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Original Research

The regulatory effects of autophagy to the CNE2 cells radio-sensitization

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Abstract: To investigate the effects of autophagy activator and autophagy inhibitor on the CNE2 radiation sensitivity of nasopharyngeal carcinoma cells. RNA interference technology was used to silence the *atg*5 gene and autophagy inhibition cell model was constructed. Rapamycin and chloroquine were treated respectively on cells with X-ray 5Gy irradiation. Cells' growth status were observed for 8 days and control group was set. The cell viability was detected by MTT assay and colony formation assay, and the cell cycle was analyzed by flow cytometry. Compared with the control group, the survival rate, clone formation rate and the survival rate of the irradiation of the other three groups were significantly lower. (P<0.05) Most cells were detected in the G0/G1 phase in the other three groups except the control group, and cells of the other two periods were less than those in the G0/G1 phase. The autophagy inhibitor or activator and *atg*5 silencing can be increased by CNE2 radiation therapy, however, the sensitization effect increase of autophagy activator is better than others.

Key words: Autophagy, nasopharyngeal carcinoma, radiotherapy sensitization, rapamycin, chloroquine.

Introduction

Autophagy is a self degradation process of cells in the absence of energy, metabolism, and so on (1). Autophagy has not only the function of maintaining the cell homeostasis, promoting cell survival, but over up-regulation of autophagy can also lead to cell death (2). Autophagy can be induced by radiotherapy, however the role of autophagy in the regulation of radiation sensitivity is not clear. In this study, the effects of combined or solo radiation therapy on nasopharyngeal carcinoma cells were studied in vitro experiments to clarify the relationship between autophagy and radiotherapy in nasopharyngeal carcinoma, provide the experimental basis for the enhancement of the sensitivity of the radiation, and find a new method for increasing the sensitivity of radiotherapy.

Materials and Methods

Materials and equipment

Fetal calf serum was obtained from Hyclone. Penicillin, streptomycin, glutamine, rapamycin, chloroquine, RPMI-1640 medium, MTT powder were obtained from Shanghai bio engineering technology services Co., Ltd. DNA extraction kit, PCR kit, Plasmid Extraction Kit, RNAi Kit were obtained from Abnova company. RT-6100C Rayto enzyme linked immunosorbent assay and PCR instrument from ABI company, three gas incubator (SANYO), laser scanning confocal microscope, linear accelerator (SIEMENS) and flow cytometry (BD) were used.

Cells culture

Human nasopharyngeal carcinoma cell line CNE2 was obtained from cancer prevention and control center of Zhongshan University in China. Cell was incubated with RPMI-1640 medium with 10% fetal calf serum, 10μg/mL penicillin and 2mmol/ml streptomycin in the saturation humidity constant temperature incubator set as 36°C, 7.5% CO2, 95% air. Cells in logarithmic growth phase were tested.

RNAi modeling

Autophagy related gene *atg*5 is a key molecule in the process of the extension of autophagy. *atg*5 RNAi included plasmid construction, transfection of plasmid and siRNA. 1. plasmid construction: cell gene DNA extraction and determination of target gene; specific primers design; PCR fragment amplified; objective fragments to recover and purify; enzyme digestion; the connection of the reaction; the construction of the plasmid; the plasmid transformation; the small amount of plasmid extraction, the identification of the recombinant plasmid; generous plasmid extraction.2. transfection of plasmid and siRNA: In 6 well plates per hole inoculated with 1 x 105 CNE2 nasopharyngeal carcinoma cells in complete medium cell growth to 40-60% fusion. Cells were transfected with recombinant DNA 4mg and liposome 10ml for 24h. Then the complete culture medium was replaced. After 48h incubation, selective medium (G418 concentration: 1-3d for 250mg/L, then 550mg/L) was used to screen resistant clone. After 18d, Individual clonal was chosen for expansion incubation to build stable *atg*5 RNAi transfected cell line.

Experimental grouping

There were four groups, control group, mTOR inhibitor rapamycin plus radiotherapy group, Chloroquine plus radiotherapy group, *atg*5 RNAi plus radiotherapy group. CNE2 cells in logarithmic growth phase were passaged, the concentration of cells was every 3×10^5/mL. Cells were seeded in 96 hole plate, 90 μL/hole. Then 1×104 cells of each group were seeded in 24 hole

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plate. Cells of 3 hole pates were digested every 24h and numbers were counted. Radiotherapy was performed by accelerator set as 6mv photon line with 5Gy. The cells'growth situation was counted for 8 days, with cell number as the ordinate, time as coss sitting to draw the growth curve.

Cell viability detected by MTT

The absorbance A value of the 490nm wave of each hole was measured by RT-6100C Rayto enzyme linked immunosorbent assay. Soft agar colony formation: 5 g/L agar was inoculated on the bottom, 3.3 g/L agar was inoculated at the top. Cells were seeded in 5 culture dish with density of 2×104 cells/dish. The average colony formation rate of 15d was calculated in the biochemical culture box at 37°C with 7.5% CO2. Clone formation rate = clone number/cell number×100%, survival rate after irradiation (SF) = the number of colonies per hole after irradiation/colony forming number in control group ×100%.

Cell cycle analyzed by flow cytometry

The cells of with 70-80% consistency were washed up two times by the PBS. Then cells were fixed with ethanol. 1×109/L cells were stained with PI and tested by flow cytometry. The distribution of each phase of the cell cycle was analyzed.

Statistical analysis

Results were analyzed by SPSS 20.0, MTT and the colony formation results were shown as $\bar{x}$ ±s. Stduent T test was used to analyze the difference between groups. The doubling time of the growth phase cells was compared with the t test and the number of samples in the combination group was also compared. P<0.05 was defined as statistically significant.

Results

The effect of autophagy activity on the sensitivity of radiotherapy in four groups

In the three experimental groups, the proliferation activity of CNE2 cells was decreased compared with the control group (P<0.05), which indicated that autophagy activity in CNE2 cells down regulated in the use of chloroquine and *atg*5 gene silencing or autophagy activity up-regulated by rapamycin, can enhance the radiosensitivity of CNE2 cells. Autophagy activity decreased in rapamycin group was significantly greater than chloroquine and *atg*5 gene silencing group, that it can more effectively enhance the radiosensitivity of CNE2, which meant that it can improve the sensitivity of CNE2 radiotherapy (Table 1).

Cell cycle in each group

Compared with the control group, there were significant differences in the other three groups of G2/M and S period (P<0.05). Compared with the control group, only rapamycin group has significant differences compared with control group (P<0.05). Rapamycin group had significant differences compared with atg5 RNAi group in G0/G1 period, also significantly different compared with chloroquine group and atg5 RNAi group in S period. There are significant differences between chloroquine group and rapamycin group in G2/M period (P<0.05). The results are listed in Table 2.

Discussion

Nasopharyngeal carcinoma is one of the most common cancers in China, which contributed in 80% of all nasopharyngeal carcinoma cases in the world. Radiotherapy is the main treatment method for nasopharyngeal carcinoma. The average survival rate of nasopharyngeal carcinoma in I-IV stage was about 50%, while in the middle and late stage was 20% to 30%, and 20% of NPC patients get carcinoma local recurrence after comprehensive treatment with radiotherapy. The local recurrence and distant metastasis of nasopharyngeal carcinoma after radiotherapy were the main cause of the death of the patients. Therefore, in addition to improving radiotherapy technology, the research on new molecular mechanism and radiation resistance should be carried on for the development of nasopharyngeal carcinoma.

Autophagy has become a hot spot in the life science research in the world. mTOR is a serine/threonine ki-

Table 1. The survival rate of CNE2 cells, clone formation rate and survival rate of the four groups were compared with the survival rate of the cells in the groups (%).

Group	Survival rate	Clone formation rate	Survival rate after irradiation
Rapamycin group	37.18±5.26[1)]	17.84[1)]	28.54±1.65[1)]
Chloroquine group	66.10±4.69[1) 2)]	40.70[1) 2)]	42.87±2.31[1) 2)]
*atg*5 RNAi group	58.32±9.13[1) 2)]	39.98[1) 2)]	43.53±2.59[1) 2)]
Control group	98.23±1.04	82.31	71.28±2.96

Note: compared with the control group[1)]P<0.05; rapamycin group was compared with the other two experimental groups[2)]P<0.05.

Table 2. Distribution of cell cycle in four groups (%).

Group	G_0/G_1 period	S period	G_2/M period
Rapamycin group	93.57±1.68[1)]	5.24±0.39[1)]	2.96±0.57[1)]
Chloroquine group	86. 73±3.35	12.46±2.65[1) 2)]	1.18±0.39[1) 2)]
*atg*5 RNAigroup	62.82±1.72[2)]	28.54±6.94[1) 2)]	7.46±0.64[1)]
Control group	39.26±5.41	35.37±1.46	25.12±2.13

Note: compared with the control group[1)]P<0.05; rapamycin group was compared with the other two experimental groups[2)]P<0.05.

nase, which is a key molecule in the initiation phase of autophagy. mTOR can affect the change of amino acid and ATP in the cells and control the autophagy. mTOR inhibits autophagy through PI3K/AKT/mTOR pathway, which promote the tumor progression. Insulin, growth factor, etc. activates Class I PI3K by tyrosine kinase receptor. PIP was then phosphorylated to PIP3, which activates AKT. Activated AKT can phosphorylate TSC2, increase the mTOR binding activity of GTP-Rheb, so that the mTOR activity is increased to inhibit the occurrence of autophagy. Rapamycin can inhibit the activity of mTOR, thus promoting autophagy. Our study showed that the combination use with rapamycin could inhibit proliferation activity and survival rate, which was consistent with the results of other research. Chang et al. (3) has also demonstrated that combination of PI3K/Akt/mTOR inhibitor with radiotherapy was a promising modality for the treatment of cancer cells to overcome radioresistance. Meanwhile, He et al. (4) has certificated that apogossypolone, which is a small-molecule inhibitor of Bcl-2, could induce radiosensitization of nasopharyngeal carcinoma cells by stimulating autophagy. Therefore, we concluded that the enhancing of autophagy would contribute to the radiosensitization increase. However other study has also demonstrated that blocked autophagy would enhance radiosensitivity of nasopharyngeal carcinoma cell line CNE-2 in vitro (5). Thus the dual regulation of autophagy seems to have both effect on the increase of radiosensitization. Chloroquine belongs to amino group, which is initially used to treat malaria and its indication was gradual expanded. In 1951, chloroquine was used in the treatment of rheumatoid arthritis. The drug can also be used for hepatic amebiasis, clonorchiasis, paragonimiasis, connective tissue disease (disk lupus erythematosus and system lupus erythematosus (SLE) and photosensitive disorders such as the hope on the disease of erythema. Chloroquine has a strong affinity with nuclear protein, quinoline ring with negative 7-chlorine radicals and DNA guanine 2-amino interattracted to send chloroquine inserted into the DNA double helix, block DNA replication and transcription. Recent studies have indicated that chloroquine can inhibit tumor by inhibiting the mechanism of autophagy and promoting apoptosis, and thus can resist tumor (6-10). Here in our study, we found that with the treatment of chloroquine, the proliferation activity and survival rate of CNE-2 were inhibited, which indicated that the radiosensitization was increased by block of autophagy. Moreover, RNA interference technology was performed in our experiment. Autophagy related gene atg5 is a key molecule in the extension phase of autophagy. atg5 complex is essential for the formation of autophagy and extension of the membrane in the process of autophagy. Interfering with the expression of atg5 gene can inhibit the formation of autophagy. Consistent with the study in other tumor cells (11,12), silencing of atg5 would also cause increase of radiosensitization. Interestingly, the extent of the radiosensitization increase was also compared between autophagy inhibitor and autophagy activator, we found that the autophagy activator had a better sensitization effect increase than autophagy inhibitor.

In conclusion, the effects of the combined therapy on nasopharyngeal carcinoma and nasopharyngeal carcinoma cells in vitro were studied in this study. It is indicated that CNE2 can increase the sensitivity of radiotherapy for nasopharyngeal carcinoma cells by enhancing or inhibiting autophagy. We provide the experimental basis for the enhanced sensitivity of radiotherapy, elucidate the role of autophagy in the treatment of nasopharyngeal carcinoma to open up a new method for the enhancement of radiotherapy. Finally, we provide potential target therapy aimed at autophagy signal pathway and experimental basis for radiotherapy resistance.

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