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Radiation dose effect of DNA repair-related gene expression in mouse white blood cells

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Background:

The aim of this study was to screen molecular biomarkers for biodosimetry from DNA repair-related gene expression profiles.

Material/Methods:

Mice were subjected to whole-body exposure with ⁶⁰Co γ rays with a dose range of 0–8 Gy at a dose rate of 0.80 Gy/min. RNA was extracted from the peripheral blood of irradiated mice at 4, 8, 12, 24 and 48hrs post-irradiation. The mRNA transcriptional changes of 11 genes related to DNA damage and repair were detected using real-time quantitative polymerase chain reaction (RT-PCR).

Results:

Of the 11 genes examined, CDKN1A (cyclin-dependent kinase inhibitor 1A or p21, Cip1) and ATM (ataxia telangiectasia mutated) expression levels were found to be heavily up- and down-regulated, respectively, with exposure dose increasing at different post-irradiation times. RAD50 (RAD50 homolog), PLK3 (polo-like kinase 3), GADD45A (growth arrest and DNA damage-inducible, alpha), DDB2 (damage-specific DNA-binding protein 2), BBC3 (BCL2-binding component 3) and IER5 (immediate early response 5) gene expression levels were found to undergo significant oscillating changes over a broad dose range of 2–8 Gy at post-exposure time points observed. Three of the genes were found not to change within the observed exposure dose and post-radiation time ranges.

Conclusions:

The results of this study add to the biodosimetry with biomarker data pool and will be helpful for constructing appropriate gene expression biomarker systems to evaluate radiation exposure doses.

key words:

irradiation • DNA repair-related gene expression • RT-PCR • biological dosimetry

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BACKGROUND

The nuclear power plant radiation accident of '3.11' in Japan refocused attention on the environmental and health effects of wide-spread use of nuclear power. Proper protective and emergency response measures should be available during use of nuclear technologies. Rapid and accurate evaluation of exposure dose plays an important role in early triage, diagnosis and medical treatment of the victims during emergency response efforts in nuclear accidents. In view of some of the serious shortcomings and limits presented by physical and chemical methods used in evaluation of radiation exposure dose [1], biodosimetry has emerged as one of the best techniques for use in individual exposure dose evaluation in many situations such as medical rescue of radiation accident victims, bio-effects research of radiation and radiation protection. Because gene expression is sensitive to environmental factors, the analysis of gene expression profiling of peripheral blood cells, particularly lymphocytes, has been used to assess the presence of certain diseases [2–8]. Accordingly, many radiobiologists have focussed on gene expression profiling analysis to find biomarkers that are suitable for assessment of individual exposure doses under different exposure conditions [9–13]. Considerable work has been done on radiation-induced gene alterations using cultured cell lines and peripheral blood cells irradiated *in vitro* or *ex vivo* [14–19]. Several genes, including GADD45, CDKN1A and BBC3, have been identified as radiation response expression genes at different exposure doses and post-irradiation times in human peripheral blood lymphocytes and tissue cells [20–22]. Recent reports have shown that gene expression signatures induced by IR are specific, durable and accurate in prediction of exposure doses in both mice and humans [23–25].

Since there is a highly relevant among exposure doses, DNA damage degree and DNA repair-related gene expression level, in this study 11 DNA damage response genes related to DNA repair (ATM, BRCA1 and RAD50), cell cycle regulation (CDKN1A, GADD45A), and apoptosis (BBC3) (Table 1) were selected as targets to screen potential bio-markers for indicating exposure dose. The radiation-induced transcription level of 11 genes was investigated in white blood cells of TBI-treated BALB/c mice with a RT-PCR method.

MATERIAL AND METHODS

Animals and exposure

Male BALB/c mice, 6–8 weeks old, provided by the Animal Center of the Second Military Medical University were divided into control (non-irradiated) and 4 exposure groups (5 mice in each group). The mice in the exposure groups were exposed to either 2, 4, 6 or 8 Gy doses of ^{60}Co γ -rays with TBI at 0.80 Gy/min dose rate and room temperature ($23\pm 2^\circ\text{C}$). The ^{60}Co γ -rays source (Shanghai Institute of Measurement and Testing Technology, SIMT China) provided a 98% uniform exposure field in 15×13 cm sides, which was measured with a graphite cavity ionization chamber. The mice were immobilized using a plastic restrainer during exposure. They were housed in a pathogen-free barrier facility (12-hour light/dark cycle) and were fed autoclaved standard rodent chow pre- and post-irradiation. All protocols were approved by the University Committee on Animal

Research, and all experiments were carried out in accordance with related guidelines. All samples were run 3 times and a total of 375 mice including control group were used.

Blood sample collection and total RNA isolation

For each mouse approximately 0.5–0.7 ml of heparin-anticoagulated blood was collected from the orbital venous vein using polypropylene tubes at each post-irradiation time point.

Total RNA was extracted from whole blood using the Axygen Blood Total RNA Miniprep Kit, No. AP-MN-BL-RNA-250 (Axygen Scientific, Hangzhou, China) following the manufacturer's recommendations. The red blood cells were removed using an AxyPrep special lysis buffer. Isolated white blood cells (WBCs) were lysed, and genomic DNA and protein were removed via precipitation. WBCs RNA was then isolated. The RNA was quantified using a NanoDrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA), and the quality of the isolated RNA was assayed with agarose gel electrophoresis by analyzing 18S and 28S RNA.

Quantitative RT-PCR

Using the SYBR Prime Script RT Reagent Kit, No. DRR037A (Takara, Dalian, China), 1 μg of total RNA was reversely transcribed to cDNA according to the manufacturer's instructions. The RT-PCR reactions were performed with the Rotor-Gene 6000 RT-PCR System using the SYBR Premix Ex Taq, No. DRR041A (Takara, Dalian, China) following the manufacturer's recommendations. All samples were run 3 times. The RT-PCR cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s and primer extension at 72°C for 30 s. The final extension occurred at 72°C for 10 min. Relative fold inductions were calculated by the Ct method with averaged relative levels of beta-actin, which was used for normalization. The sequences of primers that were used for amplification are shown in Table 2. The primers were designed and synthesized by InvitrogenTM (Shanghai).

Statistical analysis

The results are listed as the means \pm standard deviation (SD). Statistical analysis was performed with the GraphPad Prism 5.0 software package (GraphPad Software, USA). The statistical significance of differences between groups was analyzed by one-way analysis of variance with a *post-hoc* Tukey test. The significance level was set at $P=0.05$.

RESULTS

Radiation responses of CDKN1A and ATM genes

CDKN1A, which has a regulatory role in S phase DNA replication and is known to be activated by the tumor protein p53 (TP 53), had the strongest upregulation in expression level out of the 11 genes investigated in this study. As shown in Figure 1A, compared with the non-irradiated control, CDKN1A gene expression gradually increased to a maximum value of approximately 88-fold for 2 Gy ($p<0.0001$) and 130-fold for 4 Gy ($p<0.0001$) at 12 h post-irradiation, and approximately 116-fold for 6 Gy ($p<0.0001$) and 100-fold for 8

Table 1. Gene descriptions.

Symbol	Accession	Description and Function
Beta-actin	NM_007393.3	Used for normalization
CDKN1A	NM_001111099.1	Cyclin-dependent kinase inhibitor 1A (p21, Cip1); plays a regulatory role in S phase DNA replication and DNA damage repair
BBC3	NM_133234.2	BCL2 binding component 3; essential mediator of p53-dependent and p53-independent apoptosis
DDB2	NM_028119.4	Damage-specific DNA-binding protein 2; activated by UV and X-ray irradiation; it recognizes pyrimidine dimers and increased p53 expression
GADD45A	NM_007836.1	Growth arrest and DNA damage-inducible, alpha; the protein responds to environmental stresses by mediating activation of the p38/JNK pathway
IER5	NM_010500.2	Immediate early response 5; plays an important role in mediating the cellular response to mitogenic signals
PLK3	NM_013807.2	Polo-like kinase 3 (Drosophila); critical regulators of cell cycle progression, mitosis, cytokinesis and the DNA damage response
ATM	NM_007499.2	Ataxia telangiectasia mutated; activated by double-strand breaks (DSB), acting as a DNA damage sensor
BRCA1	NM_009764.3	Breast cancer 1; maintains genomic stability, plays a role in transcription, DNA repair of DSBs, and recombination
XPC	NM_009531.2	Xeroderma pigmentosum, complementation group; encodes a component of the nucleotide excision repair (NER) pathway
FDXR	NM_007997.1	Ferredoxin reductase; encodes a mitochondrial flavoprotein that initiates electron transport for cytochromes P450 receiving
RAD50	NM_009012.2	Component of the MRN complex which plays a central role in DSB repair, DNA recombination, maintenance of telomere integrity and meiosis

Table 2. RT-PCR primers.

Gene	Accession	Left primer	Right primer
Beta-actin	NM_007393.3	AGGCTGTGCTGTCCCTGTATG	ACCCAAGAAGGAAGGCTGGAAA
CDKN1A	NM_001111099.1	GTCCCACTTGGCAGCAGAATAA	GGTCGGACATCACCAGGATTG
ATM	NM_007499.2	GCACACGGATTGCTCAAGGA	GCCCATTCGGAATATGGATCAG
RAD50	NM_009012.2	CCAGGGACAGACTTGCCAAAC	TCCAAATCTGGCTACCACAACA
GADD45A	NM_007836.1	CCTGCACTGTGTGCTGTGA	CCACTGATCCATGTAGCGACTTTC
FDXR	NM_007997.1	GCCCAGCTGGCTTACACA	AGGATGGTCAGGTGCCACA
XPC	NM_009531.2	GTGGACCAAGGCACTGATGAAG	ACAGGCATGTCAGACGGTGAG
BRCA1	NM_009764.3	TCTGAAGACTGCTCGCAGAGTGATA	CCAGCACAGCTCCAGGTGA
IER5	NM_010500.2	TTCAGACGCCGAGGGTACAAC	ATCCTGCCTTCGCTCCAGA
PLK3	NM_013807.2	TCATCACGGATAACATGGAAGTGA	CACGTAGTTGGAGTGCCACA
DDB2	NM_028119.4	AAGTTGGGCAAAGCCACCTG	GTGCCATGCCAAGGACGTAG
BBC3	NM_133234.2	GACCTCAACGCGCAGTACGA	GCTCCAGGATCCCTGGGTTAA

Gy ($p < 0.0001$) at 8 h post-exposure. Then, CDKN1A gene expression gradually decreased but remained greater than 30-fold ($p < 0.05$) higher than that of the control group at 48 h post-irradiation for all exposure doses. Figure 2A and

B show the significant differences in CDKN1A gene expression induced by the different exposure doses. Significant differences can be seen by comparing the exposure groups of 2 and 6 Gy ($p < 0.05$), 4 and 6 Gy ($p < 0.0001$), and 4 and 8

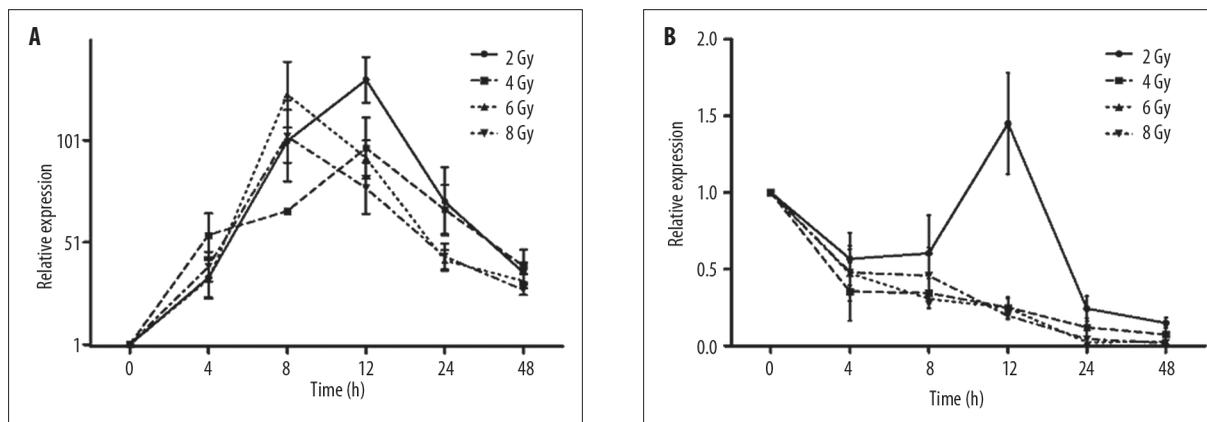


Figure 1. Temporal responses of CDKN1A (A) and ATM (B) gene expression at different exposure doses are plotted. Points denote the mean of responses in 5 different mice; error bars denote SD.

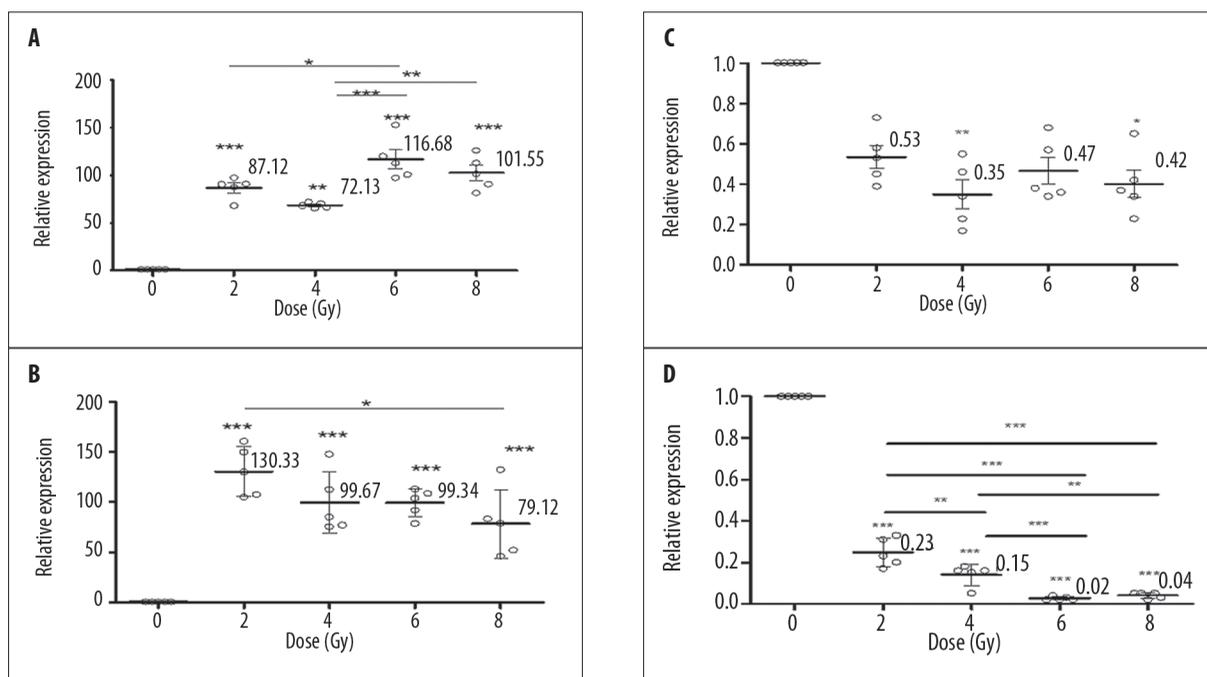


Figure 2. The dose response of CDKN1A at 8 h (A), 12 h (B) and the dose response of ATM at 4 h (C) and 24 h (D) post-irradiation. The points denote the mean responses for 5 different mice; error bars denote SD. Asterisks indicate a significant difference in the relative values of expression among the control and irradiation groups and among the different dose groups at the indicated dose point.

Gy ($p < 0.001$) at 8 h post-exposure (Figure 2A). At the 12 h post-irradiation time point, CDKN1A gene expression decreased along with an increase in exposure dose. The only significant difference was between 2 and 8 Gy.

ATM is a predominantly nuclear protein that is involved in the DNA damage repair signal transduction process. In this study, ATM gene expression decreased linearly with the exposure dose, with the exception of the 12 h post-irradiation time-point after a 2 Gy exposure (Figure 1B). Compared with non-irradiated controls, it could be determined from the time course of ATM gene expression (Figure 1B) that the gene was strongly downregulated in all exposure dose ranges at 4–48 h post-irradiation. A significant decrease was found for the 4 Gy ($p < 0.001$) and 8 Gy ($p < 0.05$) irradiation groups at 4 h post-irradiation (Figure 2C), and

highly significant changes across the dose range of 2–8 Gy ($p < 0.0001$) were found at 24 h post-irradiation (Figure 2D). Significant differences can also be seen by comparing the exposure groups of 2 and 4 Gy ($p < 0.001$), 2 and 6 Gy ($p < 0.0001$), 2 and 8 Gy ($p < 0.0001$), 4 and 6 Gy ($p < 0.0001$), and 4 and 8 Gy ($p < 0.001$) at 24 h post-exposure (Figure 2D).

Radiation responses of the other tested genes

The genes BBC3, XPC, PLK3, RAD50, DDB2, FDXR, GADD45A, BRCA1 and IER5 are largely involved in DNA repair or cell cycle regulation associated with DNA repair. The results show that genes BRCA1, XPC and FDXR had no significant change in expression level post-irradiation across all observed dose ranges and post-irradiation time-points. The time- and dose-dependent responses of 6 other

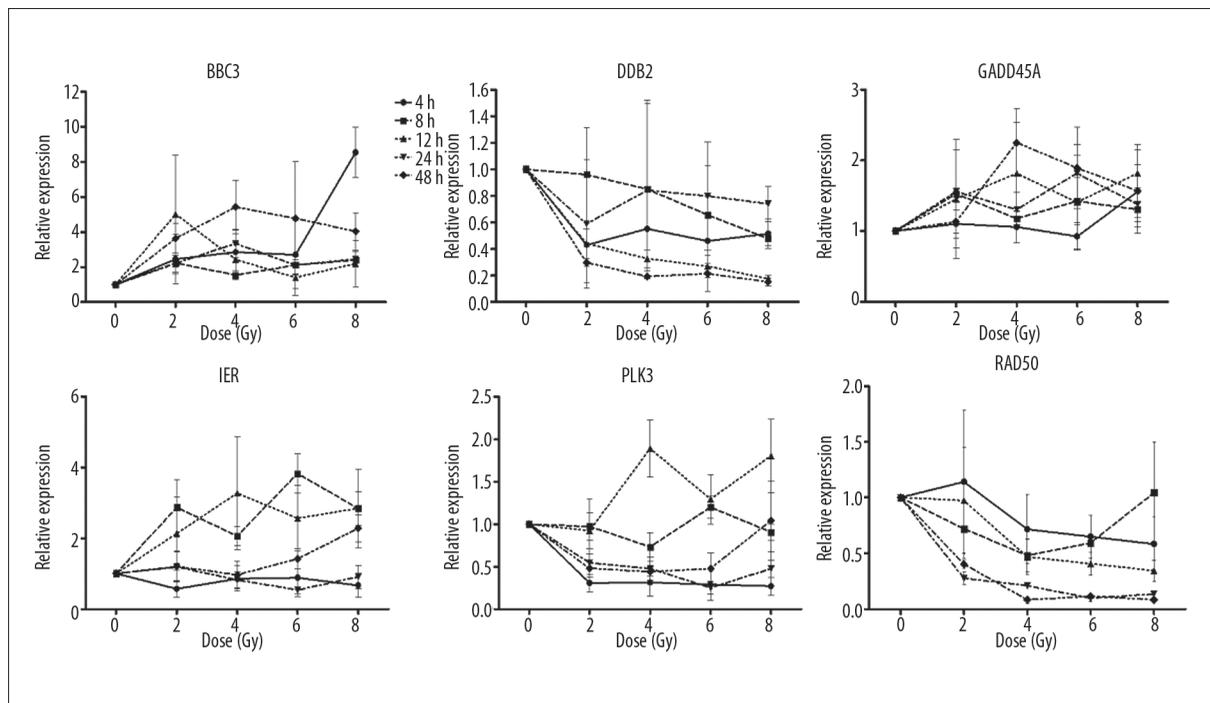


Figure 3. The dose responses of the expression levels of 6 genes after radiation exposure are plotted. The points denote the mean responses for 5 different mice; error bars denote SD.

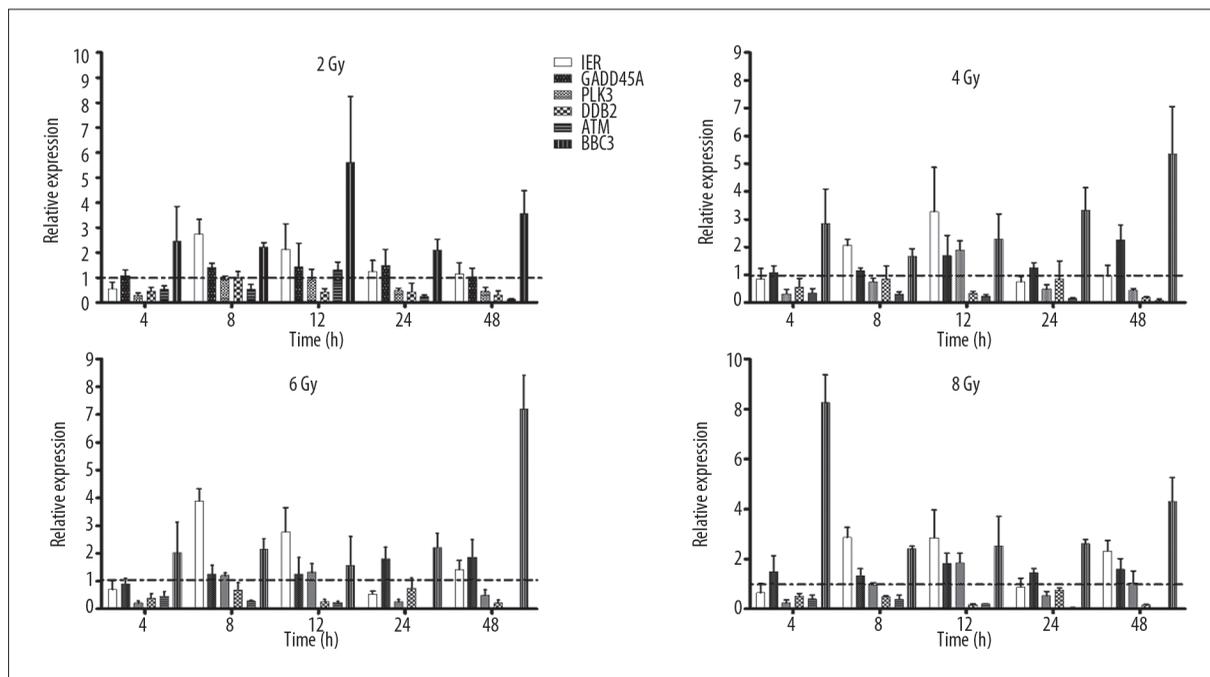


Figure 4. Relative values of IER5, GADD45A, PLK3, DDB2, BBC3 and ATM gene expression levels are plotted, and the dashed line indicates the non-irradiated control ratio. Data points denote the mean \pm SD of 5 mice per treatment.

genes (BBC3, PLK3, DDB2, GADD45A, RAD50 and IER5) are shown in Figure 3.

The changes in expression levels of these genes were upregulated and downregulated in an oscillating pattern across the observed time-points within certain dose ranges. To determine which genes could potentially characterize exposure

doses at different post-irradiation time-points, the results are charted in Figure 4. Because the change in CDKN1A expression is substantial, it is not included in Figure 4. The summary of relative expression levels of genes, which are significantly different from the control group, is shown in Table 3. These changes may serve as a useful biomarker for exposure dose evaluation.

Table 3. Summary of genes modulated by IR at different post-irradiation time points and exposure doses.

	2 Gy	4 Gy	6 Gy	8 Gy
4 h		ATM* (0.35), CDKN1A*** (54.60)	DDB2* (0.40), PLK3* (0.22)	ATM* (0.42), BBC3* (5.72), CDKN1A* (39.13)
8 h	ATM* (0.60), GADD45A* (1.53), IER5** (2.87), CDKN1A*** (86.68)	ATM** (0.34), CDKN1A*** (68.37)	ATM** (0.34), CDKN1A*** (68.37)	ATM** (0.46), IER5** (2.84), BBC3* (2.40), CDKN1A*** (102.38)
12 h	DDB2*** (0.44), CDKN1A*** (126.79)	ATM** (0.25), DDB2*** (0.33), PLK3*** (1.9), IER5* (3.27), CDKN1A*** (96.485)	ATM** (0.24), DDB2*** (0.27), CDKN1A*** (92.373)	ATM** (0.20), DDB2*** (0.18), PLK3** (1.8), CDKN1A*** (77.58)
24 h	ATM** (0.24), PLK3*** (0.55), BBC3* (2.21), CDKN1A*** (70.99), RAD50*** (0.28)	ATM*** (0.12), PLK3*** (0.48), GADD45A** (2.01), BBC3*** (2.99), CDKN1A*** (67.11), RAD50*** (0.21)	ATM*** (0.02), PLK3*** (0.25), GADD45A** (1.70), BBC3* (2.11), RAD50*** (0.09)	ATM*** (0.04), PLK3*** (0.48), GADD45A* (1.38), BBC3** (2.45), CDKN1A* (40.75), RAD50*** (0.13)
48 h	ATM*** (0.15), DDB2*** (0.30), PLK3** (0.48), CDKN1A*** (36.44), RAD50*** (0.40)	ATM*** (0.07), DDB2*** (0.19), PLK3** (0.44), GADD45A** (2.25), BBC3** (5.42), CDKN1A*** (39.33), RAD50*** (0.08)	ATM*** (0.03), DDB2*** (0.21), PLK3** (0.48), GADD45A* (1.89), BBC3** (4.78), CDKN1A*** (32.04), RAD50*** (0.11)	ATM*** (0.01), DDB2*** (0.15), GADD45A* (1.57), IER5** (2.28), BBC3* (4.04), CDKN1A* (29.61), RAD50*** (0.07)

The values in brackets represent the relative expression levels compared to the control group. Asterisks indicate a significant difference in expression values between the control and radiation treated group at the indicated dose and time point (* $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$).

DISCUSSION

Gene expression analysis following ionizing radiation as a biomarker for individual exposure dose estimation and radiation effect prediction is becoming increasing attention [26–28]. As one of the most radiosensitive targets in mammalian cells, DNA damage degree induced by IR was largely dependent on type of radiation (LET), dose deposit in the cell, and dose rate [29]. An instinctive protection mechanism in living organisms can repair DNA damage induced by IR within certain exposure dose ranges through initiating repair-related gene expression and protein activity. In this process the quantity of repair-related gene expression and protein activating should be proportional to DNA damage degree and then be logically linked with exposure dose. Therefore the information associated with DNA damage repair-related gene expression and protein activity may be seen as a huge data mine from which to derive biological markers for exposure dose evaluation with biodosimetry.

In this study significant and meaningful exposure doses and observed post-radiation time ranges in diagnosis and treatment of acute radiation sickness were selected to obtain more complete gene expression profiles following different radiation doses and different post-irradiation times. To enhance accuracy and reliability of the results, radiosensitive mouse species and an accurate irradiation source with well-distributed exposure dose field were selected. Most of the

target genes observed here were proved prior to this study as radiation response genes, and some of them were shown to have a well-defined dose-effect pattern in the changes of expression level when analysis was done with separated or cultured cells irradiated *in vitro* or *ex vivo*.

ATM, as an important protein in the cellular response to IR, plays a critical role in initiating the repair signaling pathway of DNA damage. IR could induce phosphorylation of serine 1981 of ATM protein, and phosphorylated ATM protein would pass the activating signal to downstream repair-related proteins. When DNA repair was finished, phosphorylated ATM protein would be dephosphorylated [30]. It has been reported that ATM protein level did not change during the radiation-induced repair process of DNA DSBs, but protein kinase activity of ATM might be changed following exposure doses when checking was done by a fluorescent foci observation [31–34]. In the present study, the change in transcriptional level of the ATM gene was first found to be downregulated in a dose-and post-irradiation time-dependent pattern, with the exception of the 2 Gy exposure dose at 12 h post-exposure, the reason for which requires further investigation. This finding implies that the information coming from ATM gene expression after radiation cannot be neglected as a biomarker in exposure dose indicating.

Strongly upregulated expression of the CDKN1A gene shown in this study slightly different from previous findings

that showed only a several- or 10-fold increase in CDKN1A gene expression after radiation (from 4-fold to 10- or 50-fold or greater increase) [11,16,35–38]. It is supposed that the difference of testing mode, primary design of CDKN1A gene composition and application of the primaries might be responsible for the diversity of the results. For example, Mitsuhashi et al. [39] reported that multiple primary applications in CDKN1A gene composition showed higher induction of CDKN1A mRNA than single primary and control groups when leukocytes from multiple primary breast cancer patients were irradiated. The difference of mouse strain in experiment, exposure and treatment methods of the sample are also factors resulting in diversity of CDKN1A mRNA results.

The results of the present study show that peaked expression of CDKN1A gene appeared at 12 h post-irradiation for doses of 2 and 4 Gy and at 8 h post-irradiation for doses of 6 and 8 Gy, which is well in accord with the cell cycle arrest necessity for DNA repair after radiation. As an inhibitor of cell cycle process, radiation-induced DNA repair needs more CDKN1A protein expression. The heavier DNA damage caused by high dose the more CDKN1A protein needed to satisfy the demand of DNA repair, which accompanied with a peak CDKN1A gene expression in the earlier phase of DNA repair. Combining our results with those of Dressman [25] who found only CDKN1A was in common between a PB signature of human TBI and the PB signatures of partial body irradiation exposure, suggesting that the information from CDKN1A gene expression can be a reliable and representative biomarker in estimating exposure dose within certain dose ranges.

DDB2, a damage-specific DNA-binding protein 2 that can be activated by UV and X-ray irradiation, had its gene expression level reduced to approximately 20% of the initial expression level with the radiation dose increases in this study. This result is not consistent with previous reports that showed the DDB2 gene was upregulated in both *in vivo* and *in vitro* experiments [16,25,35]. Different experimental conditions among studies might cause these differences. For example, some results come from just 1 TBI-treated patient and 6 hours to 4 days of observation time, and the study was performed by fractionated irradiation with a low dose and dose rate. In this study exposure dose was delivered once and dose rate was nearly 8-fold higher than mentioned above. Because sublethal damage repair and somewhat adaptive response exist during fractionated irradiation by low dose and dose rate, the DDB2 expression level increase was within the range of what one would expect. Recently, Stoyanova [40,41] reported that DDB2 could indirectly participate in nucleotide excision repair (NER) by regulating the cellular levels of p21^{Waf1/Cip1} (CDKN1A), and DDB2-deficient cells exhibited a significantly higher accumulation of p21^{Waf1/Cip1} (CDKN1A). They believed that DDB2 plays a critical role in attenuating the level of p21^{Waf1/Cip1} to allow apoptosis in response to DNA-damaging agents. Given their conclusions, our result that CDKN1A was highly upregulated and DDB2 was downregulated show a reasonable but unclear link when radiation response of 2 genes was evaluated.

GADD45A has previously been proposed as a potential dosimeter based on the linear dose response relationship observed in human cells that were irradiated *in vitro* [42],

but the stress-response regulation of this gene was known to be complex. Decreasing expression of this gene was observed using WBC [14,36] and in many p53 wild-type cell lines when cells were irradiated *in vitro*; however, other reports have also shown that ionizing radiation did not induce GADD45A expression change in a subset of p53 wild-type cell lines [43,44]. In our study, GADD45A expression was upregulated approximately 2–3-fold only at the 48 h post-irradiation time-point within all exposure dose ranges, and no significant changes were found at other time-points. Different experimental subjects and exposure patterns might result in different results. For example, the gene expression and regulation patterns in TBI mice might be more complex compared to the cultured or isolated cells irradiated *in vitro* or *ex vivo*. The sensitivity of analytic methods could also result in differences in quantification of gene expression [45].

In hunting for biomarkers capable of indicating individual exposure dose, linear relationship with exposure dose and sensitivity of these markers are expected, but these conditions are hard to satisfy in reality. It is possible that selecting and gathering non-linear change genes together, which induced by different exposure doses and up- or downregulated, at different post-exposure times, form many biomarker groups which contain many significantly up- or downregulated genes to distinguish and evaluate different threshold doses for medical triage and diagnosis purpose. For example, in Table 3, at 4 h post-irradiation, the gene profiles of ATM, XPC and CDKN1A, with statistically significant difference compared with unirradiated control group, can characterize a 4 Gy exposure dose, and the information coming from DDB2, XPC and PLK3 gene expression difference can characterize a 6 Gy exposure dose, and in the same way the gene expression difference information from ATM, BBC3, XPC and CDKN1A can characterize a 8 Gy exposure dose. In the situation where the gene expression profiles are the same in 2 different dose scales, such as 4 and 6Gy exposure groups at 8 h post-exposure in Table 3, further gene expression information screening is necessary until some genes are finally found with expression differences between the 2 different doses. Then this information on different genes can be selected and grouped based on the time elapsed after radiation exposure to identify and distinguish different exposure doses. Additionally, given that ATM and CDKN1A gene expression levels were significantly changed across the dose range and time course (0–48 h), it may be possible to use these 2 genes as biomarkers to distinguish people exposed to radiation from those who were not exposed, to reassure the “worried well” and to assign those with significant exposure to the appropriate medical care.

CONCLUSIONS

On the whole, although gene expression analysis is promising as a biomarker to indicate exposure dose, many problems remain to be solved before the method can be used to evaluate exposure in real-life settings. The present work represents part of the effort to establish this evaluation tool.

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