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Original Article

Biological effects of continuous low-dose irradiation from radioactive iodine-125 seeds on human adenoid cystic carcinoma cell line

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Abstract: Objective: To investigate the *in vitro* effects of iodine-125 (^{125}I) seeds on growth inhibition and induction of programmed cell death in the human adenoid cystic carcinoma cell line ACC-2. Method: Malignant human brain glioma cell lines ACC-2 were segregated into 0, 2, 4, and 6 Gy groups based on doses of ^{125}I seeds. Cell counting kit-8 (CCK-8) assay was performed to determine the effect of ^{125}I seeds on ACC-2 cell proliferation rate and flow cytometry analysis was performed to study apoptosis. Cloning was performed to observe the cell clones. Results: Clones of post-irradiated cells displayed marked growth inhibition with significant differences among groups ($P < 0.05$) and ^{125}I seeds had dose- and time-dependent inhibitory effects on proliferation in ACC-2 glioma cells cultured *in vitro*. The CCK-8 assay revealed that proliferation of ACC-2 cells irradiated with various doses of ^{125}I seeds was inhibited ($P < 0.05$). The rate of inhibition significantly increased with an increase in radiation dose and prolongation of reaction time ($P < 0.05$). Conclusions: Continuous low-dose irradiation inhibits proliferation, growth of clones, and apoptosis functions in ACC-2 cells.

Keywords: Radioactive iodine-125, human adenoid cystic carcinoma, apoptosis

Introduction

Adenoid cystic carcinoma (ACC) is the most common malignancy in head-and-neck salivary gland tissues and may occur at any age. It mostly affects middle-aged and aged populations irrespective of sex. It may occur in any salivary gland and is the third most prevalent salivary gland tumor with a proportion of more than 30% [1, 2]. ACC is mainly treated via surgical excision of lesion tissues, which are slow and continuously growing tumors. ACC easily metastasizes to distant sites, thereby drastically increasing the recurrence rate of the disease, and statistics reveal that the recurrence rate 10 years after surgical excision is greater than 70% in ACC patients with poorer long-term prognosis. Additionally, the 15-year survival rate is only 40% [3]. A few interventional chemotherapy studies yielded less-than-satisfactory results [4]. Furthermore, in recent years, ACC biology has been increasingly discussed, and clinical treatment and prognosis have been evaluated primarily via analysis of occurrence and development, biological effects, and underlying mechanism of ACC, to establish new therapies [5].

^{125}I is the most widely used clinical radioactive seed with a small effective killing range (17 mm), which lyses the ultrastructure of cancer cells by X- and γ-rays, thereby treating the cancer [6]. Presently, sealed ^{125}I seeds are commonly used for clinical treatment and do not need to be replaced after initial implantation because of their relatively lower activity [7]. The proliferative capacity of tumor cells can be effectively inhibited via continuous low-dose γ-ray irradiation of ^{125}I implanted in cancer tissues. ^{125}I seeds have the advantages of lower radiation energy (27-35 keV) and small killing range that lessen the effect on the surrounding

normal tissues, thereby protecting surrounding normal tissues while the patients receive high-intensity irradiation treatment [8]. ^{125}I seeds also have marked effects on cancer treatment via accurate positioning guided by imageology, indicating the feasibility of the use of radioactive seeds in cancer therapy.

This study aimed to discuss the biological effects of ^{125}I on ACC via continuous irradiation of ACC tumor cells with low-dose ^{125}I seeds.

Materials and methods

Cell line and reagents

Human adenoid cystic carcinoma cell line-2 (ACC-2) was purchased from Bioscience Company. ^{125}I radioactive seeds were provided by Jiangsu Huayi Chemical Co., Ltd., Dulbecco's modified Eagle's medium (DMEM), F-12K medium, 10% fetal calf serum, and trypsin were purchased from Gibco; paraformaldehyde and Giemsa, Solarbio company; cell counting kit-8 (CCK-8), Beyotine Institute of Biotechnology; OLYMPUS inverted microscope, OLYMPUS; flow cytometry FACSVerse, BD company; ELIASA Synergy 2, BioTek; cell irradiation unit, Shanghai Ibeka Ray Protection Equipment Technology Co. Ltd.

ACC-2 cell culture

ACC-2 cells were cultured at 37°C under 5% (volume fraction) CO_2 in DMEM and F-12K complete media containing 10% fetal calf serum. Cells grew via adherence with the wall and medium was replaced every two days, followed by sub-culturing till cells approached 80% confluence. The medium was discarded in a culture bottle, and the cells were washed twice with PBS. Thereafter, 1 ml of 0.25% pancreatin containing EDTA was added to loosen the cell layers, as evident from shrinking and rounding up of cells under the microscope. The cell culture bottle was agitated to disrupt cellular adherence and thereafter, 2 ml of complete DMEM was added to terminate the digestion, followed by centrifugation at 800 rpm for 5 min. The cells were re-suspended in DMEM and F12K complete media and fresh media in a ratio of 1:3, continuously cultured and sub-cultured.

Mode of irradiation

Irradiation of ^{125}I radioactive seeds was performed in accordance with previous studies [8], where 9 seeds with initial activity of 3.33×10^8 Bq and initial dose at the rate of 10.89 h/Gy was used. Also, 8 seeds were arranged and distributed in a torque loop type (3.5 cm) with the other one at the center, followed by digestion of the ACC-2 cells in the logarithmic phase, followed by preparation of a single-cell suspension (count 5×10^5/ml). The cells were then seeded in the irradiation unit with ^{125}I as the ion source, and the cells in each group were irradiated with 0, 2, 4, and 6 Gy radioactivity. The medium was supplemented with 4 ml after the completion of irradiation and thereafter the cells were continuously cultured for 10-14 d in the incubator, stained, and finally the number of cell clones was determined.

Cell cloning

After irradiation, cells were trypsinized, transferred to DMEM, and F12K media to culture for 10-14 d, and then the culture medium was discarded, the cells were rinsed twice with PBS, and a suitable amount of 4% paraformaldehyde was added to fix the cells for 15 min. The fixative was then aspirated, and a suitable amount of Giemsa working solution was added to stain for 30 min. The stain was then washed away with running water, and the cloned cells were enumerated using Image pro plus 6.0. Therefore, inhibition of the number of cell clones was determined.

Assessment of cell proliferation

Proliferation of post-irradiated cells was assessed using CCK-8 with 10 μl of CCK-8 reagent added into cell culture in each pore and cells cultured at 37°C and 5% (volume fraction) CO_2 for 48 h. The absorbance was then measured using an ELISA reader (at 450 nm) and the assessment was performed in triplicate for each pore. SDS solution (1% w/v) was added if the test was not performed in a timely manner, and was covered to prevent exposure to light and stored at 25°C (tested within 24 h).

Flow cytometry

Post-irradiation floating cells were harvested using a pipette to enumerate the cells in each group. The adhered cells were rinsed with 2 ml of PBS, and the PBS was discarded. Thereafter, 0.5 ml of 0.25% pancreatin was added and the cells were incubated (without EDTA) until they lost their adhesion, finally followed by micro-

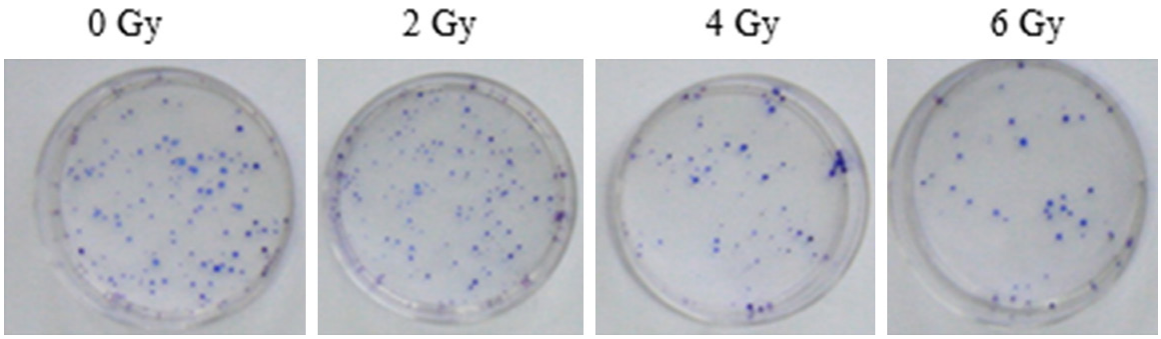

Figure 1. Effect of ^{125}I radiation on ACC-2 cell clone formation.

Table 1. Cell cloning in groups

Group	0 Gy group	2 Gy group	4 Gy group	6 Gy group	F	P
ACC-2	119.52±4.58%	105.37±6.44%*	44.79±5.92%*,#	32.69±4.58%*,#,Δ	189.27	0.01

Note: *represented that the 2 Gy, 4 Gy, and 6 Gy groups had a statistical difference compared with the 0 Gy (t=3.10, P=0.04; t=17.29, P=0.01; t=23.22, P=0.01), #represented 4 Gy and 6 Gy groups had statistical difference compared with the 2 Gy group (t=12.00, P=0.01; t=15.93, P=0.01), and Δrepresented that the 4 Gy group had a statistical difference compared with the 6 Gy group (t=2.80, P=0.05).

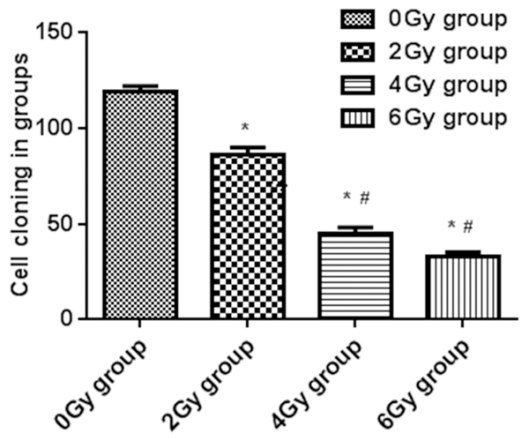

Figure 2. Comparison of cell clones. On cloning the post-irradiated ACC-2 cells, the number of cell clones in the 0 Gy group significantly increased to 119.52±4.58%, whereas the cell clones in 2 Gy, 4 Gy, and 6 Gy groups were 86.37±6.44%, 44.79±5.92%, and 32.69±4.58%, respectively, and were significantly inhibited. Furthermore, there were differences between the 4 groups (F=189.27, P=0.01). *indicates that the 2 Gy, 4 Gy, and 6 Gy groups were significantly different from the 0 Gy group; #indicates that the 4 Gy and 6 Gy groups were significantly different from the 2 Gy group.

scopic observation. The plates were agitated to completely detach the cells from the culture plate, and the cells were then resuspended in 1X buffer solution (1×10^6/ml), transferred to a clean centrifuge tube, and apoptosis detection solution (AnnexinV-FITC) was added. Cells were kept away from light for 15 min at 25°C, followed by centrifugation for 5 min at 1500 rpm. The cells were then resuspended in buffer solution, and 10 μL of PI was added and the cells were analyzed via flow cytometry with analysis performed in triplicate.

Statistical analysis

SPSS19.0 software package was used for statistical analysis for all data. Measured variables are presented as mean ± standard deviation (SD) values. Independent student's *t*-test was performed for pairwise comparison, one-way analysis of variance was performed for comparisons among groups, and the LSD *t*-test was performed for further pairwise comparisons. A *P*-value less than 0.05 was considered statistically significant.

Results

Morphological changes in cells

After irradiation with ^{125}I, growth inhibition of the cells under high-intensity irradiation was obvious, as revealed via fluorescence microscopy. ACC-2 cells exhibited apparent nuclear condensation at 4 Gy (**Figure 1**).

Comparisons among cell clones

On cloning the post-irradiated ACC-2 cells, cell clones in the 0 Gy group were significantly

Table 2. Cell inhibition rate of each group

Group	0 Gy group	2 Gy group	4 Gy group	6 Gy group	F	P
ACC-2	12.58±1.54%	63.58±2.14%	71.83±1.57%	79.54±1.05%	1043.62	0.01

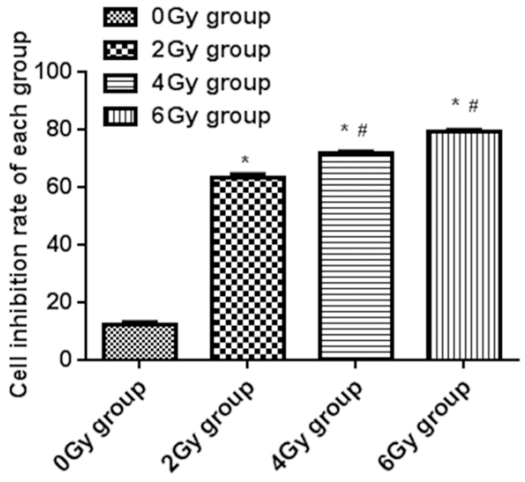

Figure 3. Comparison of proliferation among post-irradiated cells. On performing a CCK-8 assay on the post-irradiated cells in each group, cell inhibition rates in the 0 Gy, 2 Gy, 4 Gy, and 6 Gy groups were 12.58±1.54%, 63.58±2.14%, 71.83±1.57%, and 79.54±1.05%, respectively, with significant differences among the four groups (F=1043.62, P=0.01). *indicates that the 2 Gy, 4 Gy, and 6 Gy groups were significantly different from the 0 Gy group; #indicates that the 4 Gy and 6 Gy groups are significantly different from the 2 Gy group.

increased to 119.52±4.58%, whereas those in the 2, 4, and 6 Gy groups were significantly inhibited to 86.37±6.44%, 44.79±5.92%, and 32.69±4.58%, respectively. There were differences among the 4 groups (F=189.27, P=0.01), and the cell clones in the 0 Gy group displayed significant differences after pairwise comparison with other groups (P < 0.01) (**Table 1** and **Figure 2**).

Comparison of proliferation among post-irradiated cells

On performing the CCK-8 assay for proliferation of post-irradiated cells in each group, cell inhibition rates in 0, 2, 4, and 6 Gy groups were 12.58±1.54%, 63.58±2.14%, 71.83±1.57%, and 79.54±1.05%, respectively. There were significant differences among the 4 groups (F=1043.62, P=0.01). The cell inhibition rate in the 0 Gy group was significantly different from that of the 2, 4, and 6 Gy groups (t=33.50, P=0.01; t=46.66, P=0.01; t=62.22, P=0.01); cell inhibition rate in the 2 Gy group was significantly different from that in the 4 and 6 Gy groups (t=5.38, P=0.01; t=11.60, P=0.01, respectively), while that of the 4 Gy group was significantly different from that of the 6 Gy group (t=7.07, P=0.01) (**Table 2** and **Figure 3**).

Assessment of apoptosis in post-irradiated cells

As shown in **Table 3** and **Figure 4**, on flow cytometric analysis for apoptosis, the apoptosis rates of the 0, 2, 4, and 6 Gy groups were 2.77±0.59%, 5.67±1.24%, 10.58±1.54%, and 13.84±1.87% respectively, with significant differences among all four groups (F=37.84, P=0.01). The cell inhibition rate in the 0 Gy group was significantly different from that of the 2, 4, and 6 Gy groups (t=3.66, P=0.02; t=8.20, P=0.01; t=9.78, P=0.01, respectively), while that of the 2 Gy group was significantly different from that of the 4 and 6 Gy groups (t=4.30, P=0.01; t=6.31, P=0.01, respectively). However, that of the 4 Gy group was slightly but not significantly different from that of the 6 Gy group (t=2.33, P=0.08).

Discussion

A gradually growing painless malignancy, ACC, displays extracapsular invasion during early stages, and can penetrate the nerves and blood vessels, thereby easily resulting in local recurrence and distant metastases [9]. Statistics reveal that ACC mostly invades the salivary gland tissues, wherein the incidence is more than 20% in the major salivary glands, and approaches 50% in the minor salivary glands. Moreover, ACC also occurs in other organs (trachea, esophagus, skin, uterus, etc.). The lung metastatic rate 5 years after ACC surgery approaches 70%, whereas that at 10 years after ACC surgery unexpectedly approaches 100% [10]. Currently, chemoradiotherapy is considered the primary comprehensive treatment for ACC. Surgical excision, in combination with chemoradiotherapy, can significantly improve the survival rate of patients and reduce local recurrence [3]. Mendenhall, et al. [11] reported that the 5-year survival rate of

Table 3. Apoptosis in all groups

Group	0 Gy group	2 Gy group	4 Gy group	6 Gy group	F	P
ACC-2	2.77±0.59%	5.67±1.24%	10.58±1.54%	13.84±1.87%	37.84	0.01

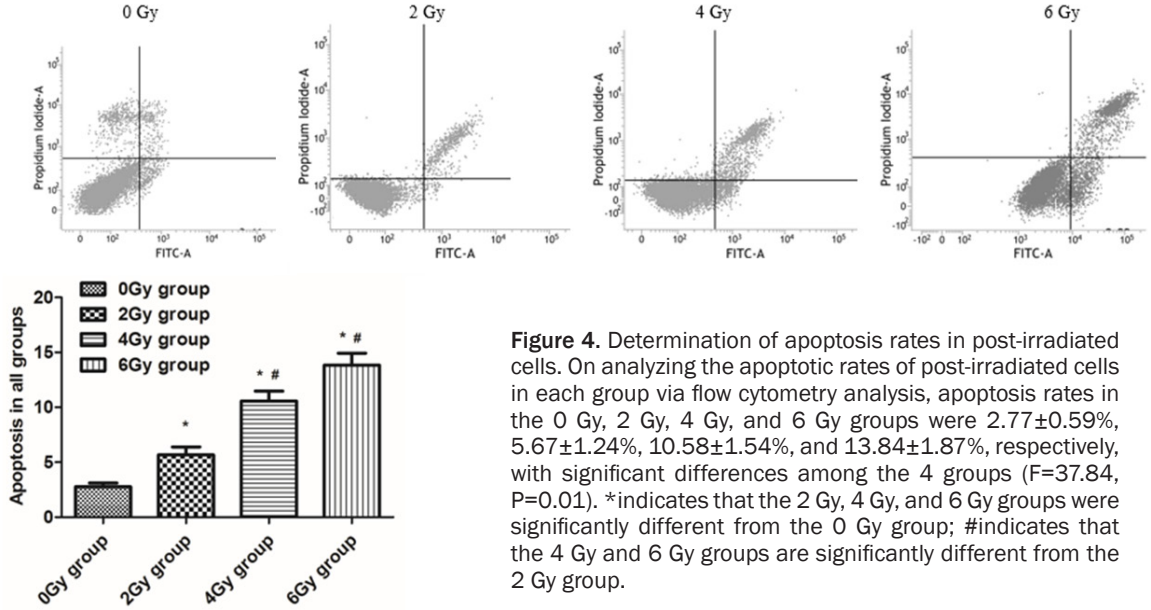

Figure 4. Determination of apoptosis rates in post-irradiated cells. On analyzing the apoptotic rates of post-irradiated cells in each group via flow cytometry analysis, apoptosis rates in the 0 Gy, 2 Gy, 4 Gy, and 6 Gy groups were 2.77±0.59%, 5.67±1.24%, 10.58±1.54%, and 13.84±1.87%, respectively, with significant differences among the 4 groups (F=37.84, P=0.01). *indicates that the 2 Gy, 4 Gy, and 6 Gy groups were significantly different from the 0 Gy group; #indicates that the 4 Gy and 6 Gy groups are significantly different from the 2 Gy group.

patients approaches 77% and 10-year survival rate approaches 55% on retrospectively assessing ACC patients who received clinical therapy assisted by radiotherapy.

For radioactive seed therapy, the radioactive source is implanted around or inside the cancer tissues. The radionuclides are released to ensure continuous radiation to eliminate the cancer cells [12]. Previously, Co and Rh radionuclides were used more commonly. However, the high-energy γ-rays produced during the release of radionuclides is very harmful and it is hence difficult to ensure the safety of the patients and the physicians, therefore it is not widely practiced clinically [13]. As the radioactive seed continuously releases low-dose γ-rays, ^{125}I is one of the most commonly used radioactive seeds clinically, which is surgically directly implanted around the tumor to continuously irradiate, thereby inhibiting tumor growth with small radioactive source volume and less side effects. With the development of imaging techniques, monitoring and guiding the implantation of ^{125}I radioactive seeds in cancer has received considerable attention [14]. Since the implantation of ^{125}I is minimally invasive, less invasion is observed in elderly patients, with no complications, and other advantages, which have been widely applied in clinical practice with better effects on the treatment of liver cancer, lung cancer, recurrent head and neck cancer, etc. [19].

In recent years, there have been advancements in the biomedical sciences, however one of the largest medical problems, cancer has puzzled medical and scientific researchers for many years. However, with the development of better technologies, there are increasing methods of treating cancers, including radioactive therapy. Presently, several studies have suggested the treatment of cancers with ^{125}I [15, 16]. However, few studies have focused on treatment of ACC with ^{125}I. Such studies mostly involve animal models [17]. In this study, an external irradiation experiment was directly performed for ACC-2 cells and the cellular biological effects were observed. Moreover, cell cloning and flow cytometry analysis were performed for the post-irradiated ACC-2 cells, which revealed that the ability to form clones among post-irradiated ACC-2 cells was significantly obliterated in a dose-dependent manner. Apoptosis, also known as programmed cell death, is regulated by genes. Thus, apoptosis plays an important

role in the process of growth and development of normal body, and in the occurrence of diseases. Presently, apoptosis is one of the major phenomena targeted in treating cancers and in inhibiting tumors via tumor cell apoptosis [18]. Flow cytometry analysis revealed that the apoptosis rate of ACC-2 cells irradiated by ^{125}I was significant, and the higher the dose, the more significant the apoptosis rate. At the end of the experiment, a cell proliferation assay with post-irradiated ACC-2 cells revealed that the tumor proliferation was higher [19]. On assessing ACC-2 cell proliferation with the CCK-8 assay, the proliferation of post-irradiated cells was dose-dependently inhibited. Therefore, ^{125}I significantly inhibits the growth of ACC.

However, this study has the following limitations. First, these experiments are basic *in vitro* experiments, whether the effect on inhibition of cancers in the study is similar to that in clinical practices needs further validation. Second, we could not delineate the mechanism and determine which genes and pathways are affected. Therefore, we intend to elucidate the underlying mechanism and validate the experimental results via a clinical pilot study with a larger sample size, in a future study. In conclusion, ^{125}I radioactive seeds inhibit the growth of ACC-2 cells by promoting apoptosis and inhibiting their proliferation and migration.

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Disclosure of conflict of interest

None.

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