Author's response to reviews

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Identification of gene-based biomarkers of alpha particle radiation exposure in human blood cells

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ABBREVIATIONS

\(^{241}\text{Am}\) Americium, \(^{222}\text{Rn}\) Radon, \(^{226}\text{Ra}\) Radium, \(^{210}\text{Po}\) Polonium, (FBS) fetal bovine serum, (TBS) triphosphate buffered saline, (TST) TBS serum triton, (PBS) phosphate buffered saline, (\(\alpha\)) alpha, (MD) mylar dish, (RPMI) Royal Park Medical Institute, (ANOVA) analysis of variance, (qRT-PCR) quantitative real-time polymerase chain reaction, (Ct) comparative threshold, (FC) fold change, (RDDs) Radiological Dispersal Devices, (LET) Linear Energy Transfer, (IPA) Ingenuity Pathway Analysis, (CBC), complete blood counts
ABSTRACT

Background: The threat of a terrorist-precipitated nuclear event places humans at risk for radiological exposures. Among the isotopes to be used, those emitting alpha (α) -particles pose the highest risk. Currently, detection methods for photon radiation exposure are being developed using genomic technology. This study was designed to determine if these methods are valid for use on exposures involving alpha -α particles. Peripheral blood mononuclear cells (PBMC) were used to mine for sensitive and robust gene-based biomarkers of alpha-particle radiation exposure. Cells were isolated from healthy individuals and were irradiated at a dose range of 0 -1.5 Gy. Genomic strategies were employed to identify transcripts that were differentially expressed relative to un-irradiated cells 24h post-exposure. Stringent statistical analysis identified strong responding genes at each of the individual doses tested. Results: Thirty-one genes were common to all doses with high expression levels ranging from 2-10 fold. This subset of genes was further validated using a complete white blood cell (WBC) population and compared to X-ray exposure using quantitative real-time PCR. The 31 gene panel was responsive in the -particle exposed WBC and was shown to exhibit a differential fold expression profile from X-irradiated cells, with no identification of -particle specific transcripts. Conclusion: This data validates existing gene panels for photon radiation for use in -particle radiation biodosimetry.
INTRODUCTION

Nuclear terrorism is an escalating global concern; our current capabilities to manage and prevent such a tragic event are limited [1]. Between 1993 and 2011, the International Atomic Energy Association (IAEA) documented 2164 nuclear material incidents or malicious acts and 588 involved the theft or loss of nuclear or radioactive materials. A further 18 of these involved plutonium or highly enriched uranium. Such illicit trafficking events highlight the potential for radioactive material falling into wrong hands and potentially being used for the fabrication of a radiological dispersal device (RDD) [2]. Los Alamos National Laboratory has conducted a thorough review of RDD source material and has postulated that four of the nine isotopes most likely to be employed are α-emitters, primarily due to their minimal shielding requirements and ease of concealment [3]. In addition, there is also the advantage of their long half lives and the resultant severe biological damage that can occur from a minimal dose of exposure.

In recent years, much work has gone towards developing strategies for radiation biodosimetry with specific focus on photon radiation [4-8]. However, α-particle exposures are also of concern and current strategies for photon radiation may not provide adequate dose estimates for these exposures. Unlike photon radiation, α-particles travel a short distance (40-70 µm) and create very dense ionizing tracks as they traverse a medium. They can typically cause an energy deposition of 160 keV·µm⁻¹ for 2.5 MeV α-particles in comparison to 2.0 keV·µm⁻¹ for low linear energy transfer (LET) X-rays[9, 10]. Therefore, α-particles produce more significant biological effects when compared to equal absorbed doses from low LET radiation (photon), which are more sparsely ionizing [11-13]. This attribute may provide a biological means to distinguish radiation types based on the magnitude of response.
Development of biomarker-based biodosimetry has been put forth as one of the key priority development areas for nuclear threat countermeasures [14] and microarray data / gene based profiling has served as timely and minimally invasive procedures to fill these priority areas [15]. There have been several studies examining the gene expression profiles of human cells using functional genomics platforms for photon radiation [16]. However, similar availability of gene tools for high LET radiation types, such as α-particles, remains limited. To date, the majority of α-particle transcriptional studies have been performed in vitro using transformed or normal cell types [17-19]. There has also been a selected few studies that have profiled genomic changes and compared the responses following exposure of cells to different radiation types [20,21]. Microarray studies in our own lab using epidermal keratinocytes exposed to both α-particle and X-ray radiation have also shown transcriptional differences [21] in responses between these radiation qualities.

In this study, genomic strategies were employed to identify biomarkers of α-particle radiation exposure. Circulating lymphocytes were isolated from normal, healthy volunteers and subjected to ex vivo exposure to α-particle radiation. Twenty-four hours post-exposure, the expression of numerous transcripts was assessed using Illumina bead array technology. The responses were compared to non-irradiated controls. Strong dose-responsive genes were then further validated in complete white blood cell population and assessed for specificity by comparison with X-irradiated cells.
MATERIALS AND METHODS

Blood Draws

All procedures were approved by Health Canada’s Research Ethics Committee. Peripheral blood from healthy, non-smoking volunteers was drawn via periphery venipuncture with informed consent from all subjects and was drawn into either 5 x 10 ml EDTA (for gene analysis) or 2 x 4 ml lithium heparin (for plasma analysis) vacutainer tubes (Becton Dickinson and Company, Franklin Lakes, NJ). A total of 6 male and 6 female donors participated. Before any further processing, a 100 μl whole blood sample was drawn to perform a complete blood count (CBC) via automatic haemocytometer (Beckman Coulter, Mississauga, ON).

Peripheral Blood Mononuclear Cell Isolation

Peripheral blood mononuclear cells (PBMCs) were used as a representative cell-type for an initial global screening of gene transcripts using microarray technology. A similar isolation procedure was employed as described by Boyum [22]. Briefly, a 15 ml of Histopaque-1077 sucrose gradient (Sigma-Aldrich, MO, USA) was pipetted into the upper chamber of an Accuspin Tube (Sigma-Aldrich). The tube was centrifuged (800 x g) for 30 sec to ensure that the Histopaque was below the frit layer. Freshly drawn whole blood was pipetted into the upper chamber of tube. The tube was then centrifuged 800 x g for 15 minutes. The band of mononuclear cells was transferred to an alternate centrifuge tube and washed with 10 ml of isotonic phosphate buffered saline (PBS) three times. Pelleted cells were then resuspended in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 100U penicillin &100μg streptomycin/ml (Sigma-Aldrich).
Total White blood Cell Isolation

Following initial experiments on microarray analysis of PBMCs, further validation studies using qPCR were conducted on the complete white blood cell (WBC) population. WBCs were isolated from whole blood using Histopaque-1119 (Sigma-Aldrich). Twenty-five milliliters of whole blood was gently poured over 15 ml of Histopaque-1119 and spun at 1000 x g for 10 minutes. This resulted in erythrocyte sedimentation below the Histopaque gradient and a total white blood cell population above the gradient. This volume (~15 ml) was then transferred to a new 50 ml tube and diluted 1:2 with phosphate buffered saline (PBS). The resulting WBC pellet was then washed twice more with 10 ml PBS and resuspended in RPMI-1640 media supplemented with 10% FBS, 2mM L-glutamine and 100U penicillin&100µg streptomycin/ml.

PBMCs and WBC Irradiations

Either Isolated PBMCs or WBCs were seeded at total cell density of 8-10 X10⁶ cells in 2ml of media and were cultured on thin Mylar based plastic dishes (MD) (Chemplex Industries, Palm City, FL, USA), which allowed for penetration of α-particles. Cells were allowed to settle for 10 minutes before performing the irradiations. Irradiations were performed at doses of 0 (control), 0.5, 1.0 or 1.5 Gy using Americium (²⁴¹Am) electroplated discs with an activity level of 66.0 kBq ± 3% (dose rate of 0.98 ± 0.01 Gy/h, LET of 127.4±0.4 keV/µm). The absorbed dose of α-particle radiation to which the cells were exposed was calculated using the GEANT4 v.9.1 Monte Carlo tool-kit [23]. Cells destined for X-ray radiation at doses of 0 (control) 2, 5 or 10 Gy were exposed using the X-RAD 320 X-ray irradiation system (Precision X-ray, Inc., North Branford, CT, USA).
at a high dose rate of 0.98 ± 0.05 Gy/min. Exposures were performed in duplicate and pooled. Twenty-four hours following irradiation, a 50 µl aliquot of cells was assessed for cellular viability using the Trypan Blue viability assay (Bio-Rad, Hercules, CA), and a 100 µl aliquot was used for a CBC via automatic haemocytometer. The remainder of the cells were spun down and the media frozen for protein analysis and the cell pellets destined for RNA extraction.

**H2AX Phosphorylation Assay**

H2AX phosphorylation was assessed using flow cytometry following a modified protocol by MacPhail et al. [24]. Thirty minutes after exposure, cell suspensions (5 × 10⁵ cells per sample) fixed with 10% formaldehyde (Fisher Scientific, USA) and incubated for 10 min at room temperature. The cells were then washed and re-suspended in 1 ml cold (−40 °C), 70% methanol (Fisher Scientific) in 1x PBS and stored at −40 °C overnight or up to two weeks. One ml of cold TBS (tris-phosphate buffered saline, 0.0154 M Trizma Hydrochloride (Sigma–Aldrich Canada), 0.5 M NaCl (Fisher Scientific), pH 7.4) was then added to each sample, mixed well, centrifuged (8 min, 400 × g, 4 °C) and re-suspended in 1 ml of cold TST (TBS serum triton, 96% TBS, 4% FBS (Sigma–Aldrich), 0.1% Triton X-100 (Sigma–Aldrich)). The samples were incubated on ice for 10 min, centrifuged (5 min, 400 × g, 4°C) and re-suspended in 200 µl of anti-γ-H2AX-fluorescein isothiocyanate (FITC) antibody (Millipore, USA) diluted 1:500 in TST. After 2 h incubation on ice in the dark, 1 mL of TBS with 2% FBS was added. The samples were then centrifuged (5 min, 400 × g, 4°C), re-suspended in 250 µl TBS with 2% FBS. Immediately prior to analysis by flow cytometry, 2 µl of 1 mg/mL propidium iodide (PI) was added to each sample. For flow cytometry analysis, data acquisition was set to analyze 2 × 10⁴ cells from the whole cell population as
identified by a forward scatter (FSC) vs. side scatter (SSC) dot plot. All debris under the FSC and SSC threshold were excluded from the analysis. The γ-H2AX response was measured by assessing the increased level of intracellular fluorescence characterized in the cells, as determined by the geometric mean of the intensity peak of the anti-γ-H2AX-FITC (channel number) of the γ-H2AX positive cells. All samples were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

RNA extractions

Twenty-four hours post-radiation exposure or negative control conditions, RNA extractions were performed on either PBMCs or WBCs. In the case of PBMCs, the cells were resuspended in 350 µl of Buffer RLT containing 1% β-Mercaptoethanol (Qiagen’s RNeasy Mini kit; Qiagen Inc, Mississauga, ON) then frozen at -80°C until processed. Frozen lysates were thawed on ice and mixed well by pipetting. The lysate was transferred directly onto a QIAshredder spin column (Qiagen Inc), placed in a 2 ml collection tube and centrifuged for 2 min at ~12,000 g. A volume of 350 µL of 70% ethanol was added. Total RNA was then extracted using the RNeasy Mini kit according to the manufacturer’s instructions (Qiagen Inc), with the addition of Qiagen’s On-Column RNase-free DNase (Qiagen Inc) to eliminate any remaining DNA contamination. In the case of the WBCs, RNA extractions were performed using QIAzol reagent (Qiagen) and following manufacturer’s instructions. Briefly, 700 µl of QIAzol reagent was added to the cell pellet and then homogenized via up-and-down pipetting of the mixture 50 times. After room temperature incubation for 15 minutes, 140 µl of chloroform was added for phase separation. The aqueous layer containing RNA was then removed and precipitated with 100% ethanol. Total RNA
was then isolated using the miRNeasy column purification kit. All total RNA sample concentrations and RNA quality were determined using both an Agilent 2100 Bioanalyzer and RNA Nanochips (Agilent Technologies Canada Inc., Mississauga, ON) and spectrophotometrically using a Nanodrop (Fisher Scientific, Ottawa, ON) (OD ratio of A260:A280). All extracted PBMC RNA samples were determined to be of good quality (RNA Integrity Number $\geq 8.0$) with minimal degradation and stored at -80°C until further analysis. WBC RNA from particle exposed samples was determined to be of good quality (RNA Integrity Number $\geq 9.2$) with three samples being excluded from sample analysis due to insufficient RNA yield.

**Genomic Profiling**

An input of 200ng of PBMC mRNA was used for whole genome analysis following the Illumina(r) Whole Genome Expression Profiling Assay Guide (11317302 Rev. A). Samples were hybridized on Illumina human-12 v2 RNA BeadChips. BeadChips were imaged and quantified with the Illumina iScan scanner and data was processed with Illumina GenomeStudio v2010.2.8.11 miRNA Profiling. An input of 100ng of WBC miRNA expression was profiled using the nCounter system (NanoString Technologies, Seattle, WA) which profiles the expression levels of 800 miRNAs. This was performed using the human miRNA expression assay (version 2) according to manufacturer’s instructions and read using the nCounter digital analyser.

**Quantitative real time-polymerase chain reaction (qPCR) validation**

Selected genes deemed significant by microarray analysis or nCounter system were further
assessed by qPCR. Total RNA (400ng mRNA 200ng miRNA) isolated from cells were reverse transcribed into complementary DNA using the RT2 First Strand Kit (Qiagen). Gene profiling was performed according to the manufacturer’s instructions using custom RT2-profiler PCR arrays (Qiagen). Reactions were prepared in 96-well plates and performed using a spectrofluorometric thermal cycler (Biorad iCycler; Hercules, CA). The relative expression of each gene was determined by using the comparative threshold (Ct) method (Schmittgen and Livak 2008). Analysis of qPCR expression profiles of data was performed using the super array biosciences web portal for data analysis of their products (SABiosciences http://www.sabiosciences.com/pcr/arrayanalysis.php).

**Customized Gene Array Panel**

A total of 96 genes were used for the development of a customized 384-well format gene array panel (Figure 1). This panel comprised of genes that were shown by microarray technology to be dose-responsive and also expressed at the medium and high dose. This panel also included negative control genes, housekeeping gene and some genes derived from the work of Paul and Amundson [25]. SABiosciences (Qiagen) designed the primers and provided a 384 well-format platform that was compatible for use on the LightCycler 480 real-time PCR system (Roche, Mississauga, ON). A high-throughput PCR platform, comprising the Caliper Zephyr Compact Liquid Handling Station, the Caliper Twister II plate handler (PerkinElmer, Woodbridge, ON) and the Lightcycler 480 was employed with custom protocols developed in the Inhalation Toxicology Laboratory of Health Canada. This system allowed for the screening of 144 samples in a one-week time-span.
Pathway Analysis

Significantly expressed genes at the high (1.5 Gy) dose of α-particle radiation were further explored by assessing their interactivity in terms of pathways, functions and networks. Gene lists were uploaded into Ingenuity Pathway Analysis (IPA) software, version 11904312 with corresponding fold change data. Analysis was performed using Ingenuity Expert Information core analysis. Functional Analysis identified the biological functions and/or diseases that were most significant to the given gene list. Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the gene list. The significance of the association between the gene list and the canonical pathway was measured in two ways. First, a ratio of the number of molecules from the gene list that map to the pathway divided by the total number of molecules that map to the canonical pathway and second, a Fisher’s exact test was used to calculate a p-value determining the probability that the association between the genes in the list and the canonical pathway is explained by chance alone. Networks were constructed to demonstrate the interactivity and molecular relationships between induced genes. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from literature, or from canonical information stored in the Ingenuity Knowledge Base.

miRNA Profiling

An input of 200 ng of WBC miRNA expression was profiled using the nCounter system
(NanoString Technologies, Seattle, WA) which profiles the expression levels of miRNAs. This was performed using the human miRNA expression assay (version 2) according to manufacturer’s instructions and read using the nCounter digital analyser.

**Statistical analysis**

Microarrays statistical analysis was performed as follows. Data background correction was done within GenomeStudio (Illumina), and then exported to the lumi R package. Data was then normalized via quantile method, rendering the distribution of probe intensities of each array in a set of arrays equivalent. Normalized data was then log2 transformed for statistical comparisons. Linear models for microarray data (LIMMA) was employed to identify differentially expressed gene signatures between the different exposure conditions [26, 27]. In brief, this method involves fitting a linear model for each gene in the data and moderating the standard error via an empirical Bayes method. This is used to estimate the moderated t-statistics/F-statistics for each gene, shrinking the standard error towards a common value. This test is similar to an ANOVA for each gene with the exception that standard deviations are moderated across genes, allowing more stable inference for each gene. Moderated standard deviations are a compromise between individual genewise standard deviations and overall pooled standard deviations. Multiple comparison false discovery rate (FDR) was evaluated using the Benjamini-Hochberg (BH) method [28].

PBMC qPCR data was analysed for statistical significance without multiple correction comparison using gene-wise ANOVA as there was a-priori reason for gene analysis. WBC qPCR data and nCounter data was analysed for statistical significance via LIMMA as described above.
Hierarchical clustering was performed using the WBC qPCR data using dChip (http://www.hsph.harvard.edu/cli/complab/dchip). This software was used to cluster the different exposure conditions by gene signature and group genes by similarity of expression patterns. The distance between genes is measured as 1 - r (Pearson correlation coefficient).
RESULTS

DNA Damage

To ensure that cells were undergoing irradiation and sustaining DNA damage, a biological assay indicative of DNA damage, the phosphorylation of H2AX, was employed. Thirty minutes post-exposure, cells were assessed for the expression of γH2AX, a marker indicative of DNA double strand breaks. A dose-dependent increase in the γH2AX signal was observed following exposure to α-particle radiation as seen by the pronounced shift in the curve and a plot of the geometric mean of this signal as a function of dose (Figure 2). Statistically significant responses were obtained at the medium and high doses tested (p<0.01) relative to the non-irradiated control treatment group. A bi-modal shaped curve was observed at the lowest dose of α-particle radiation which with increasing doses transitioned to a mono-modal curve. At the highest dose (1.5 Gy) an approximate 3-fold increase in γH2AX signal was observed relative to the control sample. As a positive control, isolated leukocytes were irradiated with X-rays at a high dose rate (1 Gy/min) and a dose range of 2-10 Gy. A plot of this response indicated X-rays to be significantly more damaging as seen by the marked increase in γH2AX signal with dose of radiation relative to alpha-particle treated cells. The α-particle exposed samples displayed lower γH2AX intensities than the X-ray exposed samples and this may be due to several factors. Firstly, the samples irradiated with X-rays were exposed to higher overall doses which will increase the probability of inducing DNA double strand breaks. Secondly, the dose rates of exposure were markedly different. The α-particle exposure system is limited to a dose rate of ~1 Gy/hr and the X-ray exposure was
performed at a dose rate of ~1 Gy/minute. This means the α-particle doses were delivered over a protracted amount of time relative to the X-rays. Thus, there is a degree of repair occurring whilst the cells are undergoing irradiation and the measured signal is lowered due to resolved γ-H2AX foci.

**Genomic Profiling**

Genomic profiling was performed on RNA extracted from isolated PBMCs 24 h post-exposure. In order to mine for reliable biomarkers, statistical stringency was prioritized to mine for reliable genes using a Benjamini Hochberg (BH) false discovery rate (FDR) correction. All differentially expressed genes were filtered on flagged spots that were of poor quality and a BH FDR corrected p-value <0.05. A summary of the gene responses at each of the doses is provided in Table 1. Overall, there was a pronounced induction of transcriptional response, with the majority of genes being up-regulated in the presence of the radiation insult. Escalating doses induced an increasing number of transcripts with 33, 77 and 154 genes differentially modified at 0.5, 1.0 and 1.5 Gy respectively. A Venn diagram was constructed to provide a quantitative representation of the similarities and differences in expression profiles at each of the doses (Figure 3). Thirty-one genes were shown to be differentially expressed at all three doses with expression levels ranging from 2-10 fold. Forty-four genes were common between differentially expressed gene sets at both the medium and high dose. The range in expression levels of these genes is summarized as a heat map which delineates the genes by degree of fold change, and comprises high, medium and low expressers (Figure 4). The 31 dose responsive genes as well as the 44 genes expressed at the
medium and high dose were further validated using quantitative
reverse-transcription polymerase chain reaction (qPCR).

**qPCR Validation**

The 44 transcripts observed to be expressed at the medium and high dose and the 31 genes expressed at all three doses were further validated using qPCR. Of these genes four were omitted, either due to name duplication from the microarray, or could not be validated due to the unavailability of effective primers. A comparison of the responses using the two technologies showed a similar trend in expression levels. As shown in Table 2, all 70 genes that exhibited a significant response across the 3 doses using microarray analysis were also observed to exhibit a similar trends using qPCR. Genes that were expressed at both the medium and high dose also displayed similar fold expression changes between the two methodologies; however, there was occasionally a discrepancy in assigning statistical significance. Approximately 20% of the total validated targets were shown to be non-significant using qPCR, potentially due to the low expression levels of these transcripts. In contrast, there were also a sub-set of 10 genes which showed statistical significance at the 0.5 Gy dose via qPCR but not microarray analysis.

**Custom qPCR panel**

The 31 dose-responsive genes alongside 55 genes identified in the literature as being highly X-ray-responsive were used to develop a customized gene array panel. This panel was used to verify the validity, specificity and overall integrity of the 31 dose-responsive genes in the entire white blood cell (WBC) population. WBCs were isolated from 12 healthy individuals and exposed to α-particles and X-rays. Total white blood cell counts were typically in the range of
5-10 x 10^6 cells/mL and enriched in neutrophils. The viability of the cells was assessed using the Trypan Blue viability assay pre- and post-irradiation. The cells remained viable (above 99%) and no significant changes in blood cell counts or populations subsets were observed post-irradiation relative to un-irradiated cells (Table 3). Twenty-four hours after irradiation, RNA was extracted and reverse transcribed to cDNA for assessment of gene expression changes using qPCR. All dose- and α-particle-responsive genes were observed to be significantly expressed at the three doses in the total WBC population. Among the 41 genes observed at the medium and high dose, 15 were shown to be un-responsive at the medium dose, however the majority of these were observed at high dose of radiation (Table 4). Box-plots of the responding genes allowed for a comparative assessment of the two radiation types and the range in inter-individual variability between transcripts (Figures 5-8). In order to better delineate expression patterns from the gene expression data derived from the WBC qPCR, further analysis was conducted through hierarchical clustering. Figure 9 shows gene expression data clustered by gene and exposure group. Inputting all qPCR data resulted in classification of exposure groups based on doses.

Pathway Analysis
Statistically significant genes expressed at the 1.5 Gyα-particle dose were entered into the IPA analysis tool [29, 30] to determine interconnectivity of the genes and associated canonical pathways. The top networks associated with this gene set included apoptosis, cell-to-cell signaling interaction, cell death and delay in cell cycle progression. The top canonical pathways associated with these genes were p53 signaling and GADD45. Several high expressing genes associated with
these pathways included CCNG1, PCNA, PMAIP1, TP53INP1, GADD45A, BBC3, CDKN1A, TNFRSF10B, MDM2, BAX, FAS, LRP5, GS5, RPMID, PRKAB1, RPS276, SPATA18, SRA1, RNU6-1, LDA3, PHLDA3 and DRAM1. All were up-regulated in expression with the exception of PACS1N1 which was down-regulated by ~2 fold following α-particle irradiation.

**MicroRNA Expression**

The screening of ~800 miRNA transcripts using NanoString profiling resulted in minimal responding targets. Only one miRNA (miR-34a) was observed to be differentially modulated (p<0.05) at all doses examined following α-particle radiation exposure. This target was dose-responsive and subsequently validated using qPCR (Table 5). miR-34a was up-regulated over 2.5 fold in all exposed samples and had a similar 3 fold induction in the 1.0 and 1.5 Gy α-particle doses. This target was not specific to α-particle exposure as expression was also observed with X-ray irradiation at all three doses tested.
DISCUSSION

The overarching goal of this research was to identify gene-based biomarkers of α-particle radiation exposure for the purposes of developing effective triage tools for use in a population exposure scenario. To date, the majority of biomarker-based radiation triage has been developed for photon radiation. Although there is a large body of work concerning radiation exposure and cytogenetic end-points, it was postulated that α-particle radiation may elicit differential cellular response due to its characteristic physical properties, which differ from photon radiation. This may potentially provide more accurate dose estimates for exposures and differentiate between radiation types. Circulating blood cells represent a sensitive target for early radiation damage and are easily accessible.

A preliminary microarray screening following the exposure of isolated PBMC to α-particles identified thirty-one dose responsive and forty-four medium and high dose responding genes. These genes were validated using qPCR and shown through pathway analysis to be associated with signaling pathways centered around p53 and GADD45A, consistent with a DNA damage response. To confirm the validity of this gene set in a more physiologically relevant population of cells, the complete white blood cell population was harvested including PBMCs. Furthermore, the ability of this gene panel to discriminate between radiation qualities was concurrently assessed using qPCR. For this purpose, a customized gene panel was constructed from the 31 dose-responsive genes, 44 medium/high responding genes and 55 genes identified in the literature as being X-ray-responsive.
These 55 genes were primarily selected from work conducted by Amuduson et al. [25]. The “in-house” gene panel comprised transcripts that were differentially expressed in the initial microarray screening and also shown to be absent in the panel proposed by Paul and Amundson, and therefore potentially $\alpha$-particle radiation specific.

The customized gene panel confirmed the validity of our initial microarray results. Primarily, that all dose-responsive $\alpha$-particle induced genes identified in PBMC were also observed to be significantly expressed at all doses in the total WBC population. However, of the 44 medium and high dose genes, 15 were shown to lack statistical significance, most likely due to the use of stringent statistical analysis. When less stringent statistical methods not accounting for multiple statistical comparisons were employed, the qPCR dataset were more comparable to the PBMC microarray results.

Further analysis of the data using box plots allowed for an assessment of gene responses with respect to each individual. All control treatment groups displayed low inter-individual variability for all differentially expressed transcripts, particularly between radiation types, highlighting the potential for these transcripts to be strong biomarkers. Furthermore, the majority of genes displayed strong dose-response trends for both $\alpha$-particle and X-ray radiation. This is further highlighted in the hierarchical clustering of the dataset. Inputting all qPCR data resulted in the classification of treatment groups dose-dependently. Of particular note is the 2 Gy X-ray dose, which was shown to cluster alone, suggesting that the gene signature expressed at this dose is
somewhat dissimilar to any of the higher doses. Subsequent clustering of the 1.0 and 1.5 Gy α-particle exposure together and the 5 and 10 Gy X-ray exposure suggests that it is possible to make distinctions between high X-ray radiation doses and low α-particle doses.

Overall, the α-particle radiation responses were not dose-dependently distinguishable from the X-ray responses. As the 96-gene panel developed in this study was constructed based on a comparative assessment of the photon gene panel developed by [25], it was anticipated that some α-particle specific gene responses would be obtained. However, the lack of distinguishable responses highlights that irrespective of LET differences, biologically the radiation types elicit similar gene responses. If doses and dose-rates were to be matched, clear distinctions between gene expression profiles may be detected. However, it would remain questionable as to whether the responses were specific to α-particle radiation or a result of LET differences resulting in a lower relative biological effectiveness for X-rays. The data shows that the Paul & Amundson gene panel may be applicable for use in scenarios involving α-particle and X-ray exposures. Future studies may assess harvest time as an option for detecting clear biological differences between the two radiation types. The repair kinetics for high LET and low LET radiation have been documented to be different [31], which may be reflected in genomic expression differences.

To date, [32] is the only other group to examine α-particle radiation induced genomic-wide transcriptional effects in isolated blood cells. This group employed a harvest time of 1hr post-irradiation using a dose range of 0.05 - 1.6 Gy of α-particle radiation. Three hundred and
thirty nine genes were shown to be differentially modified with 54% up-regulated and 46% down-regulated. In comparison to our study, the number of responding genes was much lower and the majority of the genes obtained in our study were upregulated. These differences may be attributed to experimental conditions as Turtoi et al., used varying dose-rates and a post-irradiation harvest time of 1 hour. As well, their gene responses was obtained from only one individual, while our study employed 12 individuals repeated twice with consistent results.

**Conclusion:**

In summary, genomic strategies were employed for radiation responses. Thirty-one dose responsive and forty-four medium and high dose responding genes were induced by $\alpha$-particle radiation. These genes were associated with signaling pathways centered around p53 and GADD45A, consistent with DNA damage response. Subsequent comparison with high dose-rate X-ray radiation showed that both radiation types elicited similar gene responses. It was expected that there would be $\alpha$-particle specific gene expression changes, but this was not observed. Despite the lack of obtaining discriminatory radiation responsive genes, a number of novel findings have been identified. Firstly, the published gene panel for photon radiation may be useful for $\alpha$-particle exposures. Furthermore, with respect to radiation exposure, this data has shown that the degree of expression (fold change) may be a strong parameter for developing computational models for distinguishing exposure type. These subtle differences, as shown by hierarchical clustering may be further explored. Although radiation responsive genes were identified, considerable work is needed to further validate these responses. Future work includes time and
dose sensitivity assessment and determining temporal response of the 31 dose-responsive genes identified in this study. Distinctions between α-particle radiation and X-ray radiation may become evident over a time-period. The panel of genes should also be tested in an *in vivo* environment, using radiotherapy patients undergoing either total body irradiations or α-particle radiation therapy.
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COMPETING INTEREST

The authors declare they have no competing interests.
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FIGURE LEGENDS

Figure 1: Schematic representation of the 384-well gene array that was developed for use in high-throughput qPCR validation and screening.

Figure 2: WBC H2AX phosphorylation expression after exposure to alpha-particle or X-ray radiation. A) Geometric mean of signal intensity (indicative of gamma-H2AX expression) for each of the doses and radiation types. Data is presented as means ± SD with n=12 biological replicates. ** represents p<0.01 B) Representative flow cytometric histogram overlay of gamma-H2AX expression in WBC cells at various doses of alpha-particle and X-ray exposure measured 30 min post-exposure.

Figure 3: Venn diagram showing overlap patterns of genes which were show to be significantly modulated via microarray in peripheral blood mononuclear cells (PBMCs) after various doses of alpha-particle radiation. Based on an n=12 human donors.

Figure 4: Heat map depicting the microarray fold change expression values of the genes which were found to be statistically significant at the medium and high doses and at all three doses of alpha-particle radiation exposure using microarray technology. Red colouring signifies up-regulation based on an n=12 biological replicates. The heat map is divided
into three profiles based on high (A) medium (B) and low (C) fold change induction.

Figure 5: Box-plot representation of genes responding with fold changes ranging from 5-100 fold via qPCR in isolated leukocytes exposed to alpha-particle and X-ray radiation. The high responding genes that corresponded to the heat-map are plotted with $2^{-\Delta CT}$ values along the Y-axis. The central line represents the median of the data and the box edges represent the upper (75th) and lower (25th) percentile. Whiskers denote the highest and lowest values from the data set within the upper and lower limits. Limits are defined as 1.5*50 percentile spread.

Figure 6: Box-plot representation of genes responding with fold changes ranging from 2-9 fold via qPCR in isolated leukocytes exposed to alpha-particle and X-ray radiation. The medium responding genes that corresponded to the heat-map are plotted with $2^{-\Delta CT}$ values along the Y-axis. The central line represents the median of the data and the box edges represent the upper (75th) and lower (25th) percentile. Whiskers denote the highest and lowest values from the data set within the upper and lower limits. Limits are defined as 1.5*50 percentile spread.

Figure 7: Box-plot representation of genes responding with fold changes ranging from 1-4 fold via qPCR in isolated leukocytes exposed to alpha-particle and X-ray radiation. The medium responding genes that corresponded to the heat-map are plotted with $2^{-\Delta CT}$ values along
the Y-axis. The central line represents the median of the data and the box edges represent the upper (75th) and lower (25th) percentile. Whiskers denote the highest and lowest values from the data set within the upper and lower limits. Limits are defined as 1.5*50 percentile spread.

Figure 8: Box-plot representation of genes responding with fold changes ranging from 1-2 fold via qPCR in isolated leukocytes exposed to alpha-particle and X-ray radiation. The medium responding genes that corresponded to the heat-map are plotted with 2-ΔCT values along the Y-axis. The central line represents the median of the data and the box edges represent the upper (75th) and lower (25th) percentile. Whiskers denote the highest and lowest values from the data set within the upper and lower limits. Limits are defined as 1.5*50 percentile spread.

Figure 9: Median based hierarchical clustering dataset to determine common groupings of samples and genes.
AUTHOR CONTRIBUTION

VC contributed to the conception and design of the study, acquisition of data and analysis and interpretation of data. VC drafted the manuscript and provided final approval for publication. MH contributed to the execution of experiments, data analysis and interpretation. RW contributed to H2AX data analysis and final manuscript revision.
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Figure 1
Figure 2
Figure 3

BH P<0.05
Figure 5
**Figure 7**
Figure 8
Figure 9
Additional files provided with this submission:

Additional file 1: All tables for Lymph and Leuk Blood Exposures2.pdf, 315K