

# Modulation of MMP-2 and TIMP-2 by low dose radiation in mice bearing S180 sarcoma

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## Abstract

**Objective** To investigate the inhibition of low dose radiation (LDR) on S180 sarcomas and its modulation of MMP-2 and TIMP-2 in mice.

**Methods** S180 subcutaneously implanted tumor model mice were randomly divided into two groups: control (N) and low dose radiation (LDR) groups. N mice were sacrificed after 12 h, whereas LDR mice were sacrificed after 12 (LDR-12 h), 24 (LDR-24 h), 48 (LDR-48 h), and 72 (LDR-72 h) h. Thereafter, we measured the tumor volumes. Histopathology was performed, and P-V immunohistochemistry was applied to assess MMP-2 and TIMP-2 expression.

**Results** Compared with the control group, the tumor growth was significantly inhibited in the LDR groups ( $P < 0.05$ ). MMP-2 expression was considerably reduced in LDR-24h ( $P < 0.05$ ) and LDR-48h ( $P < 0.05$ ), whereas the change of TIMP-2 was not obvious in the LDR groups ( $P > 0.05$ ) in contrast to that of the control group.

**Conclusion** LDR can effectively suppress the growth of S180 implanted tumors by reducing MMP-2, which is associated with invasion and metastasis.

**Key words:** MMP-2; TIMP-2; low dose radiation; S180 sarcoma

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As a global public health problem, malignant tumors are becoming more common, resulting in a relatively high mortality rate and a negative effect on human health. Common treatments for malignant tumors include surgery, radiotherapy, chemotherapy, endocrine treatment, and biological immunotherapy. Tubiana (1999) reported that 45% of malignant tumors could be cured by surgery (22%), radiotherapy (18%), and drugs and other methods (5%). Thus, radiotherapy is very important in the treatment of malignant tumors [1]. However, radiation could damage various cellular components, directly (molecule ionization) or indirectly (reactive oxygen species production), including DNA. For protection, the irradiated cells may have innate defense mechanisms, such as the removal of oxidative stress and damaged cells, and DNA repair that may cause tissue or organ dysfunction and malignant diseases [2-3]. According to Muller's data, low doses of radiation can cause dose-proportional detrimental effects, such as cancer and heritable genetic mutations, without a threshold dose, which has been defined as the "linear-no-threshold (LNT) hypothesis". However, the accuracy

of the LNT hypothesis for estimating cancer risks from low dose radiation by experimental and epidemiological evidence should be determined [2, 4]. The United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) (1986) reported that LDR refers to a low linear energy transfer (LET) radiation dose of  $< 0.2$  Gy or a high LET radiation dose of  $< 0.05$  Gy, and the radiation dose rate should be  $> 0.05$  mGy/min. Increasing experimental and clinical data has identified that LDR could induce comprehensive adaptive responses that could improve immunity and enhance the tolerance of normal tissue under radiation. In addition, pre-low-dose radiation for cells could increase their sensitivity to acute irradiation at high doses [5-6]. Low-dose hypersensitivity (HRS) eliminated potential mutant cells and reduced carcinogenic risks observed with LDR, and disappeared at doses  $> 0.5$  Gy [2]. Global research has shown that LDR has shown positive results in the treatment of malignant tumors.

Tumor invasion and metastasis are processes involved with tumors cells and extracellular matrix components (ECM), which provide a significant barrier to tumor cell

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invasion [7]. Numerous clinical and experimental studies have demonstrated that matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, play a key role in tumor cell invasion and metastasis due to their ability to degrade type IV collagen, a major component of the ECM [8]. As inactive pro-enzymes in a latent zymogenic form, MMP-2 and MMP-9 were activated by other MMPs or proteases and inhibited by specific inhibitors, and tissue inhibitors of metalloproteinases (TIMPs); and TIMP-2 specifically inactivated MMP-2. Consequently, the imbalance of MMP-2 and TIMP-2 may be the critical decisive factor for tumor progression and recurrence in cancer [7-8]. The overall aim of the present experiment was to study the relationship between LDR and MMP-2 and TIMP-2 expression, so that the antitumor mechanisms of LDR could be determined to provide an enhanced theoretical guide for its clinical application.

## Materials and methods

### Animals and chemicals

Male Kunming mice (purchased from Qingdao Institute for Drug Control, China), weight 20–22 g, 3–4 weeks old, were raised in specific pathogen free (SPF) conditions with unlimited food and water in a laminar flow clean bio-frame (License No. SCXK (Lu) 20030010). The monoclonal antibodies, MMP-2 (Bioss Biotechnology Co. Ltd) and TIMP-2 (Santa Cruz Biotechnology Co. Ltd) were rabbit anti-human immunoglobulin IgG1.

### Cell lines and culture

S180 sarcoma cell lines were obtained from the Medical Pharmaceutical Research Institute, Shandong Province, China, and generated for 7 days in the abdomen of the mice. After S180 sarcoma cells were passaged intraperitoneally in two passages, the abdomen dropsy was drowned off to collect the tumor cells that were made into a single cell suspension of  $6.5 \times 10^7$  mL. Later, we implanted  $6.5 \times 10^6$  S180 sarcoma cells (0.1 mL) subcutaneously into the right inguen of the mice.

### Radiation conditions

The tumor growth was observed when the mice were conventionally cultured with unlimited food and water for 7 days. Then, they were randomly divided into two groups: low dose radiation (LDR) and control (N). All the mice in the LDR group were exposed to 75 mGy of whole-body radiation via a cobalt-60 radiation machine (Xinhua Medical-equipment Company of Shandong Province). The mice were placed into wooden boxes ( $15 \times 15 \times 35$  cm), and a lead plate ( $15 \times 15$  cm) was placed between the source and box to filter the radiation at a source skin distance of 160 cm, a radiation field of  $45 \times 45$  cm, dose rate of 15.9 mGy/min, and exposure time of 4.72 min.

### Specimen collection

Seven days after the mice were implanted with tumors, the maximum horizontal diameter, a (cm), and vertical diameter, b (cm), of the tumors were measured twice with a slide gauge to determine the average tumor size, and mice with tumors that were too large ( $ab > 1.5 \text{ cm}^2$ ) or too small ( $ab < 0.40 \text{ cm}^2$ ) were excluded. The formula of  $V = 1/2 ab^2$  was used to calculate the average tumor sizes. Mice in the LDR group were exposed to 75 mGy of whole-body radiation when there was no difference in the sizes of the tumors between the LDR and N groups. Then, the mice of the two groups were sacrificed at 12, 24, 48, and 72 h.

### Tumor growth

The tumor tissue was exposed after the mice were sacrificed at 12, 24, 48, and 72 h, and the average volumes of tumors according to the above methods were calculated.

### Detection of the MMP-2 and TIMP-2 level by PowerVision two-step immunostaining

(1) The tissues were fixed in 10% neutral formalin, and tumor cells were extracted using an automatic tissue hydroextractor, then flushed with water for at least 4 h and embedded in paraffin; (2) The tissues were sliced into 1–2  $\mu\text{m}$  thick sections, and placed into a 60 °C thermotank overnight; (3) The paraffin sections were dewaxed with xylene, and hydrated with an ethanol gradient; (4) The sections were cooled at room temperature, and then placed into a potassium citrate solution (pH 6.0) in an autoclave for 5 min; (5) The sections were washed 3 times for 3 min each using a PBS buffer; (6) The sections were incubated for 80 min at 37 °C with the primary antibody (monoclonal antibodies of the rabbits); (7) The sections were washed 3 times for 3 min each using a PBS buffer; (8) The sections were incubated for 30 min at 37 °C with the second antibody; (9) The sections were washed 3 times for 3 min each using a PBS buffer; (10) The sections were dyed with a DAB stain; (10) The slides were washed with the running water, stained with hematoxylin and dehydrated with alcohol, then cleared in xylene and fixed in neutral balata. In addition, we replaced the primary antibody with a buffer as a negative control.

### Semi-quantitative assay of MMP-2 and TIMP-2

The cells were counted using a high power microscope ( $\times 200$ ). According to the light or dark colors of the immunoreactive substances and the percentage of positive cells, the expression intensity of MMP-2 and TIMP-2 was divided into the following four categories: When the percentage of positive cells was  $< 5\%$ , it was (-); when the positive cells were 5%–25% and the cells were slightly dyed, it was (+); when the positive cells were 26%–50% and tan particles were observed, it was (2+); when the

Table 1 Effects of low dose radiation on tumor growth (cm<sup>3</sup>)

Group	n	Tumor volumes			
		12 h	24 h	48 h	72 h
N	0.3875 ± 0.26	0.3980 ± 0.31	0.4155 ± 0.33	0.5246 ± 0.36	0.7173 ± 0.35
LDR	0.3966 ± 0.22	0.3841 ± 0.30 <sup>a</sup>	0.4037 ± 0.29 <sup>b</sup>	0.4157 ± 0.31 <sup>c</sup>	0.4108 ± 0.27 <sup>d</sup>

<sup>a</sup> Compared with N, *t* = 0.09, *P* > 0.05; <sup>b</sup> Compared with N, *t* = 0.08, *P* > 0.05; <sup>c</sup> Compared with N, *t* = 0.72, *P* > 0.05; <sup>d</sup> Compared with N, *t* = 2.19, *P* < 0.05

positive cells were > 50% and stained stronger, it was (3+).

### Statistical analysis

PPMS 1.5 was used for the statistical analysis. Quantitative data were expressed by mean ± standard deviation ( $\chi \pm s$ ) and analyzed with a *t*-test. Semi-quantitative data were analyzed by Ridit scoring.

## Results

### The influence of LDR on the general condition of the mice

After receiving irradiation from 12 to 72 h, it was observed that the mice in the LDR groups were in a better mental state, more active, and ate more food than those in the N group.

### The inhibition of tumor growth by LDR

After receiving low dose radiation, the tumor size was calculated at 12, 24, 48, and 72 h, as shown in Table 1. Compared with tumor size in the N mice, the tumor size of LDR mice was smaller, and there was a significant difference between the LDR-72 h and N groups (*P* < 0.05).

### Effects of low dose radiation on MMP-2 and TIMP-2

The results showed that the MMP-2- and TIMP-2-positive stained cells that displayed granular brown substances in the cell membrane and cytoplasm were mainly tumor cells and interstitial cells adjacent to the tumor cells. The positive expression of MMP-2 and TIMP-2 was uneven with sheet and focal distributions. The data from the half-quantitative assay showed that MMP-2 expression was significantly different between LDR-24 h and LDR-48 h, and N (*P* < 0.05), whereas the variation in TIMP-2 was not obvious (*P* > 0.05) in contrast to N, as shown in Tables 2 and 3, and the microscopic observation (× 400) is shown in Fig. 1–4.

## Discussion

Recently, radiation has been used as a powerful tool in the therapy of malignant tumors, but high dose radiation can have harmful effects on normal cells<sup>[9]</sup>. The LNT hypothesis, which has been accepted by regulatory agencies

Table 2 The effects of LDR on MMP-2

Irradiation time (h)	Group	Counts				Sum
		-	+	++	+++	
12	N	1	5	4	0	10
	LDR-12 h	4	3	1	0	8 <sup>a</sup>
24	N	1	5	4	0	10
	LDR-24 h	6	3	1	0	10 <sup>b</sup>
48	N	1	4	4	1	10
	LDR-48 h	6	2	2	0	10 <sup>c</sup>
72	N	2	6	2	0	10
	LDR-72 h	6	3	1	0	10 <sup>d</sup>

<sup>a</sup> Compared with N, *u* = 1.86, *P* = 0.06 > 0.05; <sup>b</sup> Compared with N, *u* = 2.30, *P* = 0.02 < 0.05; <sup>c</sup> Compared with N, *u* = 2.15, *P* = 0.03 < 0.05; <sup>d</sup> Compared with N, *u* = 1.65, *P* = 0.10 > 0.05

Table 3 The effects of LDR on TIMP-2

Irradiation time (h)	Group	Counts				Sum
		-	+	++	+++	
12	N	3	4	3	0	10
	LDR-12 h	3	3	2	0	8 <sup>a</sup>
24	N	2	4	3	1	10
	LDR-24 h	4	6	0	0	10 <sup>b</sup>
48	N	3	4	3	0	10
	LDR-48 h	2	7	1	0	10 <sup>c</sup>
72	N	2	5	1	1	10
	LDR-72 h	3	6	0	1	10 <sup>d</sup>

<sup>a</sup> Compared with N, *u* = 0.33, *P* = 0.74 > 0.05; <sup>b</sup> Compared with N, *u* = 1.81, *P* = 0.07 > 0.05; <sup>c</sup> Compared with N, *u* = 0.29, *P* = 0.77 > 0.05; <sup>d</sup> Compared with N, *u* = 0.60, *P* = 0.55 > 0.05

worldwide, indicates that every radiation dose, no matter how low, is harmful to humans without a threshold. However, based on global data, especially Japanese A-bomb survivor data, it has been widely accepted that no statistically significant risk has been demonstrated in humans when exposed to doses < 100 mGy. Consequently, LDR could be used successfully in tumor treatment because of its ability to induce a stress response in cells, which reduces the risk of spontaneous and radiation-induced disease<sup>[9–11]</sup>. Extensive research shows that mammals, including humans, exposed to low-dose radiation can induce comprehensive adaptive responses, and reduce and suppress tumorigenesis. Nevertheless, the mechanism of tumorigenesis is unclear. It could be due to the activation of cell signaling, causing the production of a series of enzymes or proteins, regulating the expression of related genes, re-

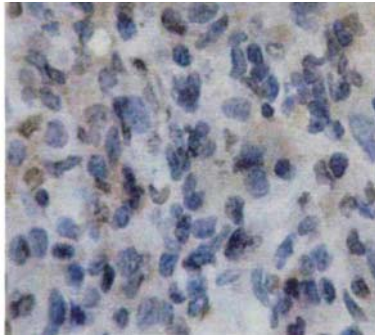


Fig. 1 The expression of MMP-2 in the control (N)

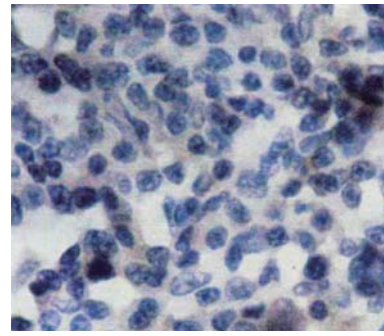


Fig. 3 The expression of TIMP-2 in the control (N)

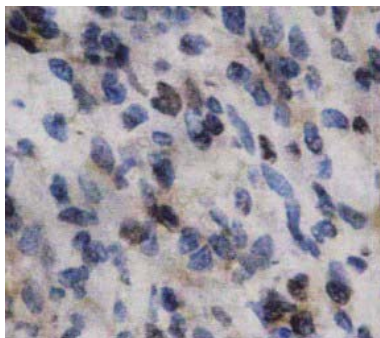


Fig. 2 The expression of MMP-2 in mice exposed to low dose radiation for 48 h (LDR-48 h)

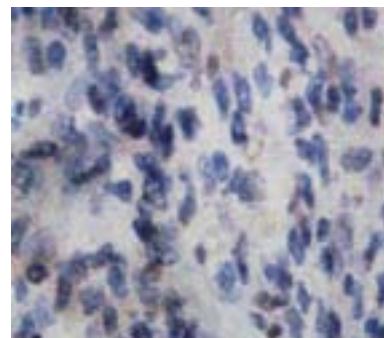


Fig. 4 The expression of TIMP-2 in mice exposed to low dose radiation for 48 h (LDR-48 h)

moving damaged cells through apoptosis, decreasing free radicals, and DNA repair after injured or increasing their sensitivity to acute irradiation at high doses<sup>[12-13]</sup>.

Previous studies by our team found that LDR stimulated the growth of normal cells but not tumor cells *in vitro* and *in vivo*<sup>[6]</sup>, and could reverse ovarian cancer cisplatin resistance by decreasing DNA damage repair and promoting apoptosis. Pre-chemotherapeutic LDR could induce anti-oxidative enzyme activities, and promote the elimination of free radicals to alleviate the damaging effects of oxidative stress to hepatic tissue caused by high-dose chemotherapeutics<sup>[14]</sup>. However, the relationship between LDR and cancer metastasis should be researched further.

The invasion and metastasis of neoplasm is a complex and multi-step continuous process that involves multiple genes, especially the MMP family. In this family, MMP-2 and MMP-9 have been widely studied and play an important role in ECM degradation. The TIMPs have the ability to block the function of MMPs, and the imbalance between MMPs and TIMPs is the principal cause of invasion and metastasis. As MMP-2, which could be specifically inhibited by TIMP-2, is closely related to the tumor invasion and metastasis<sup>[15-17]</sup>, we examined the changes of MMP-2 and TIMP-2 expression after exposure to LDR to

elucidate the relationship between LDR and cancer metastasis or invasion.

In the present study, we explored the effect of LDR on mice bearing S180 sarcomas by measuring the tumor volumes and detecting MMP-2 and TIMP-2 expression level by P-V immunohistochemistry. As expected, the results showed that tumor growth with LDR was significantly inhibited, and MMP-2 expression was considerably reduced in LDR-24 h and LDR-48 h. Therefore, LDR inhibited tumor growth and reduced MMP-2 expression. MMP-2 plays an important role in the invasion and metastasis of neoplasm, therefore, it is possible that LDR could inhibit tumor invasion and metastasis by reducing MMP-2 expression. Unfortunately, TIMP-2 expression varied after LDR exposure, but not significantly. It is possible that MMP-2 expression could be inhibited by factors other than TIMP-2, but this is unclear and further research is necessary to elucidate these relationships. However, this experiment provides a new mechanism where LDR could suppress tumor growth and inhibit tumor invasion and metastasis, which suggests that LDR would be suitable in clinical applications.

#### Conflicts of interest

The authors indicated no potential conflicts of interest.

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