Journal of Clinical and Experimental Cardiology

Yang et al., J Clin Exp Cardiolog 2017, 8:12
DOI: 10.4172/2155-9880.1000561

Research Article Open Access

Liquiritigenin Enhances the Radiosensitivity of Nasopharyngeal Carcinoma Cell by Affecting Its Autophagy

Ke Yang*, **Yujie Li, Min Yu and Xu Sun**

Zhengzhou University Affiliated Zhengzhou Central Hospital, Zhengzhou City, Henan Province, P.R. China

***Corresponding author:** Ke Yang, Zhengzhou University Affiliated Zhengzhou Central Hospital, Zhengzhou City, Henan Province, 450007, P.R. China, E-mail: 296028306@qq.com

Received date: December 11, 2017; **Accepted date:** December 13, 2017; **Published date:** December 15, 2017

Abstract

Objective: To investigate the effect of licorice on radiosensitization of nasopharyngeal carcinoma cells and its mechanism.

Methods: MTT assay was used to detect the effect of different concentrations of glycyrrhizin on the activity of nasopharyngeal carcinoma cells. The changes of autophagy were observed by transmission electron microscopy (TEM) in the treatment of nasopharyngeal carcinoma cells. Western blotting was used to detect the effect of glycyrrhizin on the level of autophagy protein in nasopharyngeal carcinoma. Flow cytometry was used to detect the changes of apoptosis rate of nasopharyngeal carcinoma cell line after radiotherapy.

Results: After successfully constructing CNE-2-RR radiation resistant cell line, MTT assay was used to detect the best inhibition rate of 20 mmol/L glycyrrhizin on nasopharyngeal carcinoma (58.86 ± 5.02)%. Transmission electron microscopy showed that the number of autophagosomes increased and the mitochondria and nuclei were abnormal in the treatment of nasopharyngeal carcinoma cells. Western Blotting was used to detect the increase of LC3-II level and the decrease of LC3-I level in nasopharyngeal carcinoma cells of 20 mmol/L concentration group.

Conclusion: Glycyrrhizin enhances its sensitivity to radiotherapy by influencing autophagy and DNA repair ability of nasopharyngeal carcinoma cells.

Keywords: Licorice; Nasopharyngeal carcinoma; Autophagy; Radiosensitivity

Introduction

Nasopharyngeal carcinoma (NPC) is a worldwide disease, and has the highest morbidity in the Southeast Asia. In China, nasopharyngeal carcinoma has a relatively high mortality in the south region [1,2]. At present, radiotherapy is a main treatment to nasopharyngeal carcinoma, and its treating effects will be improved by adjuvant treatment with chemical medicine [3,4]. However, in most cases, the radioresistance of nasopharyngeal carcinoma cells is very common, which seriously blocks the treating effects [5]. Therefore, to explore the mechanism of radioresistance and to improve the radiosensitivity of nasopharyngeal carcinoma cells are popular topics in clinical researches.

Autophagy is a process of cell disintegration and metabolism, which involves in the maintenance of cell growth and the micro-balance state of cell product synthesis, degradation, and recycling [6]. The autophagy process begins with the formation of autophagosome and autophagic vacuole, which deliver the cellular content to organelle to be gradually degraded, and then participated in the molecular recycling process [7]. Recent researches revealed that autophagy plays important roles in the immune response, inflammatory response, cardiovascular disease, malignant tumor and neurodegenerative diseases [8-10]. However, the role of autophagy in malignant tumor disease is a debatable point. Some studies showed that autophagy-related gene knock-out will accelerate the development of malignant tumor, and the autophagy-related gene deficient mice have higher sensitivity to radiotherapy and even immunotherapy [11,12].

Liquiritigenin is a flavonoids compound extracted from Glycyrrhiza, a Chinese medicine. Liquiritigenin showed various biological activities such as anti-tumor, anti-virus, anti-oxidation of free radical, and inhibitory effects of lipid peroxidation [13]. In recent years, some studies found that Liquiritigenin can enhance the chemotherapy and radiotherapy sensitivity of different cell lines, which to some extent enhanced the clinical therapeutic efficacy [14]. However, there's no research about the mechanism and effect of Liquiritigenin to the radioresistance of nasopharyngeal carcinoma cell lines.

In the present research, we treated the nasopharyngeal carcinoma cells with moderate concentrations of Liquiritigenin, and then tested the influences of radiation to the formation of autophagosome, DNA damage and cell apoptosis. We demonstrated the effects and mechanism of Liquiritigenin to the radiosensitivity of nasopharyngeal carcinoma cells, and also illustrated the correlativity of the formation of autophagosome and the radiosensitivity of nasopharyngeal carcinoma cells, which provided a new reference direction of radiotherapy.

Materials and Methods

Cell lines and reagents

The Human Nasopharyngeal Carcinoma Cell Line CNE-2 was obtained from Chinese Academy of Sciences (Shanghai, China). RPIM 1640 medium was purchased from Thermo Fisher (USA). The fetal bovine serum (FBS) was purchased from Si jiqin (Hangzhou, China). LC3 (ab48394) and GAPDH (ab8245) antibodies were both purchased from Abcam (USA). The MTT reagent and Trizol were purchased from Sigma (USA) and Ambion (Ambion Inc., Austin, TX, USA), respectively. The reverse transcription kit (FSQ-101) was obtained from TOYOBO (TOYOBO, Japan), and FITC kit was purchased from Sigma (KapaBiosystems Inc., Boston, US).

MTT assay

The cell proliferation experiment was conducted with MTT method to detect the inhibitory effects of different concentrations of Liquiritigenin to the nasopharyngeal carcinoma cell viability. The M14 cells were seeded to 96-well plates with an initial concentration of 3×10^4 cell per well. After 24 h, the cells were treated with different concentration of Liquiritigenin (5, 10, 15, 20, 25 mmol/L) for 24 h or 48 h. The cells were then incubated with MTT for 4 h, afterwards, the cells were centrifuged, the supernatant was removed, and then 150 μL DMSO was added. The absorbency (Abs) of each well was measured at 37. The CompuSyn software was applied to calculate the combinatorial index (CI) and the MTT value.

Plate clone forming assay

The CNE2 and CNE-RR cells were planted in 6-well plate, respectively, and were cultured in the incubator overnight. After the cells adhered to the plate and showed a good condition, the cells were irradiated with X-ray of 0, 2, 4, 8 Gy, and then were continuing cultured in incubator for 10-14 days. The cells were then stained with 10% Giemsa, the cell clones above 50 cells were counted, the surviving fraction was counted, and the surviving curve was drawn.

Transmission electron microscopy detect autophagosome

The transmission electron microscopy (H-600 IV, Hitachi, Tokyo, Japan) was adopted to analysis the ultrastructure image of autophagosome. The CNE-2 cells were harvested after trypsin digestion, washed with PBS for twice, and then fixed in cold glutaraldehyde (in pH 7.4, 3% dichloromethyl sulfonate buffer) for 24 h. The cells were fixed in OsO_4, dehydration with 70-100% acetone grading series, and double stained with uranyl acetate and citric acid trihydrate. The scan attachments were observed with transmission electron microscopy.

Western blotting

The CNE-2-RR cells were washed twice with cold PBS, and lysed at 4 for 15 min, and then centrifuged at 12,000 rpm for 30 min. The protein concentration was measured by BCA protein assay kit. Equivalent protein (25 μL) were loaded to 15% SDS-PAGE, and then transferred to PVDF membrane after electrophoresis. The membranes were blocked with 5% milk for 2 h, and then incubated with LC3 antibody (with a concentration ratio of 1:2000), and GAPDH antibody (with a concentration ratio of 1:800) overnight, respectively. The next day, the membranes were washed with PBS, and then incubated with fluorescence labeled goat anti-mouse IgG (with a concentration ratio of 1:5000) for 2 h, afterwards the membranes were washed with PBS, and then ECL kit was used to imaging and the fluorescence intensity of the protein was detected, the experiment was repeated for 3 times.

Comet assay detect DNA damage

The exponential phase cells in control group and Liquiritigenin group were seeded in 6-well plates with a density of 5×10^5. After cells were attached and showed a good condition, the cells were irradiated with X-ray of 0 Gy and 10 Gy, and then were cultured in the cell incubator. After the irradiation for 1 h and 6 h, respectively, the cells were harvested, digested and centrifuged. The cells were re-suspended in 1 ml cold PBS at a dark and low temperature environment. The Comet Assay Kit was obtained from Invitrogen, low melting point agarose (LMA) was melt in boiling bath for 5 min, after completely dissolved, LMA were placed at 37 for 20 min. 20 μl cell suspension and 80 μl LMA were mixed and placed in dark at 4 for 10 min. A freshly prepared and precool cell lyses buffer was gently added, and the cells were lysed overnight at 4. The water side by side the slide was took out from cell lyses buffer, and be placed at freshly prepared DNA unwinding liquids in the dark at 4 for 1 h. The glass slides were took out and were placed in the electrophoresis tank, the side with marker faced negative pole (black). 500 ml running buffer was poured gently at 21 V for 30 min in the dark environment. After rinsing completely, the cells were stained, 100 μl DAPI at work condition was added to each well, then cells were stained at room temperature for 30 min, and completely washed with distilled water for 3 times, then the slides were dried at 37 in the dark. The slides were observed in fluorescence microscope, each sample were randomly pictured 100 cells and then be preserved. The experiment results were analyzed with CASP software.

Flow cytometry detect cell apoptosis

The nasopharyngeal carcinoma cells were washed twice with PBS, centrifuged at 1800 rpm for 5 min, the cells were suspended in 500 μl binding buffer (Annexin V-FITC), and were detected in a buffer of 5 μl Annexin V-FITC and 5 μl PI. The phosphatidylserine exposed in cytoplasma membrane was detected. After incubated in light protection zone for 15 min, the cell apoptosis rate was detected by flow cytometry.

Statistical analysis

All statistical analysis was conducted with SPSS 16.0 software (SPSS, ChicagoIL). A value of $p<0.05$ was considered significant. Variance analysis was applied to compare different groups. GraphPadPrism5 was applied for making statistical diagram.

Results

The establishment of radioresistant nasopharyngeal carcinoma cell line

The nasopharyngeal carcinoma cell line CNE-2 was cultured in vitro, after the cell confluence is 60-70%, and the cells were in logarithmic growth phase, the cells were received moderate radiographic exposure. After the exposure, the cells were cultured in the incubator, the cell conditions were closely observed. After the cell states were recovered well, another exposure was conducted, above steps were repeated, and each time the dose of exposure was greater

than the last one, until the nasopharyngeal carcinoma cell line CNE-2 which has a stable tolerance capacity to radiation was established, at this time, we name the selected cells as CNE-2-RR (CNE-2 radiation resistance). The subsequent experiments were conducted with cells in good condition which passaged for 5 to 10 generation (Table 1).

	Exposure dose and times					Total	
Radiation	2 Gy	4 Gy	6 Gy	8 Gy	10 Gy	Dose	Times
X-Ray	2	2	2	2	2	60 Gy	3 Min

Table 1: The method to established the radioresistant nasopharyngeal carcinoma cells.

The inhibition of different concentrations of Liquiritigenin to nasopharyngeal carcinoma cells

The inhibition of different concentrations of Liquiritigenin to nasopharyngeal carcinoma CNE-2-RR cells was measured by MTT. The most appropriate drug action concentration of Liquiritigenin was confirmed. The experiment results showed that the growth of CNE-2-RR cells showed different extents of inhibition, and the inhibition rates was increased as the Liquiritigenin concentration increases, and in some range showed a concentration dependent ($P<0.05$, Table 2). The best inhibition rate of Liquiritigenin to CNE-2 cells was (58.86 ± 5.02)%, thus the concentration of 20 mmol/L Liquiritigenin was the best concentration.

The mass concentration of Liquiritigenin (mmol/L)	The cell inhibition rate (%)
5	8.62 ± 1.15
10	16.95 ± 2.81
15	36.77 ± 6.24
20	58.86 ± 5.02*
25	42.31 ± 2.98

Table 2: To compare the inhibition rate of Liquiritigenin in different concentrations; *$P<0.05$

Plate clone forming to detect the radioresistance of CNE2-RR cells

As shown in Figure 1A, the clone number of CNE2-RR cells is more than the radiation sensitive CNE2 cells, CNE2-RR cells showed a stronger radioresistance. The dose survival curve showed that CNE2-RR showed a slow descending trend when compared with the radiation sensitive CNE2 cells. The results showed that the sensitivity of CNE2-RR is lower than CNE2 cells, the radioresistance of CNE2-RR cells are stronger than CNE2 cells, and the radioresistance is hereditary.

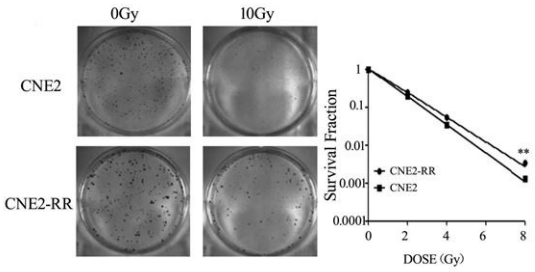

Figure 1A: Plate cloning test to detect the radiation resistance of nasopharyngeal carcinoma cells.

The accumulation situation of autophagosome in nasopharyngeal carcinoma cells induced by radiation

After the cells were treated with radiation, a concentration of 20 mmol/L Liquiritigenin was added to dispose the cells. 48 h later the autophagosome situation was contrasted. The autophagosome situation in control group and Liquiritigenin treated group were showed in Figure 1B. The subcellular structure analysis results showed after treating with Liquiritigenin for 48 h, the cell autophagosome in the control group displayed a typical morphological character. In the CNE-2 cells treated with 10 Gy radiation and Liquiritigenin combination therapy, the number of autophagosome showed an augment, and a morphological abnormality of the nucleus. In the non-Liquiritigenin-treatment after 10 Gy radiation control group, the nasopharyngeal carcinoma cells showed a normal mitochondria and cell nucleus surrounded by cytoplasm, and the autophagosome was observed sometimes.

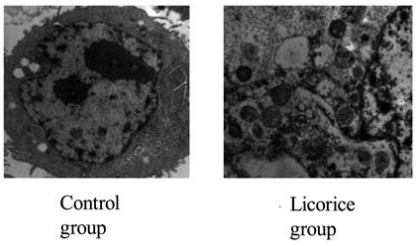

Figure 1B: Electron microscopy was used to detect the normal nuclei and mitochondrial morphology of nasopharyngeal carcinoma cells in different treatment groups.

The expression of autophagy protein in nasopharyngeal carcinoma cells was observed by Western blotting

In order to further confirm the effect of Liquiritigenin to autophagosome of nasopharyngeal carcinoma cells after the cells were received radiation, and to detect the protein LC3-I transformed to LC3-II situation in CNE-2-RR cells. The western blotting results showed that, when compared with the control group, the LC3-II/LC3-I protein level value in Liquiritigenin group showed an increase (Figure 2A). The LC3-II level is related to the extent of autophagosome

formation. The western blotting results showed that LC3-II level increased, and LC3-I level decreased ([(0.15 ± 0.01)% *vs.* (2.78 ± 0.59)%, P<0.05]), which suggested the autophagy was formed and the structure of lysosome was destructed, and the autolysosome was cumulated. The differences between two groups showed a statistical difference. The results showed that after the cells were treated with Liquiritigenin, in some extent Liquiritigenin will promote the formation of autophagosome, and increase the sensitivity of nasopharyngeal carcinoma cells to radiotherapy.

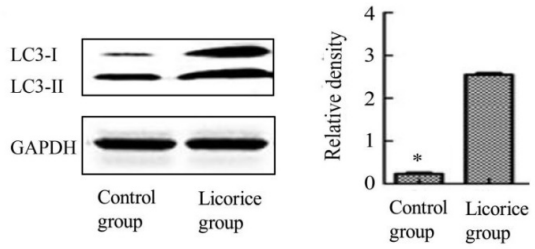

Figure 2A: Effect of glycyrrhizin on the expression of autophagocyanin in nasopharyngeal carcinoma cells.

The DNA damage situation of nasopharyngeal carcinoma cells in different groups after exposing to radiation detected by single cell gel electrophoresis

After the CNE-2-RR cells were treated with Liquiritigenin, the cells were divided into two groups: NC group and Liquiritigenin group. After 1 h or 6 h 10 Gy radiation, the nasopharyngeal carcinoma cells were collected to execute comet assay, the results were showed in Figure 2B. After the cells were radiated for 1 h, the cell nucleus in both two groups showed obvious comet tails, the comet head is large, and the comet tail showed a state of slightness. After the cells were radiated for 6 h, the cell nucleus in both two groups were shorter, which means the cells in NC group and Liquiritigenin group both have a certain degree of repair capacity. When the cells were radiated for 1 h and 6 h, the tail distance in Liquiritigenin group was longer than NC group, which suggested that the radiosensitivity of cells in the Liquiritigenin group was stronger than that in the NC group.

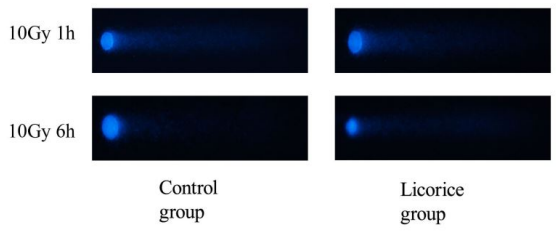

Figure 2B: Radiation induced by DNA damage in CNE-2-RR cells.

The cell apoptosis detected by flow cytometry

To investigate the radiosensitive effects of Liquiritigenin to CNE2-RR cells, the control group and Liquiritigenin group were treated with different dose of radiation. The differences of apoptosis between two different groups were detected by flow cytometry, the results were showed in Figure 3A. In the same radiation dose, the apoptosis rates of CNE-2-RR cells treated with Liquiritigenin was obviously higher than the control group, the results showed significantly differences ([0 Gy (15.32 ± 1.05)% *vs.* (42.15 ± 3.75)%, P<0.05; 10 Gy (27.16 ± 2.65) % *vs.* (58.62 ± 5.09)%, P<0.05]), which demonstrated that in the same radiation dose, the CNE2-RR cells treated with Liquiritigenin is more sensitive.

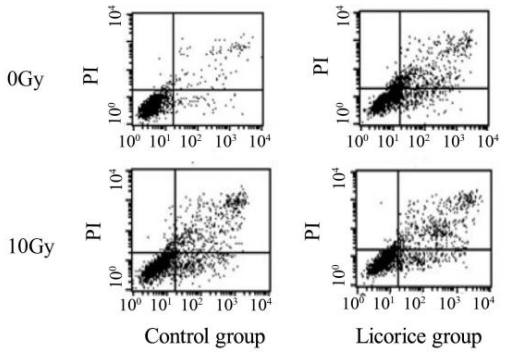

Figure 3A: Flow cytometry to detect the effect of licorice on radiosensitivity of nasopharyngeal carcinoma cells.

The DNA damage induced by ionizing radiation was detected by immunofluorescence

As we found that after the ionizing radiation, the expression of γ-H2AX protein in CNE2-RR cells was obviously lower than that in the CNE2 cells. For further observe the cells and the formation of γ-H2AX protein aggregation point, the changing situation of γ-H2AX protein aggregation point before and after ionizing radiation was detected by immunofluorescence. The results showed that ionizing radiation obviously promotes the formation of γ-H2AX protein aggregation point, but when compared with the radiosensitive CNE2 cells, the expression of γ-H2AX protein aggregation point in the radioresistant cells was obviously decreased (Figure 3B), which consistent with the flow cytometry results.

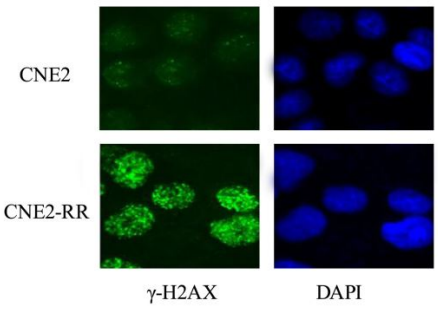

Figure 3B: Immunofluorescence Detection of DNA Damage Induced by Ionizing Radiation in Nasopharyngeal Carcinoma Cells.

The migration of cells was detected by wound healing assay

The wound healing assay results (Figure 4A) showed that in the same radiation dose, the migration rates of CNE2-RR cells in Liquiritigenin treated group was obviously lower than that in the control group, which showed a significantly difference. The results suggested that in the same radiation dose, the decline of CNE2-RR cell migration capacity was more obvious after the cells were treated with Liquiritigenin.

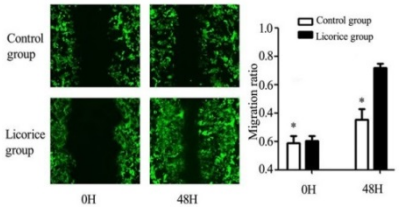

Figure 4A: Scratch healing test to detect the ability of Hotair to migrate to nasopharyngeal carcinoma cells.

The cell invasion ability was detected by Transwell invasion assay

The Transwell invasion assay results displayed (Figure 4B) that in the same radiation dose, the amounts of CNE2-RR cell invasion in Liquiritigenin treated group was evidently less than those in the control group, and the differences between the two groups were significant. The results showed that after treated with Liquiritigenin, the invasion ability of CNE2-RR cells was obviously decreased.

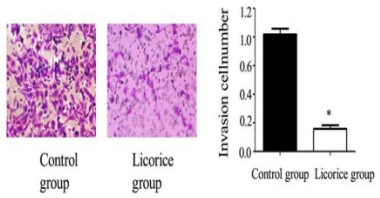

Figure 4B: Transwell invasion assay to detect the ability of Hotair to invade nasopharyngeal carcinoma cells.

Discussion

Nasopharyngeal carcinoma is one of the most common head and neck cancers that threatens human healthy globally. Nasopharyngeal carcinoma showed significant geographical aggregation, the type of this disease is mainly poorly differentiated squamous cell carcinoma [15]. According to the incomplete statistics of WHO, the incidence rate of nasopharyngeal carcinoma in China is far more than the rest areas of the world. China has a 75% of the world nasopharyngeal carcinoma patients [16]. The clinical therapeutic regimen of nasopharyngeal carcinoma patients is mainly radiotherapy, with chemotherapy and biotherapy as adjunctive therapies. However, in recent years, the number of nasopharyngeal carcinoma radiotherapy resistant patients was increased gradually, and the side effects of radiotherapy showed a growing trend [17]. Although with the improvement of radiotherapy equipment and radiotherapy techniques, the therapeutic effects of nasopharyngeal carcinoma cells have been ameliorated obviously. However, due to lack of molecule markers which can predict radiosensitivity and many nasopharyngeal carcinoma radiotherapy resistant patients in clinical practice, there are many problems remain in the clinical therapy of nasopharyngeal carcinoma. The relationships between some Chinese herb extracts and radiotherapy sensitive malignant tumor are an important direction of the basic research of the radiation oncology, but the specific mechanism of radioresistance in nasopharyngeal carcinoma patients remains unclear [18]. Thus, the mutual relation between Chinese herb extracts and radiotherapy sensitivity of malignant tumor is an important direction of solving clinical therapy and enriching radiotherapy regimen. In the nasopharyngeal carcinoma cell line, the CNE2 cells are belong to the poorly differentiated squamous-cell carcinoma, which are sensitive to the radiation, thus, in the present research, we choose CNE2 cells to build a radioresistant cell line for further research.

Cell apoptosis is a natural process of programmed cell death, and the autophagy is a natural process of programmed cell survival [19]. However, the abnormal autophagy behavior in cells will damage the cells and influence the cell function, even in some cases, the autophagy will induce cell death [20]. At present, with respect to the relationship of autophagy and tumor cells radiosensitivity, some scholars consider that in some extent, the increase of cell autophagy will enhance the effect of radiation [21]. Chen and other scholars [22] found that inhibit the autophagy of esophageal cancer cell line will increase the toxic effects of radiation to cells, and will accelerate the development of G2/M cell cycle, thus will increase the resistance of esophageal cancer cells to radiation. Some other researchers point out that, the induction of autophagy will ameliorate the efficacy of breast cancer cells, which will help improve the radiation effects of breast cancer cells [23,24]. However, there are few researches about the relationships of Chinese extract herb Liquiritigenin with the autophagy and radiosensitivity. The present research has a potential value of clinical research and application.

The results in present study demonstrate that after treated with Liquiritigenin, the nasopharyngeal carcinoma CNE-2-RR cell line can induce the increase the level of cell autophagy, the mitochondria and karyomorphism were damaged in some extent, the ratio of autophagy protein LC3-II/LC3-I showed an increasing trend. LC3-II is the only protein that exists in autophagosome and autolysosome membrane, the increasing ratio of LC3-II/LC3-I verified that the autophagy level is increasing [25]. The present research detects the number and morphology of autophagosome by transmission electron microscope, which is a golden standard to detect autophagosome [26], and further confirmed the analysis results of detecting autophagy protein by western blot. The present study showed Liquiritigenin in some extent will influence the happen behavior of autophagy after the CNE-2 cells have taken radiation, and thus stimulate the nasopharyngeal carcinoma cells to enhance the sensitivity to radiation.

The single cell gel electrophoresis is also called comet assay, which is a common technique to detect the DNA damage of tumor cells. This assay also can speculate the repair capacity of DNA damage by detecting the injury degree of double strand DNA [27]. Single cell gel electrophoresis in the present study showed that after the nasopharyngeal carcinoma cells were treated with Liquiritigenin, the repair capacity of DNA damage after radiated stimulation is inhibited in some extent, and the radiosensitivity of the cells in Liquiritigenin

group is stronger than that in the NC group. The flow cytometry results showed that after the cells were treated with Liquiritigenin, the apoptosis rate of the nasopharyngeal carcinoma cells after the radiation was obviously increased, which indicates that Liquiritigenin enhance the radiosensitivity of nasopharyngeal carcinoma cells, and promote the tumor cell apoptosis. Combining the above experiment results, we can speculate that Liquiritigenin may regulate the radiosensitivity of nasopharyngeal carcinoma cells through influencing the autophagy behavior of the cells.

In conclusion, we discussed Liquiritigenin regulate the repair capacity of DNA damage and cell apoptosis changing behavior after the radiation, and then enhance the sensitivity of nasopharyngeal carcinoma cell to radiation through influencing the autophagy behavior of nasopharyngeal carcinoma CNE-2 cell, the radioresistant cell line. The results indicated that Liquiritigenin in moderate concentration will contribute to the radiosensitization of nasopharyngeal carcinoma CNE-2 cell. The conclusions in the present research provide a new reference direction for clinical adjuvant therapy, but we need further animal and clinical studies to test the conclusions in the present research.

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