

Original Article

Cholecystokinin attenuates radiation-induced lung cancer cell apoptosis by modulating p53 gene transcription

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Abstract: The deregulation of p53 in cancer cells is one of the important factors by which cancer cells escape from the immune surveillance. Cholecystokinin (CCK) has strong bioactivity in the regulation of a number of cell activities. This study tests a hypothesis that CCK interferes with p53 expression to affect the apoptotic process in lung cancer (tumor) cells. In this study, tumor-bearing mice and A549 cells (a tumor cell line) were irradiated. The expression of CCK and p53 in tumor cells was assessed with RT-qPCR and Western blotting. The binding of p300 to the promoter of p53 was evaluated by chromatin immunoprecipitation. We observed that, with a given amount and within a given period, small doses/more sessions of irradiation markedly increased the levels of CCK in the sera and tumor cells, which were positively correlated with the tumor growth in mice and negatively correlated with tumor cell apoptosis. CCK increased the levels of histone acetyltransferase p300 and repressed the levels of nuclear factor- κ B at the p53 promoter locus in tumor cells, which suppressed the expression of p53. In conclusion, CCK plays an important role in attenuating the radiation-induced lung cancer cell apoptosis. CCK may be a novel therapeutic target in the treatment of lung cancers.

Keywords: Lung cancer, cholecystokinin, p53, apoptosis, DNA remodeling

Introduction

Lung cancers are one of the leading diseases causing human being death [1]. The therapeutic remedies of lung cancer mainly include surgery, radiotherapy, and chemotherapy [2]. However, the therapeutic effect on lung cancer is still poor currently although research in this area advanced rapidly in recent decades [3]. The pathogenesis of lung cancer is unclear. In fact, lung cancer has been a great negative impact on human health and social economy [4]. Thus, to find novel therapies or elucidate the basic mechanism of lung cancer that can contribute to development of new remedies for the treatment of lung cancer is of significance.

Radiotherapy is a common approach for the treatment of various cancers, including lung cancer [5]. One of the basic mechanisms of radiotherapy is to induce cancer cell apoptosis and death, so as to remove the tumor [6]. However, it is observed that the radioresistance

may be developed in the cancers in the course of radiotherapy soon or later [7]. Although radioresistance can be naturally developed in normal cells, it is a great drawback in the treatment of cancer; the mechanism is not fully understood yet [8].

Cholecystokinin (CCK), also known as pancreaticozymine, is synthesized and secreted by enteroendocrine cells in the duodenum [9]. The main function of CCK is to cause the release of digestive enzymes and bile from the pancreas and gallbladder, respectively. It also induces drug tolerance to opioids like morphine and heroin [10]. Published data indicate that CCK is associated with the pathogenesis of cancer; the receptors of CCK are expressed by pancreatic cancer tissues [11], colon cancer [12], prostate cancer [13] and lung cancer [14]. However, whether CCK secretion is also up-regulated in a tumor-environment and whether tumor cells are also one of the extra sources of CCK are not

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Table 1. Primers used in the present study

Molecule	Forward	Reverse
CCK	cctcggaacactggagaact	gcagtaaacacgtgctcagag
P53	tggccatctacaagcagtca	ggtacagtcagagccaacct
P300	ttgtgaagagccccatggat	gctttgcatcactgggtcaa
MOZ	tgcccaatccttctaagcc	acactactactgagcgccg
MORF	ctaatggactgtggcgctcg	ctgctcctctgggtcaaaga
HBO1	ccacgaactcctcctcatt	agttagggattgtggtaggg
SRC-3	acatgggagtcctggtcttg	ctggaaaagctgtggttccc
SRC-1	ggtaaaagggtcagggtgtgg	acaagtacggggaaggagg
ACTR	acatgggagtcctggtcttg	ctggaaaagctgtggttccc
TIF-2	gtttgccagccacttacca	aacacactgctgtttctcc
GCN5	tccccattgacctgaagacc	cctcctccttgagcttgaa
Tip60	ctacaatgtggcctgcatcc	ttgatggtgatctgtggcct
PCAF	cctctctggacttgaggca	tggtctctggccaagcatt

investigated. In a preliminary study, we noted that tumor cells produced CCK after radiotherapy (data not shown). Based on the above information, we hypothesize that tumor cells produce CCK, which can be up regulated by irradiation. In this study, we observed that tumor cells did produce CCK after radiotherapy. The autocrine CCK increased phosphor histone acetyltransferase p300 levels at the p53 promoter locus in tumor cells and suppressed the irradiation-induced tumor cell apoptosis.

Materials and methods

Cell culture

A549 cells (human lung adenocarcinoma cell lines) were purchased from Institute of Shanghai Biochemistry and Cell biology, Chinese Academy of Science (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO₂. The culture media were changed in every 1-2 days. The cell viability was assessed by Trypan blue exclusion assay. The cell culture reagents were purchased from Sigma Aldrich (St. Louis, MO).

Mice

NOD/SCID female mice (10-12 weeks old, body weight of 20-25 g) were purchased from Beijing Experimental Animal Institute (Beijing, China). The mice were maintained in a pathogen free

environment with accessing food and water freely. The experiments were approved by the Animal Ethic Committee at The Capital Medical University and carried out in accordance with the approved guidance.

Tumor mouse model

Mice were subcutaneously injected in the right flank of the abdomen with A549 cells suspension (5×10^6 cells in 0.2 mL of Hank's medium per mouse). The mice were then randomized into groups receiving one of the three radiation therapies.

Radiation therapy

Tumor mice were irradiated using a linear accelerator at an entire dose of 12 Gy/mouse, at one of the three therapies: Therapy 1 (1 Gy daily), therapy 2 (2 Gy/day in every other day) and therapy 3 (3 Gy in every 3 days). The tumor mass size was measured with a vernier caliper and recorded in every 3 days.

Histology

Tumor tissue was collected from mice immediately after sacrifice. The tissue was fixed with 4% formalin overnight and processed for paraffin sections. The sections were stained with hematoxylin and eosin. The tissue structure was observed by a pathologist.

Isolation of tumor cells

The tumor mass was excised from each mouse and cut into small pieces (2 × 2 × 2 mm). The samples were then incubated in RPMI1640 medium containing collagenase IV (0.5 mg/ml; Sigma Aldrich) for 1 h at 37°C with mild agitation. Single cells were collected by filtering through a cell strainer (70 µm in diameter) and centrifugation (1500 rpm, 10 min). The immune cells and fibrocytes mixing in the single cells were isolated out by magnetic cell sorting with reagent kits (Miltenyi Biotech) following the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

The levels of CCK in the sera were determined by ELISA with a commercial reagent kit (Antibodies-Online, Atlanta, GA) following the manufacturer's instructions.

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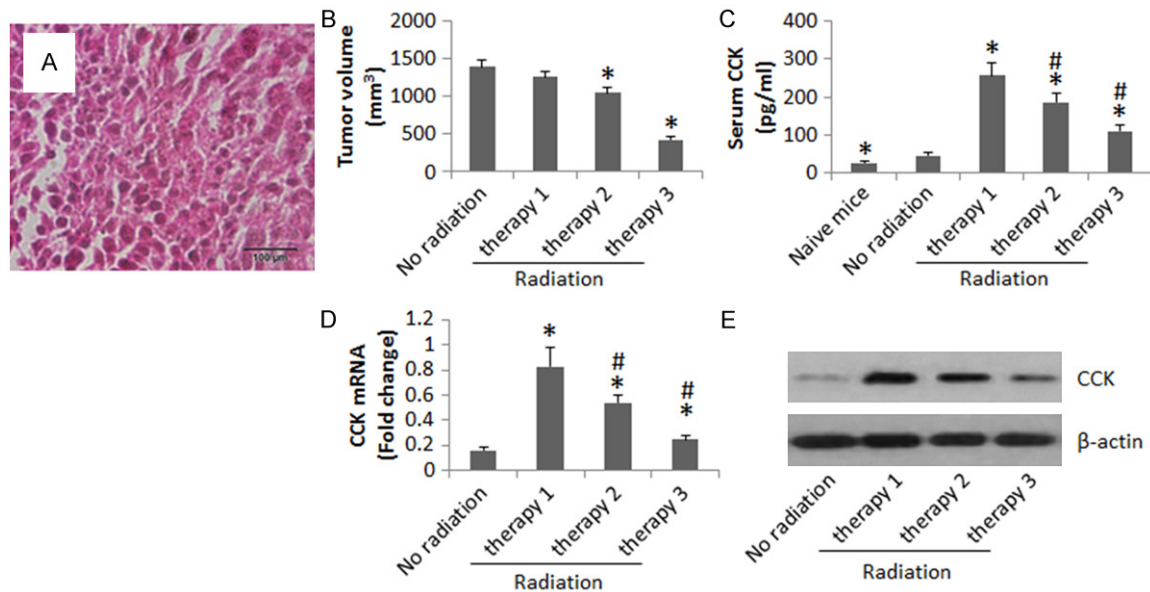


Figure 1. Radiation increases serum levels of CCK in tumor-bearing mice. Grouped tumor-bearing mice (6 mice per group) were irradiated at 12 Gys in a 12 day period. Each group was treated with one of the three therapies of irradiation. (A) A histology image from one tumor-bearing mouse representing 6 tumor-bearing mice, which shows the structure of the induced tumor tissue. (B) The bars show the tumor mass volume that was removed immediately after completion of the 12-day irradiation. (C) The bars indicate the serum CCK levels. (C, D) RNA and proteins were extracted from the isolated tumor cells, and analyzed by RT-qPCR and Western blotting. The bars indicate the CCK mRNA levels (D). The immune blots indicate the protein levels of CCK (E); the data are from one experiment representing 6 independent experiments. The data of bars are presented as mean \pm SD, which were summarized from 6 independent experiments. *, $P < 0.05$, compared to the “no radiation” group.

Real time quantitative RT-PCR (RT-qPCR)

The total RNAs were extracted from cells with TRIzol reagents. The RNA was converted to cDNA with a reverse transcription kit. The cDNA was amplified in a real time PCR device (CFX96 Touch, Bio-Rad) with the SYBR Green Master Mix. The primers used in the present study are presented in **Table 1**. The results were calculated with the $2^{-\Delta\Delta C_t}$ method and presented as folds of change against the housekeeping gene β -actin. The reagents for RT-qPCR were purchased from Invitrogen (Carlsbad, CA).

Western blotting

The total proteins were extracted from cells and quantitated by Bio Rad protein assay. The proteins were fractionated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk for 1 h at room temperature, incubated with the primary antibodies overnight at 4°C, washed with tris-buffered saline Tween 20 (TBST) for 3 times, incubated with the second antibodies for 1 h at room temperature, washed with TBST for 3

times. The membrane was developed with enhanced chemiluminescence. The results were photographed with a UVP Image System (Cambridge, UK). The integrated density of immune blots was determined by ImageJ software. The reagents for Western blotting were purchased from Invitrogen (Carlsbad, CA). The antibodies were purchased from Sana Cruz Biotech (Santa Cruz, CA).

Assessment of tumor cell apoptosis

Tumor cells were stained with propidium iodide (PI) and Annexin V reagent kit following the manufacturer’s instructions. The cells were analyzed by flow cytometry. The Annexin V⁺ or Annexin V⁺ PI⁺ cells were regarded as apoptotic cells.

RNA interference (RNAi)

The CCK gene was knocked down in A549 cells by RNAi with a shRNA kit (Santa Cruz Biotech) following the manufacturer’s instructions. The effect of knockdown was assessed by Western blotting.

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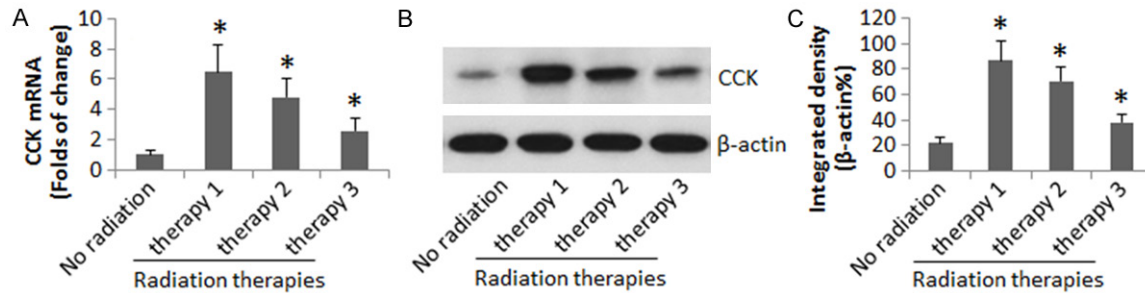


Figure 2. Irradiation induces CCK expression in tumor cells. A549 cells (a tumor cell line) were irradiated with 12 Gys with the allocation strategies denoted on the X axis. The cells were collected at the end of treatment and analyzed by RT-qPCR and Western blotting. (A) The bars indicate the CCK mRNA levels; the data were summarized from 3 independent experiments. (B) The immune blots indicate the protein levels; the data are results of one experiment representing 3 independent experiments. (C) The bars indicate the integrated density of the immune blots in (B), which were summarized from 3 independent experiments. The data of bars are presented as mean \pm SD. *, $P < 0.01$, compared to the “no radio” group.

Chromatin immunoprecipitation (ChIP)

A ChIP assay was performed using a ChIP kit (Sigma Aldrich, St. Louis., MO), according to the manufacturer's instructions. Cells were fixed with 1% formalin for 1 h at room temperature. After washing with PBS, the cells were sonicated to shear the DNA together with attached proteins into small segments (200-500 bps). The samples were precleared by incubating with protein G agarose beads for 1 h at 4°C. After centrifugation (3000 rpm, 10 min), the supernatant was incubated with antibodies of interest (or isotype IgG) overnight at 4°C, followed by addition of protein G agarose beads and incubated for another 2 h. The beads were collected by centrifugation (3000 rpm, 10 min); the attached immune complexes were eluted with an eluting buffer. The DNA was recovered by a PCR purification kit (Invitrogen, Carlsbad, CA) and analyzed by qPCR. The results were presented as folds of input. The P53 promoter primers include agagtgcattaccgttccca and tgagggttaggcaaagtcgt.

Statistics

Data are presented as mean \pm SD. Difference between two groups was determined by Student t test or ANOVA if more than two groups. A $P < 0.05$ was set as a significant criterion.

Results

Radiotherapy increases serum levels of CCK in tumor mouse model

Published data suggest that CCK is associated with the pathogenesis of cancer [11-14]. We

wondered if CCK is also involved in the radioresistance in tumor. To this end, we developed a mouse tumor model with tumor cell lines. The tumor cells grew into a tumor mass within two weeks, which showed a cancer-like histology structure (Figure 1A; reviewed by a pathologist). Tumor mice were irradiated using a linear accelerator at 12 Gy/mouse following one of three different radiation therapies: therapy 1 (1 Gy daily), therapy 2 (2 Gy/day every other day), and therapy 3 (3 Gy every 3 days). Control tumor mice were not received radiation. The tumor mass size was recorded in every 3 days. The tumor size recorded on day 12 of irradiation showed that the tumor volume was $1435.3 \pm 186.3 \text{ mm}^3$ in control tumor mice. The tumor volume was $1280.2 \pm 194.3 \text{ mm}^3$, $1007.8 \pm 157.6 \text{ mm}^3$ and $446.2 \pm 100.4 \text{ mm}^3$ in tumor mice treated with therapy 1, therapy 2 and therapy 3, respectively (Figure 1B). A correlation assay was performed with the radiation session times and the tumor size; the results showed a significant positive correlation ($r = 0.8482$, $P < 0.01$) between the tumor size and the irradiation session times. We also analyzed the correlation between the tumor size and the radiation dosage. The results showed a significant negative correlation ($r = -0.8992$, $P < 0.01$).

Blood samples were collected from each mouse immediately after cutting off the head. As analyzed by ELISA, the levels of CCK were at very low levels in naïve control mice, which were higher in tumor mice without irradiation and even higher in tumor mice after irradiation in an irradiation sessions-dependent manner (Figure

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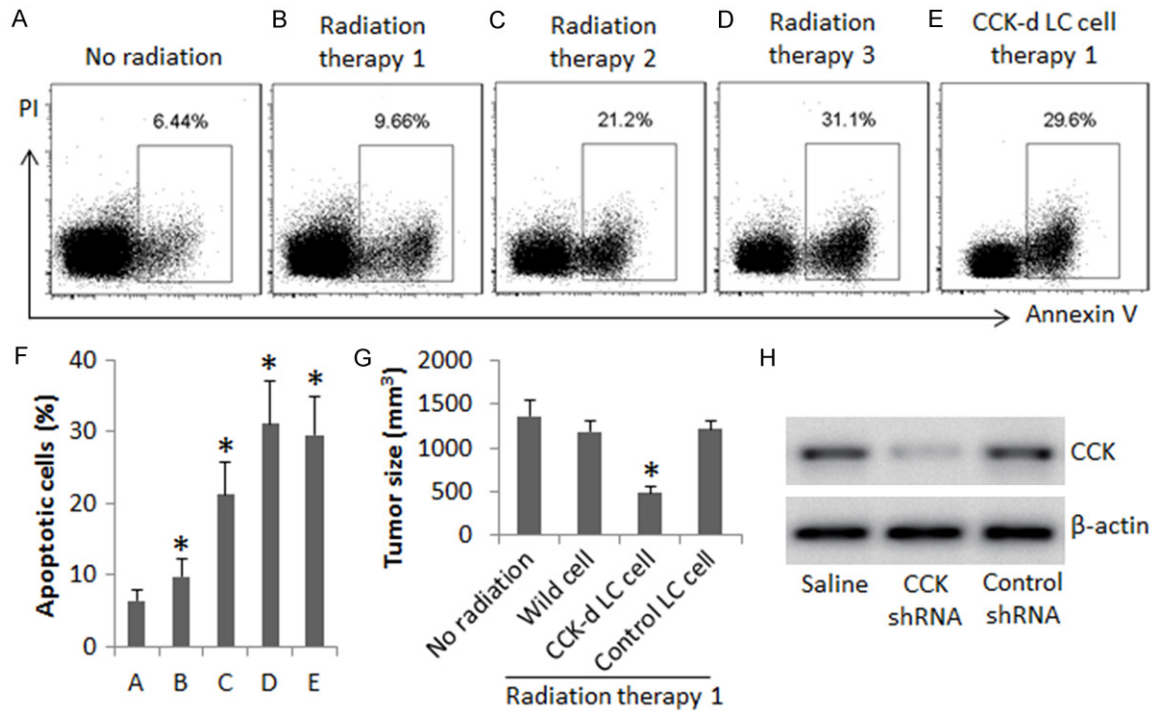


Figure 3. CCK inhibits radiation-induced tumor cell apoptosis. Single tumor cells were prepared from tumor tissue, which was excised from mice treated with radiotherapy described in **Figure 1**. (A-E) The gated dot plots indicate the frequency of apoptotic tumor cells; the sources of tumor cells are denoted above each subpanel. (F) The bars indicate the summarized data of apoptotic cells in (A-E) (The group labels are the same as those in subpanels A-E). (G) The bars indicate the tumor size from tumor-bearing mice. The treatment of tumor-bearing mice was denoted on the X axis. Wild cell or CCK-d tumor cell or control tumor cell: Mice were transplanted with wild tumor cells or CCK-deficient tumor cells or tumor cells treated with control shRNA. (H) The immune blots show the results of CCK RNAi. Each group consisted of 6 mice. The data of bars were summarized from 6 independent experiments and are presented as mean \pm SD. *, $P < 0.01$, compared to the “no radiation” group. Data of (A-E and H) are results of one experiment representing 6 independent experiments.

1B). As shown by the results of correlation assay, the serum CCK levels in irradiated tumor mice were significantly correlated with the tumor volume ($r = 0.6845$, $P < 0.01$) as well as the irradiation times ($r = 0.7658$, $P < 0.01$). We also detected the CCK mRNA and CCK proteins in the tumor tissue, which were also in an irradiation sessions-dependent manner (**Figure 1D, 1E**). The results suggest that irradiation can increase the CCK levels in the peripheral blood, which is positively correlated with the tumor volume and the sessions of irradiation times within a given length of period.

Radiation induces CCK expression in tumor cells

The results of **Figure 1** implicate that the irradiation induces tumor cells to express CCK. To test this, we irradiated A549 cells (a tumor cell line) with the same radiation strategy of **Figure**

1. The expression of CCK in tumor cells was assessed by RT-qPCR and Western blotting. The results showed that only trifle levels of CCK were detected in naïve tumor cells. The levels of CCK were significantly increased in response to irradiation, which was in the irradiation session-dependent manner with the same entire radiation dose (**Figure 2**). The results confirm that radiotherapy increases the expression of CCK in tumor cells.

CCK plays a role in the development of radioresistance of tumor cells

One of the mechanisms by which radiotherapy induces cancer cell death is to induce apoptosis in the cells. To test if CCK influenced the radiotherapy-induced cancer cell apoptosis, we excised the tumor tissue from tumor-bearing mice after completing the 12-day radiotherapy of the experiments of **Figure 1**. Tumor cells

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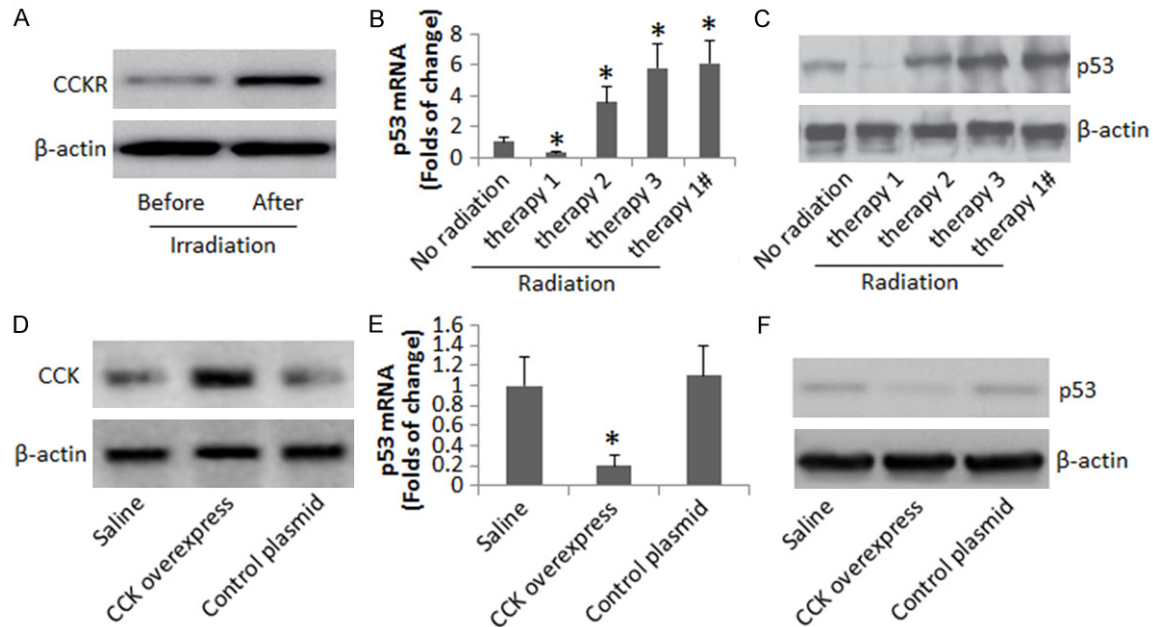


Figure 4. CCK suppresses p53 expression in tumor cells. (A) The immune blots show the protein levels of CCK receptor in A549 cells before and after irradiation (representing three independent experiments). (B, C) Cellular extracts were prepared from the isolated tumor cells of mice (6 mice per group) treated with irradiation of Figure 1 and analyzed by RT-qPCR and Western blotting. The bars indicate the p53 mRNA levels (B; summarized from 6 independent experiments). The immune blots indicate the protein levels of p53 (C; the data are results of one experiment representing 6 independent experiments). #, CCK-null mice. (D) A549 cells were transfected with a CCK plasmid. The immune blots show the CCK levels. (E, F) Cell extracts were prepared from CCK-overexpressing A549 cells and analyzed by RT-qPCR and Western blotting. The bars indicate the p53 mRNA levels (E; summarized from 3 independent experiments). The immune blots indicate the p53 protein levels (F; the data are results of one experiment representing 3 independent experiments). Data of bars are presented as mean \pm SD. *, $P < 0.01$, compared to the "No radiation" group (B) or the saline group (E).

were made from the tumor mass and analyzed by flow cytometry. The results showed that the irradiation of therapy 3 markedly induced tumor cell apoptosis, which was reduced in those treated with therapy 2, and was further reduced in those treated with therapy 1 (Figure 3A-G). A correlation assay was performed with the apoptotic data and the expression of CCK by tumor cells. The results showed a significant negative correlation between the sessions of apoptotic cells and the CCK expression in tumor cells. The results suggest that CCK inhibits the radiation-induced tumor apoptosis. To strengthen the data, we treated CCK-null mice with irradiation therapy 1. The results showed that the residual tumor mass volume was $455.67 \pm 80.48 \text{ mm}^3$, which was similar to those CCK-sufficient tumor mice treated with therapy 3 ($P > 0.05$). We also assessed the sessions of apoptotic cells in the tumor tissue of these mice. The results showed that the frequency of apoptotic tumor cells was also similar to that of

CCK-sufficient mice treated with therapy 3 (Figure 3E, 3F). The results demonstrate that CCK does play a critical role in the development of radioresistance in tumor-bearing mice. To corroborate the results, we transplanted CCK-deficient tumor cells to mice. The mice were then treated with radiation therapy 1. The results showed the radioresistance was not induced (Figure 3G, 3H).

CCK inhibits p53 expression in tumor cells

Published data indicate that suppression of p53 protein is associated with the development of radioresistance [15]. We wondered if the irradiation-induced CCK increases in tumor cells were associated with the suppression of p53. To this end, we firstly assessed the CCK receptors in tumor cells. As analyzed by Western blotting, A549 cells expressed CCK receptor, which was markedly up regulated after irradiation (Figure 4A). We then measured the p53 levels in tumor tissue after the treat-

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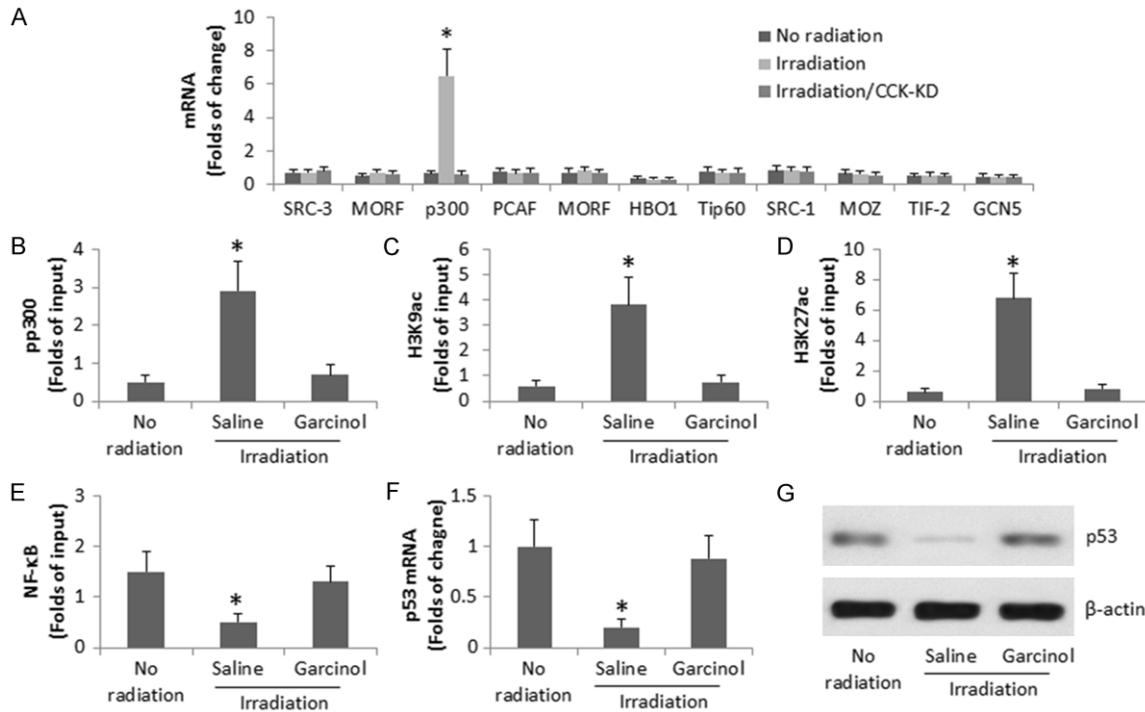


Figure 5. p300 block therapy 1 irradiation-reduced p53 gene transcription in tumor cells. (A) The bars indicate the mRNA levels of 11 HATs in tumor cells. (B-G) A549 cells were treated with the procedures as denoted on the X axis of bar graphs. The cell extracts were analyzed by ChIP (B-E), RT-qPCR (F) and Western blotting (G). (B-E) The bars indicate the levels of pp300 (B), acetylated H3K9 (H3K9ac; C), H3K27ac (D) and NF-kB (E) at the p53 promoter locus in 549 cells. (F) The bars indicate the mRNA of p53 in A549 cells. (G) The immune blots indicate the protein levels of p53 in A549 cells. Data of bars are presented as mean \pm SD. *, $P < 0.01$, compared to the "No radiation" group. The data are representatives of 3 independent experiments. Garcinol: An inhibitor of p300 (15 mM).

ment with the 3 therapies of irradiation. The results showed that the levels of p53 were low in tumor cells of the "no radiation" group, which were markedly increased in tumor cells treated with irradiation (Figure 4B, 4C), but negatively correlated with the irradiating sessions ($r = 0.6757$, $P < 0.01$). In the CCK-null mice, however, the irradiating sessions did not interfere with the expression of p53 in tumor cells (Figure 4B, 4C). The results implicate that CCK is associated with the suppression of p53 in tumor cells in radiotherapy. The data were strengthened by overexpressing CCK in A549 cells (Figure 4D), which significantly suppressed the expression of p53 in A549 cells (Figure 4E, 4F).

Histone acetyltransferase (HAT) p300 inhibitors prevent CCK-suppressed p53 expression in tumor cells

It is reported that HATs are involved in the development of radioresistance [16]. Considering HATs might be involved in the CCK-induced

radioresistance, we screened the expression of 11 HAT subtypes in tumor cells after therapy 1 irradiating. The results showed that radiotherapy increased the levels of p300 in tumor cells, which did not occur in the CCK-null tumor-bearing mice treated with irradiation (Figure 5A). We then treated A549 cells with radiation therapy 1. The cells were analyzed by ChIP. As shown by Figure 5B-G, the levels of pp300 and acetylated H3K9/H3K27 were markedly enhanced while NF-kB was markedly suppressed at the p53 promoter locus in tumor cells. The results implicate that irradiation increases CCK in tumor cells; the CCK suppresses p53 expression afterwards, in which p300 is involved. To test this inference, we added garcinol, an inhibitor of p300, to the A549 cell culture during irradiation. Indeed, the enhancement of CCK was not affected, while the levels of pp300, H3K9ac/H3K27ac and NF-kB at the p53 promoter locus were not increased, which also influenced the levels of p53 mRNA and proteins in tumor cells.

Discussion

Radioresistance is a great drawback in the treatment of cancer. To find the pathogenesis of radioresistance is of significance for the treatment of cancer. The present study revealed a previous unknown phenomenon that radiotherapy induced CCK production by tumor cells; the latter triggered the radioresistance by repressing the expression of p53. The increase in CCK in tumor cells occurred in radiation therapy 1, a condition of low radiation dose and more radiation sessions performed in a given period, but not in radiation therapy 3, a condition of high radiation dose and less radiation sessions performed in a given period. The tumor cells expressed CCK receptor. The tumor cell-produced CCK up regulated the levels of p300 at the p53 promoter locus in tumor cells and suppressed the expression of p53 in tumor cells, and thus, interfered with the radiotherapy-induced tumor cell apoptosis. Blocking p300 efficiently attenuated the CCK-induced p53 suppression.

It is accepted that radioresistance may be induced by exposure to small doses of ionizing radiation. Our data are in line with this notion by showing that within a given entire irradiation dose, the delivery session times is positively correlated with the development of radioresistance in tumor cells. Several cellular mechanisms have been proposed to associate with the development of radioresistance, such as increases in gene expression frequently leads to alteration in protein levels, DNA repair and other processes [17]. Previous reports indicated that suppression of p53 was associated with the development of radioresistance in cancer cells [18]. In that study, Tang et al found that small doses of irradiation increased a Trib1 molecule in glioma cells and suppressed the expression of p53 and reduced the glioma cell apoptosis [18]. Our data are in agreement with Tang's report by showing that p53 expression was also suppressed by small doses of irradiation. The novel aspect of our data was that the small doses of irradiation induced CCK-production by tumor cells. The autocrine CCK suppressed the gene transcription of p53 in tumor cells. The data suggest that the development of radioresistance may be a multiple cause-phenomenon.

Previous studies indicate that p53 degradation is involved in the mechanism of radioresistance in several types of tumors [19, 20]. Such p53 degradation can be reversed by some micro RNA to restore the radio-sensitivity [21]. Our data showed another aspect of modulating p53 production to recover the lost radio-sensitivity. The data demonstrate that blocking CCK or p300 almost abolished the small doses of irradiation-induced radioresistance in tumor cells, demonstrating a therapeutic potential in the prevention of radioresistance by using these inhibitors.

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Disclosure of conflict of interest

None.

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