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Metabolomic Analysis of Mice Exposed to Gamma Radiation Reveals a Systemic Understanding of Total-Body Exposure

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Abstract

Diagnostic markers are needed for accidental or deliberate radiation exposure that could cause acute and chronic radiation toxicity. Biomarkers of temporal, dose-dependent, aging-attenuated and multiple radiation exposures have been previously described by others. However, the physiological origin and biochemical networks that generate these biomarkers and their association at the molecular level have yet to be explored. Hence, the discovery and identification of total-body-irradiation-induced tissue specific biomarkers remains an enormous challenge within radiation biodosimetry research. To determine the tissue level response of total-body exposure (6 Gy), metabolomics analysis was carried out on radiosensitive tissues bone marrow, ileum, liver, muscle and lung as well as serum and on urine within 12 h postirradiation. Differences in the metabolic signatures between the sham and gamma-irradiated groups were analyzed by hydrophilic interaction liquid chromatography (HILIC)-based ultra-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS). A panel of 67 biomarkers identified in radiosensitive tissues and biofluids (serum and urine) at a 6 Gy dose. Among the identified biomarkers, 3-methylglutaryl carnitine (3-MGC) was found to be a novel metabolite in liver, serum and urine that could potentially be an early radiation response marker. The degree of metabolic changes among different tissues showed perturbations in pathways including DNA methylation, energy, nucleic acid, amino acid, glutathione and bile acid metabolism. These results highlight metabolomics as a potential novel approach to understand functional alterations in the metabolome that could be adapted for use in the rapid assessment of radiation exposure and triage protocols in the case of nuclear incidents.

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INTRODUCTION

Humans and other living organisms can be exposed to ionizing radiation by accidental damage of nuclear power plants and by potential terrorist attacks using radiological or nuclear devices. These intentional or unintentional exposures can have profound impact on the radiosensitive tissues leading to acute radiation sickness (ACS) (1). Conventional biomarkers of experimental radiation-induced damage including dicentric chromosomes, micronucleus formation, γ -H2AX phosphorylation and changes in gene expression, such as xeroderma pigmentosum (*Xpc*), cyclin-dependent kinase inhibitor 1A (*Cdkn1a*), growth arrest and DNA-damage-inducible 45 alpha (*Gadd45a*) and mouse 3T3 cell double minute 3 (*Mdm3*) (2, 3), have been proposed as biomarkers for radiation exposure but these are largely low-throughput technologies that require large sample volumes, inaccessible tissues and significant sample processing time (4). Currently, human exposure to radiation is evaluated based upon the onset and duration of nausea, vomiting, whole blood cell counts and neurological symptoms (5, 6). Hence, novel high-throughput biodosimetry devices should be of great value for the medical management of radiation exposure incidents for the purpose of triage applications. To understand the consequences of radiation exposure, a radiation biodosimetry study was initiated to develop rapid, sensitive assays, diagnostic platforms that are accurate, would be useful to confirm exposure, predict exposure levels, to gain an organ level understanding in victims, to provide triage and definitive care of exposed population.

Radiation metabolomics as a potential measure of radiation biodosimetry was initiated to monitor the biological responses to radiation dose using state-of-the-art of mass spectrometry-based metabolomics (7, 8). Radiation-induced metabolome changes and their biological complexity were studied with UPLC-ESI-QTOFMS in TK6 and BJ cell lines (9) and in the urine of rodents (10–13) and non-human primates (14, 15) by various doses and at different time points. Additionally, the effect of ionizing radiation on aging and multiple previous exposures was studied by employing metabolomic approaches in the mouse model (16). Taken together, these studies identified urinary metabolic signatures that were minimally-invasive markers of radiation exposure, many of which are predominantly found across exposed species. The signatures identified were essentially derived from nucleic acid damage, oxidative stress, fatty acid β -oxidation pathways and polyamine metabolism. However, the understanding of intrinsic biological response of the absorbed dose is critical for improving triage and medical care in an exposed population since the severity of radiation injury depends on the definite dose that is unpredictable during both accidental and deliberate radiation exposures.

The potential damage from an absorbed dose depends on the type of radiation and the sensitivity of different tissues and organs. Exposure to gamma radiation alters hematopoietic, gastrointestinal and neurological organ systems (17) leading to acute radiation syndrome ARS. Knowledge of the acute response of radiation damage in different organs and tissues is inadequate. Hence, the overall goal of the current study was to determine if biomarkers were organ specific. The current study sought to understand the effect of total-body exposure (6 Gy) by comparing sham-exposed tissues and/or organs with varying degree of radiosensitivity and biofluids (serum and urine) to discover the signatures

of acute tissue-specific and minimally-invasive radiation-induced damage using radiation biodosimetry and metabolomic analysis.

MATERIALS AND METHODS

Chemicals

All chemical standards were purchased from Sigma-Aldrich (St. Louis, MO) or Avanti Polar Lipids (Alabaster, AL). High performance liquid chromatography (HPLC) grade solvents were obtained from Fisher Scientific (Hampton, NH).

Animals

Animal handling and experimental protocols were approved by the National Institutes of Health (NIH), Animal Care and Use Committee. Male C57BL/6N mice (4–6 weeks old) were purchased from Charles River Laboratories and randomly assigned to the sham and irradiated groups. All mice were maintained under a 12 h light/dark cycle with standard NIH31 diet and water provided *ad libitum*. Mice in the sham and irradiated groups were co-housed and acclimatized to metabolic cages twice for at least 18–20 h prior to each exposure.

Rationale for Radiation Exposure

Previous metabolomics observations from our group showed, elevated biomarkers displayed a time-dependent excretion, peaking in urine at 8–12 h postirradiation but returning to baseline by 36 h postirradiation (11). Hence, we have selected a 12 h time point, to understand the metabolic changes in the mouse metabolome as an early response.

Radiation Dosing to Mice

Authorization for using the gamma irradiator and safety guidelines was obtained from the Division of Radiation Safety (DRS), Office of Research Services (ORS), National Institutes of Health (NIH). The mice were moved to a dosimetry room with a Gammacell 40 Exactor (MDS Nordion, Ottawa, Canada) and whole-body irradiated with a category 2 radioactive quantity of Cesium-137 (^{137}Cs) at a dose rate of 0.92 Gy/min for a total dose of 6 Gy. The sham-irradiated animals were placed in the irradiator for the same amount of time, but without exposure to the source. Mice were carefully handled by placing them individually in pie-cage chambers.

Urine, Serum and Tissue Collection

The sham and irradiated mice were transferred back to a regular holding room and placed individually in metabolic cages for 12 h for the collection of urine. All the samples were labeled and kept on ice, later urine samples were centrifuged at 14,000g for 20 min at 4°C and the clear supernatant transferred to polypropylene tubes. Blood was drawn post euthanasia from inferior vena cava. The whole blood was transferred to the BD Microtainer® blood collection tubes. After collection, blood was allowed to clot by leaving it undisturbed at room temperature for at least 15–30 min. Later, the tubes were centrifuged to remove the

clot by centrifuging at 1,000–2,000*g* for 10 min in a refrigerated centrifuge. The serum was separated with the Pasteur pipette and immediately transferred polypropylene tubes.

All mice were euthanized by CO₂ asphyxiation for collection of blood and radiosensitive tissues, specifically, bone marrow, ileum, liver, skeletal muscle and lung. Serum was separated using serum separators (BD Bioscience). Bone marrow cells extracted from the femur were treated with ammonium-chloride-potassium lysis buffer followed by centrifugation for red blood cell (RBC) lysis. The supernatant containing lysed RBC was discarded and the cell pellet was saved. Radiosensitive tissues were flash frozen in liquid nitrogen and stored along with urine, serum and bone marrow cells at –80°C for comparative global metabolomics.

Sample Preparation for UPLC-ESI-QTOFMS

Urine, serum and bone marrow samples were thawed while the tissues were cut to approximately 20–30 mg pieces for metabolite extraction. Sample extractions from various tissues were simultaneously performed on the same day. Metabolite separation process in tissues and serum was carried out by a modified Folch biphasic extraction method (aqueous and organic phase) described elsewhere (18). Briefly, tissues were homogenized (Precellys®24 homogenizer) with dry-ice-cold acetonitrile:water:chloroform (65:30:5 v:v:v) containing 10 μ M of 2-aminopimelic acid and 5 μ M chlorpropamide. After 2–3 cycles, tissue homogenates were centrifuged at 14,000*g* for 20 min at 4°C to separate aqueous and organic layers. Extracts from two phases were transferred into 1 ml deep 96-well plates separately for HILIC UPLC. A 5 μ l aliquot of each sample was injected into the UPLC system (Waters Corporation, Milford, MA). Urine (50 μ l) was transferred to a microcentrifuge tube containing 250 μ l acetonitrile: water:methanol (65:30:5 v:v:v) on dry-ice and 10 μ M 2-aminopimelic acid and 5 μ M chlorpropamide. After a 1 min vortex, samples were centrifuged at 14,000*g* for 20 min at 4°C to remove precipitated protein. The supernatants (~125 μ l) were diluted with acetonitrile: water:methanol (65:30:5 v:v:v) containing 5 μ M (*R,S*)-2-(difluoromethyl)-ornithine hydrochloride hydrate, then transferred to a UPLC vial for HILIC analysis. Quality control (QC) samples were prepared separately for each group containing 5 μ l of each sample. An Acquity UPLC amide column (Waters Corporation, Milford, MA) was used to separate chemical constituents at 35°C. The mobile phase flow rate was 0.5 ml/min with an aqueous acetonitrile gradient containing 0.1% formic acid over a 10 min run (0% acetonitrile for 0.5 min to 20% acetonitrile by 5 min to 95% acetonitrile by 9 min, then equilibration at 100% water for 1 min before the next injection). The Waters Xevo G2-mass spectrometer was operated in both the positive and negative electrospray ionization mode. Capillary voltage and cone voltage were maintained at 3 kV and 30 V, respectively. Source temperature and desolvation temperature were set at 120 and 550°C, respectively. Nitrogen was used as both cone gas (50 l/h) and desolvation gas (950 l/h) and argon was used as the collision gas. For accurate mass measurements, the QTOFMS was calibrated with sodium formate solution (range *m/z* 100–1,000) and monitored by the intermittent injection of the lock mass sulfadimethoxine ($[M+H]^+ = m/z$ 311.0814) in real-time. All samples were analyzed in a randomized fashion to avoid artifacts related to injection order and changes in instrument efficiency. QC pooled samples and standard mix were also injected recurrently during the run to monitor the stability of the

system. Mass chromatograms and mass spectral data were acquired and processed by MassLynx software (Waters Corporation) in centroid format.

Multivariate Data Analysis and Biomarker Identification

The differences in the systemic metabolic alterations between sham and irradiated groups were compared and analyzed using multivariate data analysis (MKS Instruments AB, Sweden). The data matrix generated by mass spectrometry was normalized to protein concentration for bone marrow cells, tissue weight for all radiosensitive tissues, total ion current for serum, creatinine ion abundance for urine. Normalized data was subjected to unsupervised principal components analysis (PCA) for visualizing group clustering after irradiation in radiosensitive tissues, serum and urine separately. Supervised orthogonal projection to latent structures-discriminant analysis (OPLS-DA) identified potential ions/features that contributed to group separation after irradiation. The corresponding features between groups were calculated by two-tailed Mann-Whitney U test ($P < 0.05$).

Identification and Quantitation of Biomarkers in Tissues, Serum and Urine

Significant features from multivariate data analysis were identified by comparing retention time and m/z of authentic standards with fragmentation patterns using an ACQUITY UPLC system coupled with a XEVO-TQ triple-quadrupole tandem mass spectrometer (Waters Corporation) previously described elsewhere (14, 16). Identified biomarkers were expressed quantitatively as fold changes (FC), with relative abundances calculated after normalization to protein concentration (BMC), tissue weight (other tissues) or creatinine (urine), in which last case the creatinine ion (114.067 at 1.01 min in ESI+ mode) was used.

Metabolic Pathway Analysis

Two methods were used to analyze the metabolic context of the data. The first method used was metabolite set enrichment analysis (MSEA) performed using the MetaboAnalyst 3.0 online algorithm (<http://www.metaboanalyst.ca>). In the second, the SetRank algorithm (Simillion *et al.*, manuscript submitted) was adapted to perform Gene Set Enrichment Analysis (GSEA) on the sets of significant compounds for each tissue. All compounds were mapped to Chemical Entities of Biological Interest (ChEBI) identifiers. Next, compound pathway databases were retrieved from the Reactome and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. The principle of GSEA is to detect over-represented gene sets — i.e., pathways — in a foreground set of elements — here metabolites — compared to some background set. To avoid bias towards pathways containing soluble molecules, we constructed a background set as follows. For all compounds identified in our dataset, the logP, logS and molecular mass values were retrieved from the Human Metabolome Database (HMDB). For each of these properties a minimum and maximum threshold was defined by respectively subtracting 10% from the lowest value observed or adding 10% to the highest value observed. The background set was then constructed by including all compounds with pathway annotation in both the KEGG and Reactome databases for which all three properties fall within the specified range. The SetRank algorithm was then run in “unranked” mode, meaning that a Fisher’s exact test was applied for every pathway in both KEGG and Reactome, comparing the number of metabolites in the list of identified compounds to the number in the background test. The P value threshold for significance was

set to 0.01, and the FDR threshold for filtering after multiple correction was set to 0.05. This procedure was repeated for each of the tissue-specific lists of metabolites, as well on the union of these (marked as “global” in the results).

Statistical Analysis

All the experiments were conducted with at least 6 mice in each group. Statistical significance was calculated by two-tailed Mann-Whitney *U* test for comparing sham and irradiated groups using GraphPad Software Prism 6 (San Diego, CA). All values were presented as mean $\pm$ standard deviation. Fold changes of individual metabolites were obtained from the average of relative abundance from the sham group. The differences were considered significant when $P < 0.05$. Biomarker robustness for individual urinary and serum metabolite was analyzed by receiver operating characteristic (ROC) analysis using MetaboAnalyst 3.0 software. The ROC area under the curve (AUC) was used to assess and integrate the sensitivity and specificity of the biomarkers which shows classification performance.

RESULTS

The current study investigated the metabolic signatures at the tissue level 12 h after 6 Gy total-body irradiation. Metabolomic analysis was carried with HILIC coupled ESI-QTOFMS and followed by multivariate data analysis to identify polar metabolites.

Metabolomics and Multivariate Data Analysis

The data matrix of bone marrow, ileum, liver, muscle, lung, serum and urine showed clear differences in the metabolome as a result of radiation exposure. PCA analysis showed clustering of groups according to sham and gamma irradiation indicating overall differences in the metabolomic signatures (Fig. 1A–G). Radiation-induced perturbations were further evaluated in different tissues using OPLS-DA (Fig. 1 A–G). To identify potential candidate ions contributing to separation of the two groups, S-plots of the OPLS-DA model were generated for each tissue.

Biomarker Quantitation and Fold Changes

Comparative metabolomic profiling of tissues, serum and urine was performed 12 h postirradiation on the group that received a 6 Gy dose of gamma radiation compared with the sham-irradiated animals. Significant biological changes with respect to metabolic pathways and radiation exposure were noted in tissues, serum and urine (Table 1A–G and Figs. 2–8). These biomarkers of radiation response have significant implications for overall metabolism and thus are potential indicators of biological response to radiation, as discussed below.

Bone marrow—Proliferating hematopoietic stem cells and progenitor cells of bone marrow are the highly radiosensitive cells in the body. Exposure to gamma radiation can cause DNA damage in continuously dividing hematopoietic cells (19). A number of gamma-radiation signatures were found in the bone marrow 12 h postirradiation (Table 1A; Fig. 2). Notably, 5-oxoproline, which is a degradation product of glutathione, produced and used in

the gamma-glutamyl cycle was increased by 1.4-fold ($P < 0.007$) (20). All other metabolites, which belong to various essential metabolic pathways such as purine/pyrimidine metabolism where hypoxanthine, uridine, deoxyuridine, cytosine, AMP and ADP were found to be lower by 2.7-, 2.0-, 10-, 2.0-, 1.4- and 2.5-fold, respectively. Metabolites of the glutathione pathway including GSH and GSSG, were decreased by 1.5- ($P < 0.007$) and 3.4- ($P < 0.007$) fold, respectively. Metabolites such as betaine (1.4-fold, $P < 0.007$), proline betaine (5.6-fold, $P < 0.004$), carnitine (1.4-fold, $P < 0.007$), taurine (2.3-fold, $P < 0.007$), glycerophosphocholine (1.8-fold, $P < 0.007$) and dimethylarginine (2.5-fold, $P < 0.01$) were also depleted. The amino acids proline, aspartic acid and asparagine were depleted by 1.7- ($P < 0.004$), 2- ($P < 0.009$) and 2-fold ($P < 0.007$), respectively.

Ileum—Gastrointestinal epithelial cells are highly sensitive to gamma radiation. Epithelial cell degeneration due to radiation injury in the gut leads to long-term adverse consequences (21). In the current study, six elevated metabolites and one depleted metabolite were identified after gamma irradiation (Table 1B; Fig. 3). The elevated metabolites were deoxyinosine (2.8-fold, $P < 0.004$), taurine (1.4-fold, $P < 0.01$), taurocholic acid (5-fold, $P < 0.0006$), UDP-*N*-acetylglucosamine (UDP-GlcNAc) (2-fold, $P < 0.004$), glutamine (1.6-fold, $P < 0.004$) and glutamate (2-fold, $P < 0.008$). The only detected depleted ion in the ileum after gamma irradiation was cytosine (5.6-fold, $P < 0.002$).

Liver—The liver performs many critical metabolic functions, including synthesis of critical serum proteins including blood coagulation factors, synthesis of cholesterol and steroid hormones, glycogen synthesis, metabolism of drugs and redox maintenance is low radiosensitive. Elevated hepatic metabolites after gamma irradiation included uridine (3.3-fold, $P < 0.002$), reduced glutathione (1.4-fold, $P < 0.004$), oxidized glutathione (2.3-fold, $P < 0.001$), 5-oxoproline (1.2-fold, $P < 0.01$) and glutamate (1.3-fold, $P < 0.002$) (Table 1C; Fig. 4). Elevated metabolites also included S-adenosylmethionine (1.4-fold, $P < 0.04$) and 3-methylglutarylcamitine (5-fold, $P < 0.0006$). Among the depleted hepatic metabolites after gamma irradiation were hypoxanthine (1.6-fold, $P < 0.004$), xanthine (1.7-fold, $P < 0.002$), inosine monophosphate (2-fold, $P < 0.0006$) and inosine (2.7-fold, $P < 0.01$) and all purine catabolic intermediates involved in uric acid production. Other metabolites, such as *N*-acetylglutamine (1.3-fold, $P < 0.008$), *N*-adenosylhomocysteine (3-fold, $P < 0.002$), betaine (2.4-fold, $P < 0.0006$) and taurine (1.4-fold, $P < 0.01$) were also depleted as an indication of altered liver metabolism after gamma irradiation.

Muscle—In the current study, four metabolites were identified that reflect the early responses of skeletal muscle to gamma irradiation (Table 1D; Fig. 5A). Elevated markers were UDP-glucose (2.3-fold, $P < 0.009$), phenylalanine (2-fold, $P < 0.0006$) and creatine (2-fold, $P < 0.01$). Choline was depleted by 2.5-fold ($P < 0.002$) in quadriceps muscle.

Lung—In the lung, the elevation of glycerophosphocholine (1.2-fold, $P < 0.01$) and depletion of proline betaine (3.2-fold, $P < 0.008$) was observed (Table 1E; Fig. 5B).

Serum—HILIC-MS-derived metabolic signatures of radiation exposure in mouse serum showed significant elevation of acylcarnitines, specifically, acetylcarnitine (3-fold, $P < 0.004$), propionylcarnitine (2.5-fold, $P < 0.0006$) and 3-methylglutarylcamitine (5-fold, $P < 0.004$).

0.002) metabolites (Table 1F; Fig. 5C). Additionally, cytosine (1.5-fold, $P < 0.004$), proline betaine (1.3-fold, $P < 0.006$) and glutamine (1.4-fold, $P < 0.006$) were also elevated after gamma irradiation. There were no significantly depleted markers found in serum.

Urine—Metabolites in the urine from mice exposed to gamma radiation were mostly derived from purine and pyrimidine metabolism, fatty acid β -oxidation and amino acid metabolism (Table 1G; Fig. 6). Metabolic signatures in urine are potential endpoints of the biological response to gamma-radiation-induced tissue damage. Nine elevated and five depleted metabolites were identified in urine. The upregulated markers include uridine, deoxyuridine, 5-methylcytosine and cytosine derived from pyrimidine metabolism, and notably reflects direct nucleic acid damage after gamma irradiation. Elevated concentrations of acetylcarnitine, butyrylcarnitine and propionylcarnitine that reflect altered energy metabolism were also detected after gamma irradiation. Increased levels of taurine and creatine were also observed, which were reported in the earlier studies as radiation-induced markers (14). Depleted urinary biomarkers of radiation exposure were pantothenic acid, xanthurenic acid, arginine, glutamine and inosine.

Biological Context of the Metabolomic Findings

Figure 8 represents a summary of all the observed metabolite alterations, both up- and down-regulated, in each tissue examined. In particular, the central circle contains all the metabolic findings derived from the analysis of urine and, overlapping with each tissue, show the metabolites that were altered in that tissue and also altered in urine. The purpose of this analysis was an attempt to understand better the tissue of origin of each urinary metabolite that was perturbed by exposure to gamma radiation. Examples of the utility of this data representation concerns, for example, uridine and 3-methylglutaryl carnitine, which were elevated in urine and liver. This strongly suggested that these two urinary metabolites were elevated due to an effect of radiation on the liver. The same applied to creatine from muscle and taurine from ileum (Fig. 8). Therefore, Fig. 8 provides a map by which urinary biomarkers of radiation exposure can be traced back to their tissue of origin.

To investigate this further, two types of pathway analysis were conducted. Metabolite set enrichment analysis (MSEA) for each tissue studied, except for lung where there was an insufficient number of metabolites found, and for serum and urine are shown in Figs. 9–11. These analyses provide insights into coordinated changes among groups of metabolites. For bone marrow cells, the effect of radiation was to provoke the most statistically significant changes in glutathione metabolism, the urea cycle, protein biosynthesis and ammonia recycling (Fig. 9A). In contrast, for ileum, radiation most significantly affected taurine and hypotaurine metabolism (Fig. 9B). For liver, statistically significant changes occurred in betaine, purine, methionine and glutathione metabolism (Fig. 10A). For muscle, the most significant alterations occurred in nucleotide sugar, betaine, phenylalanine and tyrosine, together with starch and sucrose metabolism (Fig. 10B). Serum was found to reflect oxidation of branched-chain fatty acids and β -oxidation of very long fatty acids (Fig. 11A). Finally, urine reflected mainly pyrimidine metabolism, oxidation of branched-chain fatty acids, protein biosynthesis, the urea cycle, arginine and proline metabolism, taurine and hypotaurine metabolism, together with pantothenic acid and coenzyme A biosynthesis (Fig.

11B). The urinary changes reflected tissue metabolomic alterations in all tissues except the lung, surprisingly since the lung is categorized as very sensitive to radiation-induced injury (22). These data may also provide clues to the biochemical processes in target tissues that are affected by gamma radiation.

A second type of pathway analysis employed used the SetRank algorithm. This analysis, like MSEA above is based upon gene enrichment analysis used in transcriptomics. It uses not only the Human Metabolome Database like MSEA, but also the Reactome and KEGG databases. Therefore, more biological findings are produced, based upon gene annotations in addition to metabolite annotations. This methodology is greedy and therefore there were only sufficient data to conduct analyses on liver and ileum, together with a global analysis, as shown in Fig. 12. This heat map does not represent fold changes, rather statistical significances. The liver data generated many hits, including several methylation reactions that may represent epigenetic changes induced by gamma radiation. The ileum had a much clearer pattern, mostly centering around the finding of elevated taurine.

Validation of the Urinary and Serum Biomarker Robustness

ROC curves were built comparing irradiated with sham-irradiated mice to determine the predictive value and robustness of marker metabolites as potential radiation-induced biomarkers. An AUC score of >0.8 was designated as a robust marker of gamma-radiation exposure. ROC analysis was done for each urine and serum biomarker 12 h after a dose of gamma radiation (6 Gy) (Fig. 7). Out of 14 urinary biomarkers, deoxyuridine, uridine, 5-methylcytosine, taurine, acetylcarnitine, butyrylcarnitine, 3-methylglutaryl carnitine, pantothenic acid and xanthurenic acid had accurate predictive ability with AUCs of 1.0. Other biomarkers creatine, inosine and arginine had AUCs >0.9 , whereas propionylcarnitine and glutamine, had a AUCs of 0.898, 0.826, respectively. Similarly, serum biomarkers acetylcarnitine, propionylcarnitine, 3-methylglutaryl carnitine, glutamine and proline betaine showed accurate predictive ability with AUCs >0.95 . The diagnostic markers with the highest AUC value (>0.8) could significantly increase the diagnostic performance as minimally invasive gamma-radiation biomarkers.

DISCUSSION

The focus of this study was to detect tissue specific metabolic biomarkers of gamma-radiation exposure, as well as helping identify the radiation biomarker associated with the tissue of origin of both serum and urinary. Triage of large populations potentially exposed to accidental or deliberate release of ionizing radiation will most probably involve analysis of blood and/or urine. Naturally, tissue specimens will not be available. However, it may be possible to interpret urinary or serum biomarkers in terms of their tissues of origin and therefore the targets of radiation damage in that individual.

Radiation related accidental deaths have been reported due to the extensive loss of the hematopoietic system (23). Bone marrow is the first affected tissue in the ARS (23, 24). Hence, candidate biomarkers of bone marrow damage may provide a more mechanistic view of hematopoietic system damage by gamma radiation. After a 6 Gy dose of gamma radiation the metabolic origins of candidate bone marrow biomarkers were from nucleic acid damage

and amino acid metabolism. Additionally, depleted reduced glutathione and oxidized glutathione represents loss of antioxidant defense. We report here the first study on bone marrow cells to identify the potential biomarkers in response to radiation-induced injury.

The continuously dividing intestinal epithelium is highly radiosensitive to gamma-radiation (25). Concentrations of deoxyinosine and cytosine were evidence of DNA and RNA damage that occurred at the tissue level (26). Exposure to gamma radiation induces changes in histone post-translational (27, 28) modifications including γ -H2AX formation (27) and *O*-GlcNAc modification (28) that in turn affect DNA damage response and genome stability. Radiation exposure is known to affect all critical purine, pyrimidine, bile acid biosynthesis, glutathione metabolic pathways, and elevated UDP-GlcNAc (substrate for *O*-GlcNAc transferases) could be a sign of nutrient sensor for radiation injury to the ileum and leads to gastrointestinal syndrome (25).

Although the liver has a fairly low radiosensitivity to ionizing radiation, the absorbed radiation dose could potentially alter some of the critical metabolic functions of the liver. Radiation is capable of inducing alterations in DNA methylation dependent gene expression patterns and cell metabolism (29). The methionine cycle metabolites, *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) were altered in irradiated mice, indicating the potential for epigenetic changes. The cellular SAM:SAH ratio was shown to define cellular methylation potential, and importantly aberrant DNA methylation patterns would induce changes in cell signaling events (30). The presence of 3-MGC was found in the serum and urine, and hence could be considered as a systemic biomarker of early radiation response of the liver. The 3-MGC could be a sign of the failure of ketone body formation from fatty acid β -oxidation through the enzyme HMG-CoA lyase (31). The elevation of acylcarnitines may have a more generalized effect on mitochondrial β -oxidation of fatty acids. Glutathione is an essential antioxidant that regulates cellular redox and functions in phase II conjugation pathways by enzymatic transferase -dependent reactions. In this study, glutamate, a precursor for glutathione synthesis, was elevated in the ileum of gamma-irradiated mice. However, both reduced and oxidized glutathione were depleted in the bone marrow, indicating altered cellular redox balance after radiation injury (32). The metabolites of purine catabolism were also a measure of DNA damage and/or altered redox balance (15).

The current findings in quadriceps muscle tissue showed the elevation of nucleotide sugar UDP-glucose, which functions as a precursor for the synthesis of complex carbohydrates, lipopolysaccharides, and glycosphingolipids (33). Recent studies on gamma-irradiated human skeletal muscle-derived cell line HMCL-7304, also showed elevated intracellular glucose metabolism (34). However, due to the technical limitation, no evidence of increased glucose or aerobic glycolysis was apparent in muscle tissue *in vivo*. Elevated creatine in muscle tissue after gamma irradiation results from hydrolysis of phosphocreatine. It is produced primarily in the liver, kidney and pancreas and is taken up by muscle cells, converted to phosphocreatine which assists in the regeneration of ATP from ADP after muscle contraction (35).

The lung is very sensitive to radiation injury (22). A time lag of 12 h postirradiation showed glycerophosphocholine as significantly elevated metabolite among sham and gamma-

irradiated mice. The presence of choline phospholipids was also reported in lungs of mice exposed to silica dust (36). Furthermore, betaine analogue proline betaine is a dietary metabolite mostly derived from rodent diet supplementation (10, 37).

Minimally invasive serum biomarkers are quantifiable and showed significant alterations in nucleic acid, amino acids and energy metabolism in response to gamma radiation. Increased levels of acylcarnitines in the blood reveal an impact of gamma radiation on energy homeostasis. Elevated acylcarnitines that are essential cofactors for mitochondrial fatty acid β -oxidation and are required for cellular energy homeostasis (38). Additionally, a recent review also described the functional implications of carnitines such as its antioxidant and radioprotective properties (39). Further, evidence of DNA and/or RNA damage was noted in serum as revealed by an elevation of the pyrimidine cytosine. The amino acid glutamine has many essential roles in cells, was also elevated in gamma-irradiated mouse serum. Glutamine is a major source of energy after glucose (40) in rapidly dividing cells and perhaps an alternative cellular energy source to fatty acid β -oxidation, which was presumed to be impaired by the appearance of elevated acylcarnitines.

Radiation-induced DNA damage activates a number of DNA damage response and repair signaling cascades that control cell cycle arrest, and DNA repair (41). Elevated uridine and deoxyuridine in the urine likely reflecting nucleic acid damage may be the result of radiation-induced apoptosis or necrosis (42), or ER stress and autophagy (43). Exposure to gamma radiation also results in DNA modification such as production of 5-methylcytosine which correlates with the biological responses to radiation. DNA methylation changes were enriched in pathways involved in cell cycle, DNA repair and apoptosis at a 6 Gy dose (29).

Pathway analysis added context to the list of altered metabolites found in various tissues after the animals received whole-body irradiation, with several metabolic pathways highlighted that could be tissue-specific targets of gamma radiation, for bone marrow, ileum, liver and muscle. In addition, pathway analysis interpreted the acylcarnitine findings in serum as measures of increased oxidation of both branched-chain (BCFAs) and very long-chain fatty acids (VLCFAs). It has long been recognized that VLCFAs are oxidized by peroxisomes, since their CoA esters cannot be formed in mitochondria (44). BCFAs are also oxidized by peroxisomes (45) and therefore it seems highly likely that peroxisomal metabolism may be stimulated by gamma radiation, leading to the increased concentrations of acylcarnitines, in serum and urine. These biomarkers most likely arise from liver as evidenced by experiments with transgenic mice expressing the human catalase gene, largely in hepatic and renal peroxisomes (46). These mice demonstrated enhanced sensitivity to gamma radiation, which was taken to be mediated via transgenic peroxisomal catalase expression (46). Additional context added by pathway analysis was that the most statistically significant pathways identified globally involved DNA and histone methylation by the polycomb repressive complex 2 (PRC2). PRC is known to be recruited to sites of DNA damage, contributing to DNA double-strand break repair (47). The potential involvement of DNA and histone methylation in response to gamma radiation was deduced through pathway analysis of the metabolomic data.

Ionizing radiation stimulates cellular and metabolic alterations that are measurable in tissues that could potentially provide early markers of radiation exposure. Global metabolomic analysis by UPLC-ESI-QTOFMS is a reliable and comprehensive method for identifying radiation-induced responses at a given time or dose. In the current study, a 6 Gy total-body exposure revealed significantly altered metabolite levels that affected biochemical pathways (Table 1) in radiosensitive tissues, serum and urine. The identified markers in individual radiosensitive tissue helps to understand the mechanism of radiation injury and associated biochemical pathways. Nucleic acid damage metabolites were detected in bone marrow, liver, ileum, but not muscle and lung. Notably, taurine, which was previously reported as radiation marker in mouse, rat and nonhuman primates (10, 13, 14), was found altered in bone marrow, liver, ileum and urine. Important physiological functions of taurine include bile acid conjugation, anti-oxidant properties and ROS scavenging (10). In the tissue metabolome, taurine and taurocholic acid were elevated in the ileum and depleted in bone marrow and liver. Creatine was also detected and validated as a biomarker for 6.5 Gy dose and it was also shown in mice and non-human primates (14) muscle and bone marrow at 24 h postirradiation. It is clear that creatine was essentially derived from muscle tissue as a result of changes in muscle energy metabolism. A panel of carnitine metabolism markers was also excreted into urine and were also mirrored in the serum. This pool of carnitines in urine and serum directly points to altered energy homeostasis after total-body irradiation. Radiation-depleted markers indicated alteration of amino acid metabolism. Urinary markers were mostly from purine and pyrimidine metabolism, fatty acid β -oxidation and amino acid metabolism. Taken together, urinary biomarkers were useful end points of tissue perturbations after gamma irradiation.

In conclusion, the results of the current study characterized metabolic consequences of radiation toxicity at the tissue level in mice that were exposed to 6 Gy of gamma radiation. The gamma-radiation response exhibited global metabolic changes in various tissues which have an important role in developing treatment strategies. The overall study highlights the identification of 3-methylglu-tarilcarnitine in the serum, liver and urine. This potential biomarker would be useful in detection of early radiation response of the liver as well as detecting radiation injury and/or toxicity. In terms of the mechanisms of gamma radiation on tissue damage, the urine contained the greatest wealth of information, pointing specifically to alterations in energy metabolism and nucleic acid damage. Therefore, urine as a readily accessible biofluid is recommended as the richest source for identification of gamma-radiation-induced damage to the organism. Development of a map that could reveal from a urine analysis the topology of radiation damage to individual tissues is a positive advance in the field of radiation metabolomics.

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FIG. 1.

Effect of total-body gamma irradiation on global metabolic signatures. Scores scatter plot for principal components analysis (PCA) of normalized data obtained from (panel A) bone marrow, (panel B) ileum, (panel C) liver, (panel D) muscle, (panel E) lung, (panel F) serum and (panel G) urine 12 h postirradiation. R2X and Q2 values were bone marrow (0.525; 0.211), ileum (0.483; 0.144), liver (0.449; 0.113), muscle (0.430; 0.079), lung (0.588; 0.348), serum (0.355; 0.044) and urine (0.616; 0.375). Empty and filled boxes represent sham [(□) 0 Gy] and gamma irradiated [(■) 6 Gy] mice, respectively. Potential elevated and

depleted features derived from the orthogonal partial least squares discriminant analysis (OPLSDA) of corresponding tissue are shown next to the PCA scores scatter plot.

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FIG. 2.

Changes in bone marrow markers in response to gamma radiation (6 Gy). Box and whiskers plots showing protein concentration-normalized fold change (FC) for individual biomarkers were calculated with respect to the average of the abundances of the metabolites in the sham group. P values were calculated by two-tailed Mann-Whitney U test. *** $P < 0.0001$, ** $P < 0.001$ and * $P < 0.01$.

FIG. 3.

Changes in ileum markers in response to gamma radiation (6 Gy). Box and whiskers plots showing tissue weight-normalized fold change (FC) for individual biomarkers was calculated with respect to the average of the abundances of the metabolites in the sham-irradiated group. *P* values were calculated by two-tailed Mann-Whitney U test. ****P* < 0.0001, ***P* < 0.001 and **P* < 0.01.

FIG. 4.

Changes in liver markers in response to gamma radiation (6 Gy). Box and whiskers plots showing tissue weight-normalized fold change (FC) for individual biomarkers were calculated with respect to the average of the abundances of the metabolites in the sham group. P values were calculated by two-tailed Mann-Whitney U test. *** $P < 0.0001$, ** $P < 0.001$ and * $P < 0.01$.

FIG. 5. Changes in (panel A) muscle, (panel B) lung and (panel C) serum markers in response gamma radiation (6 Gy). Box and whiskers plots showing variations in the total ionic currents normalized serum circulating biomarkers. *P* values were calculated by two-tailed Mann-Whitney U test. ****P* < 0.0001, ***P* < 0.001 and **P* < 0.01.

FIG. 6.

Changes in urine markers in response to gamma radiation (6 Gy). Box and whiskers plots for creatinine-normalized concentrations of individual biomarkers in urine. P values were calculated by two-tailed Mann-Whitney U test. *** $P < 0.0001$, ** $P < 0.001$ and * $P < 0.01$.

FIG. 7.

ROC curve based on logistic regression analysis of radiation (6 Gy), urinary (panel A) and serum (panel B) biomarkers compared with sham irradiation. AUCs and 95% CI for individual biomarkers in urine and serum are represented.

FIG. 8.

Gamma-radiation-induced biomarkers of bone marrow, lung, ileum, liver, muscle, serum and urine are shown. The metabolites shown in the light blue circles are specific to a particular tissue/serum and the markers that are shown in the dark blue circle were shared commonly with urine. The trends of quantitatively elevated and depleted metabolites are indicated with red and blue arrows, respectively.

FIG. 9.

Metabolite set enrichment analysis for bone marrow (panel A) and ileum (panel B).

FIG. 10.
Metabolite set enrichment analysis for liver (panel A) and muscle (panel B).

FIG. 11. Metabolite set enrichment analysis for serum (panel A) and urine (panel B). Abbreviations used: FA, fatty acid; VLCFA, very-long-chain fatty acid.

FIG. 12.

Gene set enrichment analysis using the SetRank algorithm. The heat map shows the statistical significance [$-\log_{10}(P)$] for pathway alterations in ileum, liver and globally across all samples analyzed.

TABLE 1
Metabolic Signatures of Tissue, Serum and Urine in Mice Exposed to Total-Body Gamma Radiation

Source	6 Gy biomarkers	p(Corr)[I]	ESI	RT	m/z	Trend	P value	FC
A								
BMC	Oxoproline	0.856	POS	4.26	130.1160	↑	0.0079	1.36
BMC	ADP	-0.912	POS	5.66	428.0318	↓	0.0048	-2.5
BMC	AMP	-0.727	POS	5.24	348.0711	↓	0.0159	-1.35
BMC	Hypoxanthine	-0.877	POS	1.06	137.0459	↓	0.0079	-2.27
BMC	Cytosine	-0.914	NEG	3.86	110.0340	↓	0.0043	-1.96
BMC	Uridine	-0.831	NEG	1.02	243.0617	↓	0.0079	-2.04
BMC	Deoxyuridine	-0.793	NEG	2.00	263.0463	↓	0.0022	-10
BMC	Glutathione, oxidized	-0.862	POS	6.21	613.1579	↓	0.0079	-3.44
BMC	Glutathione, reduced	-0.751	POS	4.84	308.0917	↓	0.0079	-1.51
BMC	Glycerophosphocholine	-0.956	POS	4.39	258.1145	↓	0.0079	-1.81
BMC	Betaine	-0.812	POS	2.44	118.0866	↓	0.0079	-1.38
BMC	Creatine	-0.799	POS	3.89	132.0768	↓	0.0087	-1.31
BMC	Proline betaine	-0.706	POS	2.37	144.1025	↓	0.0043	-5.55
BMC	Taurine	-0.855	POS	3.25	126.0060	↓	0.0079	-2.32
BMC	Carnitine	-0.859	POS	3.89	162.1130	↓	0.0079	-1.4
BMC	ADMA	-0.744	POS	5.34	203.1516	↓	0.0159	-2.5
BMC	Proline	-0.753	NEG	3.32	114.0546	↓	0.0043	-1.69
BMC	Aspartic acid	-0.687	POS	4.85	134.0547	↓	0.0095	-2.04
BMC	Asparagine	-0.705	NEG	4.35	131.0461	↓	0.0079	-2
B								
Ileum	Deoxyinosine	0.732	NEG	5.13	251.0776	↑	0.0043	2.82
Ileum	Taurine	0.766	NEG	2.51	124.0068	↑	0.0152	1.38
Ileum	Taurocholic acid	0.724	NEG	0.54	514.3004	↑	0.0006	3.08
Ileum	UDP-GlcNAc	0.826	NEG	3.29	606.0757	↑	0.0043	1.76
Ileum	Glutamine	0.752	NEG	4.24	145.0620	↑	0.0043	1.59
Ileum	Glutamate	0.781	NEG	2.42	146.0434	↑	0.0087	1.87
Ileum	Cytosine	-0.852	POS	1.96	112.0500	↓	0.0022	-5.55

Source	6 Gy biomarkers	p(Corr)[1]	ESI	RT	m/z	Trend	P value	FC
C								
Liver	Uridine	0.672	NEG	1.00	243.0616	↑	0.0022	3.31
Liver	Oxoproline	0.870	NEG	4.85	128.0306	↑	0.0152	1.21
Liver	Glutamate	0.723	POS	4.70	148.0616	↑	0.0022	1.25
Liver	Glutathione, reduced	0.935	POS	4.83	308.0918	↑	0.0022	1.43
Liver	Glutathione, oxidized	0.714	POS	6.02	613.1579	↑	0.0012	2.28
Liver	S-Adenosylmethionine	0.779	POS	5.39	399.1451	↑	0.0411	1.36
Liver	3-Methylglutaryl carnitine	0.633	POS	4.62	290.1617	↑	0.0006	4.93
Liver	Hypoxanthine	-0.844	NEG	1.03	135.0310	↓	0.0043	-1.58
Liver	Xanthine	-0.835	POS	5.01	152.031	↓	0.0022	-1.69
Liver	Inosine	-0.735	NEG	1.66	267.0724	↓	0.0012	-2.7
Liver	Inosine monophosphate	-0.728	NEG	5.44	347.0542	↓	0.0006	-1.9
Liver	N-Acetylglutamine	-0.828	NEG	3.45	187.0715	↓	0.0087	-1.29
Liver	S-Adenosylhomocysteine	-0.777	NEG	4.34	383.1283	↓	0.0022	-2.85
Liver	Betaine	-0.957	POS	2.36	118.0866	↓	0.0006	-2.43
Liver	Taurine	-0.707	POS	3.25	126.0237	↓	0.0152	-1.35
D								
Muscle	UDP-Glucose	0.713	NEG	5.20	565.0765	↑	0.0095	2.3
Muscle	Phenylalanine	0.785	NEG	2.05	164.0777	↑	0.0006	1.8
Muscle	Creatine	0.706	POS	3.75	132.0768	↑	0.0159	1.75
Muscle	Choline	-0.704	POS	1.91	104.1072	↓	0.0022	-2.5
E								
Lung	Glycerophosphocholine	0.709	POS	4.35	258.1108	↑	0.0159	1.22
Lung	Proline Betaine	-0.683	POS	2.29	144.1024	↓	0.0087	-3.22
F								
Serum	Propionyl carnitine	0.701	POS	2.56	218.1387	↑	0.0006	3.21
Serum	Acetyl carnitine	0.702	POS	3.06	204.1227	↑	0.0041	1.28
Serum	3-Methylglutaryl carnitine	0.724	POS	4.62	290.1617	↑	0.0025	5.03
Serum	Cytosine	0.681	POS	1.23	112.0439	↑	0.0043	1.5
Serum	Proline Betaine	0.723	POS	2.29	144.1021	↑	0.0006	1.28
Serum	Glutamine	0.755	NEG	4.17	145.0620	↑	0.0006	1.39

Source	6 Gy biomarkers	p(Corr)[I]	ESI	RT	m/z	Trend	P value	FC
G								
Urine	Deoxyuridine	0.927	NEG	0.68	227.0671	↑	0.0012	9.58
Urine	Uridine	0.928	NEG	1.01	243.0616	↑	0.0022	5
Urine	5-Methylcytosine	0.839	POS	1.36	126.0650	↑	0.0022	1.98
Urine	Taurine	0.798	NEG	4.24	124.0068	↑	0.0012	3.16
Urine	Creatine	0.699	POS	3.79	132.0768	↑	0.0025	1.38
Urine	Acetylcarbitine	0.695	POS	3.02	204.1227	↑	0.0012	9.73
Urine	Butyrylcarbitine	0.802	POS	2.12	232.1547	↑	0.0023	3.3
Urine	Propionylcarbitine	0.747	POS	1.11	218.1393	↑	0.0043	7.82
Urine	3-Methylglutarylcarbitine	0.733	POS	4.62	290.1617	↑	0.0012	2.24
Urine	Inosine	-0.716	NEG	1.51	267.0724	↓	0.0411	-1.55
Urine	Glutamine	-0.765	NEG	4.19	145.0667	↓	0.0051	-2.03
Urine	Arginine	-0.715	POS	5.96	175.1195	↓	0.014	-2.7
Urine	Pantothenic acid	-0.896	POS	2.27	220.1178	↓	0.0012	-1.73
Urine	Xanthurenic acid	-0.800	POS	1.01	206.0455	↓	0.0043	-2.39

Notes. Individual biomarkers, p(Corr)[I] values, ionization mode, RT, m/z, trend, P values and FCs for (A) bone marrow cells (BMC), (B) ileum, (C) liver, (D) muscle, (E) lung, (F) serum and (G) urine were described. These molecular signatures were detected 12 h after gamma irradiation. Degree of correlation to the OPLS-DA model (p(Corr)[I]), electrospray ionization mode (ESI), retention time in min (RT), mass/charge ratio (m/z), fold change (FC).