RESEARCH ARTICLE

Radio-Sensitization by *Piper longumine* of Human Breast Adenoma MDA-MB-231 Cells *in Vitro*

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Abstract

Background: The current study investigated the effects of *Piper longumine* on radio-sensitization of human breast cancer MDA-MB-231 cells and underlying mechanisms. Materials and Methods: Human breast cancer MDA-MB-231 cells were cultured in vitro and those in logarithmic growth phase were selected for experiments divided into four groups: control, X-ray exposed, *Piper longumine*, and *Piper longumine* combined with X-rays. Conogenic assays were performed to determine the radio-sensitizing effects. Cell survival curves were fitted by single-hit multi-target model and then the survival fraction (SF), average lethal dose (D_0), quasi-threshold dose (D_q) and sensitive enhancement ratio (SER) were calculated. Cell apoptosis was analyzed by flow cytometry (FCM).Western blot assays were employed for expression of apoptosis-related proteins (Bc1-2 and Bax) after treatment with *Piper longumine* and/or X-ray radiation. The intracellular reactive oxygen species (ROS) level was detected by FCM with a DCFH-DA probe. Results: The cloning formation capacity was decreased in the group of piperlongumine plus radiation, which displayed the values of SF2, D0, Dq significantly lower than those of radiation alone group and the sensitive enhancement ratio (SER) of D0 was1.22 and 1.29, respectively. The cell apoptosis rate was increased by the combination treatment of *Piper longumine* and radiation. *Piper longumine* increased the radiation-induced intracellular levels of ROS. Compared with the control group and individual group, the combination group demonstrated significantly decreased expression of Bcl-2 with increased Bax. Conclusions: *Piper longumine* at a non-cytotoxic concentration can enhance the radio-sensitivity of MDA-MB-231cells, which may be related to its regulation of apoptosis-related protein expression and the increase of intracellular ROS level, thus increasing radiation-induced apoptosis.

Keywords: *Piper*longumine - MDA-MB-231cells line - radiation enhancement - apoptosis - Bax - Bcl-2

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Introduction

Triple-negative breast cancer (TNBC), a subtype of breast cancers, is defined by lack of both protein expression of estrogen receptor (ER), progesterone receptor (PR) and over-expression of human epidermal growth factor receptor-2 (HER-2). TNBC accounts for about 15% of breast cancer and often happens to women aging below 40 years, having special biological features such as high pathologic grade, poor prognosis, short 5-year survival, strong tumor invasion, high incidence of local recurrence and distant metastasis (Bauer et al., 2007). Because of the negative expression of ER, PR and lack of over-expression of HER-2, TNBC doesn't respond to both endocrine therapy and targeted therapy of trastuzumab. Moreover, it has been observed that TNBC often resists radiation and responds poorly to conventional radiation therapy (RT). There are few effective treatment modalities, so the management of advanced TNBC is very challenging.

Piper species are used as spices and traditional medicine by many people in Asia and the Pacific islands; they are especially prevalent in Indian medicine. Preparations of *Piper* species have exhibited anti-inflammatory, anti-feedant, insecticidal and anti-hypertensive activities. *Piper longumine* (PL) is a natural alkaloid component of several *Piper* species (Figure 1) isolated from *Piper* longum Linn roots. Through its complex functions, it exerts insecticidal (de Moraes et al., 2011), anti-fungal (Lee et al., 2007), antinociceptive (Rodrigues et al., 2009), antidepressant (Bezerra et al., 2007), and anti-platelet aggregation properties (Park et al., 2007; Fontenele et al., 2009).

In recent years, PL has been recognized as a promising anti-cancer agent. Its antitumor functions have been investigated in breast cancer (Yao et al., 2013), HL-60 leukemia (Kong et al., 2008), and prostate cancer cells line (Bezerra et al., 2007) by the way of changing cell cycles and inducing apoptosis through elevating reactive oxygen species (ROS) production. As far as we know, the radio-sensitivity of cancer cells is intimately connected

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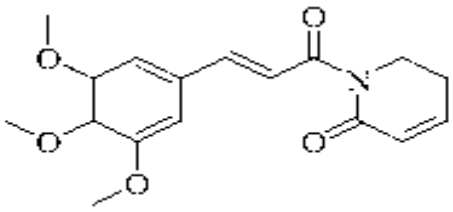

Figure 1.Chemical Structure of *Piper*longumine ($C_{17}H_{19}NO_5$)

with the apoptosis and cell cycle. Despite widespread investigation into the direct antitumor effect of PL (Bezerra et al., 2007; Kong et al., 2008; Yao et al., 2013), there is little data regarding the effect of PL as a radio-sensitizer for antitumor therapy and the literature is also silent on the enhancement of radio-sensitivity of PL on breast adenoma cells. In addition, no further studies have been performed on the molecular mechanisms of increased apoptosis by the co-treatment of PL and radiation. A safe and effective radio-sensitizing agent may allow a decrease in the radiation dose and side effects associated with RT for advanced TNBC. Thus we believe the radio-dose enhancing effect of PL on breast adenoma cells needs to be investigated.

In this article, we focus on three aspects: 1) Whether PL has the potential to enhance radio-sensitivity of MDA-MB-231 cells; 2) Whether it is accomplished by increasing apoptosis; 3) The underlying further molecular mechanisms of radio-sensitivity.

Materials and Methods

Reagents

*Piper*longumine {5, 6-dihydro-1-[1-oxo-3- (3, 4, 5-trimethoxyphenyl)-2-propenyl]-2- (1H)-pyridinone} ($C_{17}H_{19}NO_5$, MW317.3) was extracted, isolated and purified in Laboratory of Chemistry Engineering of East China Normal University, China. It was dissolved in sterilized DMSO which concentration was 5mmol/L and diluted in serum-free fresh cell medium to the desired concentration for all the experiments (Final concentration of DMSO was less than 0.1%). High glucose medium DMEM, 0.25% trypsin, PBS, penicillin-streptomycin solution and Giemsa solution were obtained from Gibco Company. Fetal calf serum (FCS) was purchased from Sijiqing Company, Hangzhou. DMSO, Annexin V-FITC /PI cell apoptosis kit were purchased from Kaiji Company, Nanjing. Reactive oxygen kit was obtained from Biyuntian Company. Mouse monoclonal antibody against Bcl-2 and Bax, mouse monoclonal antibody against β-actin, and horseradish peroxidase (HRP)-conjugated anti-mouse antibody were purchased from Santa Cruz Company. Flow cytometry (FACS Calibur, Beckman Coulter Company). Linear accelerate (ELEKTA Company).

Cell line and cell culture conditions

Human breast cancer MDA-MB-231 cells were provided by the Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. These cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100μg/ml streptomycin in a humidified 5 % CO_2 atmosphere containing at 37°C.

Cell radiation

Cells were divided into two groups: single radiation group and PL plus radiation group. The radiation rate is 300cGy/min. All radiation was carried out using 6MV X-ray linear accelerator with 100cm source skin distance (SSD) and 15cm*15cm exposure field. The absorbed dose was 6Gy.

Cell clonogenic survival assay

Logarithmically growing cells were re-suspended and plated in wells of six-well dishes with culture containing medium alone or medium supplemented with PL (1 μmol/L and 2.5 μmol/L) at different number of cells (200, 500, 1000, 2000, and 4000) in accordance with laddered doses of radiation (0, 2, 4, 6, and 8 Gy). After treatment with PL for 24h, it was removed, and cells were radiated, then cells were incubated in a continually humidified atmosphere. After 14 days, when cell colonies were formed, the visible colonies with more than 50 cells were counted. Plating efficiency (PE) was calculated relative to the control group (0Gy), and survival fraction (SF) of each group was determined using the equation: SF=colony number/ (plating cell number×PE). The dose-survival curve was fitted by the single-hit multi-target statistical model $SF=1-(1-e^{-D/D0})^N$ using SPSS17.0 to calculate several survival parameters including SF2 (surviving fraction of 2 Gy), D_0 (mean lethal dose or final slope), and Dq (quasi-threshold dose). Three parallel samples were set at each radiation dosage.

Flow cytometry

Cells were divided into four groups as follows: 1) control group: no treatment; 2) 1 μmol/L PL alone group; 3) 6Gy X-ray radiation alone group; 4) 6Gy X-ray radiation plus 1 μmol/L PL group. To confirm the apoptosis of PL on MDA-MB-231cells, dual-staining [propidium iodide (PI) and annexin-V (AV)] flow cytometry was used to measure the externalization of phosphatidylserine (PS). Aliquots (5×10^6) of MDA-MB-231 cells were treated as described above for 24h. Controls were treated with DMSO only. Following washing and trypsinization, cell samples were collected by centrifugation (1200rpm, 5min, 4˚C) and double-stained using the apoptosis detection kit according to the manufacturer's instructions. Cells were incubated for 30 min at 25˚C in 100 μl 1×buffer solution, 5 μl FITC-Annexin V and 5 μl PI, and a further 400 μl of 1×solution buffer was added. The green fluorescence of Annexin V-FITC-bound PS and the red fluorescence of DNA-bound PI in individual cells were measured using a BD FACSCalibur. Cell populations were classified as: AV-/PI-, viable cells; AV+/PI-, early apoptotic cells; AV+/PI+, late apoptotic cells; and AV-/PI+, residual damaged cells.

Study of protein expression

Cells were divided into four groups as follows: 1) control group: no treatment; 2) 6Gy X-ray radiation

alone group; 3) 2.5 μmol/L PL alone group; 4) 6Gy X-ray radiation plus 2.5 μmol/L PL group. Total protein concentration was determined by BCA method, using bovine serum albumin (BSA) as standard. Twenty micrograms of total cell lysate was boiled for 5 min. Equal amounts of protein in the cell extracts were fractionated by 10% SDS-PAGE denaturing gels, then electro-transferred to the PVDF membranes and transfer of proteins was confirmed by Ponceau-S staining. The membranes were blocked in 20 mmol/l Tris-buffered saline-0.1%Tween-20 (TBST) buffer containing 5% skim-milk at 4°C overnight and probed with specific primary antibodies (Mouse monoclonal antibody against Bcl-2 and Bax and mouse monoclonal antibody against β-actin, 1:1000 dilution) overnight at 4°C. After washing with TBST for 1.5 h, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:1000 dilution) for 1h at room temperature and washed with TBST for 40 min. Protein-antibodies immuno-reactive complexes were visualized using an enhanced chemiluminescene (ECL) Western blotting detection reagent according to the manufacturer's recommendations. The experiment was repeated three times.

Study of ROS levels

2', 7'-dichlorofluorescent diacetate (DCFH-DA), one kind of non-marking and oxidation-sensitive fluorescent probe, which is not fluorescent, can freely pass through cell membrane into the cells and be hydrolyzed into DCFH by esterase. DCFH can not transparent cell membrane, and the intracellular reactive oxygen species (ROS) can oxidize the non-fluorescent DCFH to generate fluorescent DCF. So the fluorescence intensity of DCF reflects the level of intracellular ROS. Cells were divided into four groups as follows: 1) control group: no treatment; 2) 1 μmol/L PL alone group; 3) 6Gy X-ray radiation alone group; 4) 6Gy X-ray radiation plus 1 μmol/L PL group. MDA-MB-231 cells in the exponential growth phase were seeded in a 6-well culture plate at the concentration of 5×10^5 cells/ml. After treating these cells as described above for 24 h, the culture medium was sucked out of the 6 well-plate and washed three times with PBS, then 1×10^6 cells treated as described above were collected and washed with serum-free medium to remove extracellular fluorescence agent. Then the cells were incubated with DCFH-DA (10 μmol/L) at 37°C in the dark for 20 min to estimate the ROS level. The cells were harvested with EDTA-free trypsin, washed three times with PBS and analyzed immediately for fluorescence intensity detection by flow cytometry (excitation wavelength 488 nm, emission wavelength 525 nm). All experiments were conducted in triplicate.

Statistical analysis

Statistical analysis was performed on SPSS17.0 statistical package. Each of our experiments was performed in triplicate with results expressed as mean±standard error (SE). Student's t test was used to assess statistical significance within groups and two-way ANOVA was used to assess statistical significance between groups. Statistical significance was set at $p<0.05$.

Results

Clonogenic survival assay

The clonogenic survival assay was used to determine the enhancement of radio-sensitivity by PL. The survival curve of control and PL-treated cells after radiation was shown in Figure 2. The radio-biological parameters of each group were shown in Table 1.

Apoptosis of MDA-MB-231 cells by flow cytometry

Increasing evidences suggest that apoptosis has a determinant role for radio-sensitivity. In our study, the fraction of MDA-MB-231 cells undergoing apoptosis was determined by flow cytometry. We double stained cells with Annexin V for externalized PS as the symbol of early apoptosis (lower-right quadrant, LR) and with PI as the mark of late apoptosis (upper-right quadrant, UR) in the scattered point diagram of flow cytometry. Therefore, the sum of LR and UR represents the apoptotic rate. Figure 3 and Table 2 shows the fraction of MDA-MB-231 cells

Table 1.Radiation Biology Parameters by Single-hit Multi-Target Model

PL	D_0(Gy)	D_q(Gy)	SF_2	SER_{D0}	SER_{Dq}	SER_{SF2}
0 μmol/L	2.48	1.35	0.78			
1 μmol/L	2.04	0.88	0.69	1.22	1.53	1.13
2.5 μmol/L	1.92	0.64	0.58	1.29	2.11	1.34

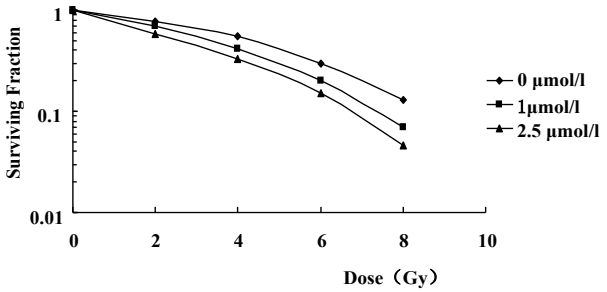

Figure 2. The Clonogenic Survival Assay with a Combination of PL (1 μmol/l and 2.5 μmol/l) with Laddered doses of Radiation (0, 2, 4, 6, and 8 Gy) of 6 MV X-ray

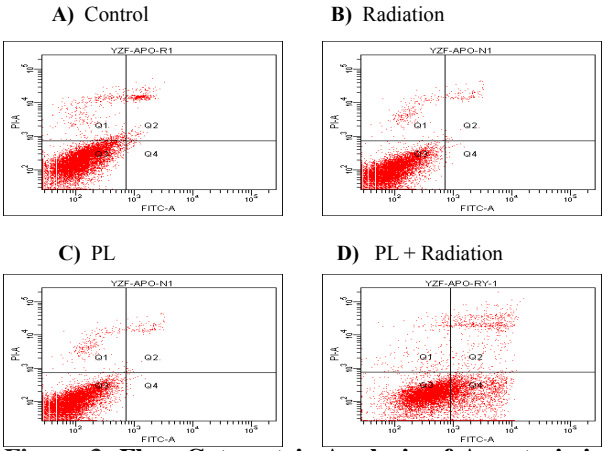

Figure 3. Flow Cytometric Analysis of Apoptosis in MDA-MB-231cells with Annexin V and PI Staining. The figure shows apoptosis in MDA-MB-231cells: early apoptotic cells (lower-right quadrant, Annexin V+/PI-) and late apoptotic cells (upper-right quadrant, Annexin V+/PI+). **A)** control, **B)** Radiation alone, **C)** PL alone, **D)** PL+ Radiation

undergoing apoptosis in each group. There is a significant difference between PL plus X-ray radiation group and individual treatment group (p<0.05). The data indicate that PL helped radiation inhibiting MDA-MB-231 cell growth by increasing cell apoptosis.

Expression of apoptosis-related proteins in enhancement of radio-sensitivity by PL

To further explore the molecular mechanisms involved in the increased apoptosis mediated by PL, we tested the expression of several critical proteins related to apoptosis, including Bcl-2 and Bax (Figure 4 and Table 3). Western blot analysis showed that there was a significant up-regulation of Bax, after PL treatment with or without radiation. However, the expression of Bcl-2 was significantly down-regulated after treatment with PL. The result implies that PL plays a role in apoptosis by regulating the expression of the Bcl-2 family of proteins and activating the mitochondrial apoptotic pathway.

Table 2. Apoptosis in Each Group

Groups	damaged cells	late apoptosis	viable cell	early apoptosis
Control	0.45±0.01	0.85±0.03	97.65±2.55	1.05±0.02
Radiation	0.98±0.06	3.20±0.65	93.30±1.48	2.60±0.48
PL	1.55±0.05	4.50±0.35	90.95±3.42	3.00±0.46
PL+Radiation	2.58±0.08	18.40±1.05*#	62.82±2.45	16.20±2.45*#

Compared to PL, *p<0.05, Compared to radiation, #p<0.05

Table 3. The Expression of Apoptosis-Related Protein Bcl-2 and Bax

Groups	Bcl-2	Bax	Bcl-2/ Bax
Control	1.55±0.09	0.42±0.02	3.69±0.15
X-ray	1.43±0.02*	0.74±0.1*	1.93±0.27*
PL	1.19±0.03*#	0.82±0.07*#	1.45±0.05*#
PL+X-ray	0.72±0.08*#△	0.48±0.05*#△	0.49±0.07*#△

Compared to control group, *p<0.05; Compared to X-ray group, #p<0.05; Compared to PL group △p<0.05

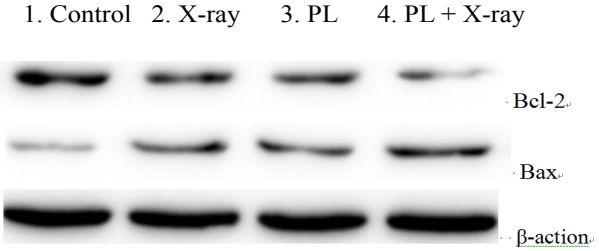

Figure 4. Western Blot of Protein Expression of Bcl-2 and Bax, Lane 1 Control, Lane 2 X-ray Alone, Lane 3 PL alone, Lane 4 X-ray+PL. Loading control was performed with an antibody for β-actin

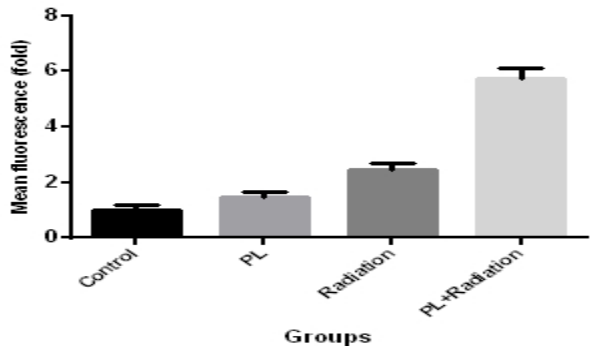

Figure 5. The ROS Levels in Each Group

Induction of intracellular ROS

We determined the effect of PL on intracellular ROS levels in MDA-MB-231 cells through flow cytometry using the redox-sensitive fluorescent probe DCFH-DA. As demonstrated in Figure 5, the ROS levels of MDA-MB-231 cells (corresponding to the fluorescence intensity) increased significantly after treatment with PL plus radiation for 1h. Compared with that in DMSO control group, the ROS level increased by 1.40, 3.10, 5.60 times in cells treated with PL, radiation, PL plus radiation, respectively. There was statistically significant difference compared with the control group. There was also statistically significant difference between PL+ radiation and the individual treatment group.

Discussion

Breast cancer is a common malignancy of women, and the current treatment is surgery, with postoperative radiotherapy and chemotherapy according to pathology and clinical conditions. P53 gene mutation frequency is as high as 58% in breast cancer patients who have family history of breast cancer, while P53 aberration frequency in patients with advanced TNBC is as high as 70%-80%, which leads to decreased sensitivity to radiotherapy of TNBC cells (Sekine et al., 2009). Since TNBC cells contains mutant p53, the radio-sensitivity of this type of breast cancer is inferior to other types of breast cancer clinically (Dent et al., 2007), therefore, developing efficient and low toxicity radio-sensitizing drugs is an important subject on radiation therapy research of TNBC.

Recent studies have shown that PL is cytotoxic to a variety of tumor cells through inhibiting the proliferation and inducing the apoptosis of various human tumor cells significantly, while having no significant toxicity to human normal cells (Raj et al., 2011; Yao et al., 2013). As the radio-sensitivity of tumor cells is closely related to the biological behavior of proliferation and apoptosis, we suggest that PL may have radio-sensitivity enhancement on tumor cells. We chose MDA-MB-231cells as the target because their sensitivity to radiation is resistant and it is essential to increase the radio-sensitivity to improve the clinical efficiency of RT of TNBC. This paper is to investigate whether PL has radio-sensitization through detecting the effect of RT in combination with PL on the clone formation of MDA-MB-231cells and to explore its possible molecular mechanism.

Our preliminary study showed PL could inhibit the proliferation of MDA-MB-231 cells *in vitro*, with certain concentration and time-dependent manner, which showed the survival rate of MDA-MB-231 cells was 91.48% upon 1 μmol/L PL administration for 24h (Yao et al., 2013). This concentration of PL produced no significant inhibition on the proliferation, so we chose the concentration of 1 μmol/L as the sensitizer concentration in this research, while selecting the concentration of 2.5 μmol/L to observe whether the radio-sensitization was in concentration-dependence.

DNA is the main target to kill tumor cells for X-rays

radiation, so single-hit multi-target model SF=1- (1-e-$^{D/D0}$) ^{N} is widely used to measure cell radio-sensitivity in the field of radiobiology (Yao et al., 2013), which is the gold standard currently to evaluate whether certain drug has radio-sensitivity. Our results showed that radiation biology parameters (D_0, D_q and SF_2) in radiation plus PL group were lower than those in radiation alone group (Table 1). In the present study, we confirmed that PL could inhibit MDA-MB-231 cell growth using clonogenic assay. PL in combination with RT produced significant synergistic inhibition of MDA-MB-231 cell growth. The dose-survival curve of PL-treated MDA-MB-231 cells exhibited a narrow shoulder (indicating decrease in D_q) and a greater slope rate (indicating decrease in D_0). Through analysis of cell survival curves, we found that the D_q value of PL combined with RT is smaller, the curves had no apparent shoulder area, which indicate that the PL increased the sensitivity of the MDA-MB-231 cells to RT. At the same time, the D_0 value is relatively lower suggesting that the reasonable lower doses of X-ray can also kill tumor cells when coupled with PL. The decreased SF_2 value of the PL-treated MDA-MB-231 cells indicated the radio-sensitization effect of PL on MDA-MB-231 cells. The sensitive enhancement ratio (SER D0) of PL treatment (1 μmol/L and 2.5 μmol/L) was 1.22 and 1.29, respectively, which suggested that PL had radio-sensitization effect on MDA-MB-231cells (Figure 2).

Based on the above results, the present study was aimed to verify the increased radio-sensitivity mediated by PL, and to further explore the mechanisms underlying this effect. Apoptosis is the main form of death when the cells are exposed to radiation and one important cause of radiation resistance is the resistance to radiation-induced apoptosis of tumor cells (Corvo et al., 2001). Cellular apoptosis represents an important process that may influence the ultimate response of a particular tumor to RT. Activation of apoptosis is widely used as a potential anticancer strategy. If one drug could increase radiation-induced apoptosis, it is expected to increase the sensitivity of tumor cells to RT. More and more research found that when tumor cells were exposed to RT, the increasing apoptotic response was positively correlated with the enhancement of radio-sensitivity (Meyn et al., 1993). Thus the induction of apoptosis by affecting the relevant regulatory pathways of apoptosis may increase the radio-sensitivity of tumor cells.

Annexin-V has a high affinity for PS and binds to cells with exposed PS. In non-apoptotic cells, most PS molecules are localized on the inner leaflet of the plasma membrane, but shortly after the onset of apoptosis, PS redistributes to the outer layer of the membrane (Ji et al., 2011). Cells in the early stages of apoptosis usually bind A V-FITC in the absence of PI uptake (lower-right quadrant, Figure 3), whereas those in the late stages of apoptosis bind A V-FITC and exhibit PI uptake (upper-right quadrant, Figure 3). Use of Annexin-V in combination with PI allowed the distinction of early apoptotic and necrotic cells from viable cells (Yu et al., 2011). In this study, Annexin-V/PI double staining flow cytometry was used to detect the apoptosis with the treatment of PL and/or radiation. We found that PL treatment alone did not increase the rate of cell apoptosis whereas the co-treatment of PL and radiation significantly increased late apoptosis and early apoptosis (Figure 3, Table 2). Our previous study found that PL made MDA-MB-231 cells be arrested in G_0/G_1 phase. As the irradiated cells failed to fully repair and was prone to apoptosis in the period of cell cycle, so the proportion of apoptotic cells was significantly increased (Yao et al., 2013).

Thus, we speculate PL may influence the regulation of apoptosis-related pathways to increase the radiation-induced apoptosis, although PL of low concentration and low cytotoxic induces no obvious apoptosis of MDA-MB-231 cells. Regulatory mechanism of apoptosis plays an important role in the regulation of cellular radio-sensitivity. In the apoptosis induced by a variety of death signals, the Bcl-2 family proteins play an important role. The Bcl-2 is an inhibitory factor can inhibit cell apoptosis (Song et al., 2014). Bcl-2 (apoptosis-suppressor gene) and Bax (apoptosis-trigger gene) play anti-apoptotic and pro-apoptotic roles, respectively. Hara and colleges found that high expression of Bcl-2 inhibited radiation-inducing apoptosis, thus reducing the radio-sensitivity of tumor cells. The decreased expression of Bcl-2 might release the inhibition effects of Bcl-2 on the tumor cell apoptosis, therefore promoting the apoptosis of cancer cells (Zhang et al., 2014). Radiation-induced down-regulation of Bcl-2 and up-regulation of Bax is associated with increased radio-sensitivity, therefore the ratio of Bcl-2/Bax is closely related to radio-sensitivity and it becomes one of the vital prognostic factors for clinical RT (Hara et al., 2005). Kong and colleges confirmed PL promoted the apoptosis of prostate cancer cells and further study found that Bax expression was not changed significantly, but Bcl-2 expression was obviously decreased, thus the ratio of Bax/Bcl-2 decreased (Kong et al., 2008).

Our primary study suggested PL significantly down-regulated Bcl-2 expression and up-regulated the expression of Bax (Yao et al., 2013). Whether the increase of apoptosis resulting from the combination of PL and RT was related to the regulation of the expression of Bcl-2 family proteins? In the present study, we detected the expression Bax and Bcl-2 with treatment of PL or/and radiation by Western blot. The results showed that the expression of Bax in PL and RT co-administration group was significantly higher than that in the individual treatment group, whereas the expression of Bcl-2 was significantly lower than the individual treatment group, the ratio of Bcl-2/Bax decreased (Figure 4, Table 3). The cell radio-sensitivity has passive correlation with the relative expression level of Bcl-2/Bax, indicating PL exhibits radio-sensitizing effects probably by regulating the expression of Bcl-2 family proteins.

Ionizing radiation produces abnormally elevated levels of ROS through indirect effect, which can cause large amounts of DNA damage, thereby increasing the killing effect of radiation on the tumor. Some studies suggest that oxidative stress is a common factor in the regulation of apoptosis, and its participation plays a key role in the process of apoptosis (Zou et al., 2004). Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called ROS, and

their elimination by protective mechanisms, referred to as antioxidants (Durackova., 2010). ROS are products of a normal cellular metabolism and play vital roles in the stimulation of signaling pathways in plant and animal cells in response to changes in intra- and extracellular environmental conditions (Jabs., 1999). In order to control the balance between production and removal of ROS, normal cells have a series of effective antioxidants in protecting cells from radicals (Ueda et al., 2002). This antioxidant system includes enzymatic antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase catalase as well as nonenzymatic antioxidants, such as glutathione (GSH). GSH is a tri-peptide and the major endogenous antioxidant produced by cells and helps to protect cells from ROS such as free radicals and peroxides (Pompella et al., 2003). GSH can directly supply hydrogen and plays a vital role for the elimination of radiation-induced intracellular ROS, which determines the intrinsic radiation sensitivity of cells (Grant., 2001). It is now well established that ROS can damage DNA and that GSH can protect against this type of damage. On the other hand, elevated GSH levels are observed in various types of cancerous cells and solid tumors, and this tends to make these cells and tissues more resistant to radiotherapy (Valko et al., 2007).

Based on the above theory, we speculate that if enough ROS is produced by PL to consume antioxidants such as GSH before the tumor cells are exposed to X-rays, the radiation killing effect on tumor cells will undoubtedly increase, thus showing radio-sensitizing effect. Our preliminary research found the level of intracellular ROS increased in the course of PL-induced apoptosis of MDA-MB-231 cells in concentration-dependent manner (Yao et al., 2013). Whether radio-sensitization of PL was associated with the elevated levels of intracellular ROS? We observed the effect of treatment with PL and/or radiation on the intracellular ROS levels in MDA-MB-231 cells through flow cytometry using the redox-sensitive fluorescent probe DCFH-DA. Our results showed that the ROS levels increased in either radiation alone group or single PL-treatment group, however no statistically significant difference compared to cells of the control group ($p>0.05$), while the level of intracellular ROS in the co-treatment of PL and radiation group was significantly higher than the individual group and control group ($p<0.05$), which confirmed that PL played an important role in sensitizing effect through improving the radiation-induced ROS levels in the MDA-MB-231 cells (Figure 5).

As we know, ROS generated by radiation can trigger the apoptosis signal transduction pathway. Based on our results, we speculate the elevated ROS induced by PL may further accelerate the process of apoptosis once radiation-induced apoptosis starts, thereby increasing the sensitivity of tumor cells to radiation. Therefore, our results may confirm that both PL and X-ray radiation produce excessive ROS in the tumor cells, thus weakening the intracellular antioxidant system, which protect cells against radiation. Thus we propose the hypothesis that there seems to be certain synergies effect between PL and radiation. Some scholars found Bcl-2 could protect lipid membrane and maintain the redox state of the cell through the elevated level of cellular GSH, therefore they proposed Bcl-2 might be a kind of anti-oxidant, which inhibited cell death through inhibiting the generation of ROS (Mrtrailler-Ruchonnet et al., 2007). Over production of ROS induces oxidative damage including: lipid peroxidation, protein oxidation and DNA damage which results in modulation of Bcl-2 family proteins and dissipation of mitochondrial membrane potential, which are characteristic features of mitochondrial apoptosis (Meng et al., 2012). Our results showed that PL plus radiation significantly reduced the expression of Bcl-2 while obviously increased intracellular ROS levels and the apoptosis. We speculate that there seems to certain intrinsic connection between reactive oxygen stress pathway and the regulation of apoptosis pathway which might be worthy of further study.

In conclusion, PL significantly enhanced radio-sensitization possibly via regulating the expression of the Bcl-2 family proteins and activating the mitochondrial apoptotic pathway and the stress response pathway to ROS, thereby increasing radiation-induced apoptosis in breast cancer cells. This provides a rationale for combining PL and RT in the treatment of human triple-negative breast cancer. An ideal radiation sensitizer must have no apparent toxicity to normal tissues. Such characteristics as its non-specific anti-tumor effect and no effect on normal cells can make PL seem to have more obvious advantages than other drugs, which is of potential clinical interest. However, there is still a large amount of study remaining to be done: investigating the radio-sensitizing functions of PL in normal cells and animal models, which can yield the basic theory for the clinical application of PL in the future.

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