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DOSE-DEPENDENT AND GENDER-RELATED RADIATION-INDUCED TRANSCRIPTION ALTERATIONS OF *GADD45A* AND *IER5* IN HUMAN LYMPHOCYTES EXPOSED TO GAMMA RAY EMITTED BY ^{60}Co

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Growth arrest DNA damage-inducible 45a gene (*Gadd45a*) and immediate early response gene 5 (*Ier5*) have been emphasised as ideal radiation biomarkers in several reports. However, some aspects of radiation-induced transcriptional alterations of these genes are unknown. In this study, gender-dependency and dose-dependency as two factors that may affect radiation-induced transcription of *Gadd45a* and *Ier5* genes were investigated. Human lymphocyte cells from six healthy voluntary blood donors (three women and three men) were irradiated *in vitro* with doses of 0.5–4.0 Gy from a ^{60}Co source and RNA isolated 4 h later using the High Pure RNA Isolation Kit. Dose and gender dependency of radiation-induced transcriptional alterations of *Gadd45a* and *Ier5* genes were studied by quantitative real-time polymerase chain reaction. The results showed that as a whole, *Gadd45a* and *Ier5* gave responses to gamma rays, while the responses were independent of radiation doses. Therefore, regardless of radiation dose, *Gadd45a* and *Ier5* can be considered potential radiation biomarkers. Besides, although radiation-induced transcriptional alterations of *Gadd45a* in female and male lymphocyte samples were insignificant at 0.5 Gy, at other doses, their quantities in female samples were at a significantly higher level than in male samples. Radiation-induced transcription of *Ier5* of females samples had a reduction in comparison with male samples at 1 and 2 Gy, but at doses of 0.5 and 4 Gy, females were significantly more susceptible to radiation-induced transcriptional alteration of *Ier5*.

INTRODUCTION

At present, the development of gene expression profiles as a novel biomarker approach is introduced by most researchers and current findings strongly support the usefulness of gene expression profiles for radiation biodosimetry[1–3]. This approach could provide both an estimate of physical radiation dose and an indication of the extent of individual injury or future risk. Specially, the development of some techniques and instruments such as Comet assay, microarray and real-time polymerase chain reaction (RT–PCR) assay make it possible to quantitatively monitor changes in radiation-responsive gene expression as a radiation biomarker. There are extensive efforts to identify candidate radiation-responsive gene biomarker. In recent studies of gene expression, it has been found that growth arrest DNA damage-inducible 45a gene (*Gadd45a*) and immediate early response gene 5 (*Ier5*) are genes extremely responsive to ionising radiation. As a result, assay of *Gadd45a* and *Ier5* gene expression alterations is possibly useful for the assessment of absorbed radiation dose in radiological casualties. In other words, they can be candidates as precise and specific radiation biomarkers[4–6].

On the basis of strong evidences, it is clear that DNA is the principal target for ionising radiation. Consequently, most of the common biological side effects and cellular injuries of ionising radiation begin logically with DNA damage[7, 8]. Therefore, ionising radiation induces cascades of signal transduction pathways for maintaining cellular homeostasis and protecting cells from hazardous effects of ionising radiation. Mammalian cells exhibit complex, but intricate cellular responses to ionising radiation as a genotoxic stressor, including cell cycle checkpoints, DNA repair and apoptosis[9, 10].

Most of the outstanding characteristics of *Gadd45a* and *Ire5* genes are related to repair process of damaged DNA. The principal mechanism of

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repair is the excision repair process and it is predominant in DNA molecules that have suffered damage to ionising radiation. Basically, *Gadd45a* as a DNA damage responsive gene is a p53-regulated gene, which is known to participate in the nucleotide excision repair and cell–cycle regulation. Interactions of Gadd45a protein with several other proteins play a central role in DNA repair, cell cycle control and apoptosis. Besides *Gadd45a*, *Ier5* is a novel member of the slow-kinetics immediate-early gene family that is present in a wide variety of tissues. The exact biological function of *Ier5* gene is not defined; however, studies have shown the up-regulation of the *Ier5* gene following ionising radiation[11–14].

Since some transcriptional factors and experimental conditions may affect the gene expression, the present study was designed to reveal gender-dependency and dose-dependency of *Gadd45a* and *Ier5* gene expression in irradiated human peripheral blood lymphocytes. Gamma rays emitted from a ^{60}Co radioisotope was used as a radiation source and there were some reasons for such a choice. Firstly, ^{60}Co has been extensively exploited in various fields. This radioisotope with a 5.27 y half-life emits two high-energy gamma photons (1.173 and 1.332 MeV) in each decay. Secondly, ^{60}Co is used medically for radiation therapy as implants and as an external source of radiation exposure. Thirdly, it is used industrially in levelling gauges and gamma-ray welding seams and other structural elements to detect flaws. Finally, ^{60}Co is used for food irradiation as a sterilisation process[15, 16]. For these widespread applications of ^{60}Co, it was selected for the present investigation.

MATERIALS AND METHODS

Cell culture and irradiation

Ten microlitres of peripheral blood was obtained from six healthy voluntary blood donors (three women and three men, aged 26 ± 2 y) in heparinised Vacutainer tubes (Becton Dickinson). Lymphocytes were separated on Ficoll density gradient by centrifugation, (Lymphoprep, Inno-train, Frankfurt, Germany) at $\times 23$ *g* for 20 min at 4°C, and then washed with Hanks buffer.

Peripheral blood mononuclear cells were isolated from the fresh blood by centrifugation over density solution, Lymphoprep, (Inno-train). The Buffy coat layers were washed in Hanks buffer and resuspended at a density of 1.5×10^6 to 2×10^6 million cells per flask in RPMI1640 (Invitrogen, Karlsruhe, Germany). The medium was supplemented with 10 % heat-inactivated (56°C for 45 min) foetal bovine serum (Karlsruhe, Germany), 10 % 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Sigma-Aldrich, Steinheim, Germany), 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

The lymphocytes were distributed in five 25-cm^2 flasks and transferred to the Secondary Standard Dosimetry Laboratory in Iran Atomic Energy Organization for irradiation. Lymphocytes were irradiated at doses of 0 (as a control or reference), 0.5, 1, 2 and 4 Gy, using Picker-V9-Co-60 (USA). The ^{60}Co-gamma ray source to sample tubes was 80 cm with a dose rate of 74 cGy min^{-1} at room temperature (23 ± 1°C). The samples were exposed to the radiation field for 40, 80, 160 and 320 s, the absorbed dose as a result of which was 0.5, 1, 2 and 4 Gy, respectively.

The total RNA was extracted 4 h after irradiation, using the High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany) and RNA quality and quantity were determined using 1.5 % agaros gel electrophoresis and spectrophotometer, respectively, which showed high quality and quantity for RNA samples.

Real-time quantitative PCR

In order to investigate changes in *Gadd45a* or *Ier5* gene expression, the real-time quantitative PCR (RT-qPCR) technique was used. Template total RNA was reverse transcribed with haexamer primers according to the manufacture's protocol, using the First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). The primers and cDNA sample qualities were analysed using PCR and agarose gel electrophoresis. Quantitative real-time PCR was carried out in a Roter-Gene 6000 instrument (Corbett Life Science, Sydney, Australia) using Fast Start SYBR Green Master (Roche Applied Science, Mannheim, Germany). Relative gene expression changes between cDNA samples were determined by the $\Delta\Delta C_t$ method according to the formula: $\Delta\Delta C_t = (C_{t\ \text{sample}} - C_{t\ \text{ref}})_{\text{control}} - (C_{t\ \text{sample}} - C_{t\ \text{ref}})_{\text{irrdiated}}$, and the estimated expression ratio is equal to $2^{\Delta\Delta Ct}$, in which the *GAPDH* gene was used as an internal control (C_t is the critical cycle number). The primers sequences that were used for Gadd45a and Ier5 in Real time PCR experiments, are illustrated in Table 1.

Table 1. The primer sequences that were used in real-time PCR.

Genes	Primers sequences
Gadd45a	F: TCAGCGCACGATCACTGTC
	R: CCAGCAGGCACAACACCAC
Ier5	F: ATCAGCATCTTCGGTTCCAG
	R: TCCAGGGGTTCATGTCTCTC
GAPDH	F: GAGTCAACGGATTTGGTCGT
	R: TTGATTTTGGAGGGATCTCG

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Statistical analysis

At first, to determine $\Delta\Delta C_t$ for the different samples (control and irradiated), the C_t real-time PCR results were grouped and the average of gene expression was calculated. Thereafter, for data analysis and comparison differences between various groups, two statistical methods including analysis of variance (ANOVA), three-way ANOVA were chosen using the SPSS16.0 software. In fact, at the first stage, the significance of variation between all the groups was comprehensively analysed by using ANOVA, which allowed looking at all the groups simultaneously. Finally, for the assessment of the simultaneous effects of dose and gender on the gene expression of *Ier5* or *Gadd45a*, in other words, exploration of the really link between radiation dose and gender, 3-way ANOVA calculations were performed[17, 18]. All the results are reported as mean $\pm$ SE and $p < 0.05$ were considered significant in all the statistical analyses[19, 20].

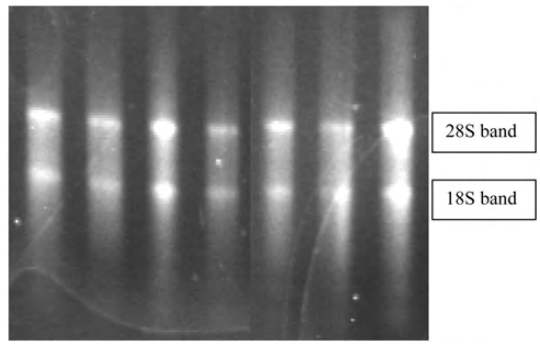

Figure 1. Gel electrophoresis patterns of RNA from human lymphocytes treated with gamma rays and visualised with ethidium bromide. Equal amounts of extracted RNA (5 μl) from various samples were used in the gel electrophoresis assays. RNAs were collected 4 h after irradiation at 0 (as the control), 0.5, 1, 2 and 4 Gy gamma rays emitted from ^{60}Co radioisotope (from right to left, respectively). The experiment was repeated at 0 and 4 Gy.

RESULTS

To assess the *Gadd45a* and *Ier5* gene expression by ionizing irradiation in human lymphocyte cells, RT-PCR analysis was performed using unirradiated (control group) total RNA isolated from human lymphocyte cells, as well a those irradiated with different doses of gamma ray emission from ^{60}Co radioisotope.

In order to obtain information about RNA yield and to evaluate the overall quality of RNA preparation, gel electrophoresis was performed. In fact, agarose gel electrophoresis is sufficient to judge the integrity and overall quality of a total RNA isolated from human lymphocytes samples by inspection of the 28S and 18S RNA bands. For this purpose, gel electrophoresis analysis of RNA was carried out using agarose gel 1.5 %. In Figure 1, the bands of 18S and 28S RNA are clearly visible in the samples. As a result, these sharp bands showed that total extracted RNAs were undamaged. In addition, the quantitative evaluation of RNA in various prepared samples was done with a UV-Visible spectrophotometer at 260 and 280 nm. The ratio of optical densities at 260 and 280 nm (A260/A280) showed that the 28S band was approximately twice as intense as the 18S band. This 2:1 ratio (28S:18S) was an additional good indication that the prepared RNAs were intact [21, 22].

Figures 2 and 3 illustrate alterations of *Gadd45a* expression in human lymphocytes obtained from female and male samples, respectively. The zero columns are related to unirradiated samples (it is considered as a control group) and data obtained from other doses of radiation (0.5, 1, 2 and 4 Gy) are identified in both figures and compared with the

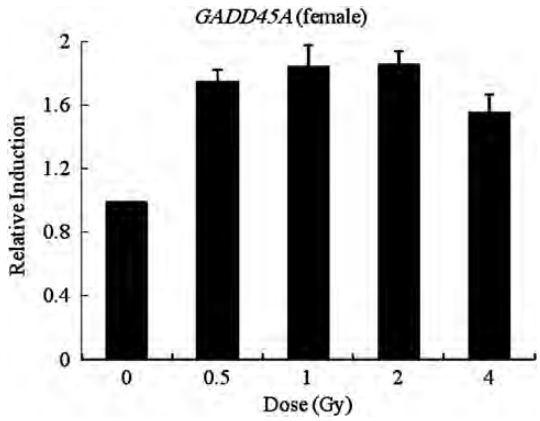

Figure 2. Alterations of *Gadd45a* expression in human lymphocytes obtained from female samples. Cells were irradiated with 0 (as a control), 0.5, 1, 2 and 4 Gy and RNAs isolated 4 h after irradiation. Each column shows the means and standard errors (error bars) of three independent experiments.

control groups. The RT–PCR data were statistically analysed and as a whole, *Gadd45a* gave significant ($p < 0.05$) responses to radiation. In fact, in the presence of gamma rays, *Gadd45a* was up-regulated, both in female and in male samples. To identify the dose-dependency of *Gadd45a* gene expression, by omitting the reference data (zero columns), the RT–PCR data at different doses were statistically analysed. The results showed that the differences between various doses of gamma rays were insignificant ($p < 0.05$) in both genders.

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There was also possibility that the gender could influence the expression of *Gadd45a.* To determine the effect of sex on *Gadd45a* expression, the RT–PCR data were statistically analysed on the basis of gender. Figure 4 illustrates the alterations of *Gadd45a* expression in human lymphocytes obtained from female (black columns) and male (grey columns) samples. Although the alteration of *Gadd45a* in female and male lymphocyte samples was insignificant at a radiation dose of 0.5 Gy ($p < 0.05$), it was strongly significant at other doses. In other words, female lymphocytes samples exposed to various doses of gamma radiation showed significantly higher levels of *Gadd45a* gene expression in comparison with radiation-exposed lymphocytes of male samples. Consequently, at doses higher than 0.5 Gy, exposure of human lymphocytes to gamma rays induced a gender-dependent *Gadd45a* gene expression as a radiation response.

Besides *Gadd45a*, the mRNA levels of *Ier5* were determined by RT–PCR in human lymphocytes from female and male samples exposed to gamma rays emitted from ^{60}Co. The relative *Ier5* gene expression was evaluated on the basis of both the dose-dependent and gender-related radiation responses over the dose range 0.5–4 Gy. The radiation-induced transcriptional profiles of *Ier5* in lymphocytes of female and male samples are shown in Figures 5 and 6, respectively. As shown in these figures, altogether doses

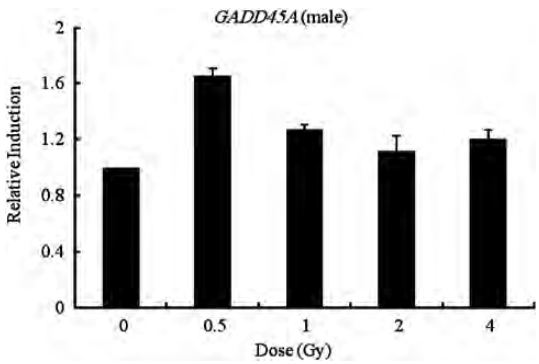

Figure 3. Alterations of *Gadd45a* expression in human lymphocytes obtained from male samples. Cells were irradiated with 0 (as a control), 0.5, 1, 2 and 4 Gy and RNAs isolated 4 h after irradiation. Each column shows the means and standard errors (error bars) of three independent experiments.

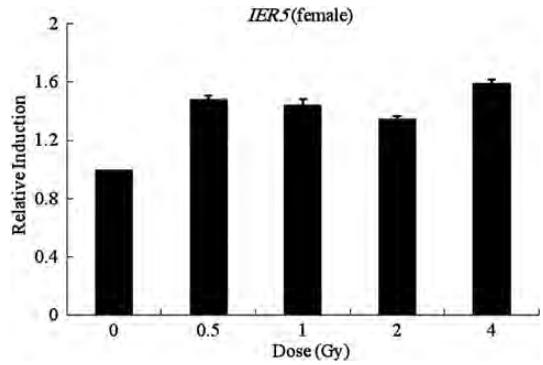

Figure 5. Alterations of *Ier5* expression in human lymphocytes obtained from female samples. Cells were irradiated with 0 (as a control), 0.5, 1, 2 and 4 Gy and RNAs isolated 4 h after irradiation. Each column shows the means and standard errors (error bars) of three independent experiments.

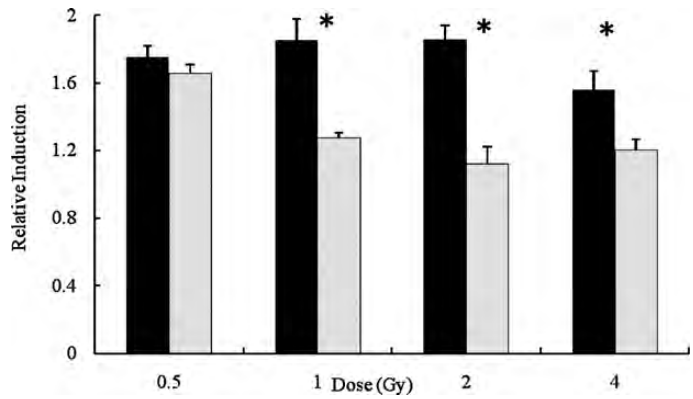

Figure 4. Alterations of *Gadd45a* expression in human lymphocytes obtained from female (black columns) and male (grey columns) samples. Cells were irradiated with 0 (as a control), 0.5, 1, 2 and 4 Gy and RNAs isolated 4 h after irradiation. Each column shows the means and standard errors (error bars) of three independent experiments. The asterisks represent the significance of differences between the mean values of *Gadd45a* expression ($p < 0.05$).

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of gamma rays brought about up-regulation of *Ier5* gene in both genders ($p < 0.05$). However, ignoring the data of the control sample, when the transcriptional profiles of *Ier5* were compared with one another at rest doses of radiation, interestingly the average values of the responses were insignificant ($p < 0.05$). In other words, it means that the gene expression of *Ier5* as a radiation response is dose-independent and responded to radiation identically over the dose range 0.5–4 Gy.

The radiation-induced transcriptional profiles of *Ier5* were rearranged on the basis of gender and illustrated in Figure 7. To ascertain the gender role on alterations of *Ier5* gene expression, statistical analysis of quantitative RT–PCR data was performed. The results showed that with the exception of 0.5 Gy, at other doses, the variation of average *Ier5* expression in female and male samples was statistically significant ($p < 0.05$) and it means that the radiation-induced expression of this gene was sex-related. While the average *Ier5* expression was greater in the male than in female samples, by contrary, at doses 1 and 2 Gy, this value was greater in the female than in the male samples at doses 0.5 and 4 Gy.

Finally, in order to determine the effect of radiation doses on expression of *Gadd45a* and *Ier5*, the mean values of mRNA level of each gene were compared with one another at different doses. For this aim, without regarding the sex-related genes expressions, the mean values of each genes expression level were considered. Figure 8 shows the changes in the mean value of radiation-induced transcriptional level of *Gadd45a* (grey columns) and *Ier5* (black columns) at different doses. When mean value variations of genes expression were statistically compared with one another, apart from dose 0.5 Gy, the differences were insignificant at other doses (N.S).

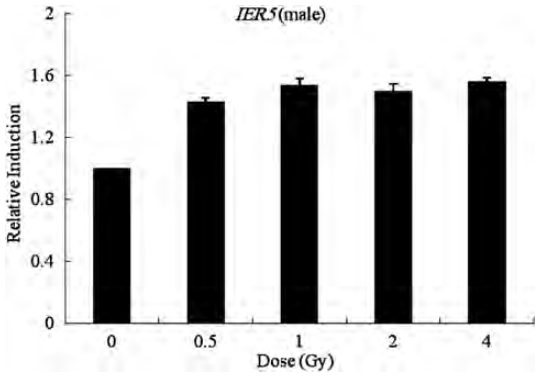

Figure 6. Alterations of *Ier5* expression in human lymphocytes obtained from male samples. Cells were irradiated with 0 (as a control), 0.5, 1, 2 and 4 Gy and RNAs isolated 4 h after irradiation. Each column shows the means and standard errors (error bars) of three independent experiments.

DISCUSSION

Gadd45a and Ier5 as radiation biomarkers

For many reasons, researchers have been interested in developing novel biodosimetry methods. For instance, in nuclear or radiological accidents[23], extended space exploration missions[24] and a cosmic or solar incident in which earth would be directly exposed by a great deal of various types of radiation[25], surely, traditional biodosimetry methods

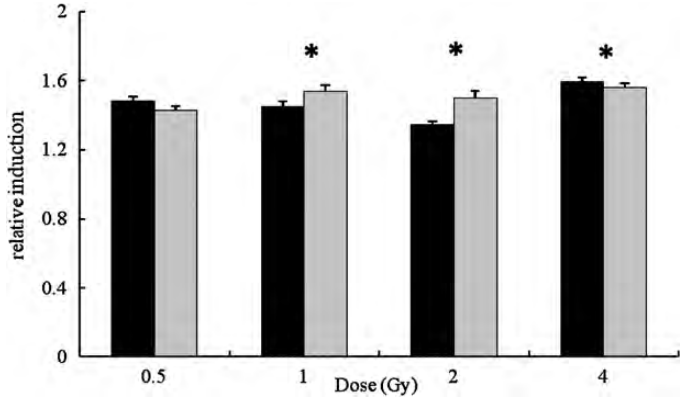

Figure 7. Alterations of *Ier5* expression in human lymphocytes obtained from female (black columns) and male (grey columns) samples. Cells were irradiated with 0 (as a control), 0.5, 1, 2 and 4 Gy and RNAs isolated 4 h after irradiation. Each column shows the means and standard errors (error bars) of three independent experiments. The asterisks represent the significance of differences between the mean values of *Ier5* expression ($p < 0.05$).

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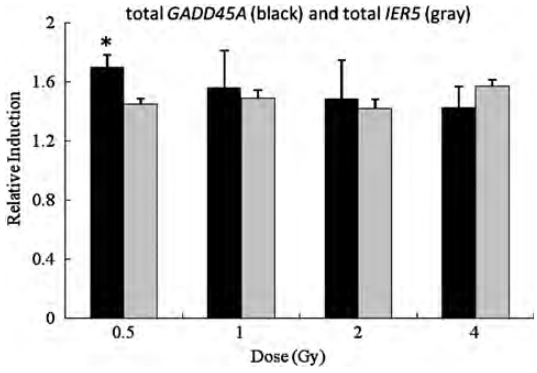

Figure 8. Alterations of *Gadd45a* and *Ier5* expression in human lymphocytes obtained from three female and three male samples (n=6 for each gene expression). Cells were irradiated with 0 (as a control), 0.5, 1, 2 and 4 Gy and RNAs isolated 4 h after irradiation. Each column shows the means and standard errors (error bars) of six independent experiments. The asterisk represents the significance of differences between the mean values of genes expressions levels at 0.5 Gy ($p < 0.05$).

would not be sufficient for responding to such an enormous number of victims. Consequently, it is essential to develop some high-throughput; rapid and fieldable assays for biodosimetry methods. At present, many groups of researchers have recommended utilising gene expression profiles as an excellent biodosimetry method for medical triage of large-scale radiological casualties. For this purpose, a great number of genes in which their name and character are accessible in various references have been recently introduced as radiation biomarkers by numerous groups of investigators[26–28]. In relation to this, in recent times, *Gadd45a* and *Ier5* have been emphasised as ideal biomarkers for radiation biodosimetry in several reports[17]. However, some transcriptional aspects of these genes, such as effects of radiation dose and gender of irradiated casualties, have been blurred. In this study, the roles of dose and gender in the gene expression profile of *Gadd45a* and *Ier5* in irradiated human lymphocytes were characterised using RT–PCR. It is worthwhile to note that *Gadd45a* and *Ier5* can be used together with a few genes such as *Cdkn1a, Ddb2, Fcgr1a* and *Cxcl10* for dose assessment of radiological casualties[18]. The transcriptional alteration measurement of 4–7 gene may be prolonged and for this reason it would not suitable for radiological events in which rapid biodosimetry is necessary. To solve this problem, various techniques have been developed for automatic gene expression measurements. Some of these techniques are Qiagen BioRobot 8000, chip-based capillary electrophoresis (CE) systems, PCR-CE microdevice, Agilent whole-genome microarray and automated multistep genetic assay[25, 29, 30].

Assessment of dose-dependency of Gadd45a and Ier5

Gene expression profile analysis of *Gadd45a* and *Ier5* both in female and male samples clearly demonstrated higher levels of induction at all radiation doses relative to the control (unirradiated cells) (Figures 2, 3, 5 and 6). The over-expression made by this analysis was in agreement with most of the reports published thus far; therefore, *Gadd45a* and *Ier5* can be considered potential radiation biomarkers. However, ignoring the control group, the mean value variations of *Gadd45a* and *Ier5* induction among other groups were statistically insignificant. Consequently, in spite of over-expression of *Gadd45a* and *Ier5* after exposed to gamma rays, even at low doses (based on some reports, even <0.5 Gy), dose-dependency of radiation-induced transcriptional alterations of both genes was not observed at the range of radiation doses utilised in this study. These findings are completely confirmed by some reports. For instance, in the case of *Gadd45a,* it is interesting to note that radiation-responsive changes in the Gadd45a protein levels in human blood cells were found to be independent of radiation dose after exposure to ^{60}Co gamma rays, at the range 0–4 Gy[31]. In addition, dose dependency of the gene expression pattern of *Ier5* was investigated by some researchers. The results revealed that although the radiation-induced transcriptional alterations of *Ier5* were statistically significant, their variations were independent of radiation doses. Hence, in spite of some reports that have insisted on the dose-dependence of radiation-induced expression of *Gadd45a* and *Ier5*, this view particularly at low doses remains unknown and appear to be required more investigations. As a result, since the lack of a relationship between radiation dose and expressions of genes, *Gadd45*a and *Ier5* can be exclusively considered radiation biomarkers and not biological dosemeters.

Assessment of gender dependency of Gadd45a and Ier5

Analysis of the radiation-induced transcription by gender indicated statistically significant differences in gene expression of females versus males for both the *Gadd45a* and *Ier5* genes. Radiation-induced alterations levels of *Gadd45a* males had a statistically significant reduction in the gene expression compared with females (Figure 4), whereas, *Ier5* females had a statistically significant reduction in the gene expression compared with males only at medium doses of 1 and 2 Gy (Figure 7). Nevertheless, at doses of 0.5 and 4 Gy, females were significantly more susceptible to radiation-induced transcriptional alterations of *Ier5* than males. Interestingly, gender-dependent responses of *Gadd45a* and *Ier5* have been reported in the presence of some other genotoxic agents. For example, gender-dependent response of *Gadd45a* expression has been observed in haematopoietic stem

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cells after chronic exposure to benzene metabolites[32]. In addition, gender-related *Gadd45a* expression has been reported by other investigators in the presence of some carcinogenesis agents such as dimethylbenzanthracene[33]. Benzene metabolites and dimethylbenzanthracene have been classified by studies of ionising radiation both as cell stressors and highly genotoxic agents that can cause DNA damage by forming reactive oxygen species; therefore, these results were completely predictable. In the case of *Ier5*, although gender-dependent of *Ier5* expression has not been crucially approved, some researchers have preferred using only one sex of human or animal sample in their studies to eliminate the effect of gender on gene expression patterns[34]. However, there are some reports that confirm some individual differences such as sex could influence radiation-induced expression profile of *Ier5* [35].

Assessment of radiation dose, gender and dose and gender dependency of Gadd45a and Ier5

Three-way ANOVA was carried out to check for the effect of dose, gender and dose and gender on radiation-induced transcription of *Gadd45a* and *Ier5* genes. The statistic analysis showed that the interaction among, doses, genders and genes expressions were significant ($p < 0.05$).

Finally, apart from sex type, radiation-induced transcriptional alteration profiles of *Gadd45a* were totally compared with *Ier5* profiles at different doses. Among the transcriptional alteration profiles of *Gadd45a* and *Ier5*, only differences in gene expression pattern at a dose of 0.5 Gy were statistically significant (Figure 8). The *Gadd45a* and *Ier5* responses to radiation were identical at doses higher than 0.5 Gy, suggesting that each of these genes utilized as radiation biomarkers have the same sensitivity for biodosimetry purposes. Whereas, at low doses because of more radiation-induced transcriptional alterations of *Gadd45a* relative to *Ier5*, it seems that exploiting of *Gadd45a* as a radiation biomarker is more favourite than *Ire5*. Since besides accuracy, precision, resolution, reproducibility and detection limit, sensitivity is one of the most important analytical parameters[36], and hence, the results reported in this article are very valuable and must be regarded in radiation biodosimetry. In addition, according to our findings for avoiding any false-positive or false-negative interpretation of the results, the gender of casualty must be considered for dose assessment in radiological events.

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