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Original Article

Over-expression of PLAU regulates the radiosensitivity of esophageal cancer cell

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Abstract: Purpose: Radiotherapy is an effective treatment for esophageal cancers (EC), but the major clinical challenge of this therapy is the radiation resistance. Moreover, the mechanisms of radioresistance has not been fully elucidated so far. The gene coding for urokinase-plasminogen activator (PLAU) have been confirmed to be implicated in abroad spectrum of pathophysiological processes. However, the correlation between PLAU expression and radiosensitivity of EC cells is still unclear. The purpose of this study was to explore the effects of PLAU on the radiosensitivity of EC cells. Methods: In this study, the expression of PLAU protein specimens of patients with EC before and after irradiation was investigated by immunohistochemistry. Results: The result demonstrated that the PLAU protein expression was over-expression in tumor tissues compared with adjacent non-cancerous tissues and the expression of PLAU was obviously increased after irradiation in TE-2 cells. PLAU was associated with radiotherapy response, in which over-expression of PLAU was observed more frequently in the radioresistant group than in the effective group. Then the PLAU expression was inhibited by lentivirus-mediated siRNA targeting PLAU. And this inhibition of PLAU increased the radiosensitivity and apoptosis, while decreased the colony formation of TE-2 cells. Conclusions: Taken together, our data suggested that PLAU might play an important role in radioresistance of EC, and inhibition of PLAU can increase the radiosensitivity of TE-2 cells by enhancing apoptosis, and decreasing colony formation.

Keywords: Esophageal cancers, PLAU, radiosensitivity

Introduction

Esophageal cancers (EC) is one of the most common malignancies and the leading causes of death due to cancer worldwide [1]. The morbidity and mortality of EC are very high with a rank of eighth and sixth, respectively [2]. EC is a multistep, multifactorial disease and the result of complex interplay between genetic and environmental factors [3]. Radiotherapy is the mainstay in the treatment of EC, this method has a major problem of local failure which is said that around 40-60% of patients with EC would be persistent or recurrent [4]. Besides, the radioresistance in cancer cells after radiotherapy is not only one of main source of local recurrence but also a big obstacle for the using of this therapy. Nevertheless, the proper molecular targeted therapy combined with irradiation and effective measures to identify patients with radioresistance in ESCC were still rarely. Moreover, the mechanisms of the occurrence of ESCC radioresistance are not fully under stood.

Urokinase-plasminogen activator (PLAU), also named uPA, is located on chromosome 10q24 and involved in many processes such as angiogenesis, growth factor activation, mobilization, ECM remodeling, invasion and metastasis in tumors [5-7]. It was also confirmed to be overexpressed in many cancer cells [8-10]. According to the study of Fukuda et al., PLAU was up-regulated in radioresistant sublines of oesophageal cancer cell lines [11]. However, its detailed effects and mechanism of PLAU on the radiosensitivity had never reported.

In this study, we investigated the expression of PLAU in EC cell line TE-2 before and after irradiation. And the effects of PAU on radiosensitivity as well as its possible mechanism were also estimated. This study was expected to provide

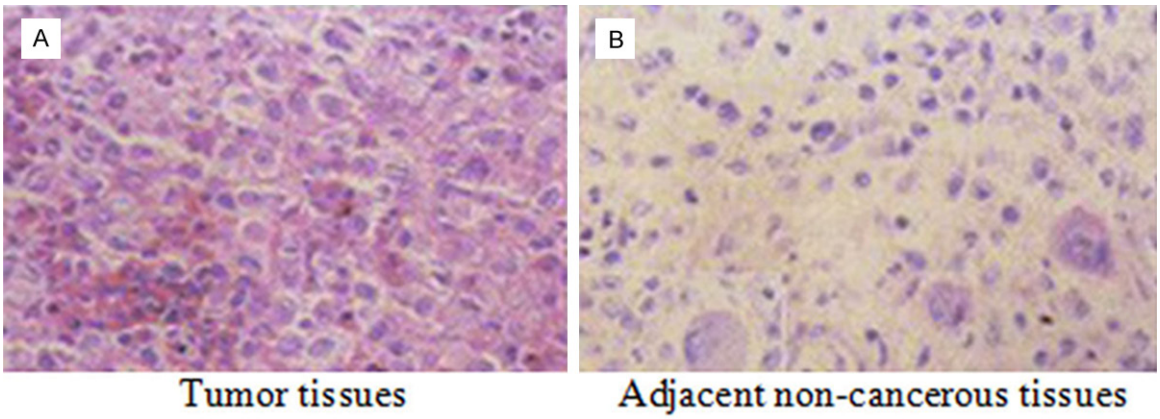

Figure 1. The expression level of PLAU protein in tumor tissues and adjacent non-cancerous tissues which was detected by immunohistochemical staining. The positive rate of PLAU expression in tumor tissues was significantly higher than those in adjacent non-cancerous tissues ($P<0.05$).

Table 1. Expressions of PLAU protein in EC tissue and in adjacent non-cancerous tissues

Group	Cases (n)	PLAU expression		χ^2	*P*
		Positive expression (n)	Negative expression (n)		
Tumor tissues	94	72	22	15.867	0.000
Adjacent tissues	94	8	86		

Table 2. Correlation of *PLAU* expression level and response to radiotherapy

Treatment response	All cases (n=94)	*PLAU* expression		χ^2	*P*
		Positive expression (n=72)	Negative expression (n=22)		
Effective	64	45	19	3.615	0.028
Resistant	30	27	3		

a new indicator for the radiosensitivity of EC cells to irradiation.

Materials and methods

Patients and samples

94 patients diagnosed as EC during 2009-2010 at Shaanxi General Hospital of CAPF were collected and the study was permitted by the Ethnic Committee of the hospital. All patients had never received any treatment before sampling and signed written informed consent in advance. The tissues and adjacent non-cancerous tissues were obtained from patients with EC and frozen in liquid nitrogen, immediately. Then the samples were stored at -80°C for RNA and protein extraction.

Immunohistochemical staining

The tissues and adjacent tissues from 94 patients with EC were fixed with formalin and embedded with paraffin. The sections were cut into 5 μm and deparafinized, rehydrated, and immersed in 3% hydrogen peroxide solution for 10 min. Then the sections were heated in EDTA buffer (pH 8.0) at 95°C for 25 min, and cooled at room temperature for 60 min. 10% normal goat serum was added into block the sections at 37°C for 30 min, and then incubated with rabbit polyclonal antibody against *PLAU* for overnight at 37°C. The sections were washed with PBS, and then incubated with biotinylated second antibody for 30 min at 37°C, followed by streptavidin-peroxidase incubation at 37°C for 30 min. Then the sections were stained with a mixture of DAB solution while hematoxylin was used to counterstain. The immunohistochemical slides were scored according to the percentage of tumor cells exhibiting nuclear staining. The extent of *PLAU*-positive staining over 10% was presented as "+" while less than 10% were attributed to negative group (-).

Cell lines and cell culture

Human EC cell lines TE-2 were obtained from the Cell Resource Center for Biomedical

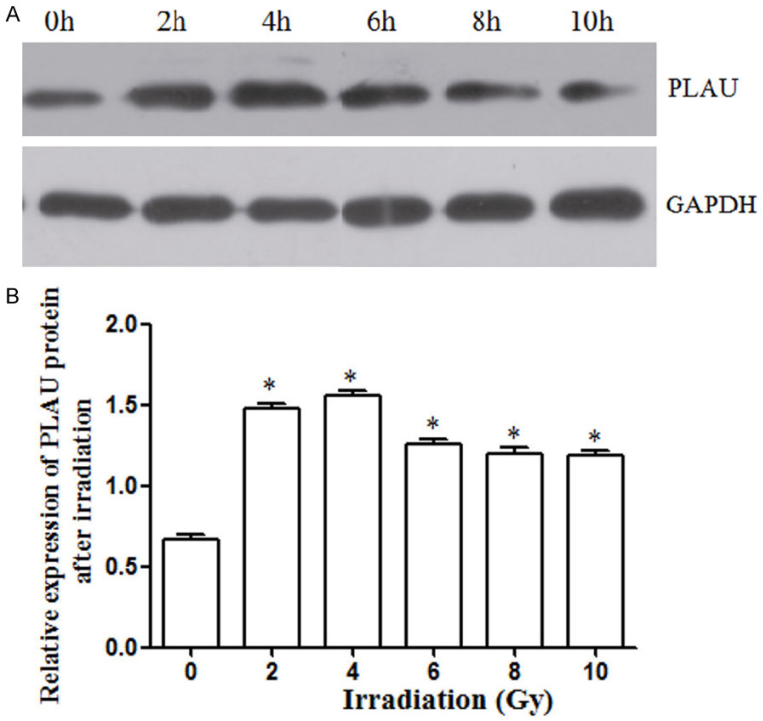

Figure 2. The expression of PLAU was increased after irradiation in TE-2 cells. A. The level of PLAU protein detected by Western blot after irradiation at dose of 8-Gy. B. The relative expression of PLAU protein was displayed, which normalized to GAPDH. Elevated PLAU protein was observed after irradiation, Columns mean, bars ± SD, **P*<0.05 versus group 0 h.

Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan) and cultured in RPMI-1640 (Life Technologies, Grand Island, NY, USA) with 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37°C.

Cell transfection

Lentiviral vectors pGLVH1/GFP+Puro for *PLAU* were provided by GenePharma (Shanghai, China). The TE-2 cells were seeded in 96-well plates and transfected with the *PLAU*-siRNA lentivirusesu (*PLAU*-siRNA group) and empty vectors (negative control group) using Lipofectamine 2000 reagent according to the manufacturer's instructions, respectively. The untreated cells were taken as blank control. 48 h later, the cells were harvested.

Western blot analysis

Total protein was isolated from cell lines TE-2, respectively. Then the protein was separated by SDS-PAGE gels. Transferred the brands on gels into nitrocellulose membrane. The membranes were blocked with 5% non-fat milk (Bio-Rad) and incubated with primary anti-PLAU antibody at 4°C overnight. Washed membrane and added into horseradish peroxidase-conjugated secondary antibody. Reaction with the enhanced chemiluminescence kit (Forevergen Biosciences, China) was used for detecting quantification of PLAU proteins. GAPDH was taken as internal controls.

Irradiation

The cells were seeded into 96-well plates and irradiated with 0, 2, 4, 6 or 8 Gy X-ray irradiation by linear accelerators at a dose rate of 1.15 Gy/min using a 250-KV orthovoltage until the following day (Philips, Amsterdam, The Netherlands).

Cell apoptosis assays

After transfecting with *PLAU*-siRNA and empty vector for 48 h, the cells were harvested and fixed with 2.5% glutaraldehyde for 30 minutes after irradiating X-rays for 12 h. Then the cell were conducted with routine embedment and section. The apoptosis rates were determined via Annexin V-FITC and PI staining flow cytometry.

Colony forming assay

The radiosensitivity of cells was analyzed bu colony forming assay. The transfected cells were resuspended and seeded into six-well plates at 200-6,000 cells/well depending on the dose of radiation. The cells were irradiated with 0, 2, 4, 6 or 8 Gy X-ray irradiation by linear accelerators at a dose rate of 1.15 Gy/min. After 7~10 days, the colony formation was formed. Then the cells were subsequently fixed and stained using crystal violet. The colonies number of the cells were counted.

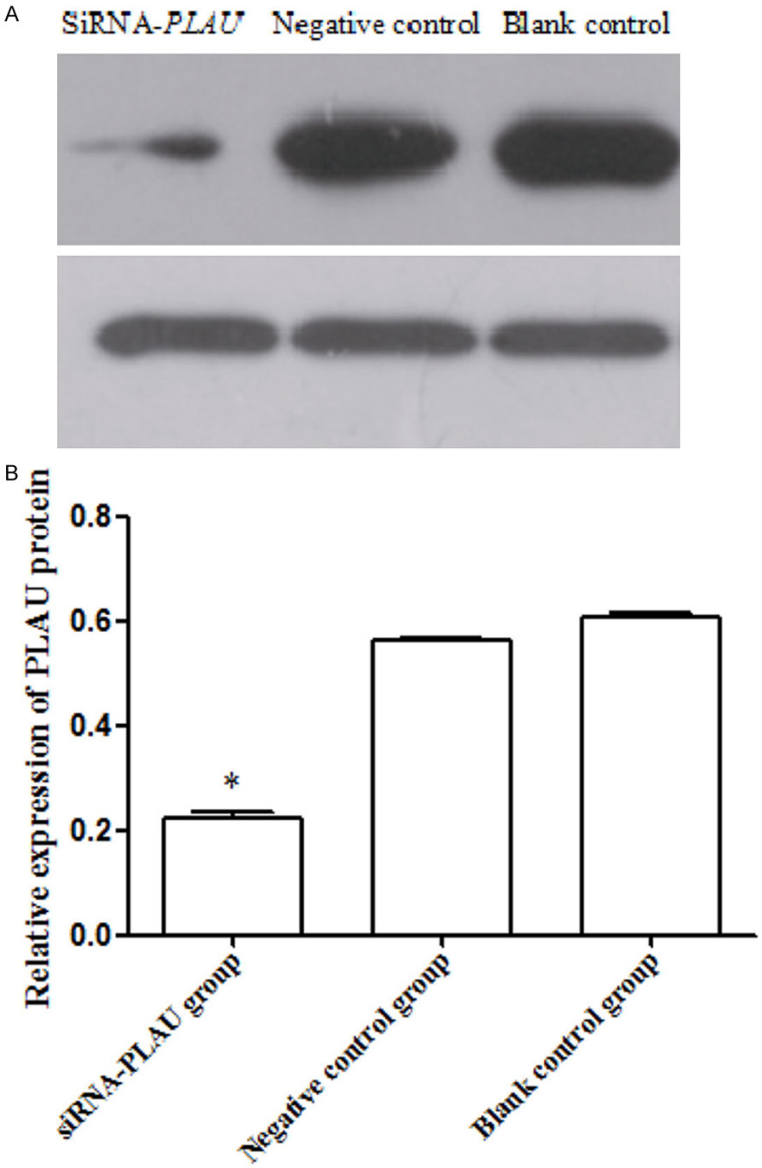

Figure 3. The levels of PLAU protein in transfected TE-2 cells. A. Total proteins were isolated from TE-2 cells at 48 h after transfection, and the expression of PLAU protein was detected by Western blot analysis. B. The relative expression of PLAU protein was shown, which normalized to GAPDH. The expression of PLAU in siRNA-PLAU group was significantly decreased compared with negative control group and blank control group. Columns mean, bars ± SD, **P*<0.01 versus Blank control group and negative control group.

Statistical analysis

All data were stated as Mean ± SD. Statistical analysis was conducted with SPSS version 13.0 software. Experimental results were analyzed using students' t-test or one-way ANOVA. All cell culture experiments were performed in triplicate. *P*<0.05 was considered to be statistically significant.

Results

The expression of PLAU in EC tissues

The expression of *PLAU* was detected in EC tumor tissues and adjacent tissues by immunohistochemical staining. As shown in **Figure 1** and **Table 1**, the positive rate of *PLAU* was 76.6% (72 positive in 94 patients) while the negative rate was 23.4% (22 negative in 94 patients). This result demonstrated the *PLAU* was over-expression in EC tissues compared with in adjacent non-cancerous tissues. These revealed that *PLAU* was related to EC and it might be an oncogene in EC.

Correlation between PLAU expression and radiotherapy response

Among the 94 patients in this study, there were patients in the effective group while others were in resistant group. The therapeutic response rate was 68.1% which indicated that PLAU expression was significantly correlated with radiotherapy response (*P*<0.05, **Table 2**).

PLAU expression was increased after irradiation in TE-2 cells

To explore the correlation between PLAU expression and the irradiation response of EC cells, we examined the PLAU expression in TE-2 cells with a dose of 8 Gy at 0, 2, 4, 8 and 12 h. The PLAU expression was obviously up-regulated after receiving irradiation which might showed that PLAU expression could influence

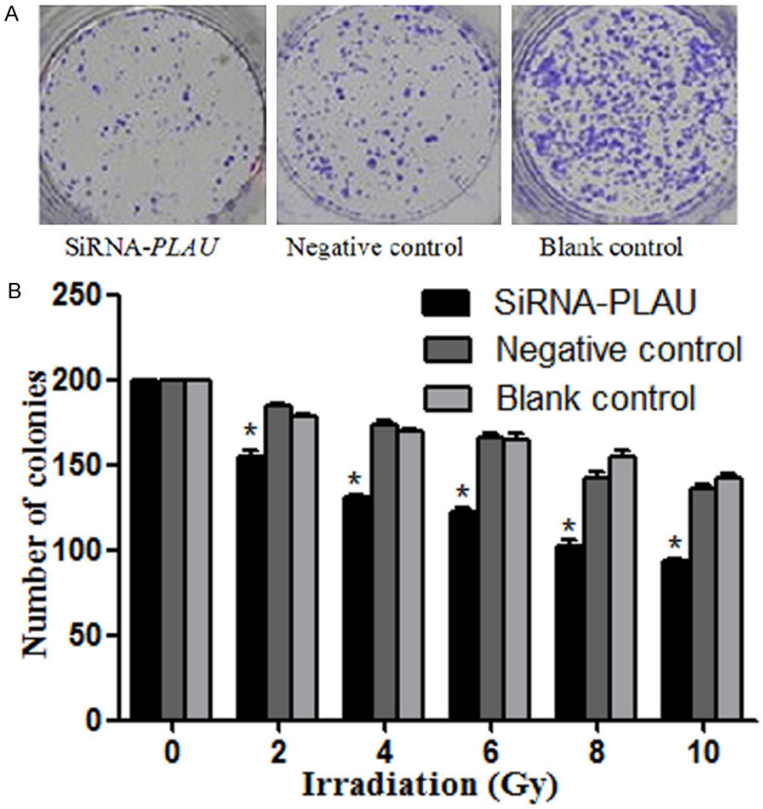

Figure 4. The effects of PLAU on colony formation. A. The colony forming assay was taken to estimate the colony ability of TE-2 cells after transfection. B. The colonies number was obviously less in siRNA-PLAU group than in negative control group and blank control group. Columns mean, bars ± SD, **P*<0.01 versus group 0 Gy.

the radiosensitivity of TE-2 cells (**Figure 2A** and **2B**).

The effects of PLAU on the radiosensitivity of EC cells

To analyzed the effects of *PLAU* on the sensitivity of EC cells to irradiation, we measured the colony forming rate. Firstly, we examined the inhibited effect of lentiviral on the expression of *PLAU*. The down-regulated expression of *PLAU* was found in cells of siRNA-*PLAU* group compared that in negative control group and blank control group (**Figure 3A** and **3B**). Then the colony forming assay was made after varying doses of irradiation exposure. The colonies number of the cells in siRNA-*PLAU* group were significantly less than those in negative control and blank control group with a dose-dependent manner of irradiation (**Figure 4A** and **4B**). These results indicated the down-regulation of *PLAU* expression could increase the radiosensitivity of TE-2 cells to irrradiation.

Down-regulation of PLAU increased irradiation-induced apoptosis of EC cells

The apoptosis rate of TE-2 cells was examined to estimate the possible mechanism of the influence of *PLAU* on radiosensitivity of EC cells. The TE-2 cells were treated with irradiation with a dose of 8 Gy. 12 h later, the apoptosis rate of TE-2 cells was detected by flow cytometric assay. The outcome manifested that the apoptosis rate of TE-2 cells transfected with siRNA-*PLAU* was notably increased compared with those in negative control and blank control groups (**Figure 5A** and **5B**).

Discussion

In current study, we found PLAU was up-regulated in tumor tissues compared with adjacent non-cancerous tissues. And the over-expression of PLAU decreased the radiosensitivity of TE-2 cells of EC. Moreover, knocking down PLAU expression using lentivirus-mediated siRNA targeting PLAU induced their susceptibility to radiation-induced apoptosis. These findings suggest that PLAU act as inhibitor of radiosensitivity in EC.

Radiotherapy is an optimal treatment of EC. Recent studies proved it provides a choice of definitive chemoradiotherapy as their first-line and primary treatments for patients with EC [12]. However, not all patients have susceptibility of radiotherapy and some may be show either no response or experience adverse effects of this therapy [13]. Once the latter situation occurred, the timely treatment may have been delayed and even led to the death of patients. Radioresistance is another condition of patients with EC faced in radiotherapy. The existence of radioresistant often causes local recurrence of tumors which weakened the effectiveness of radiotherapy [11]. And the factors give rise to radioresistant include tumour size, hypoxia, intrinsic radiosensitivity cell cycle

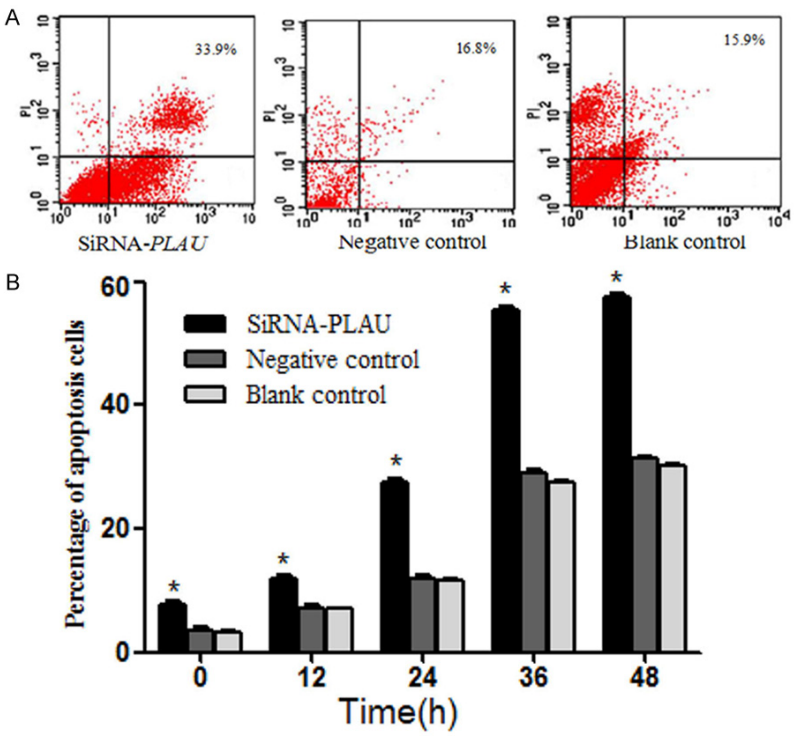

Figure 5. The effects of PLAU on apoptosis after irradiation. A. After irradiation, cell apoptosis was detected by flow cytometry analysis at different time points. B. The graph indicated 8-Gy irradiation induced apoptosis of TE-2 cells. While, cells in siRNA-PLAU group increased apoptosis after irradiation than that in negative control group and blank control group. Columns, mean; bars ± SD, **P*<0.05 versus negative control group and blank control group.

arrest, apoptosis, and differentially expressed genes between radioresistant cells and radiosensitive cells, and so on [11, 14, 15]. Thus, exploring the differentially expressed genes and estimating their expression may be helpful for esophageal cancer therapy. It is also meaningful for the identification of reliable markers of radiosensitivity and the key molecules that enhance radiosensitivity in EC cells.

In previous studies, there were many reports about the regulation of radiosensitivity of EC cells by some genes or other special moleculars [14, 16-22]. PLAU is a multifunctional serine protease and had been explored in many diseases such as quebec platelet disorder, colon cancer, colorectal cancer and alzheimer's disease [23-26]. Most studies focused on its assistant regulation role or gene polymorphism. Recently, several studies about the effect of PLAU on therapy sensitivity have been covered. For instance, PLAU genes were found to be involved in apoptosis evasion and might contribute to radioresistance in the mesenchymal stem cells [27]. Jerhammar et al., identified the role of PLAU as a hub gene in ingenuity pathway analysis network and was important to intrinsic radiosensitivity in head and neck squamous cell carcinoma [28]. These finding revealed that PLAU is a key radiosensitizer in cancer cells.

As patients can still develop recurrent cancer because of radioresistant [29]. The understanding of the molecular mechanisms of cancer underlying radiation sensitivity or resistance is necessary. Cell apoptosis is an autonomic ordered programmed cell death in order to maintain homeostasis, which is controlled by serial genes. It involves complicated regulatory mechanism. The disorder of apoptosis may causes the occurrence and development of many diseases and it may be caused by many factors. Among them, radiation which is one of the main therapy method s in cancers plays an vital role. Therefore, the research of apoptosis in EC cells after irradiation might benefit for in the understanding of the mechanisms of PLAU-related radiosentivity. Through flow cytometry, we preliminary inferred that PLAU regulated the radiosentivity of EC cells via controlling cell apoptosis.

In conclusion. PLAU is over-expression in EC patients and its regulation can decrease the radiosensitivity of EC cells to irradiation. Meanwhile, these effects of PLAU in EC may be realized via regulating cell apoptosis. Due to the limitation of sample size and other unfavorable factors, further replication experiments will be need in future.

Disclosure of conflict of interest

None.

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