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Effects of radiation dose on the stemness-related genes expression in colorectal cancer cell line

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► ***Original article***

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ABSTRACT

Background: Accumulating reports suggest that radiation may change gene expression in cancer cells and promote cell migration and invasion, as well as inducing cancer stem cell (CSC). However, the correlation between these processes and radiation dose has not been shown yet. Therefore, the present study aimed to evaluate the effect of low, medium, and high doses of X-ray on expressing three genes involved in CSC induction in colon cancer cell line (HT-29). ***Materials and Methods:*** The cells cultured in flasks were irradiated with X- rays in different doses including 0.1, 2.5, 5, and 10 Gy. Then, the expression of Oct4, CD44, and ALDH1 genes was measured using real-time PCR. PCR efficiency was evaluated for each gene using Linreg PCR software, and relative changes for mRNA were calculated based on the ΔΔCt method. ***Results:*** CD44 gene expression increased equally at all doses. Oct4 and ALDH1 gene expression were not affected by 10 Gy, but low and moderate doses increased them equally. ***Conclusion:*** The effects of low and moderate doses on increasing the expression of stem-related genes are equal. In addition, the effect of the high dose on increasing CD44 gene expression was equal to the low and moderate doses.

Keywords: *Radiation, colorectal cancer, gene expression, real-time PCR.*

INTRODUCTION

The accumulation of genetic alteration is considered as one of the essential causes of cancer incidence. These alterations can disrupt signaling pathways, which lead to initiation, promotion, and maintenance of tumor cells, as well as tumor recurrence or metastasis after treatment process [1, 2]. In addition, altered signaling pathways and genomic instability can change tumor metabolism and cause cancer cells to evade tumor suppressors, resist cell death mechanisms, promote inflammation, and induce angiogenesis. Acquisition of epithelial-mesenchymal transition (EMT), and activation of invasion and metastasis pathways in cancer cells develop cancer stem-like phenotype, which results in tumor relapse and therapy resistance [3-6].

Based on reliable evidence, a network of signaling pathways including growth factors such as transforming growth factor-beta (TGF-β) or epidermal growth factor (EGF) and their related signaling proteins such as Wnt, Notch, Hedgehog, and NF-kB may change in response to radiation at the molecular level. These alterations may result in expressing different genes such as cancer stem cell (CSC) related

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ones [5, 7, 8], which is the leading cause of cancer recurrence and has been observed after irradiation in different cancers such as lung [9], breast [5, 10], colorectal [4], cervical [11] and squamous carcinoma [12, 13]. Some studies indicated that was correlation was observed between the radiation dose and the expression of the genes involved in EMT and CSC induction in post-irradiated cancer cells [7, 14], while no correlation was reported in others [15]. Thus, the effect of radiation dose on the expression of Oct4, CD44, and ALDH1 genes (involved in CSC formation) was evaluated in a colorectal cancer cell line (HT-29).

MATERIALS AND METHODS

Cell line and cell culture

First, the HT-29 colorectal cell line, provided from Pasteur Institute (Tehran, Iran) grown in Roswell Park Memorial Institute 1640 (Bioidea, Tehran, Iran) was supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 u/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, Mo, USA). The cells were incubated at a humidified 5% CO_2 atmosphere at 37°C and sub-cultured, when required, by using 0.25% trypsin-0.5 mM EDTA (Gibco, Grand Island, NY, USA).

Irradiation

HT-29 cells were plated in the 12.5 cm^2 tissue culture flask (Jet Biofil, China). The 70% confluent cells were irradiated with various single doses of X-ray including 0.1, 2.5, 5 and 10 Gy, which was emitted from an X-ray unit (Philips, serial number 2.625, Netherland, dose rate: 1.365 Gy/min with 100 kVp and 8 mA) at the room temperature. The cells without any radiation were used as a control group.

RNA extraction

To evaluate the effect of post-irradiation time on gene expression, the total RNA of the cells was exposed to 2.5 Gy of X-ray and their relevant RNA in the control group was extracted 6, 20 and 48 hours after irradiation (Yekta Tajhiz Azma kit, Tehran, Iran). The procedure was performed according to the manufacturer's instructions.

The total RNA of the cells exposed to various doses of X-ray (0, 0.1, 2.5, 5, and 10 Gy) was extracted 20 hours after irradiation to evaluate the effect of radiation dose on gene expression. Then, the extracted RNA was checked for concentration, purity, and integrity using nanodrop® spectrophotometer (Thermo Fisher Scientific, lnc) and agarose gel electrophoresis. In addition, 1 µg of total RNA was treated with RNase-free DNase I and inactivated by EDTA using Thermo Scientific kit (Massachusetts, USA). Finally, the extracted RNAs were stored at -80°C until synthesizing cDNA.

cDNA synthesis and RT-PCR

According to the manufacturer's instructions, treated RNAs were reversely transcribed into cDNA using Suprime Script RTase, Oligo-dT, and dNTPs (Genet Bio, Korea).To confirm the fidelity of synthesized cDNA, polymerase chain reaction was performed by Ampliqon Taq DNA polymerase Master Mix RED kit (Denmark). GAPDH primers were used in this reaction, and the final products were loaded on 2% agarose gel. Table 1 indicates the cycling conditions of polymerase chain reaction.

Quantitative real-time PCR

Finally, the Ampliqon SYBER Green PCR kit (Denmark) was used to perform real-time polymerase chain reaction (real-time PCR) for CD44, ALDH1, and Oct4 genes. Then, Light Cycler 96 System (Roche, Basal, Switzerland) was used to perform real-time PCR. Table 2 indicates the specific primer sequences. The Ct number of all genes was normalized to GAPDH in each sample. In addition, PCR efficiency was measured for each gene using Linreg PCR software, and relative changes for mRNA were calculated based on the ΔΔCt method.

Statistical analysis

The data were statistically analyzed using Graph Pad Prism version 8.0. The normality of the quantitative data was checked by

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Shapiro-Wilk test. Furthermore, one-way ANOVA or Kruskal-Wallis test was used for analyzing the difference in gene expression profile. All results were shown as mean ± SD in at least three independent experiments run in duplicate, and P value <0.05 was considered as the significant value.

RESULTS

Gene expression of HT-29 cells after irradiation with 2.5 Gy X-rays at different post-irradiation times

As shown in figure 1, the expression of all genes almost increases due to radiation, although some are not statistically different from non-irradiated cells (control group). In addition, a delay for 20 hours occurs after increasing gene expression of Oct4. The mRNA level of ALDH1approximately doubled 20 hours after irradiation although it was not statistically different from the control group. Accordingly, the expression of the genes was examined 20 hours after exposure to different doses of X-ray in the rest of the study.

Expression of CD44, ALDH1, and Oct4 genes after exposure to different doses of X-ray

Radiation resulted in upregulating both CSC genes including CD44 and ALDH1. However, these genes were overexpressed differently when exposed to various doses of X-ray. As shown in figure 2, the expression of CD44 increased significantly at low, medium, and high doses of X-ray, while the over-expression of ALDH1 was statistically significant only at doses of 0.1 and 2.5 Gy. In addition, the radiation indicated a significant increase in Oct4 expression at 0.1 and 2.5 Gy doses. However, no change occurred in the expression of Oct4 gene at 5 and 10 Gy doses.

Table1. Polymerase Chain Reaction Cycling.

Steps	Temperature (˚C)	Time (s)	Cycle
Initial denaturation	95	5	1
Denaturation	95	30	40
Annealing	58	30	
Elongation	72	30	

Table 2. The List of Primer Sequences and their Product Size Used for Real-Time PCR Analysis.

Primer Name	Primer Sequence (5' to 3')	Product Size
GAPDH-forward	GACCACTTTGTCAAGCTCATTTCC	150
GAPDH -reverse	GTGAGGGTCTCTCTCTTCCTCTTGT	
CD44 -forward	CGGACACCATGGACAAGTTT	176
CD44- reverse	GAAAGCCTTGCAGAGGTCAG	
ALDH1-forward	CTGCTGGCGACAATGGAGT	111
ALDH1-reverse	GTCAGCCCAACCTGCACAG	
Oct4 -forward	GAACATGTGTAAGCTGCGGCC	270
Oct4 -reverse	CCCTTCTGGCGCCGGTTAC	

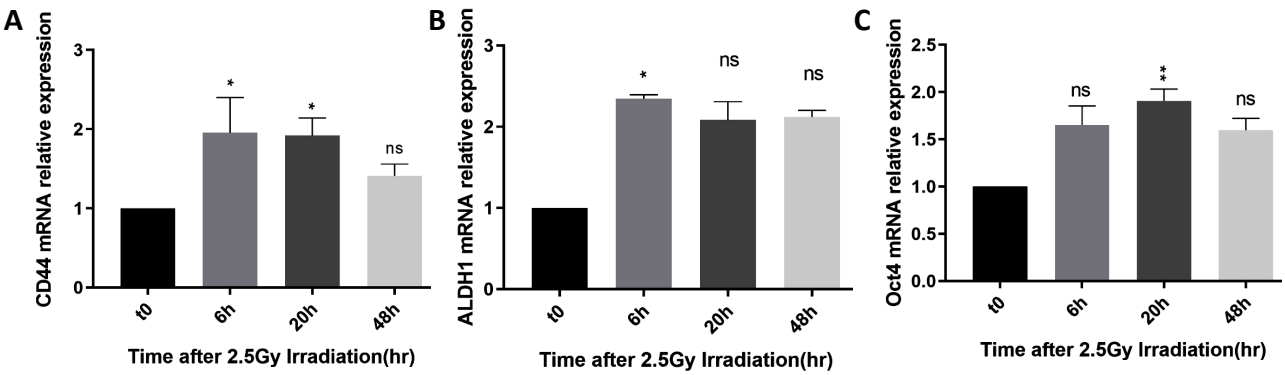

Figure 1. Upregulation of CD44 **(A)**, ALDH1 **(B)** and Oct4 **(C)** genes at different post-irradiation times. Gene expression values obtained from irradiated cells were compared with control group which was standardized to a value of 1. The experiment was performed at least three times in duplicate and the results were presented as mean±SD.

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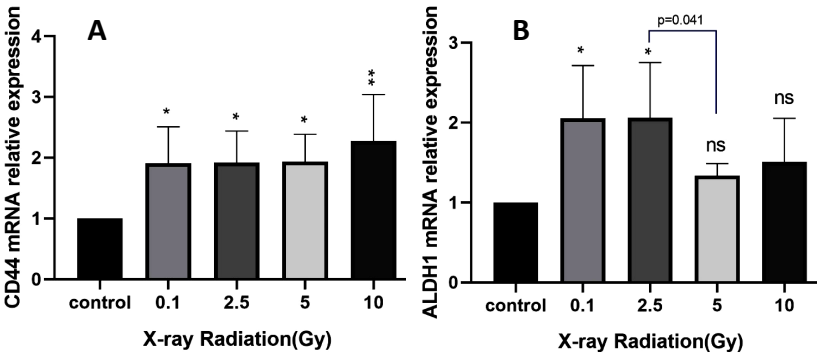

Figure 2. Upregulation of CD44 **(A)** and ALDH1 **(B)** genes after irradiation of HT-29 cells with different single doses of X-ray. Gene expression values obtained from irradiated cells were compared with control group which was standardized to a value of 1. The experiment were performed at least three times in duplicate and results were presented as mean±SD. (*P<0.05 and **P<0.01).

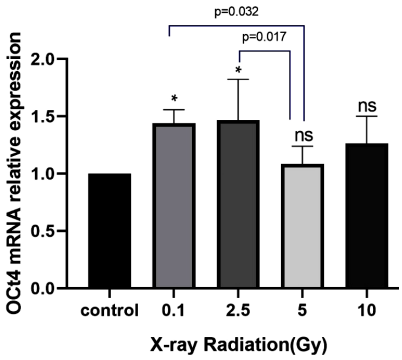

Figure 3. Upregulation of oct4 gene after irradiation of HT-29 cells with different single doses of X-ray. Gene expression values obtained from irradiated cells were compared with control group which was standardized to a value of 1. The experiment were performed at least three times in duplicate and results were presented as mean ±SD. (*P<0.05).

DISCUSSION

Ionizing radiation is considered as one of the most effective methods for cancer treatment. More than 50% of the patients suffered from cancer receive radiation therapy as a part of their therapeutic process, either alone or in combination with other modalities [16, 17]. However, tumor relapse and metastasis of cancer occur in a large number of the patients who received radiation therapy. Some studies indicated that some genes promote invasion, enhance metastasis potential, and induce cancer stem cell (CSC) gene expression in non-stem cancer cells in response to radiation [4, 13, 18-21]. However, the effect of different radiation doses on expressing CSC related genes has not been considered yet. Thus, the present study aimed to evaluate the effects of low, medium, and high doses of X-rays on expressing Oct4, CD44, and ALDH1genes involved in inducing EMT and CSC in an invasive colorectal cancer cell line (HT-29).

The results of the present study indicated that the CD44 overexpression does not depend on radiation dose as it is over-expressed in response to radiation without any significant difference among various doses. The expression of ALDH1 gene increased at 0.1 and 2.5 Gy doses, but no significant change occurred at 5 and 10 Gy as high doses. It seems that the gene expression of ALDH1 relies on radiation dose. The same happened for Oct4 gene expression. In fact, its expression relied on radiation dose without any effect in high doses.

CD44 protein is known as a CSC marker in various cancers such as colorectal cancer [22-26]. CD44 is a transmembrane glycoprotein interacting with its prominent receptor hyaluronic acid and activating different signaling pathways which can contribute too many cellular processes including cell growth, survival, differentiation, and motility [27]. In cancer cells, the expression of CD44 up-regulates and enhances cellular aggregation and tumor cell growth, and facilitates the proliferation process during radiation-induced accelerated repopulation [27-30]. Aldehyde dehydrogenase (ALDH1) is known as a CSC marker. Like CD44, it represents enhanced expression in different cancer cells with high proliferation and clonogenic capability [31]. In addition, it performs a protective role against oxidative stress, conserves cancer cells against the toxicity of radiation-induced reactive oxygen species (ROS) production, and promotes radio-resistance [32]. The results of the previous studies indicated that the higher level of ALDH1 is associated with lymph node and liver metastasis in the patients with colorectal cancer [31, 33]. Octamer-binding transcription factor 4 (Oct4) gene as a central

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regulator of pluripotency plays a leading role in self-renewing embryonic stem cells [34, 35]. Some studies indicated that the high expression of Oct4 can induce malignancy and stem-like properties in cancer cells [36, 37]. Chang *et al.* demonstrated that the increased mRNA level of Oct4 can elevate the expression of cytokines IL-8 and IL-32 and promote stem-like features in colorectal cancer cells [38]. Saigusa et al. reported that the enhanced level of Oct4 may develop distant recurrence and poor disease-free survival in rectal cancer patients treated with preoperative chemo-radiation therapy [39]. Consequently, the overexpression of these genes should be considered as a critical alarm of radiation outcome failure due to radiation.

Since CD44 is considered as a putative marker of CSC induction in colorectal cancer and its gene is overexpressed at all doses, the lack of upregulating ALDH1 and Oct4 at high doses (5 and 10 Gy) does not necessarily indicate that CSC induction does not occur at these doses. Various signaling pathways such as Wnt/β-catenin, Hdgehog, notch, JAK/STAT and TGF-β, some cytokines, and various microRNAs are involved in regulating the genes related to CSC induction. In addition, the elevated expression of the genes involved in Wnt pathway is associated with pluripotency-related genes [40, 41]. Further, different responses of the genes considered in the present study into the radiation dose may be related to the different mechanisms controlling their regulation. On the other hand, a crucial role of Wnt/β-catenin signaling pathway in up-regulating both ALDH1 and CD44 genes in colorectal cancer cell lines was observed in some studies [32, 42, 43], which indicated that the same mechanism regulates their expression. However, they were expressed differently at high dose in this study. Therefore, further studies should be conducted to evaluate which mechanism plays a central role in upregulating CD44, ALDH1, and Oct4 genes separately.

In line with the results of the present study, Shao et al. reported that the expression of Oct4 increased in post-irradiated HT-29 cells with different doses of 1, 2, and 3 Gy of X-ray. However, doses more than 3 Gy was not considered in the present study. In addition, they found that the high expression of Oct4 may lead to the resistance to radiation in colorectal cancer cells [44]. In another study, Ghisolfi et al. indicated that 2 and 4 Gy gamma radiation significantly increased spherogenesis in HepG2 and Huh7 cells, while the number of spheres failed to increase significantly at the doses of 6, 8, and 10 Gy. Further, they measured the expression of Oct3/4 and Sox2 genes. Only Oct3/4 gene overexpressed in HepG2 cells, while the expression of Sox2 gene increased significantly at 4Gy in Huh7 cells [15].

In another study, Lagadec *et al.* indicated that the number of ALDH-positive cells increased after irradiating SUM159PT cells with both 4 and 8 Gy doses. However, an increase in the number of ALDH1-positive cell was significantly higher at 8 Gy compared to that of 4 Gy. Further, they indicated that the number of $CD24^{-/low}/CD44^{high}$ cells (indicating CSC phenotype) increased in MCF-7 and T47D breast cancer cells as a result of 4 and 8 Gy irradiation [7]. Furthermore, the expression of SOX2 gene increased just at 8 Gy.

Regarding low-dose irradiation, some studies addressed the preventive effect of low-dose irradiation on CSC induction. Savickiene *et al.* demonstrated that low-dose of gamma-irradiation (1-100 cGy) caused 25% of HL-60 cells undergoing differentiation, while only 3-5% of these cells underwent spontaneous differentiation [45]. Additionally, Kaushik et al. observed that low-dose radiation suppressed EMT and CSC induction in breast cancer cells by inhibiting the Jak1/STAT3 signaling pathway [46], which are inconsistent with the results of the present study in which all three genes are overexpressed at 0.1 Gy.

CONCLUSION

The results of the present study indicated that different doses of X-ray may effectively upregulate the expression of CD44, ALDH1, and Oct4, which are genes with a central role in CSC induction in colorectal cancer cells. The results suggest that even low-dose irradiation can upregulate the expression of these genes.

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This study mainly focuses on the effects of irradiation on gene expression. Further studies can be conducted to evaluate the number of CSCs directly and investigate whether the above alterations in gene expression can promote CSC or EMT phenotype in tumor cells.

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***Conflicts of interest*: Declared none.**

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