Letter to the Editor

X-ray-induced Expression Changes of TNFSF4 Gene in Human Peripheral Blood*

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This study examined ionizing radiation-induced tumor necrosis factor (ligand) superfamily, member 4 (TNFSF4) mRNA expression changes in human peripheral blood cells and their distribution in a normal population. The results showed that expression level of TNFSF4 mRNA exhibited a dose-dependent response after different irradiation doses, but that was independent of incubation time post-irradiation. Moreover, it was not affected by age and gender in 51 healthy donors. Our studies indicate that TNFSF4 can be considered as a candidate gene to develop a new biodosimeter.

Human tumor necrosis factor (ligand) superfamily, member 4 (TNFSF4) was first described as a gp34 molecule that is preferentially expressed on the cell surface of human T-cell lines transformed by human T-cell leukemia virus type I using a monoclonal antibody (mAb)[1]. It has been previously reported that the expression level of TNFSF4 was associated with the early response of acute kidney injury, the develop of systemic sclerosis, the risk of incident myocardial infarction and cerebral infarction, primary Sjogren's syndrome and primary biliary cirrhosis, etc[2-6]. However, only a few experiments have evaluated ionizing radiation-induced TNFSF4 mRNA expression changes. Whole blood samples from five donors exposed to ionizing radiation using a Gamma Cell 1000 Elite Irradiator at an average rate of 7.6 Gy per minute at the following doses: 0 (sham), 2, 6, and 12 Gy, Pogosova-Agadjanyan et al.[7] found that the expression of TNFSF4 mRNA in CD3+ lymphocytes, mononuclear cells, and white blood cell fraction showed linear dose-dependent responses at 24 h. In addition, MOLT-4 cells exposed to 5 Gy gamma-radiation using a cobalt-60 treatment unit at an average rate of 0.45 Gy per minute, fold changes of TNFSF4 mRNA at 3, 6, 12, and 24 h were respectively 5.7, 9.5, 5.9, and 9.3[8]. Some other previous study also found that the TNFSF4 mRNA expression was up-regulated after exposure[9]. These results suggested that the expression level of TNFSF4 mRNA indeed changed after irradiation. But up to now, no study has elaborated on whether TNFSF4 mRNA expression displays a dose-dependent response at other time points apart from 24 h or a time-dependent response at different time points after the same dose irradiation except 5 Gy. Likewise, we also haven't had knowledge of the distribution of the expression levels of TNFSF4 mRNA in a normal population. Therefore, we examined the expression changes of TNFSF4 in peripheral blood across a wide range of doses (0-12 Gy) at 6, 12, and 24 h and their distribution in normal populations using real-time quantitative PCR (qRT-PCR), hoping to provide some basal data of TNFSF4 expression changes for establishing a new ionizing radiation (IR) biodosimeter.

First, the radiation-induced TNFSF4 mRNA expression changes were validated in peripheralblood samples taken from 4 healthy volunteer donors (3 males and 1 female) aged 23-31 years. All subjects had no history of chronic disease, substance abuse or toxic chemical exposure, radiation exposure or viral infection before the study. Meanwhile, 51 healthy volunteer donors (24 males and 27 females) aged 21-79 years with their eligibilities evaluated using questionnaires and regular medical procedures. The scope of the study was explained to each subject and written informed consents were obtained. The Ethics Committee of HIOM approved all experiments in this study.

Second, whole blood samples were irradiated with X-ray at room temperature using MEVATRON medical electron linear accelerator (First Affiliated Hospital of Zhengzhou University, China) at an

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average rate of 100 cGy/min at the following doses: 0, 1, 3, 5, 8, 10, and 12 Gy. The homogeneous irradiation field was 40×40 cm. After irradiation, the cells were maintained in a 37 ˚C constant-temperature incubator and cultured in a 12 mL RPMI-1640 medium (HyClone, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (SunBao Biotech, Shanghai, China), 160 U/mL gentamicin, and then harvested at 6, 12, and 24 h to prepare cell isolation. White blood cells from all samples including those directly from a normal population were separated as previously described[10]. RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions. The quality and concentration of each RNA sample were assessed using NanoVue™ (GE Healthcare, USA).

A total of 1000 ng RNA was reverse-transcribed with a random primer using reverse transcriptase M-MMLV (Takara, Dalian, China) according to the manufacturer's instructions in all qRT-PCR studies. Each reverse transcription product was diluted to 40 μL with sterilized double distilled water. Then, 50 ng cDNA equivalents per detector was applied for one qRT-PCR reaction each mixed with the components of SYBR® Premix Ex Taq™ (Takara, Dalian, China) for gene expression detection following the manufacturer's instructions. The forward primers (GAPDH: 5'-GGAGAAGGCTGGGGCTCAT-3'; TNFSF4: 5'-TCTTCAGGTATCACATCGGTATCC-3') and reverse primers (GAPDH: 5'-TGGGTGGCAGTGATGGCA-3'; TNFSF4: 5'-GGCTCCTCATCCTTCTGGTAAT-3') were designed according to the MITOMAP Human Cambridge Sequence data (www.mitomap.org), and then synthesized by Gene Core Bio Technologies Co. Ltd., Shanghai, China. The qRT-PCR reactions were performed with the Applied Biosystems 7500 RT-PCR System (Applied Biosystems, CA, USA) and the data were evaluated using the Sequence Detection Software 1.3.1 (Applied Biosystems, CA, USA) combined with Excel® (Microsoft, Unterschleissheim, Germany) for statistical evaluation. The fluorescence signals were normalized by the internal control dye (ROX II). The threshold cycle values (Ct) of the samples were normalized to the Ct of endogenous control glyceraldehyde 3 phosphate dehydrogenase 1 (GAPDH). The gene expression was detected using the $2^{-\Delta\Delta CT}$ method. All samples were run in triplicate with appropriate blank controls. The data were expressed as mean±SD and analyzed using IBM SPSS software Version 21.0 (SPSS, Chicago, USA). $P<0.05$ was considered statistically significant. All reported P values were two sided. The TNFSF4 mRNA expression in a normal population was analyzed by one-way ANOVA. Repeated measurement ANOVA and curve fitting were used to determine the statistical significance of irradiated blood samples.

Repeated measurement ANOVA showed that there was significant statistical difference among different doses (F=5.765, P=0.013; Table 1; Figure 1A). The relationship between the TNFSF4 expression level and exposure dose (0-12 Gy) was delineated with a linear model at 6 h or 12 h after irradiation (y=0.154x+0.978, R^2=0.546, F=9.364, P=0.006; y=0.326x+1.781, R^2=0.498, F=6.282, P=0.021; where y is the relative expression level of TNFSF4 mRNA after exposure, and x is the radiation dose), but that fitted a linear exponential model at 24 h ($y=1.378e^{0.091x}$, R^2=0.113, F=2.687, P=0.116; where y is the relative expression level of TNFSF4 mRNA after exposure, and x is the radiation dose). Therefore, the correlation between radiation doses and the expression changes of TNFSF4 mRNA at 6 h or 12 h was

Table 1. X-ray Irradiation-induced Expression Changes of TNFSF4 mRNA

Dose (Gy)	Relative Induction Level		
	6 h	12 h	24 h
0	1.0097±0.4226	0.3790±0.3306	0.5113±0.6385
1	1.2285±0.7703	1.1113±0.4480	3.5070±3.2484
3	1.6683±0.1979	6.5560±3.8905	5.7553±4.5212
5	1.1123±1.2283	3.9210±2.4849	1.5963±0.8296
8	1.6787±0.4499	4.4950±0.0000	3.8940±3.3860
10	2.9880±1.6040	5.0555±3.2878	6.6457±2.6463
12	2.7595±1.7289	5.0295±2.9734	2.5270±1.0901

better than that at 24 h following exposure. However, Pogosova-Agadjanyan et al.[7] found that the expression of TNFSF4 mRNA exhibited a significant linear dose-dependent manner at 24 h after irradiation. It could be largely due to the differences of irradiation source, dose rate, or irradiation doses, though the differences of donor information, culture conditions or cell types may also play a certain role. Moreover, it was reported that the expression changes of TNFSF4 mRNA at 3, 6, 12, and 24 h after 5 Gy irradiation were independent of incubation time post-irradiation[8]. In the present study, the incubation time post-irradiation was not significantly different (F=0.246, *P*=0.745; Table 1, Figure 1B), though there was a trend of up-regulation with the increasing incubation time. Therefore, it was considered that the expression changes of TNFSF4 gene after *in vitro* irradiation could be mainly due to dose-response effect. Furthermore, some scholars also took into account the expression changes of TNFSF4 mRNA after whole body irradiation. 10-to 11-wk-old C57BL/6 female mice received whole-body irradiation with the dose of 0.01 and 0.1 Gy at an average dose of 1.2 cGy/day using protons ($^1H^{1+}$), the expression levels of TNFSF4 mRNA in CD4+ T cells were both up-regulated on day 0 (<2 h after irradiation), and fold changes were respectively 8.18 and 6.28[9]. These results indicated that the expression changes of TNFSF4 mRNA could be induced by IR, regardless of *in vitro* or *in vivo* irradiation, and exhibited a certain dose-dependent correlation. So it might be an ideal biomarker for IR.

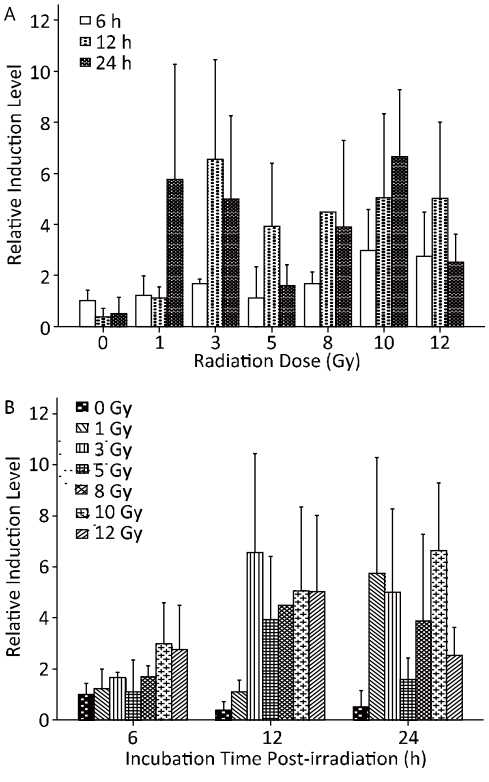

Figure 1. Radiation-induced dose-dependent expression change (A) and time-dependent expression change (B) of TNFSF4 gene in peripheral blood. Error bars represent the standard deviation (*n*=4 donors).

Ideally, genes selected for biodosimeter models will exhibit dose-dependent changes that are independent of age, gender, time, cell type and/or other potential inter-individual confounders. Dosimetry assays using such targets can be widely implemented and interpreted across various human populations and under potentially infinite exposure scenarios[7]. Therefore, we evaluated the distribution of the expression levels of TNFSF4 among age groups and genders of a normal population. In the present study, the expression levels of TNFSF4 in a normal population were not associated with age (F=1.198, *P*=0.326) or gender (F=0.211, *P*=0.648) (Supplementary Table 1). Distribution of expression levels of TNFSF4 among different ages of a normal population is shown in Figure 2.

In summary, the expression changes of TNFSF4 mRNA weren't relevant to incubation time post-irradiation, but highly dependent upon the irradiation dose after irradiation. Furthermore, we also found that the expression levels of TNFSF4 mRNA were not associated with age and gender in a normal population. So it can be used as a molecular biomarker for IR to develop a new biodosimeter.

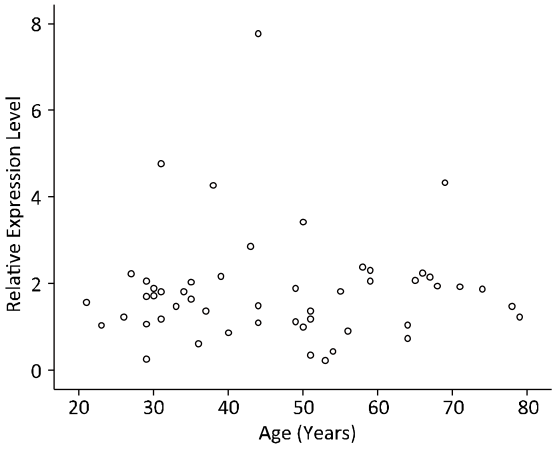

Figure 2. Distribution of TNFSF4 mRNA in peripheral blood from 51 healthy donors.

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Supplemental Table 1. Expression Levels of TNFSF4 mRNA in Peripheral Blood from a Normal Population

Group	Cases	Fold change
Age (y)		
21-	10	1.4698±0.5874
31-	12	1.9964±1.2647
41-	8	2.5763±2.2759
51-	10	1.2973±0.8176
61-	7	2.0674±1.1557
71-79	4	1.6220±0.3325
Gender		
Male	24	1.7399±1.0708
Female	27	1.9052±1.4424
Total	51	1.8274±1.2713