The Direct Effects of Different Irradiation Methods on The Survival of Laryngeal Squamous Carcinoma Hep2 Cells

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Short report

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Abstract

Radiotherapy plays an important role in the treatment of laryngeal squamous cell carcinoma. However, radiation resistance is an important cause of radiotherapy failure. To determine the direct effects of different irradiation methods on the survival of Hep2 cells, three different irradiation methods were used in vitro to explore the effects on the colony formation, cycle and apoptosis, showing that different irradiation methods had inhibitory effects on the colony formation of Hep2 cells. Radioactive ^{125}I continuous low dose rate radiation (CLDR) has a stronger inhibitory function, and single dose radiation (SDR) is also superior to fractionated dose radiation (FDR). The G1 phase decreased more significantly, while the G2/M phase ratio also increased significantly in CLDR group. The CLDR group was more significant increase in the early apoptosis and general apoptosis. During these process, the production of higher γ-H2AX, Cyclin D1, Cdc2, NF-κB p21, CyclinB1 and lower p-Cdc25c in CLDR group were observed. Taken together, the continuous low-dose rate irradiation of ^{125}I seeds can significantly increase the apoptosis rate of Hep2 cells, resulting in continuous G2/M phase arrest and significantly inhibiting the proliferation ability of Hep2 cells

Introduction

Laryngeal squamous cell carcinoma is one of the most common malignant tumors, accounting for 14% of head and neck tumors, while squamous carcinoma is the most common type of laryngeal cancer, accounting for about 93–99% of laryngeal cancer [1,2]. Over the past 40 years, the incidence of laryngeal squamous cell carcinoma has declined steadily, while the 5-year survival rate has not improved significantly, especially in the advanced stage, where early symptoms are not significant and about 60% of patients are not diagnosed until the advanced stage (stage III or IV) [3,4]. Due to the characteristics of occultation, invasiveness, recurrence and metastasis, it has low sensitivity to chemo/radiotherapy, strong resistance and poor curative effect. Radiotherapy plays an important role in the treatment of laryngeal squamous cell carcinoma [5]. Radiotherapy includes in vitro irradiation and in vivo irradiation, in which in vitro irradiation can be divided into long distance irradiation and short distance irradiation according to radiation distance. However, radiation resistance is an important cause of radiotherapy failure [6]. Treatment resistance and recurrence have always been the difficulties and unresolved problems in the treatment of head and neck squamous cell carcinoma. Therefore, it is of great significance and necessity to explore the effect of radiotherapy on the survival of laryngeal squamous cell carcinoma cells.

In present study, we found that continuous low-dose rate irradiation of ^{125}I seeds could significantly increase the apoptosis rate of Hep2 cells, resulting in continuous G2/M phase arrest and significantly inhibiting the proliferation ability of Hep2 cells.

Material And Methods

Cells and reagents

Human laryngeal squamous carcinoma cell line Hep2 obtained from ATCC, were cultured in RPMI1640 (Hyclone, US) containing 10%FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Corning, US) at 37°C in a humidified atmosphere of 5% CO2. The cells at logarithmic growth stage were digested by trypsin to produce a single cell suspension, which was planted in a 35 mm culture dish, and then the cells were cultured for 24 h for irradiation with high dose rate single dose radiation (SDR), high dose rate fractionated dose radiation (FDR), and iodine-125 seeds continuous low dose rate radiation (CLDR). The initial dose rate of particle irradiation was 2.77 cGy/h. When the irradiation dose was 4 Gy and 6 Gy, 150.0h and 229.4h were needed respectively. RS2000 X-ray bioradiometer (Rad Source Technologies, USA) was used for high dose rate irradiation with a dose rate of 6312 cGy/h. The single irradiation group was the total dose completed by one irradiation. The fractional irradiation group received irradiation every 24 h, with each dose of 2 Gy.

Colony Forming Assay

Hep2 cells were taken and planted in a 35mm culture dish, which were irradiated for 24 h and then further cultured after the irradiation of 4 Gy and 6 Gy. The cell planting date was set as the 1st day. From the 14th day, the cells of each group were fixed with methanol for 10 min, and the cells were stained with Giemsa-staining solution for 5 min, and then the colony number was counted under conventional microscope. One colony unit was obtained with more than 50 cells and its colony rate (PE) and survival fraction at 2 Grey (SF2) at each clinical irradiation dose. In addition, the calculation formula of PE is : (number of clones/number of cells inoculated) ×100%;The calculation formula of SF2 is :(PE in the irradiation group/PE in the control group) ×100%.

Apoptosis and Cell Cycle Analysis

At 24h, 48h and 72h after irradiation of 4Gy, cells were digested and all of them were prepared into single-cell suspension with a concentration of 5×105 cells per ml. Apoptosis was detected using Annexin-V-FITC Apoptosis Detection Kit (Biolegend, USA) by flow cytometry, according to the manual. For cell cycle analysis, cell suspension was taken, washed twice with pre-cooled PBS, and the pre-cooled 2 ml 75% alcohol was fixed, and then placed at 4℃ overnight. The residual alcohol was washed with PBS twice, and the cells were resuspended with 300μl of PBS. PI and RNaseA were added until the final concentration was 50 ug/mL. The cells were incubated at 37℃ for 30 min in dark. The cell cycle was then measured by flow cytometry (BD celesta, USA).

Immunoblotting analysis

The cells were washed with PBS, lysed with PBS containing protease and phosphatase inhibitors cocktail, and then centrifuged to remove the nuclear fraction. The supernatant was used as the cytoplasmic fraction. To obtain the nuclear fraction for immunoblotting analysis, the cell pellet was processed with the EpiQuik Nuclear Extraction Kit I (Epigentek, USA) according to the manual. The soluble proteins from each sample were separated by SDS-PAGE in a 10% polyacrylamide gel and then transferred onto a PVDF membrane (Millipore, USA). The membrane was blocked with 5% BSA and incubated with the target

antibodies. All bound antibodies were incubated with HRP-conjugated anti-rabbit IgG secondary antibody (abcam,USA). The bands were detected by using Immobilon Western (Millipore, USA) and the Las-1000 mini image analyzer (Fujifilm, Tokyo, Japan).

Statistical analysis

Data are described as the mean ± SEM. Statistical analysis and significance were measured by One-way ANOVA. All data were performed using Origin professional software version 2018 (Origin Lab Software, USA). In all comparisons, p values less than 0.05 are considered a statistically significant difference. Statistical analysis was performed using SPSS Statistics (SPSS Inc. Chicago, IL, USA).

Results

To determine the direct effects of different irradiation methods on the survival of Hep2 cells, three different irradiation methods were used in vitro to explore the effects on the colony formation, cycle and apoptosis. The results of colony forming assays showed that different irradiation methods had inhibitory effects on the colony formation of Hep2 cells. At 6 Gy, CLDR has a stronger inhibitory function ($p < 0.05$), and SDR is also superior to FDR (Figure A, B). Further study of the cell cycle found that compared with the control, after 6 Gy irradiation, the other three groups were uniformly blocked in G1 phase to varying degrees, and the proportion of cells in G2/M phase increased. It is worth mentioning that among the time points of CLDR group, the G1 phase decreased more significantly, while the G2/M phase ratio also increased significantly, especially at 48 h (Figure C, D). How do these three affect Hep2 apoptosis? We found that the early apoptosis and general apoptosis of SDR, FDR and CLDR were significantly increased compared with control, and the CLDR group was more significant ($P < 0.001$), while there was no marked difference between SDR and FDR. The phenomena above were more significant at 24 h, but decreased at 72 h, which may be related to DNA damage repair (Figure E, F). During these process, the expression of γ-H2AX, Caspase3 and CyclinB1 were significantly increased at 24 h, 48 h and 72 h. The production of γ-H2AX and CyclinB1 in CLDR group were higher than those in FDR group, while Cdc2 increased more significantly in CLDR group (Figure G). Compared with control, the expression of NF- B P21 in the other three groups were up-regulated, and the production of these two proteins in the FDR group and CLDR group were all higher than those in the SDR group. For CyclinD1, CLDR and FDR were markedly elevated than SDR, while CLDR was significantly increased than FDR at 72 h. However, for p-Cdc25c, the CLDR group was lower (Figure H).

Discussion

Radiotherapy plays an important role in the treatment of laryngeal squamous cell carcinoma. However, radiation resistance is an important cause of radiotherapy failure. Treatment resistance and recurrence have always been the difficulties and unresolved problems in the treatment of head and neck squamous cell carcinoma [6]. Therefore, it is of great significance and necessity to explore the effect of radiotherapy on the survival of laryngeal squamous cell carcinoma cells. Radioactive ^{125}I seeds are more and more

widely used in the treatment of laryngeal squamous cell carcinoma, which has the advantages of less damage and local high dose [7]. Single seeds irradiation can cause DNA damage, cell cycle arrest and cell apoptosis, and thus inhibit the growth of tumor cells. Radiation can cause DNA double-strand break (DSB), phosphorylation of histone H2AX on serine 139 to form γ-H2AX, and aggregation at the site of DNA break [8]. γ-H2AX can be detected as soon as 20 seconds after irradiation of cells (with DNA DSB formation), and half maximum accumulation of γ-H2AX occurs in one minute, which is involved in the steps leading to chromatin decondensation after DNA DSB [9]. Thus, γ-H2AX generally reflects the presence of DSB in DNA [10]. The current study found that higher levels of γ-H2AX in the CLDR group of Hep2 cells after irradiation, indicating that continuous low-dose irradiation may cause more significant DNA damage, leading to weakened colony formation ability. This phenomenon was also verified by apoptotic detection, and the results above showed consistency. The Caspase 3 protein is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis [11]. The present study found that the expression of Caspase 3 protein in the CLDR group was markedly elevated, which also suggested that the apoptosis of Hep2 cells was on the increase. When DNA damage occurs, the cell cycle is blocked and DNA damage repair is initiated, with the involvement of NF- κB and Cyclin. DNA damage can up-regulate p21 transcription. Furthermore, Cyclin D/CDK4 is bound and inhibited, so p21 is an important factor in inhibiting the entry of G1/S phase [12,13]. Current results showed that p21 and Cyclin D1 in the CLDR group, suggesting that CLDR may inhibit cells to enter G1/S phase. Cyclin B1 is a regulatory protein involved in mitosis. The gene product complexes with Cdk1 to form the maturation-promoting factor during G2/M phase of the cell cycle. Just prior to mitosis, a large amount of cyclin B1 is present in the cell, but it is inactive due to phosphorylation of Cdk1 by the Wee1 kinase. The complex is activated by dephosphorylation by the phosphatase Cdc25 [14]. Cdc25 is always present in the cell but must be activated by phosphorylation. Active Cdk1 is also capable of phosphorylating and activating Cdc25 and thus promote its own activation, resulting in a positive feedback loop [15]. In this study, after three types of different irradiation, Hep2 cells showed a decrease in the proportion of G1 phase and an increase in the proportion of G2/M phase, with the CLDR group showing the most significant change. After irradiation, the expression of Cyclin B1 was the most significantly up-regulated in CLDR group, while the protein level of p-Cdc25c was the lowest, indicating that cells may be continuously blocked in the G2 phase, probably because ^{125}I seeds sustained low dose rate irradiation inhibited the activation of Cyclin B1/Cdk1 complex. As cells in the G2/M phase are more sensitive to irradiation [16,17], our study suggest that continuous low-dose rate irradiation of ^{125}I seeds can significantly increase the apoptosis rate of Hep2 cells, resulting in continuous G2/M phase arrest and significantly inhibiting the proliferation ability of Hep2 cells (decreased colony formation ability). In addition, the results above also remind us that the use of target drugs to block cells into G2/M phase during radiotherapy may significantly improve the level of apoptosis, thereby improving radiotherapy sensitivity and reducing radiation resistance.

Declarations

Ethical Approval and Consent to participate

Not applicable

Consent for publication

Yes

Availability of supporting data

Not applicable

Authors' contributions

Junjie Wang conceived the manuscript; Suqing Tian and Li Huang contributed equally to perform the experiments.

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Competing interests

The authors declare no competing interests.

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Figures

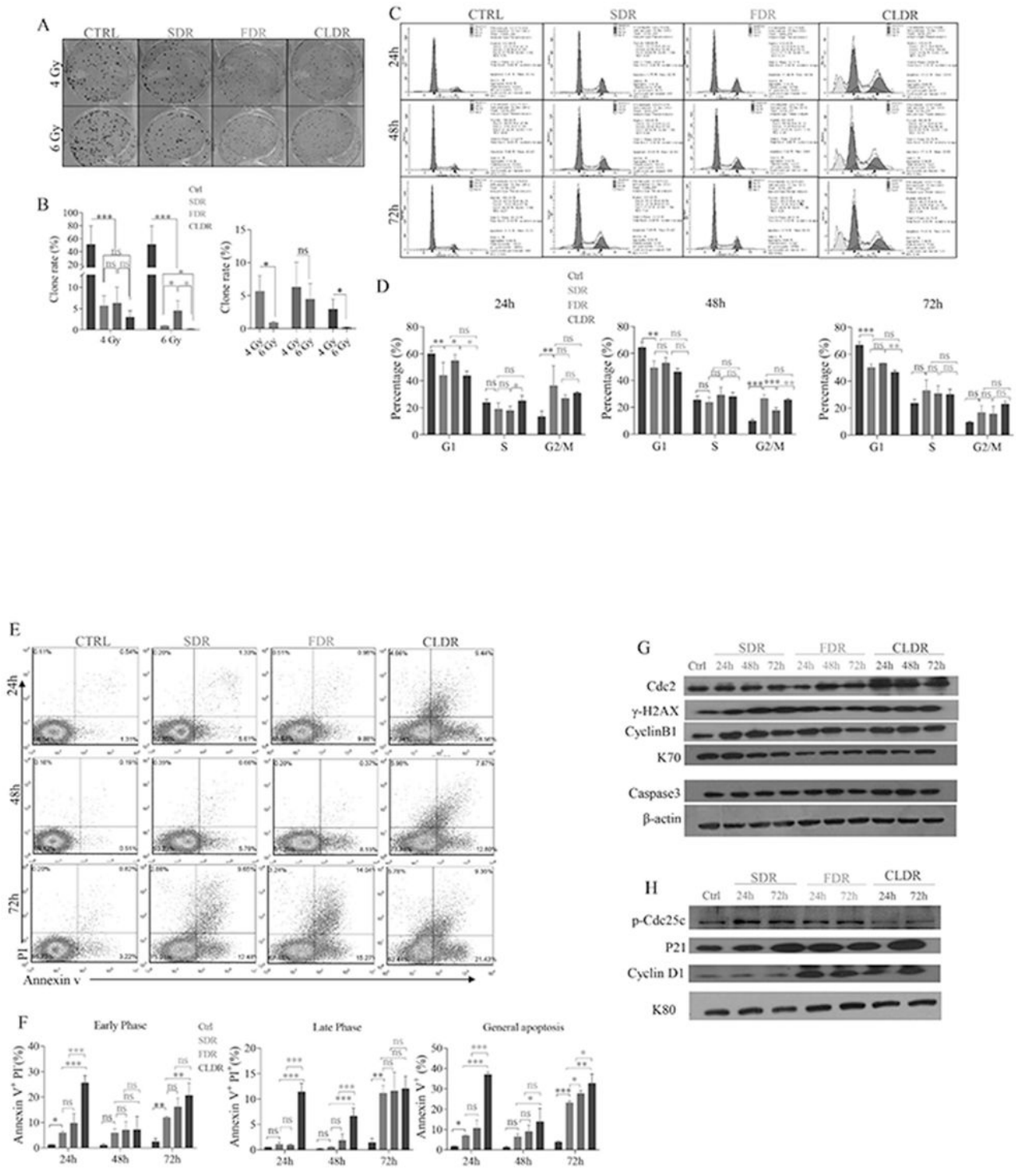

Figure 1

Different irradiation methods on the survival of Hep2 cells. Hep2 cells were taken and planted in a 35mm culture dish, which were irradiated for 24 h and then further cultured after the irradiation of 4 Gyand 6 Gy. The cells were stained with Giemsa. A, the representative pictures of Giemsa dyeing. B, statistical analysis of colony formation. C, cell cycle Histogram by FACS. D, statistical analysis of cell cycle. E, the

representative pictures of apoptosis detected by FACS. F, statistical analysis of apoptosis. G,H, Immunoblotting analysis.