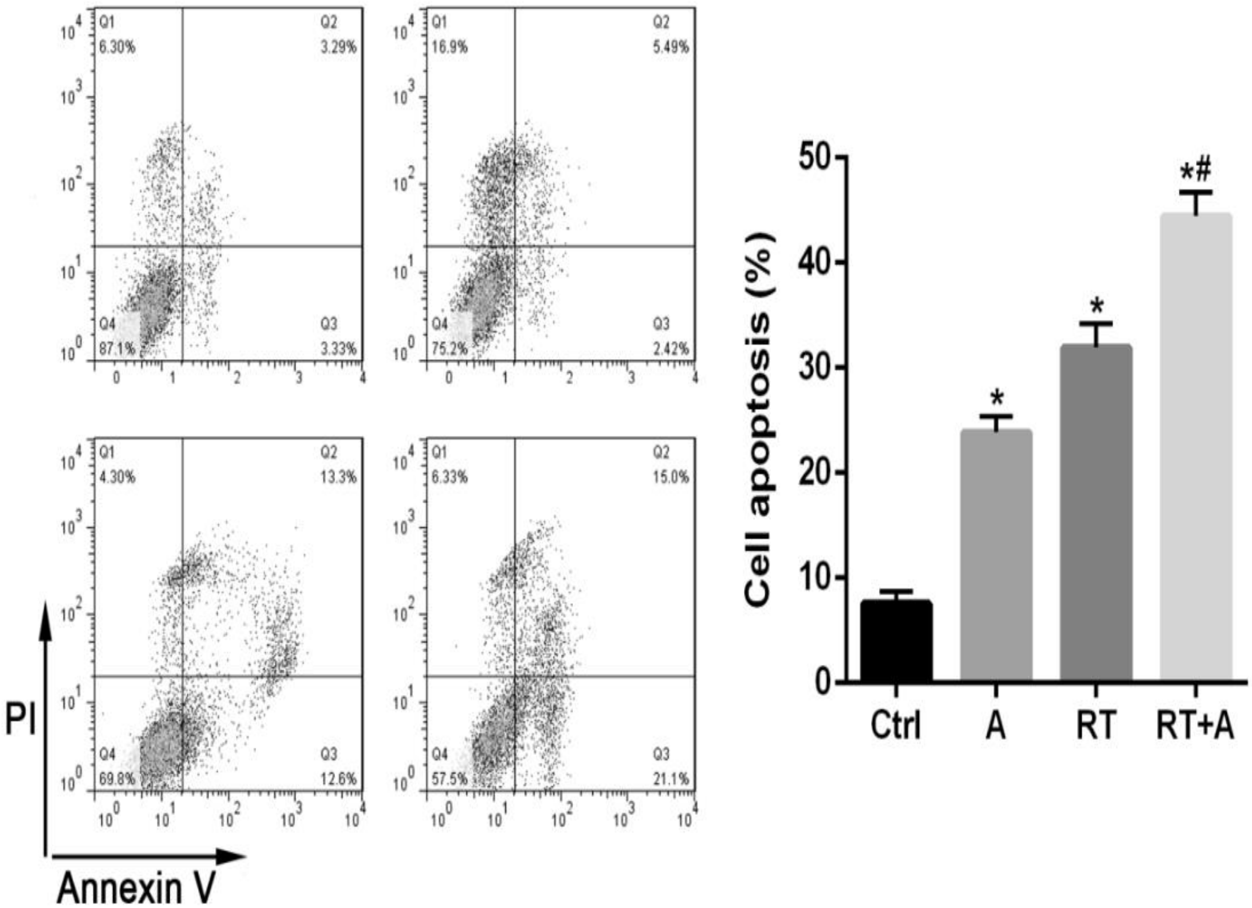
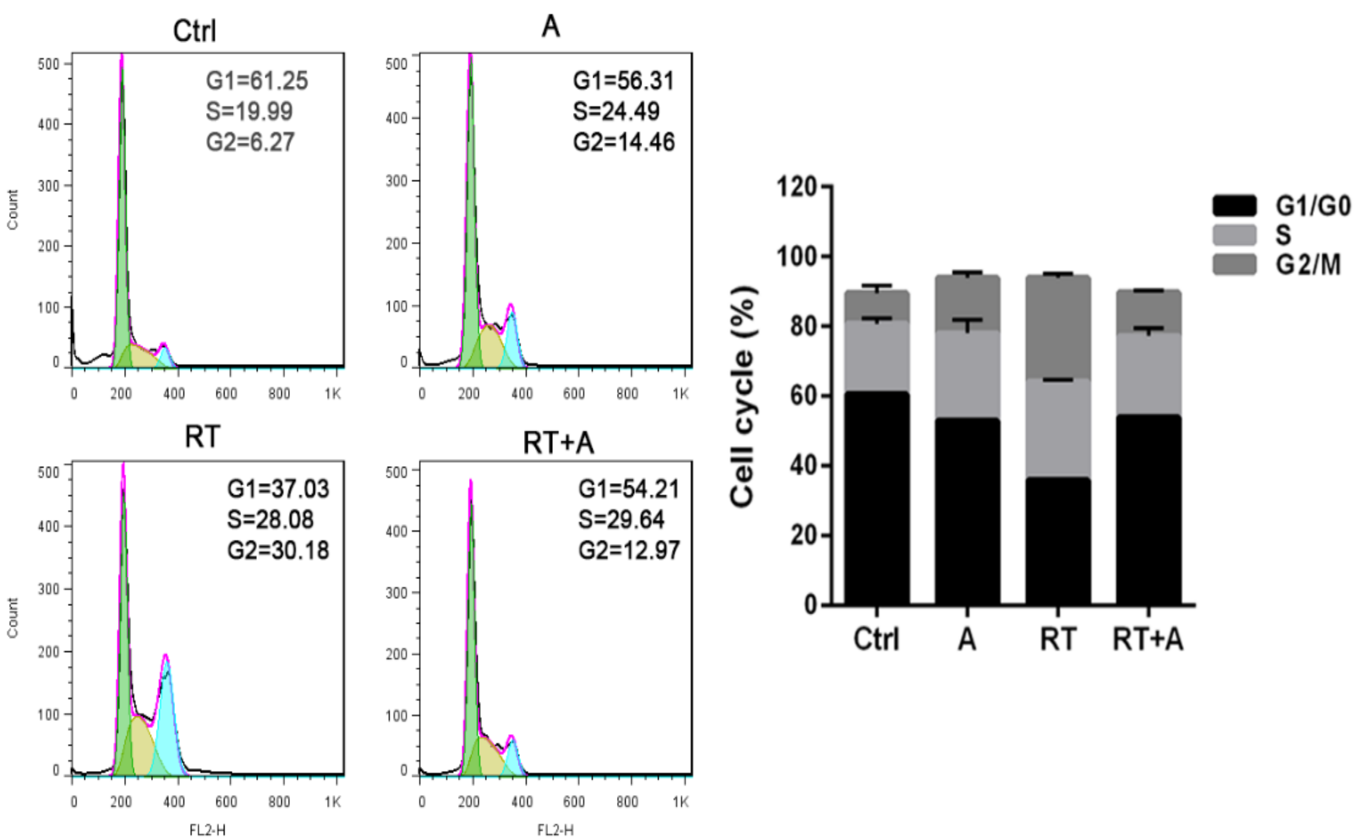
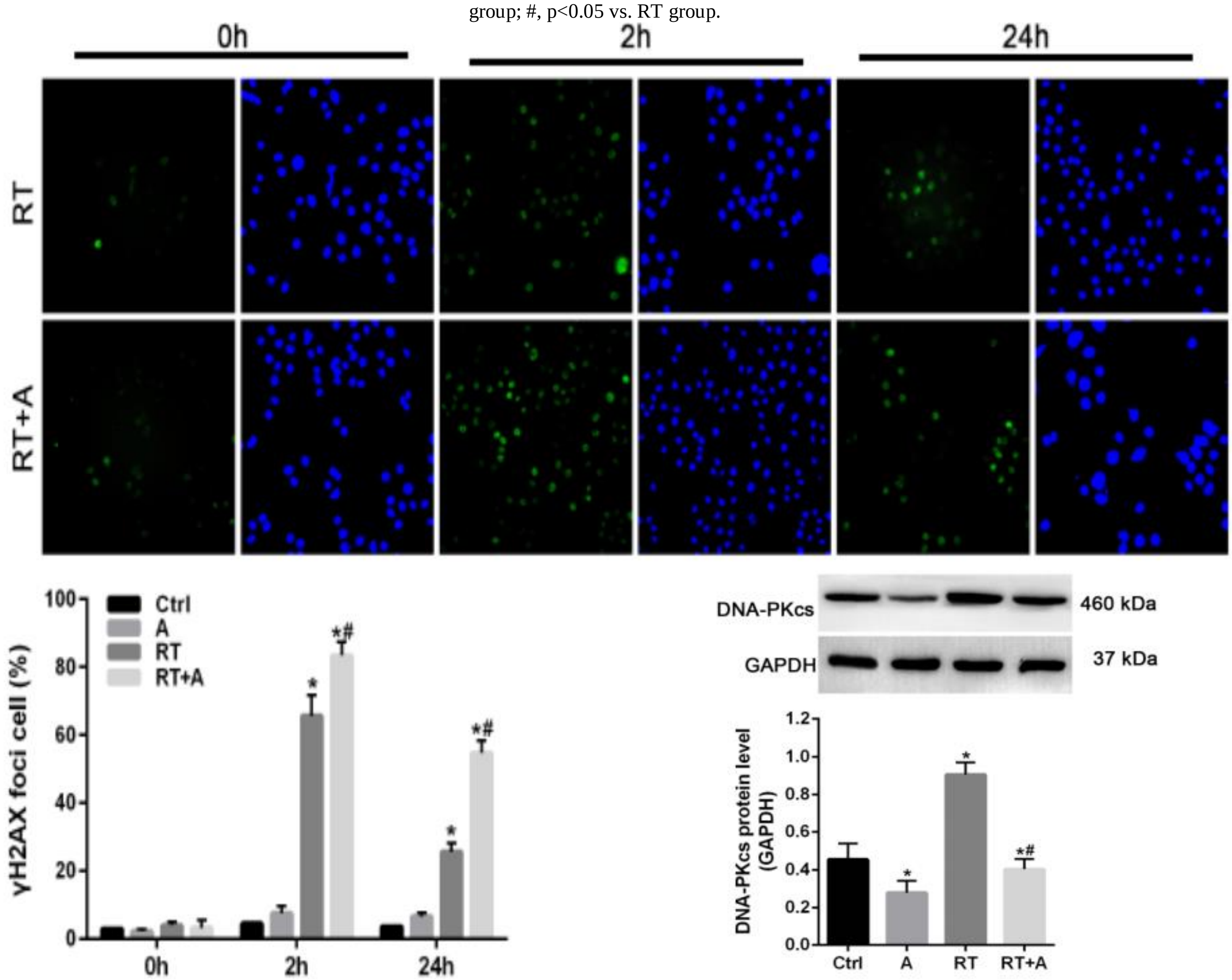


Figure 4: Effects of anlotinib and/or combined with radiation on cell cycle redistribution of A549 cells



DOI: 10.55522/jmpas.V12I6.5821 ISSN NO. 2320 – 7418

Figure 5: Effects of anlotinib and/or combined with radiation on DNA damage respond in A549 cells (A) Fluorescence of γ-H2AX foci in A549 cells treated with anlotinib and/or combined with radiation. (B) The level of DNA-PKcs expression in A549 cells treated with anlotinib and/or combined with radiation. *, p<0.05 vs. Ctrl group; #, p<0.05 vs. RT group.



CONCLUSION

In summary, we have proved for the first time that Anlotinib increases the radiosensitivity of non-small cell lung cancer cells A549 in vitro. The mechanism may inhibit EGFR from entering the nucleus and combining with DNA-PK, which affects DNA damage repair. Further explore the mechanism of anlotinib in radio-sensitization, which may provide a potential strategy for anlotinib combined with radiotherapy in the treatment of NSCLC.

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