RESEARCH ARTICLE

Gene Expression Biodosimetry: Quantitative Assessment of Radiation Dose with Total Body Exposure of Rats

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Abstract

<u>Background</u>: Accurate dose assessment and correct identification of irradiated from non-irradiated people are goals of biological dosimetry in radiation accidents. <u>Objectives</u>: Changes in the FDXR and the RAD51 gene expression (GE) levels were here analyzed in response to total body exposure (TBE) to a 6 MV x-ray beam in rats. We determined the accuracy for absolute quantification of GE to predict the dose at 24 hours. <u>Materials and Methods</u>: For this *in vivo* experimental study, using simple randomized sampling, peripheral blood samples were collected from a total of 20 Wistar rats at 24 hours following exposure of total body to 6 MV X-ray beam energy with doses (0.2, 0.5, 2 and 4 Gy) for TBE in Linac Varian 2100C/D (Varian, USA) in Golestan Hospital, in Ahvaz, Iran. Also, 9 rats was irradiated with a 6MV X-ray beam at doses of 1, 2, 3 Gy in 6MV energy as a validation group. A sham group was also included. After RNA extraction and DNA synthesis, GE changes were measured by the QRT-PCR technique and an absolute quantification strategy by taqman methodology in peripheral blood from rats. ROC analysis was used to distinguish irradiated from non-irradiated samples (qualitative dose assessment) at a dose of 2 Gy. <u>Results</u>: The best fits for mean of responses were polynomial equations with a R^2 of 0.98 and 0.90 (for FDXR and RAD51 dose response curves, respectively). Dose response of the FDXR gene produced a better mean dose estimation of irradiated "validation" samples compared to the RAD51 gene at doses of 1, 2 and 3 Gy. FDXR gene expression separated the irradiated rats from controls with a sensitivity, specificity and accuracy of 87.5%, 83.5% and 81.3%, respectively, 24 hours after dose of 2 Gy. These values were significantly ($p<0.05$) higher than the 75%, 75% and 75%, respectively, obtained using gene expression of RAD51 analysis at a dose of 2 Gy. <u>Conclusions</u>: Collectively, these data suggest that absolute quantification by gel purified quantitative RT-PCR can be used to measure the mRNA copies for GE biodosimetry studies at comparable accuracy to similar methods. In the case of TBE with 6MV energy, FDXR gene expression analysis is more precise than that with RAD51 for quantitative and qualitative dose assessment.

Keywords: Gene expression - biodosimetry - rats - X-rays - lymphocytes

Introduction

In a situation of increasing concern about the possibility of large-scale acute exposure the ability to assess the extent of exposure is essential for decreasing morbidity and mortality through medical intervention (Bazan et al. 2014; Forrester and Sprung. 2014; Min et al. 2014; Tsuyama et al. 2014). Cytogenetic studies that employ dicentric assays, premature chromosome condensation and micronucleus assays are time and labor-intensive (Horn et al. 2011; Romm et al.; Senthamizhchelvan et al., 2009).

Previously, the development of GE profiles in peripheral blood lymphocytes (PBLs) was recommended as an alternative approach for biological dosimetry. The changes in the GE and quantity of RNA transcripts in

cells that have been exposed to ionizing radiation suggest the possibility of that the GE analysis providing not only qualitative assessments of dose but also quantitative dose determination. It may provide high-throughput assessments of radiation exposure in a large number of exposed individuals (Filiano et al. 2011).

There are two approaches for analyzing the GE that have been used extensively for biodosimetry: microarray and quantitative RT-PCR (Tucker et al. 2014). The measurement of changes in the GE by quantitative RT-PCR is more sensitive than that using the microarray method (Kabacik et al. 2011). Moreover, dose assessment with transcriptional responses to ionizing radiation by real-time PCR could be described with accuracy, sensitivity, specificity and high-throughput assessments comparable

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to the cytogenetic method (Badie et al. 2013).

To our knowledge, the absolute quantitative strategy of real-time PCR has rarely been used for the GE biodosimetry. In this study, we used absolute quantitative real-time PCR to investigate the response to ionization radiation of the specific genes regulated transcriptionally by p53 and involved in DNA repair in the blood of healthy rats exposed in vivo. For this purpose, the expression level of RAD51 and ferodoxin reductase (FDXR) gene was analyzed. The aims of this study were to quantitatively assess doses (0, 0.2, 0.5, 2, and 4 Gy) and qualitatively assess dose (2 Gy), using gel-purified of RT-PCR method, 24 hours after total body irradiation of the rats.

Materials and Methods

Animal and Medical Ethics. In this in vivo experimental study, using simple randomized sampling, a total number of 32 female Wistar rats (8-10 weeks old) with the mean weight of 190 ±30 g were obtained from the animal house at the Jundishapur University of Medical Science (Ahvaz, Iran) from 2013-2014 in Iran. The rats were anesthetized with a ketamine/xylazine mixture (85 mg/kg ketamine and 10 mg/kg xylazine). The present study was approved by the medical ethics committee at the Ahvaz Jundishapur University of Medical Sciences (ethical code No. U-92133).

Total body Irradiation of the rats. The irradiation was performed at room temperature in a dose rate of 0.3 Gy/min using Varian 2100C/D Linac (Varian USA) in Golestan Hospital, Ahvaz, Iran. A total of 36 rats were included in the study. Four groups (5 rats per training group) were total body irradiated with 0.2, 0.5, 2, and 4 Gy of 6 MV X-ray beams. The eight rats control-sham group was anesthetized, but without radiation. The validation (unknown) group was consisted of 9 rats (3 rats per group using doses of 1, 2 and 3 Gy). All rats were irradiated at 100cm source to surface distance (SSD) and field size of 20×20cm.

Twenty-for hours after irradiation, 500 μl of peripheral blood was collected from all rats through intra-cardiac puncture. This represent a practical window of time for medical decision making in a mass-casualty situation. The control rats were sham-exposed by keeping them under anesthesia and irradiation conditions. The collected blood was transferred into 2.5-mL EDTA-containing tubes (FL, Italy).

RNA Purification and cDNA synthesis. The RNA was prepared from 500μl of whole blood by the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. The RNA was then quantified using a NanoDrop-2000 spectrophotometer (Thermo, USA) by analyzing the ratio of A260/A280 and A260/A230 and its integrity was confirmed by 2% agarose gel electrophoresis.

Using the High-Capacity cDNA Archive Kit according to the manufacturer's instructions, 0.7 μg of the total RNA was reverse transcribed to cDNA according to the manufacturer's instructions; for quality control purposes, cDNA was subjected to RT-PCR using 18S primers, followed by agarose gel electrophoresis.

The standard curve preparation: The standard curve was drawn by PCR amplification of the total cDNA from lymphocyte cells using RAD51 and FDXR primers. The PCR Product was loaded onto 2% agarose gel for 30 min, stained by DNA safe stain solution (CinnaGen, Tehran, Iran) and visualized by UV-trans illuminator (Vilber Lourmat, Paris, France). A 100-bp and 50-bp size marker were used for RAD51 and FDXR genes, respectively. The correct size bands (110 bp for RAD51and 95 bp for FDXR) were cut from the gel and purified using gel purification kit (Bioneer, Daejoon, South Korea). The concentration of standards were measured using the NanoDrop. The copy numbers per μl were determined (approximately 1011 and 1010 copy numbers per μl for RAD51 and FDXR, respectively) and ten-fold serial dilutions were then prepared, ranging from 102 to 107 copies per μl for FDXR and 102 to 106 copies per μl for RAD51 mRNA. The volume of PCR reaction was 20 μl, thus the range of copy numbers/reaction were 5 to 2×10^6 for FDXR and 5 to 2×10^5 for RAD51.

Concentration of gel purified PCR product for FDXR and RAD51 were 11 ng/μl and 17 ng/μl, respectively and the molecular weights of each copy number from FDXR and RAD51 genes were 58744.8 (9.75×10-11 ng) and 68003.02 Daltons (1.13×10-10 ng), respectively. Therefore, the copy number for each gene was calculated.

Quantitative Real-time PCR by Taqman strategy. PCR amplification was performed using step one real-time PCR machine (Applied Bio system). Cycling conditions for RAD51 gene were as follows: 48°C for 30 second and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The conditions for FDXR gene were as follows: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 20 second and 60°C for 1 min. Primers and probes were prepared from Bioneer Inc. (Daejeon, South Korea). Probes were synthesized with 6-carboxyfluoresin (FAM) at the 5' end and TAMRA quencher at the 3' end. All reactions were performed in duplicate using Takara master mix (Japan), primer and probe set for RAD51 and FDXR genes at 200 nM concentration and 1 μl of cDNA in 20 μl reaction volume. The list of primers and probes are given in Table 1. Ct values were converted to transcript quantity using standard curves obtained by serial dilution of PCR amplified cDNA fragments. Absolute GE levels was calculated and the internal control gene was 18S.

Exclusion criteria: Coagulated blood samples, extracted RNA samples with low quantity, cDNA samples with low quality and quantity, data with more than 0.5 Ct difference between copy numbers in technical duplicate, data for standard of runs with efficiency less than 90% and more than 105% and R2<0.98 were excluded from the study.

Statistical analysis: Curve fitting for TBE was done by using standard regression analysis programs in Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

ROC curve analysis was performed using the statistical package for the social sciences, version 16.0 (SPSS, Inc. Chicago, IL, USA). It was used to find out the best cut-off copy numbers of genes for assessment of 8 irradiated in

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dose of 2Gy from non-irradiated samples (p<0.05).

Results

Real-time PCR data

Quantitative real-time PCR was performed on all samples and plotted as an average response ratio. The results, were presented as copy numbers/reaction. The PCR product was confirmed by size using electrophoretic separation onto 2% agarose gel (supplementary1). The linear dynamic range of the standard curves covered five orders of magnitude (a range from $\times 10^2$ to $\times 10^6$ numbers of cDNA standard copies). For all runs, PCR efficiencies were between 89% and 105% for FDXR and RAD51 genes with $R^2 > 0.98$, respectively. The Ct for FDXR and RAD51 genes was initiated from the cycles of 18 and 23 for the first concentration of standard samples and ended in the cycles of 32 and 34 for the last concentration of standard samples, respectively. The data from a run

of real-time PCR for the FDXR gene are presented in supplementary 2. The GE was measured for TBE at 24 hours after exposure to doses of 0, 0.2, 0.5, 2 and 4 Gy of 6 MV beam energy of x-ray. The mean responses in 20 rats are shown for FDXR and RAD51 (Figure 1-A and 1-B). The best fits for these models were polynomial equations with an R2 of 0.97.

Dose-response curve: quantitative dose assessment

To check the dose estimation curves, 9 rats were irradiated at doses 1, 2 and 3 Gy. For each dose 3 rats were exposed and assayed (Table 2). The accuracy of the dose prediction with dose response curve of FDXR gene at doses 1, 2 and 3 Gy were 87.5%, 78.5% and 74%, respectively. For RAD51 gene, validation samples with doses 1, 2 and 3 Gy were predicted with 49.2%, 71.5% and 42.7% accuracy, respectively. Accordingly, these data suggest that, in case of TBE, analysis of the FDXR expression is more accurate than the RAD51 expression

Table 1. Oligonucleotide Primers and Probes Used in this Study

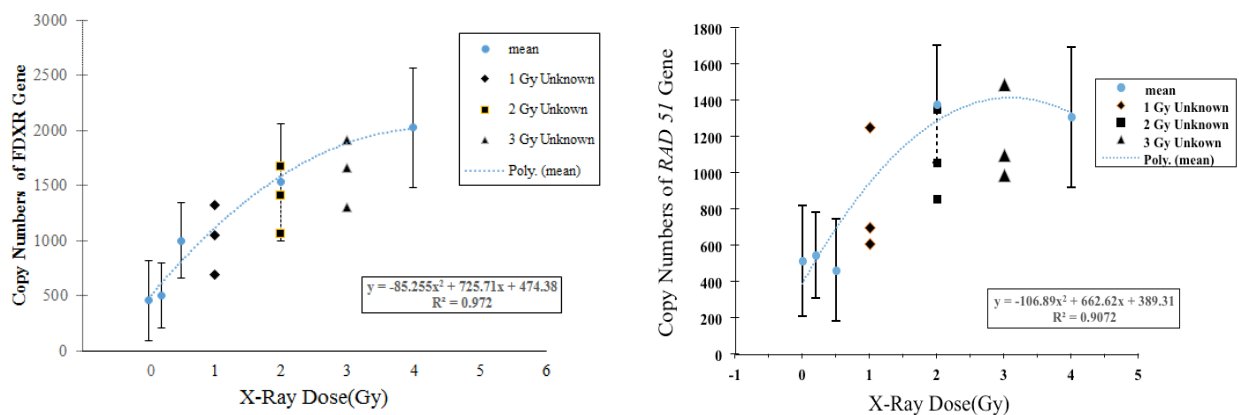| Genes	Accession. No	PCR Primers	Probes	Amplicon
RAD51	NM_001109204.1	GAGACTGGGTCTATCACAGAG (Fwd) TCATCCCCACCTCGGTCAATG (Rev)	CTGGGAAGACACAGATCTGTC	110
FDXR	NM_024153.1	GAGAAGCTGGTGGATCGAAG (Fwd) CCAACCCAGCACAGCTCTTC (Rev)	GAGATGCTGCAGCTGCTGGG	95
18S	NR_046237.1	GCCGCTAGAGGTGAAATTCTTG (Fwd) GAAAACATTCTTGGCAAATGCTT (Rev)	TGGTGAAGCAGGCGGCCGAG	71

Figure 1. Dose estimation curves at 24 hours after TBE of rat for 6 MV beam energy of x-ray: (A) FDXR (B) RAD51. Bars indicate real-time quantitative PCR data (3 samples per training group and 8 samples for sham group). A polynomial fit has the best R2 value for these models. Validation samples from 9 irradiated rats were analyzed at 24 hours to training against dose-response curve. For each dose 3 rats were exposed and radiation doses were 1, 2 and 3 Gy. Copy number values of each rat entered into polynomial equation to find the estimated dose. Mean estimated dose and standard deviation were presented in Table 2

Table 2. Dose Estimation data by FDXR and RAD51 GE Levels from 9 Rats for doses of 1, 2 and 3 Gy

	FDXR		RAD51	
Nominal dose(Gy)	Predicted Dose(Gy)	Average Predicted Dose (Gy) ± SD	Predicted Dose(Gy)	Average Predicted Dose (Gy) ± SD
1	0.88		0.51	
1	0.31	0.87± 0.54	0.35	0.49 ± 0.13
1	1.39		0.61	
2	2.23		2.21	
2	1.25	1.46 ± 0.68	1.27	1.43 ± 0.72
2	0.9		0.81	
3	1.36		1.1	
3	2.18	2.22 ± 0.88	1.18	1.28 ± 0.24
3	3.12		1.56	

Table 3. The Results of Regression Analysis for the dose Response Curve of FDXR and RAD51 Genes and p-value for dose Prediction of Validation Samples in 6 Beam Energy

Gene/Energy	Equation	R^2
FDXR	$y = -85.255x^2 + 725.71x + 474.38$	0.97
RAD51	$y = -106.89x^2 + 662.62x + 389.31$	0.9

†Significant dose prediction using dose response curves of both genes in the same doses in 6 photon beam (p<0.05); *Non- significant difference between the copy numbers of genes in the same doses in 6 and 18 MV photon beam

for dose estimation of 6MV energy beam.

ROC curve analysis: qualitative dose assessment

Using ROC curve analysis, the best sensitivity and specificity were found in the response of FDXR and RAD51 genes to identify non-irradiated from irradiated samples in dose of 2 Gy in 6MV beam energy for TBE (Table 3). The cut-off copy numbers per reaction were found for qualitative dose assessment of rats with the highest sensitivity and specificity (ROC curves has shown in supplementary 3 and 4). Gene expression analysis using ROC curve for FDXR and RAD51 showed that the best specificity and sensitivity of 87.5% and 75% in the cut-off number of 975 and 1059.5. The accuracies of 81.25% and 75% were obtained, respectively, for the FDXR and RAD51 gene expression analysis using the cut-of numbers in dose of 2 Gy at 24 hours. In qualitative dose assessment for TBE with 6MV X-ray Photons, the sensitivity and specificity of GE level for the FDXR were higher than that of the RAD51 in dose of 2 Gy (p<0.05).

Discussion

In this *in vivo* study, to estimate the accuracy of gel-purified RT-PCR method in qualitative and quantitative dose assessment. The rats were exposed to the total body irradiation of different doses in 6 MV energy. To create a standard for absolute quantification, PCR amplification product of total cDNA from lymphocyte cells using RAD51 and FDXR primers was used. After qRT-PCR, amplicons were separated using gel electrophoresis and dilution series finally allowed the absolute quantification, thus, converting CT-values into copy numbers. Using a gel-purified RT-PCR product as a standard sample, the non-specific bands were removed. The gel purification step is a simple experimental work which can increase the accuracy in virtual concentration determination of the FDXR and RAD51 amplicons in standard solution.

We compared our findings with a previously used gene in GE biodosimetry, FDXR (Boldt et al. 2012; Kabacik et al. 2011; Knops et al. 2012; Manning et al. 2013; Manning and Rothkamm; Paul et al.; Paul and Amundson, 2008), which is regulated by p53 family and with our previous in vitro study (Khodamoradi et al. In Press). In our pervious study, we found the dose response curve of the FDXR and RAD51 genes in response to different doses and 6 and 18 MV beam energies in human PBLs. Our dose assessment model for TBE of the rats with the FDXR and RAD51 genes were polynomial. Polynomial model for the RAD51 gene was consistent with our in vitro study in 6 MV energy. This model for the FDXR gene was consistent with the in vitro study of Manning et al. (2013) whereas it was disagreeable with our in vitro study in 6 MV energy. Current study exhibited the increasing rate of copy numbers in doses from 0 to 2 Gy and then, a slow reduction over 2 Gy (Figure 1-A and B) but, the saturation was not showed in our in vitro study in high dose of 6 MV energy. Moreover, the association of increase in the copy numbers with increasing of doses was noticeable for the FDXR gene. Saturation in the in vivo dose response curve of the FDXR gene may be related to the in vivo condition. It seems that the apoptosis mechanism under the in vitro condition was activated more than that of the in vivo condition.

The more slope of the dose response of FDXR gene compared to RAD51 (shown in Figure 1-A and B) was consistent with the hypothesis that the FDXR gene is an appropriate gene for biological dosimetry, as mentioned in our pervious paper. The saturation of the dose-response curve of RAD51 was shown at doses over 2 Gy in Figure 1-B. The biological interpretation of the plateau in the FDXR and RAD51 dose-response curves was explained in our previous study. The possible interpretation was that sub-lethal DNA damages were created by the low dose photons and the expression level of the FDXR and RAD51 genes were increased in response to these damages. Sub-lethal DNA damages were repaired by DNA repair mechanisms. After high dose radiation, lethal damage was induced and cell death mechanisms (apoptosis and mitotic death) led to cell death before 24 hours as well as decrease in the levels of mRNA transcripts.

A same accuracy was obtained for dose prediction by the absolute quantification technique using the RAD51 and FDXR genes at dose 1 and 2 Gy (Table 2). At 3 Gy, the accuracy of FDXR gene was more than the RAD51 gene. The average accuracy of the dose prediction by the FDXR and RAD51 genes was 78.7% and 73.5%, respectively. Collectively, a higher accuracy was observed for the FDXR gene compared to RAD51 gene. Whole genome microarray analysis predicted the dose of validation samples with an accuracy higher than 90% (Tucker JD, et al. 2014). These data suggest that GE analysis of the FDXR for quantitative dose assessment in TBE is more reliable than the expression analysis of the RAD51 gene.

In conventional radiotherapy, patients received 2 Gy per dose in each session. Therefore, o estimate the specificity, sensitivity and accuracy of the FDXR and RAD51 genes, we used 2 Gy for gene expression analysis. The cut-off for the number of copies of FDXR and RAD51 genes was used as a measure to identify the irradiated from non-irradiated individuals.

According to qualitative dose assessment, a higher sensitivity and specificity was observed for the ROC curve analysis for FDXR gene in TBE than RAD51gene. As a result, the FDXR gene is more reliable than the RAD51 gene for qualitative dose assessment of total body irradiation of the rat.

Due to the low sample size, it was not possible to use any type of regression analysis such as multiple regression or a similar approach utilizing data from both

genes to predict the absorbed radiation dose. It is possible, to measure simultaneously the expression level of the principal genes in NHEJ mechanism in association with RAD51 gene and used them for biological dosimetry.

Briefly, for quantitative and qualitative dose assessment in total body irradiation of the rat, with increasing radiation doses in biological dosimetry, the FDXR represent a more accurate response than the RAD51 gene.

In conclusion, the absolute quantification by a quantitative RT-PCR can be used to measure the mRNA copies for the studies of GE biodosimetry. The RAD51 and FDXR genes expression in response to TBE of high-energy x-ray were changed polynominally. Quantitative and qualitative dose assessment the FDXR expression analysis was more accurate than that of the expression analysis of RAD51 gene at 24 hours after TBE.

Acknowledgements

References

Badie C, Kabacik S, Balagurunathan Y, et al (2013).Laboratory intercomparison of gene expression assays. *Radiation research*, **180**, 138-48.

Bazan JG, Chang P, Balog R, et al (2014). Novel human radiation exposure biomarker panel applicable for population triage. *International Journal of Radiation Oncology* Biology* Physics*, **90**, 612-9.

Boldt S, Knops K, Kriehuber R, et al (2012). A frequency-based gene selection method to identify robust biomarkers for radiation dose prediction. *International journal of radiation biology*, **88**, 267-76.

Filiano AN, Fathallah-Shaykh HM, Fiveash J, et al (2011). Gene expression analysis in radiotherapy patients and C57BL/6 mice as a measure of exposure to ionizing radiation. *Radiation research*, **176**, 49-61.

Forrester HB, Sprung CN (2014). Intragenic controls utilizing radiation-induced alternative transcript regions improves gene expression biodosimetry. *Radiation research*, **181**, 314-23.

Horn S, Barnard S, Rothkamm K (2011). Gamma-H2AX-based dose estimation for whole and partial body radiation exposure. *PLoS One*, **6**, 25113.

Kabacik S, Mackay A, Tamber N, et al (2011). Gene expression following ionising radiation: identification of biomarkers for dose estimation and prediction of individual response. *International journal of radiation biology*, **87**, 115-29.

Khodamoradi E, Saberi A, Tahmasebi M, et al (In Press). Dose response curves of FDXR and RAD51 genes in 6 and 18 MV Beam energies in human peripheral blood lymphocytes. *Iran red crescent medical j.*

Knops, K., et al. (2012) Gene expression in low-and high-dose-irradiated human peripheral blood lymphocytes: possible applications for biodosimetry. *Radiation research*, **178**, 304-12.

Manning G, Kabacik S, Finnon P, et al (2013). High and low dose responses of transcriptional biomarkers in ex vivo X-irradiated human blood. *International journal of radiation biology*, **89**, 512-522.

Manning G, Rothkamm K (2013). Deoxyribonucleic acid damage-associated biomarkers of ionising radiation: current status and future relevance for radiology and radiotherapy. *The British journal of radiology*, **86**, 20130173.

Min X-Y, Zhang X-H, Zhou Q-P, et al (2014). Development of serum zinc as a biological dosimeter in mice. *International journal of radiation biology*, **90**, 909-913.

Paul S, Amundson SA (2008). Development of gene expression signatures for practical radiation biodosimetry. *International Journal of Radiation Oncology* Biology* Physics*, **71**, 1236-44.

Paul S, Barker CA, Turner HC, et al (2011). Prediction of in vivo radiation dose status in radiotherapy patients using ex vivo and in vivo gene expression signatures. *Radiation research*, **175**, 257-65.

Romm H, Ainsbury E, Barnard S, et al (2013). Automatic scoring of dicentric chromosomes as a tool in large scale radiation accidents. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, **756**, 174-183.

Senthamizhchelvan S, Pant G, Rath G, et al (2009). Biodosimetry using micronucleus assay in acute partial body therapeutic irradiation. *Physica Medica*, **25**, 82-87.

Tsuyama N, Mizuno H, Katafuchi A, et al (2014). Identification of low-dose responsive metabolites in X-irradiated human B lymphoblastoid cells and fibroblasts. *Journal of radiation research*, rru078.

Tucker JD, Joiner MC, Thomas RA, et al (2014). Accurate gene expression-based biodosimetry using a minimal set of human gene transcripts. *International Journal of Radiation Oncology* Biology* Physics*, **88**, 933-9.