Inter-individual Differences in Radiosensitivity based on CDKN1A, GADD45A, DDB2, BCL2 and TRX Gene Expression in Human Lymphocytes

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Abstract

Transcriptomic signatures of radioresponsive genes are recently explored as a powerful tool for biodosimetry. As cytogenetics remains the gold standard and a robust approach for wide-scale testing during radiologic emergencies, the utility of molecular signatures remains to be adequately validated. The present study analyzed the expression profiles of five highly responsive genes to radiation: B-cell lymphoma 2 (BCL2), DNA damage-binding protein 2 (DDB2), cyclin-dependent kinase inhibitor 1A (CDKN1A), growth arrest and DNA-damage-inducible alpha (GADD45A) and thioredoxin (TRX). Ex vivo exposure of peripheral blood lymphocytes from 17 healthy subjects, aged 21 to 53 years, at 2 Gy radiation appeared to mobilize the index genes involved in cell cycle arrest (GADD45A and CDKN1A) and the binding to DNA lesion to facilitate excision repair (DDB2). However, the transcription of pro-survival protein BCL2 and redox repair antioxidant protein TRX were not as reliable as molecular biodosimeters considering the variability among individual responses. Nevertheless, the significant correlations observed among the genes emphasize their synchronized roles during DNA damage and redox response. Gender-based differences in gene expression were not detected. These findings indicated the diverse transcriptional regulation of p53-dependent pathways in radiation-exposed lymphocytes. Further validation in more patients during healthy and diseased states could contribute to the clinical application of gene-based radiation biodosimetry.

***Keywords**:* biodosimetry, radioresponse, lymphocytes, p53-dependent pathways, transcriptome

1. Introduction

Exposure to ionizing radiation induces measurable cellular damage that is proportional to the radiation dose. These measurements may involve

deoxyribonucleic acid (DNA) changes in lymphocytes, dicentric chromosomes, chromosome translocations and chromosome fragments, among others (Crespo *et al.*, 2011; International Atomic Energy Agency, 2011). They are called biodosimeters because they allow the measurement of the received radiation dose of an individual by interpolation of a population-established dose-response curve. The reliability of the dose information from an exposed person is premised on the uniformity of radiation response within a given population. However, there is a growing focus nowadays on characterizing individual differences in these biological endpoints to obtain better information to safeguard radiation protection limits and for use in precision medicine involving radiation therapy (Müller *et al.*, 2001). Genetic variation is a likely source for radiosensitivity variation observed between individuals (Pollard and Gatti, 2009). For a more radiosensitive person, the radiation protection limits might place the individual at higher risk for stochastic effects. From the standpoint of precision medicine, patients may benefit from personalized radiotherapy to evade possible severe adverse effects (Scaife *et al.*, 2015).

Although traditional cytogenetic methods remain the gold standard for biodosimetry, recently, there have been interests in evaluating molecular endpoints (Amundson *et al.*, 2004; Paul and Amundson, 2008; Turtoi *et al.*, 2008; Kabacik *et al.*, 2011; Pernot *et al.*, 2012). Several of these studies investigated the utility of measuring changes in both proteomic and genomic endpoints (Turtoi *et al.*, 2010), messenger ribonucleic acid (mRNA) transcripts in lymphocytes or whole blood concerning the applied dose of radiation although there is less emphasis on the impact of individual differences in lymphocyte radiation sensitivity to the studied mRNA biodosimeters (Grace *et al.*, 2003; Paul and Amundson, 2008; Joiner *et al.*, 2011; Manning *et al.*, 2011; Budworth *et al.*, 2012; Nongrum *et al.*, 2017). Some studies have already evaluated inter-individual variation in radiation sensitivity in some biological endpoints like DNA damage, comet and micronucleus assay, and gamma H2AX (Bishay *et al.*, 2001; Müller *et al.*, 2001; Greve *et al.*, 2012; Royba *et al.*, 2017).

In this study, the variability was analyzed among healthy individuals in terms of transcriptional responses of lymphocytes after exposure to gamma radiation. The transcriptional behavior of five candidate genes that are highly responsive to radiation was characterized, namely B-cell lymphoma 2 (BCL2), DNA damage-binding protein 2 (DDB2), cyclin-dependent kinase inhibitor 1A (CDKN1A), growth arrest and DNA-damage-inducible alpha

(GADD45A) and thioredoxin (TRX). As these genes have specific roles in addressing damage or oxidative stress following radiation exposure, the results describing the inter-individual variation in radiation-induced gene expression among healthy individuals may provide additional evidence reinforcing these genes as potential biomarkers for intrinsic radiation sensitivity.

2. Methodology

2.1 Sample Collection and Irradiation

Ethics approval was secured from Jose Reyes Memorial Medical Center Ethics Review Board with protocol number 2019-064. The peripheral blood samples were collected once from nine females (22 to 53 years old) and eight males (21 to 42 years old) consenting healthy donors (n = 17). Whole blood samples were stored in lithium heparin-anticoagulated tubes. Each sample was split into two 1-mL portions. One portion was exposed to 2-Gy gamma radiation at a dose rate of 13.56 Gy/h at the Gammacell 220 Facility of the Philippine Nuclear Research Institute (PNRI), Philippines as previously described by Caraos *et al.* (2019). The other portion served as the non-irradiated sample. Immediately after irradiation, the samples were brought back to the laboratory for lymphocyte isolation and subsequent ribonucleic acid (RNA) extraction.

2.2 Lymphocyte Isolation

The lymphocytes were isolated via density gradient centrifugation. Briefly, each sample was first mixed with an equal volume of RPMI-1640 medium supplemented with 5% fetal calf serum (FCS) (Gibco, United States) before being layered on top of a 3-mL Lymphoprep™ column (Stemcell Technologies, Canada). The lymphocyte isolation proceeded following the manufacturer's instructions. Isolated lymphocytes in each sample were counted by performing the white blood cell count macrodilution method on an improved Neubauer hemocytometer (Sigma Aldrich, Germany). Twenty µL of lymphocyte suspension was mixed with 380 µL of Turk's fluid (Medic Diagnostics, Philippines), and then 15 µL of this suspension was charged onto one chamber of the improved Neubauer hemocytometer. Lymphocytes were counted on the four white blood cell (WBC) squares from which the number of cells per microliter was calculated.

2.3 RNA Extraction

The total RNA extraction was carried out from isolated lymphocytes using a silica spin column-based EZNA Blood RNA kit (Omega Biotek, United States) by following the manufacturer's instructions. RNA quality and quantity were then evaluated with a spectrophotometer (DS-11, DeNovix Inc., United States). Only samples with 260/280 nM ratios equal to or between 1.8 and 2.2 were processed for the first-strand cDNA synthesis.

2.4 Reverse Transcription

Qualified samples were reverse transcribed with Sensifast cDNA synthesis kit (Bioline, United Kingdom) in a 20-µL reaction consisting of the recommended 250 ng of total RNA, 1 unit of reverse transcriptase, 4 µL of 5x TransAmp buffer and nuclease-free water. A thermal cycler (C1000 Touch Cycler, Bio-Rad, United States) was programmed for the transcription reaction as follows: 25 °C for 10 min (annealing), 42 °C for 15 min (reverse transcription) and 85 °C for 5 min (inactivation).

2.5 Real-Time Polymerase Chain Reaction (RT-PCR)

RT-PCR was undertaken in duplicate for each sample in a thermal cycler equipped with CFX 96 real-time system (Bio-Rad, United States) using iTaq™ Universal SYBR9® Green Supermix (Bio-Rad, United States). The reaction consisted of 0.5 ng cDNA, 5 µL of the supermix, 300 nM each of the forward and reverse primers, and nuclease-free water to complete the 10-µL volume requirement. The cycling conditions were initial denaturation and polymerase activation at 95 °C for 1 min, then 40 cycles of denaturation at 95 °C for 30 s, annealing at 61.2 °C for 35 s, plate read at 79 °C for 4 s and a melt curve analysis at 75 to 95 °C. Amplification curves with more than one melt peak were excluded from the study. Relative quantification of genes of interest was calculated automatically by the Bio-Rad CFX Manager software version 3.1 based on the modified ΔCq method of Pfaffl, wherein the quantity of target genes in the irradiated samples was expressed as fold change concerning its non-irradiated sample and normalized to a housekeeping gene, β-2-microglobulin. Table 1 lists the sequences of the previously published primers used in this study (Grace *et al.*, 2003; Oh *et al.*, 2004; Watanabe *et al.*, 2010; Li *et al.*, 2014).

Table 1. Primers used for RT-PCR

Genes	Forward	Reverse
β-2-microglobulin	5'-CGGGCATTCCTGAAGCTGA-3'	5'-GGATGGATGAAACCCAGACACATAG-3'
BCL-2	5'-TGTATGAACTGACAATGTGCAAGA-3'	5'-CACCTGGCAGCG TAGGGTAA-3'
CDKN1A	5'-ATGTCAGAACCGGCTGGGGAT-3'	5'-TAGGGCTTCCTCTTGGAGAAG-3'
DDB2	5'-CATGATCTTCGCATAGAGCACAGT-3'	5'-GGGACTCCTGCTCCTCTTGTT-3'
GADD45A	5'-TGCTCAGCAAAGCCCTGAGT-3'	5-GCAGGCACAACACCACGTTA-3'
TRX	5'-CTGCTTTTCAGGAAGCCTTG-3'	5'-TGTTGGCATGCATTTGACTT-3'

2.3 Statistical Analysis

Statistical tests were carried out with Real Statistics Resource Pack software (release 7.6). Shapiro-Wilk test was used to test for normality of distribution of data in each gene followed by Grubb's test to identify outliers. Mann-Whitney U test was then employed to determine if the fold change values significantly differed between males and females. Pearson's correlation followed by linear regression analysis was then carried out to assess relationships between genes' expression values and the degree of their association.

3. Results and Discussion

To discern the individualized radiosensitivity of peripheral lymphocytes that can serve as molecular biodosimeters, a set of literature-based signature genes was selected with regards to radiation response, namely BCL2, DDB2, CDKN1A, GADD45A and TRX. BCL2, DDB2, CDKN1A and GADD45 are downstream targets of the p53 pathway activated once the sensing genes detect DNA damage brought about by ionizing radiation. At the same time, TRX is part of the oxidative stress pathway, which can be radiation-induced. As shown in Figure 1, when p53 is initiated, several cellular responses are lodged to address the crisis. Both GADD45A and CDKN1A/p21, through binding cyclin-CDK1 complexes, inhibit cell cycle progression to allow for cellular repair at different stages, one at the G1-S and the other at the G2-M checkpoint (Grace and Blakely, 2007; Kreis *et al.*, 2019). Their immediate and prolonged upregulation in response to in vitro irradiation doses of up to 10 Gy have been documented (Jen and Cheung, 2003; Grace and Blakely, 2007; Kabacik *et al.*, 2011). However, reduced CDKN1A expression has been associated with sensitivity to radiotherapy (Badie *et al.*, 2008). BCL2 comes from the family of apoptotic proteins that regulate the caspase pathway. It forms heterodimers with a family member, Bax to prevent caspase activation; thus, its

upregulation was seen only until 3 Gy favors survival (Grace and Blakely, 2007). A lower BCL2/Bax ratio was previously associated with radiation sensitivity (Palumbo *et al.*, 2019). DDB2 is one of the DNA damage binding proteins that localize at the site of damage to recruit excision repair proteins. TRX, on the other hand, is responsible for the reduction of oxidized proteins and is one of the endogenous antioxidants. Both immediately upregulate post-irradiation until 24 h and have never been demonstrated to be linked to radiation sensitivity (Hoshi *et al.*, 1997; Jen and Cheung, 2003; Grace and Blakely, 2007; Li *et al.*, 2017; Caraos *et al.*, 2019).

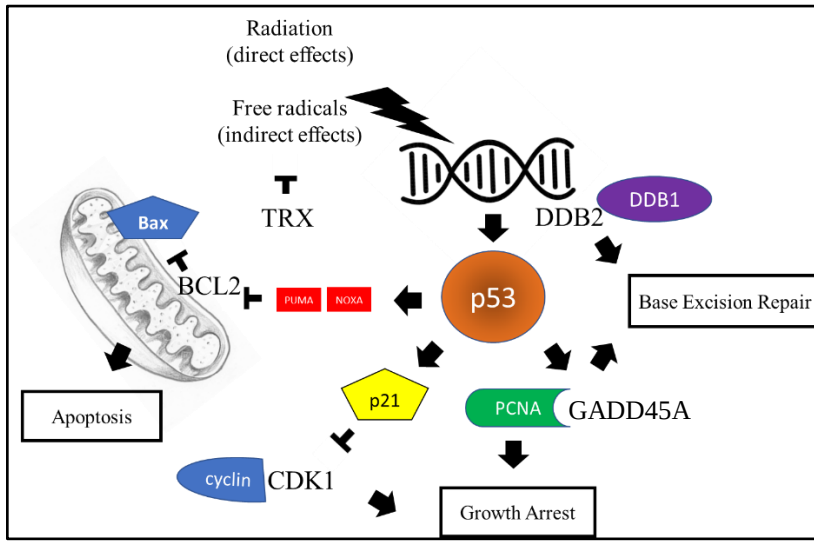


Figure 1. The p53-dependent pathway of CDKN1A, GADD45A, BCL2, TRX and DDB2

Indirect effect of radiation causes damage to critical macromolecules addressed by redox repair involving TRX. DDB2-DDB1 or GADD45A-PCNA complex mediates base excision repair of direct DNA damage. Failed repair pathways converge with p53 which induce GADD45A and up-regulate p21 protein leading to growth arrest. Apoptosis occurs through BCL2 inhibition and activation of pro-apoptotic Bax through p53 upregulated modulator of apoptosis (PUMA)-induced nicotinamide adenine dinucleotide phosphate oxidase activator (NOXA).

3.1 Variation in Radiation-Induced Transcriptional Responses between Individuals

Following ex vivo exposure to 2-Gy gamma radiation, 100% of the lymphocyte samples were observed to have upregulated GADD45A (1.04 to

38 fold) and DDB2 (1.2 to 15 fold) genes, while 16 out of 17 (95%) of samples showed CDKN1A upregulation (1.3 to 11 fold). On the other hand, only 65% among the samples had upregulated BCL2 (1.2 to 7.9 fold) and TRX (1.05 to 27 fold). Of the five samples that displayed downregulation, the lowest observed reduction in transcripts was 95% for one BCL2 and 75% for one TRX sample with reference to its non-irradiated levels.

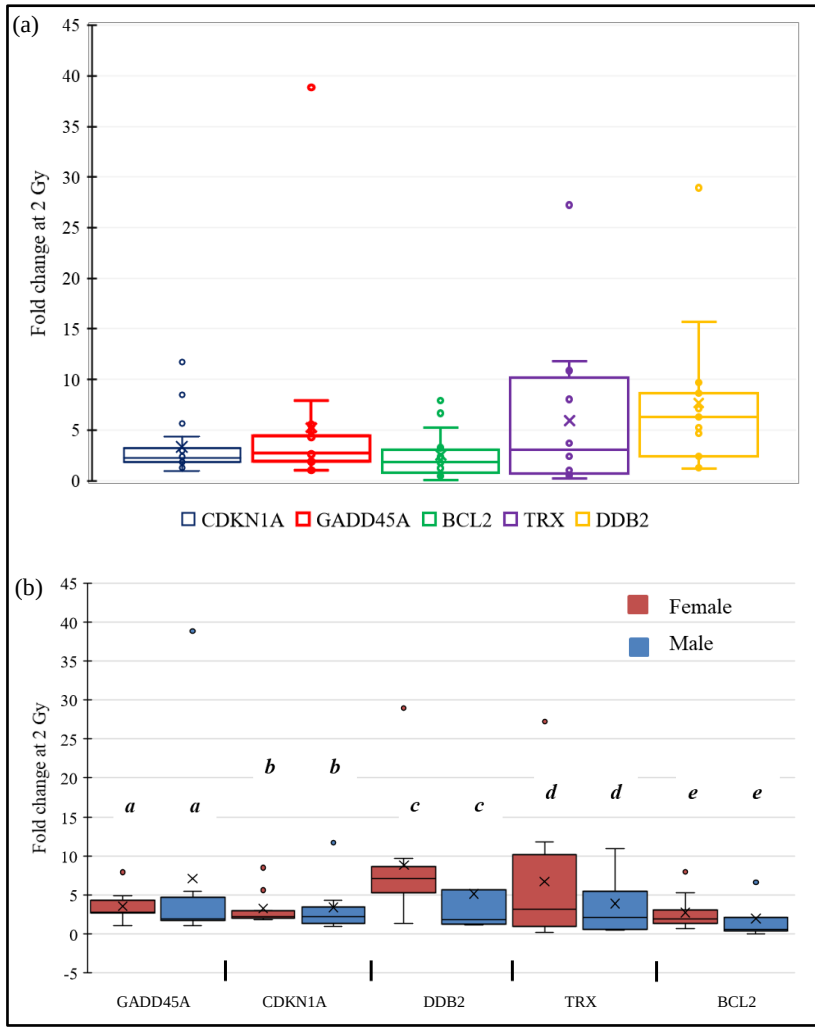
To determine if the transcriptional responses observed for each gene followed a normal distribution, the Shapiro-Wilk test revealed that the transcriptional responses of all genes did not follow the normal distribution ($p < 0.05$). Grubbs' test was then used to identify potential outliers, and these are illustrated as data points (dots) outside of the box plots for each gene in Figure 2a. Notably, the most extreme transcriptional responses of CDKN1A (11-fold) and GADD45A (38-fold) were from the same sample. Likewise, the outliers of BCL2 (seven-fold), TRX (27-fold), and DDB2 (28-fold) were contributed by only one individual.

A considerable amount of variability in TRX transcriptional responses between individuals can be observed compared with the other genes based on the broad interquartile range, which accounted for 50% of the samples and ranged from 0.59 to 10.87-fold. DDB2 followed closely with an interquartile range of 2.4 to eight-fold. On the other hand, there was slight variation among the transcriptional responses of individuals for the other three genes based on the interquartile range.

When transcriptional response differences based on gender were tested with the Mann-Whitney U test, it was revealed that no significant differences between male and female gene expression responses existed in any of the genes, as shown in Figure 2b ($p > 0.05$). Even when outliers were excluded from the testing, no significant differences were detected between male and female gene responses.

In this study, it was found that 2-Gy irradiation immediately induced the upregulation of GADD45A and DDB2 in lymphocytes of all healthy individuals. When the outliers are excluded and the median expression values, three- and six-fold, respectively, are considered, the results are comparable to that of Brzóska and Kruszewski (2015), wherein GADD45A and DBB2 increased to four- and eight-fold, respectively, at 6 h. Similarly, both transcripts went up to five-fold at 24 h in the study of Grace *et al.* (2003). As with both these studies, there was a slight variation observed between

individuals for GADD45A expression in the present study, making it suitable for a biodosimeter.



Dots represent each individual and X marks the mean. Groups with the same small letters did not significantly differ ($p > 0.05$).

Figure 2. Radiation-induced mRNA expression of CDKN1A, GADD45A, BCL2, TRX and DDB2 in lymphocytes from healthy individuals after 2-Gy ex vivo gamma irradiation: fold change of target genes at 2 Gy relative to non-irradiated lymphocytes and normalized to a housekeeping gene, β-2-microglobulin (a) and gender-based comparison of target genes expression at 2 Gy (b)

The response of CDKN1A transcript levels agrees with the studies of Brzóska and Kruszewski (2015) at 6 h and Budworth *et al.* (2012) at 24 h, which were upregulated six- and three-fold changes, respectively. There were three individuals with high CDKN1A (> five-fold). Still, it could not be ascertained as to whether these pointed out possible radiosensitive responses because it is contrary to the results of Badie *et al.* (2008), which suggested CDKN1A reduction to be associated with sensitivity.

The genes which elicited varying responses among healthy individuals were BCL2 and TRX transcripts, wherein 35% was downregulated. TRX expression seemed similar to previous studies ranging until 15-fold within 24 h (Hoshi *et al.*, 1997; Caraos *et al.*, 2019). It is interesting to note that BCL2 transcription likewise varied in a 24-h period after irradiation (Grace *et al.*, 2003; Grace and Blakely, 2007; Brzóska and Kruszewski, 2015). It has been widely accepted that p53 regulates the BCL2 family of proteins, and this is said to be proportionate to the amount of DNA damage (Basu and Haldar, 1998). Still, more recent studies have shown that BCL2 might also be involved in oxidative stress regulation whose initiation of BCL2 may be independent of p53 (Susnow *et al.*, 2009). It is possible that since ionizing radiation induces both pathways, there is a mixture of responses presented by the individuals.

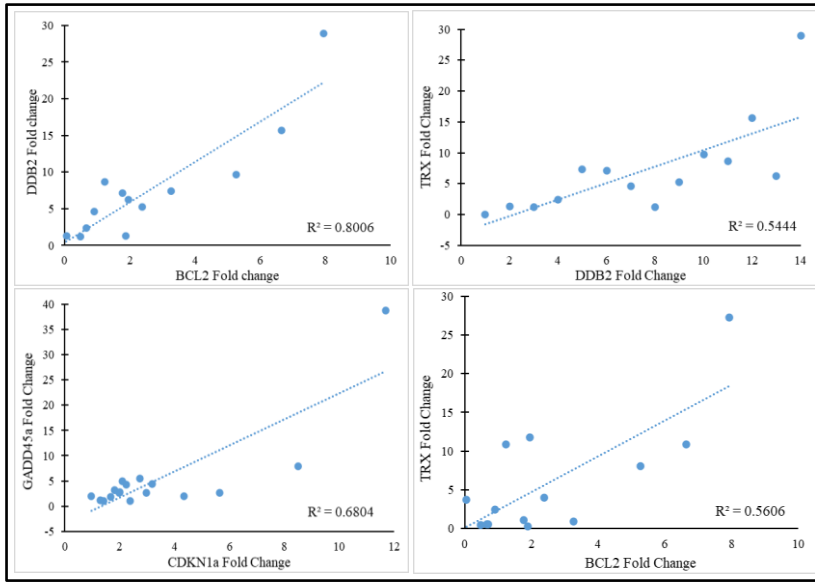
3.2 Associations between Genes' Transcriptional Responses

Possible associations of the different genes to one another based on how they initiated radiation-induced transcripts were tested with Pearson's correlation, and the coefficients are summarized in Table 2. Strong positive correlations were indicated in GADD45A and CDKN1A, BCL2 and DDB2, and DDB2 and TRX, whose Pearson's R is between 0.82 and 0.90. A moderate positive correlation was likewise seen in TRX and BCL2 (R = 0.75). These results suggest that how one gene is induced after 2-Gy irradiation is similar, up to a certain degree, to the magnitude of expression of another gene.

Table 2. Pearson's correlation coefficients between genes' transcriptional responses

	CDKN1A	GADD45A	BCL2	TRX	DDB2
CDKN1A	1.00				
GADD45A	0.82	1.00			
BCL2	0.51	0.57	1.00		
TRX	0.14	0.22	0.75	1.00	
DDB2	0.28	0.38	0.89	0.90	1.00

However, despite these strong associations, only the association between DDB2 and BCL2 could be accounted for by the majority ($R^2 = 0.80$, $p < 0.05$) of the data (Figure 3). Albeit acceptable, the percentage of the data accounting for the correlation of the three other pairs of genes was far lesser at 68% or below.



The mRNA expression at 2 Gy of the different genes was tested for correlation with one another. Gene pairs with moderate to strong correlation based on Pearson's coefficients found in Table 2 were then subjected to linear regression analysis ($p < 0.05$).

Figure 3. Linear regression analysis of gene pairs with moderate to strong correlation

4. Conclusion and Recommendation

Considering the underlying mechanisms, radiation exposure at 2 Gy appeared to have a strong impact on the p53-dependent pathways involved in cell cycle arrest (GADD45A and CDKN1A) and the binding to DNA lesion to facilitate excision repair (DDB2). Upregulation of the anti-apoptotic mitochondrial protein BCL2 and the redox-associated antioxidant protein TRX were not as prominent as molecular markers of radiation exposure given the variable response among the tissues sampled. However, significant correlations were observed among the genes highlighting their parallel roles in the DNA damage and redox response. No gender differences in gene expression were found.

Such gene regulation can ultimately influence tissue health status and propensity for an individual to experience the stochastic effects of radiation exposure such as cancer formation, accelerated aging and tissue damage. The present study's available data suggested that the quality and extent of these gene-based biodosimeters might be dependent on various physical and biological factors. Hence, there is a need for more research that takes into consideration the type of exposure, health status and genetic variability of the individual.

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