

Investigating Radiosensitization Mechanisms of Hematoporphyrin Derivatives in Lung Adenocarcinoma Cells

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Abstract: Background: Radiotherapy effectiveness is drastically reduced in malignant tumors with low radiosensitivity. Combining hematoporphyrin derivatives (HPDs) with radiotherapy may have radiosensitizing effects, but radiosensitization mechanisms of HPDs in lung adenocarcinoma remains unclear. This study used in vitro experiments to verify whether HPDs could increase the sensitivity of lung adenocarcinoma cells to radiotherapy, and to determine how HPDs induce radiosensitization. Methods: Cloning experiments were performed on lung adenocarcinoma (A549) cells under three conditions: untreated, X-ray radiation alone, and HPDs combined with X-ray radiation. We analyzed results from previous research screening target gene HSP90AA1, then used western blotting to detect autophagosome formation. Differences in AKT, mTOR, and LC3 expression before and after X-ray/HPD treatment in A549 cells were analyzed. Results: Optimal HPD concentration and X-ray dose were 10 and 10 Gy, respectively. The combination of HPDs and X-ray inhibited proliferation and promoted apoptosis of A549 cells in a dose-dependent manner. Western blotting revealed few autophagosomes in the control group, whereas autophagosomes increased significantly in A549 cells after X-ray irradiation. Combining HPDs and X-ray decreased autophagosome numbers. Compared to X-ray only, HPDs + X-ray resulted in decreased LC3II expression and LC3II/LC3I ratio. Additionally, the LC3II/LC3I ratio was higher in the X-ray group than in the control group. Conclusions: The combination of X-ray irradiation and HPDs inhibited cell proliferation and induced radiosensitization in A549 cells. The radiosensitizing effect of HSP90AA1 may be related to autophagy. Thus, HSP90AA1 is a potential biomarker for enhanced radiosensitivity after HPD treatment.

Keywords: Lung Adenocarcinoma, X-ray, Radiosensitization, Hematoporphyrin Derivatives, Autophagy

1. Introduction

Lung cancer is one of the most common malignant tumors. China has seen annual increases in morbidity and mortality linked to lung cancer [1]. Lung adenocarcinoma has a higher incidence than non-small cell lung cancer [2]. Currently, various treatment methods (i.e., surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy) are available for lung cancer [3], with radiotherapy among the most important. However, the efficacy and clinical benefit of

radiotherapy are poor for certain radiotherapy-insensitive tumors (e.g., adenocarcinoma and sarcoma). Thus, radiosensitizers with low toxicity are used to improve treatment outcomes of such tumors [4].

Hematoporphyrin derivatives (HPDs) are metabolites of the reaction between hematoporphyrin and a mixture of sulfuric and acetic acids. The radiosensitizing effects of porphyrins were identified as early as 1955. Specifically, radiotherapy combined with HPDs significantly improved local control rate in patients with rhabdomyosarcoma, fibrosarcoma, carcinoid, and squamous cell carcinoma [5]. Subsequently,

Kostron *et al.* confirmed the radiosensitizing potential of HPDs in a rat glioma model, with HPD-treated animals exhibiting a 40% reduction in tumor growth compared with control animals [6]. However, the radiosensitization mechanism of HPDs in lung adenocarcinoma cells has not yet been investigated.

In this study, we hypothesized that heat shock protein 90AA1 (HSP90AA1) is the key to HPD radiosensitization effects in lung adenocarcinoma. HSP90AA1 is an important molecular chaperone; it is the most abundantly expressed and conserved protein in eukaryotic cells. High-throughput analysis suggests that HSP90AA1 promotes tumor progression and metastasis, so its expression may be related to lung adenocarcinoma cells [7]. Furthermore, the HSP90AA1 gene could play a role in the radiosensitization effects of HPDs [7]. However, little empirical data are available to support this hypothesis. Thus, the role of HSP90AA1 still requires experimental verification to support the feasibility of applying HPDs in radiotherapy for lung adenocarcinoma. This study used *in vitro* experiments to explore whether HPDs exert radiosensitization effects in lung adenocarcinoma cells, and if so, how HSP90AA1 expression influences those effects.

2. Materials and Methods

2.1. Materials

Human lung adenocarcinoma cell line A549 was provided by the Cell Bank of the Biological Laboratory of the Chinese Academy of Sciences.

The primary instrument used was an X-ray irradiator machine (RS2000, Rad Source Company). Its specifications are as follows: single focus, fixed anode, tungsten target, cermet tube; thickness of beryllium window: 4 mm; beam angle: 45 degrees; X-ray tube life: 2000 h; dose output range 0.1–30 Gy/min; dose uniformity $\geq 95\%$. The environmental dose is $\leq 60 \mu\text{R/h}$ at a distance of 5 cm from any reachable surface of the machine. Irradiation time is 9 s to 1 h 59 min 59 s. Dose rate is 1.0–1.1 Gy/min.

The main reagents were Giemsa staining solution, DMEM, penicillin/streptomycin, and fetal bovine serum.

Photosensitizers were HPDs and hematoporphyrin monomethyl ether, purchased from Shanghai Hanxiang Biotechnology Co., Ltd.

2.2. Methods

2.2.1. Cell Culture

Lung adenocarcinoma A549 cells were cultured in DMEM basal medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Culture conditions in the incubator were 37°C, 5% CO₂, and [insert percentage] humidity. The medium was changed daily, and routine subculture was performed. Cells in the logarithmic growth phase were used for subsequent experiments.

2.2.2. Clone Formation Experiments

A549 cells in the logarithmic growth phase were collected

for conventional digestion and centrifugation. A549 cells were divided into three groups: blank control, X-ray irradiation, and X-ray irradiation plus HPDs. The same number ($1-3 \times 10^3$) of A549 cells per group was seeded in a six-well plate, and three replicate wells were set up in parallel for each group. After 2 weeks, macroscopic clones appeared in the wells. At this stage, the medium was discarded from the six-well plate, which was washed three times with phosphate buffered saline (PBS). Methanol (5 mL pure or 1:3 v:v) was added to fix the clones for 15 min before being discarded. Cells were stained with Giemsa staining solution for 20 min. After discarding the stain, cells were washed three times with PBS. Colony formation rate was calculated via counting the number of clones visible to the naked eye on a gridded transparent film. Clones were counted three times and the average was calculated. Clone formation rate = (number of clones/number of seeded cells) $\times 100$.

2.2.3. Cell Viability Test

After A549 cells were treated, the cell culture medium was removed and cells were rinsed with PBS, then digested with trypsin. Complete medium was added to terminate digestion when microscopic examination indicated that cells became rounded and no longer adherent. Samples were centrifuged at 1000 rpm for 5 min, the supernatant was discarded, and 1 mL of complete medium was added for resuspension. Cells were counted in a cell counting plate and incubated overnight at 37°C in a 5% CO₂ incubator, then treated with different HPD concentrations. They were then subjected to a cell viability test.

2.2.4. Protein Extraction and Western Blotting

A549 cells in each group were treated with 3×10^5 HPDs and lysed with RIPA buffer (Beyotime, Shanghai, China). The lysate was centrifuged at 4°C and 12000 rpm for 20 min. Total protein concentration was determined from the supernatant using a bicinchoninic acid (BCA) assay (Beyotime, Shanghai, China). Total protein (20 μg) per group was subjected to polyacrylamide gel electrophoresis, then transferred to polyvinylidene fluoride (PVDF) membranes. Primary antibodies used were mouse monoclonal anti-HSP90AA1 (1:1000; Abcam), rabbit polyclonal AKT (1:500; Abcam), and anti-mTOR (1:1000; Abcam). The secondary antibody was horseradish peroxidase-conjugated goat antirabbit/mouse antibody (1:5000; Boster, Wuhan, China). The test was repeated three times. Gray values of the final protein band were analyzed in ImageJ.

2.2.5. X-ray Irradiation

A549 cells in the logarithmic growth phase were inoculated into culture flasks. After reaching 80% confluency, cells were irradiated using an X-ray irradiator at doses of 5 Gy, 10 Gy, or 15 Gy (dose rate: 1 Gy/min). After 24 h of treatment, cell proliferation and apoptosis were measured, and the optimal irradiation dose was determined.

2.2.6. Statistical Methods

Data are presented as means \pm standard deviation and

tabulated. All statistical tests were performed in SPSS 16.0, and TanonImage was used for grayscale analysis of protein data. Grayscale analysis results were statistically analyzed in GraphPad Prism (San Diego, CA). Significance was set at $P < 0.05$.

3. Results

3.1. Cell Proliferation Activity Under Various HPD Concentrations

A549 cells were treated with 0, 2.5, 5, 10, 20, 30, 60, and 120 $\mu\text{g/mL}$ HPDs to monitor effects on proliferation. Compared with the blank control, 2.5 $\mu\text{g/mL}$ HPD treatment did not have a significant effect on A549 cell proliferation. Higher HPD concentrations (5, 10, 20, and 30 $\mu\text{g/mL}$) inhibited proliferation, but not at significant levels ($P > 0.05$). However, 60 and 120 $\mu\text{g/mL}$ HPDs significantly inhibited cell proliferation, with 120 $\mu\text{g/mL}$ having a stronger effect (Figure 1, $P < 0.05$).

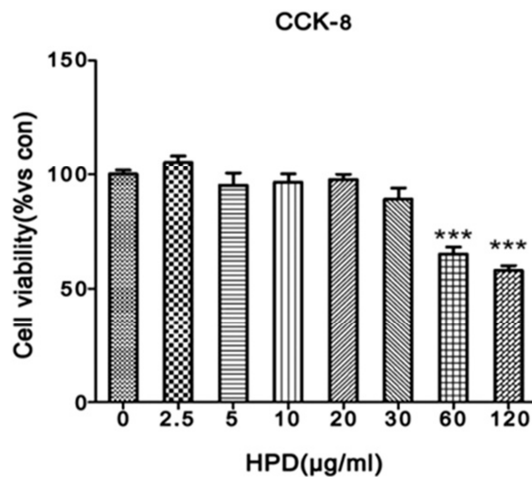


Figure 1. Effects of variable HPD concentrations on A549 cell proliferation. HPD, hematoporphyrin derivative. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.2. Cell Clone Formation Under Various Treatment Conditions

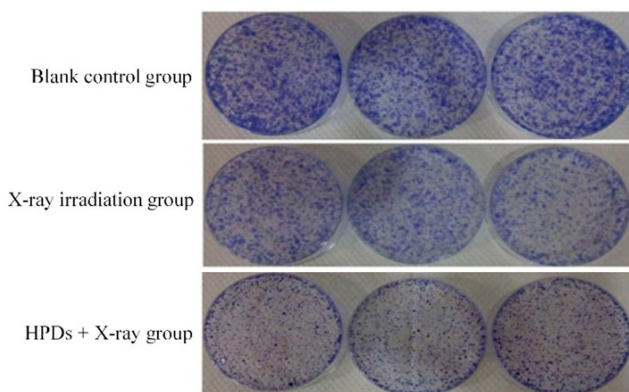


Figure 2. Effects of X-ray irradiation alone and HPDs + X-ray irradiation on A549 cell clone formation.

Figure 2 shows the results of cell clone formation.

Compared with the blank control, 10 Gy of X-ray radiation decreased A549 clone formation (Figure 2). Additionally, 10 $\mu\text{g/mL}$ HPD + 10 Gy X-ray decreased clone formation more than 10 Gy X-ray alone. Clone formation rates in the three groups were 62%, 38%, and 19.67%, respectively. These results indicate that 10 $\mu\text{g/mL}$ HPD significantly inhibited A549 colony formation.

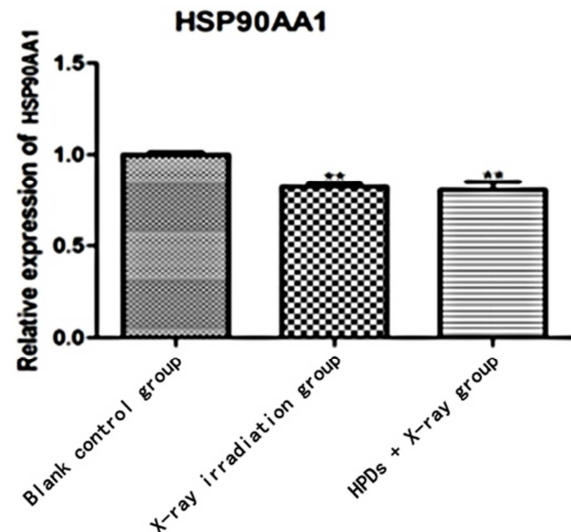


Figure 3. HSPAA1 expression detected with qPCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

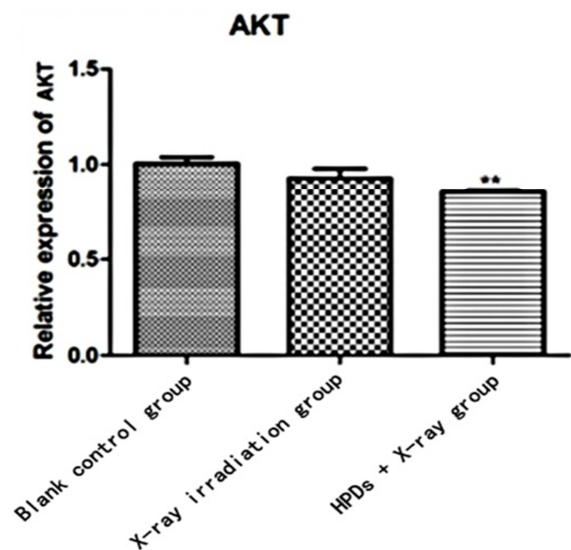


Figure 4. AKT expression detected with qPCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.3. qPCR Detection of HSP90AA1 and AKT Gene Expression

We used qPCR to detect HSP90AA1 expression in A549 after the cells were treated with 10 Gy X-ray or 10 $\mu\text{g/mL}$ HPD + 10 Gy X-ray. Figures 3 and 4 respectively show HSP90AA1 and AKT gene expression in A549 cells. Compared with the blank control, X-ray alone and HPDs + X-ray decreased both HSP90AA1 and AKT gene expression. Treatment with HPDs + X-ray caused the largest decrease.

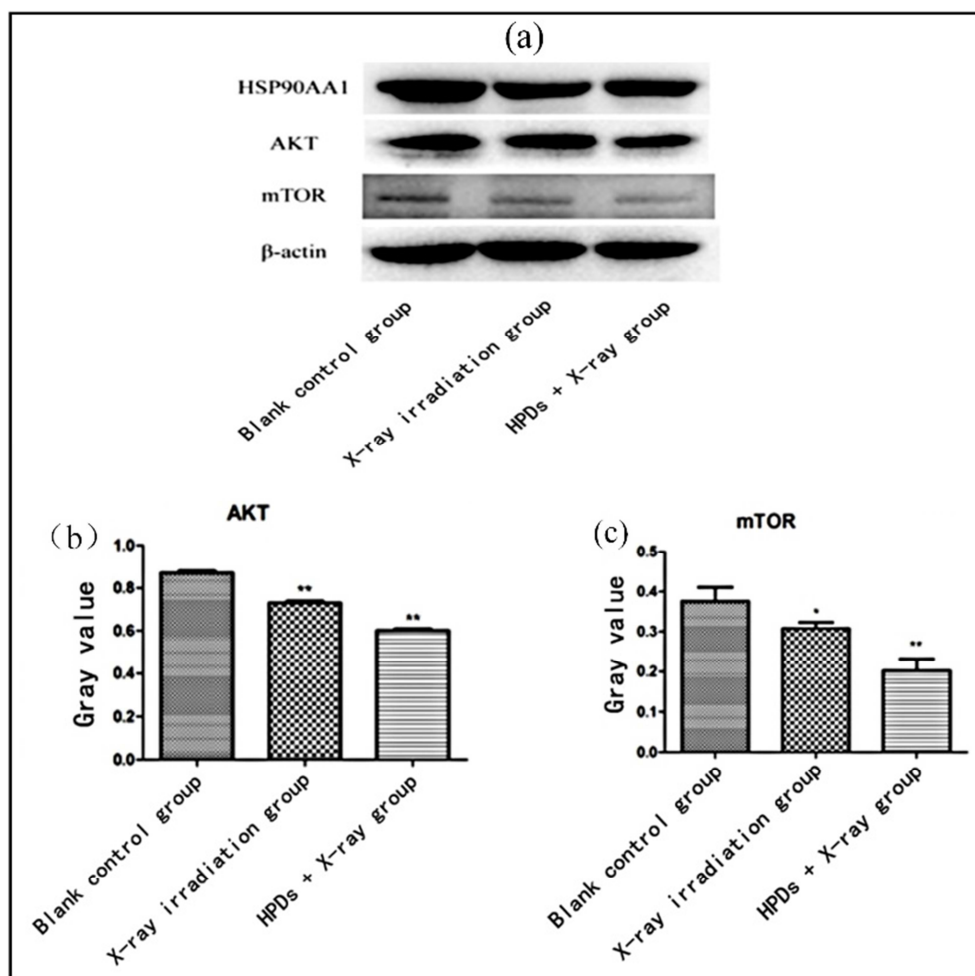


Figure 5. HSP90AA1, AKT and mTOR expression measured with western blots.

3.4. Western Blotting of HSP90AA1, AKT, and mTOR Protein Expression

We used western blots (WB) to detect the expression of related proteins in A549 cells (Figure 5). The X-ray alone group had decreased HSP90AA1, AKT, and mTOR expression than control, and this downregulation was even more evident in the HPDs + X-ray group.

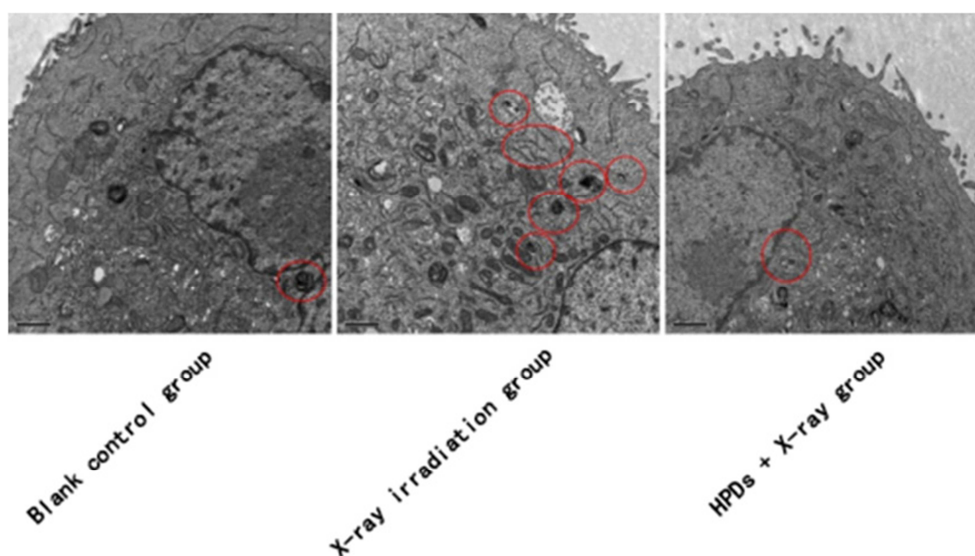


Figure 6. Cell autophagosomes visualized under an electron microscope.

3.5. Autophagosome Formation Under Transmission Electron Microscopy

We used transmission electron microscopy to examine the effect of X-ray/HPD treatment on autophagosome formation in A549 cells (Figure 6). Few autophagosomes were present in

control A549 cells, whereas X-ray irradiation increased the number of autophagosomes (Figure 6). In contrast, HPDs + X-ray irradiation decreased autophagosomes because HPDs inhibited autophagy-related HSP90AA1 expression.

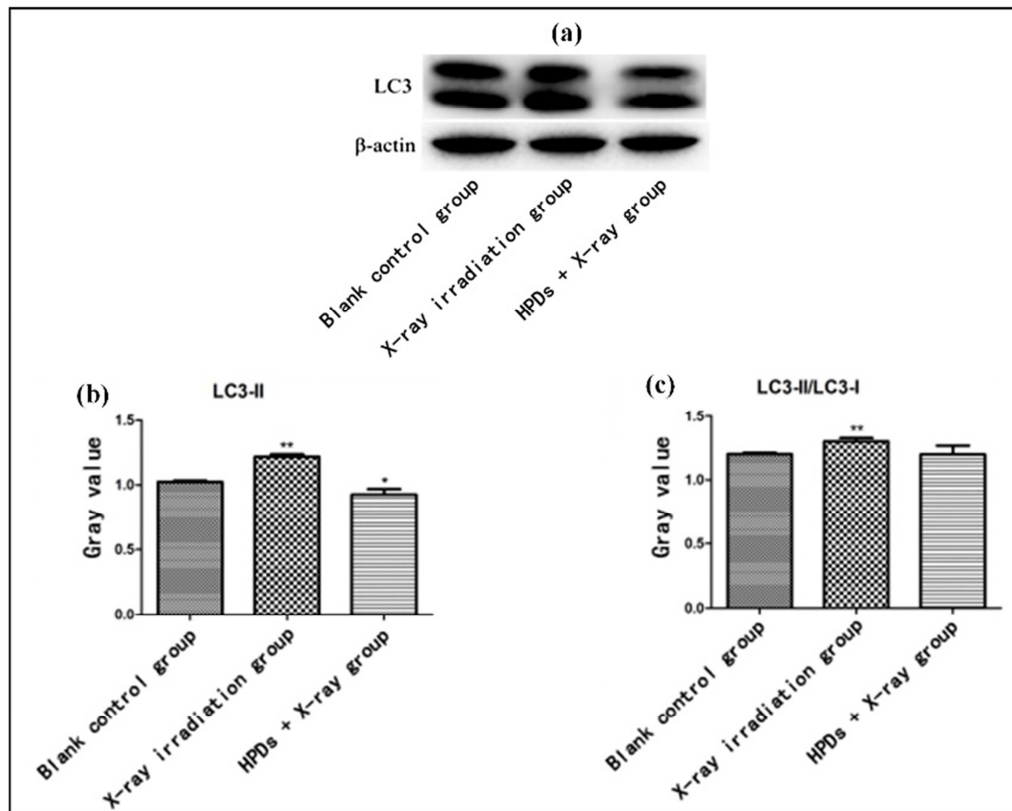


Figure 7. a) Western blot of autophagy-related protein LC3 expression. b) Changes to LC3II expression in the HPDs + X-ray group. c) Changes to LC3II/LC3I ratio in HPDs + X-ray group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.6. Western Blot Detection of Autophagy-Related Protein LC3 Expression Changes

Figure 7 shows WB results of autophagy-related LC3 expression. Treatment with HPDs + X-ray decreased LC3II expression and the LC3II/LC3I ratio more than control or X-ray treatment alone, while X-ray alone resulted in higher LC3II expression and LC3II/LC3I than control. Thus, HPDs exert an X-ray sensitizing effect on A549 cells through altering HSP90AA1 expression and influencing autophagy.

4. Discussion

Lung cancer incidence is increasing annually. More than half of such patients receive radiotherapy. With gradual improvements to lung cancer treatments, overall patient survival rate has significantly increased, but the 5 year survival rate remains $<10\%$ [8]. Some patients with lung cancer have low radiosensitivity, rendering radiotherapy ineffective. Increasing radiosensitivity of these patients is an urgent clinical problem.

HPDs have been applied in photodynamics therapy (PDT) and sonodynamic therapy (SDT). Compared with other treatments (i.e., surgery, radiotherapy, chemotherapy, targeted therapy), PDT and SDT adopt photo/acoustic sensitizers that are selectively concentrated in malignant tumors. They are activated through lasers or ultrasonication to treat local primary or recurrent tumors [9]. As one of the earliest photo/acoustic sensitizers for PDT and SDT, HPD is among the most widely used sensitizers in clinical practice. [10] Studies have shown that HPDs have a synergistic effect in combination with radiation.

The results of our CCK-8 and clone formation experiments showed that HPDs combined with X-ray irradiation significantly inhibited A549 cell proliferation, decreased clone formation, and induced tumor cell apoptosis. Since $10 \mu\text{g/mL}$ HPDs and 10 Gy X-ray irradiation were the lowest concentration and dose that exerted an inhibitory effect, we selected them as the treatment criteria for subsequent experiments. In Europe, HPDs are commonly used as sensitizers for treating advanced lung cancer [11].

The radiosensitization effect of HPDs mainly has two

mechanisms. First, HPDs enhance radioactivity through reacting with cytotoxic activating molecules, such as OH radicals generated via the interaction between ionizing radiation and water [12]. Second, HPDs inhibit repair processes that tend to mitigate radiation-induced cellular damage. Generally, ionizing radiation causes lethal or sublethal injuries. Sublethal injuries either progress to lethal injuries or stimulate repair mechanisms. When combined with ionizing radiation, HPDs decrease the likelihood of successful repair. Radiosensitization using HPDs is a complex process involving multiple steps and signaling pathways. Further research will hopefully determine the mechanism of HPD-mediated radiosensitization.

Previous studies have confirmed that HPD-PDT inhibits A549 cell proliferation and induces apoptosis, effectively killing adenocarcinoma cells [13]. Here, RNA sequencing of A549 cells showed that HSP90AA1, AKT, and mTOR were downregulated under X-ray treatment. Thus, HSP90AA1 may be related to HPD-induced radiosensitivity in lung adenocarcinoma cells.

HSPs are involved in natural defense responses. Their high expression enables cells to withstand lethal conditions. HSPs are evolutionarily conserved and ubiquitously expressed in all organisms and different subcellular compartments [14]. In eukaryotic cells, HSPs have multiple roles but primarily function as molecular chaperones [15]. When cell homeostasis is disrupted, HSPs promote protein folding and maintenance of host protein structure and function [13]. Furthermore, HSPs are involved in important cellular processes, such as protein assembly, secretion, trafficking, translocation, and degradation, as well as transcription factor regulation, especially in repairing misfolded proteins [14]. In particular, HSP90 is positively correlated with lung cancer occurrence and development [16]. A recent study showed that HSP90 affects the biological behavior of lung cancer cells through blocking the AKT/mTOR pathway, providing a theoretical basis for studying HPD mechanisms [17].

A previous study found that the frequency of HSP90AA1 mutations increased significantly in Turkish patients with non-small cell lung cancer [18]. HSP90 expression is negatively correlated with overall survival in this form of lung cancer. Therefore, HSP90 inhibitors significantly block A549 cell proliferation [19], suggesting that HSP90AA1 may be closely related to the radiosensitivity of HPD-induced non-small cell lung cancer.

The contribution of autophagy to cancer development remains controversial, mainly because autophagy may suppress or promote tumors depending on the biological environment [20]. Previous studies have reported that HSP90AA1 participates in lymphocyte autophagy through the LAMP2A-lysosomal interaction. In this study, WB results indicated that LC3II expression and LC3II/LC3I ratio decreased in with HPDs + X-ray treatment. Additionally, X-ray alone resulted in significantly higher LC3II expression than the control group. These results further demonstrated that X-ray/HPD treatment of A549 cells influenced autophagy through regulating HSP90AA1 expression.

This study had some limitations. First, it was limited to in vitro experiments, without accompanying in vivo experiments. The exact mechanism of radiosensitization using HPDs also requires further verification. Based on the preliminary results of this study, we will continue to investigate the HSP90AA1/Akt/mTOR signaling pathway using molecular biological techniques and animal experiments. We will also further explore the radiosensitization effects of HPDs in treatments for lung cancer.

5. Conclusions

This study used in vitro experiments to investigate radiosensitization mechanisms of HPDs in lung adenocarcinoma cells. Our results demonstrated that treating A549 cells with X-ray + HPDs decreased HSP90AA1 expression. This downregulation limited autophagosome formation and inhibited autophagy in A549 cells. The down-regulation of HSP90AA1 directly reduces the number of adenocarcinoma cells and plays an anti-tumor effect. Moreover, we found that HSP90AA1 expression is related to the Akt/mTOR pathway which is related to the occurrence and development of tumors, suggesting that HSP90AA1 can further enhance the sensitivity of radiotherapy and increase the death of tumor cells. Thus, we conclude that HPDs have potential as new sensitizers for radiotherapy of lung adenocarcinoma.

Author Contribution

Hongtao Yin contributed in conducting in vitro experiments, data processing, writing the manuscript; Hongxu Zhang and Chunbo Wang contributed in designing vitro experiments and research workflow; Wencheng Shao contributed in writing and revising the manuscript.

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Conflict of Interest

All the authors do not have any possible conflicts of interest.

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