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Elucidation of gastrointestinal dysfunction in response to irradiation using metabolomics

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\textbf{ABSTRACT}

Gastrointestinal toxicity is frequently observed secondary to accidental or therapeutic radiation exposure. However, the variation in the intestinal metabolites after abdominal radiation exposure remains ambiguous. In the present study, C57BL/6 mice were exposed to 0, 2, and 20 Gy irradiation dose. The Head and chest of each mouse were covered with a lead shield before x-ray irradiation. 24 h post-irradiation treatment, intestinal tissue of each mouse was excised and prepared for metabolites measurement using gas chromatography-mass spectrometry (GC-MS). Our comprehensive analysis of metabolites in the intestinal tissues detected 44 metabolites after irradiation, including amino acids, carbohydrates, organic acids, and sugars. Amino acid levels in the intestinal tissue gradually rose, dependent on the radiation dose, perhaps as an indication of oxidative stress. Our findings raise the possibility that amino acid metabolism may be a potential target for the development of treatments to alleviate or mitigate the harmful effects of oxidative stress-related gastrointestinal toxicity due to radiation exposure.

1. Introduction

Radiotherapy is well known as the mainstay treatment for several types of malignancies [1]. Approximately half of the cancer patients undergo radiation therapy and it plays a role in curing 40% of these cases [2]. Recent technological advances in the field of radiation therapy has boosted its impact and ability to target cancerous tissues. However, many patients still suffer from side effects [3]. Irradiation of pelvic tumors in particular damages the nearby healthy tissues involved in digestion [4]. The small intestine is particularly vulnerable to incidental irradiation during the targeting of tumors in the pelvis. Nearly all patients exposed to radiation for pelvic or abdominal cancers exhibit gastrointestinal tract symptoms [5]. Previous studies elucidated the response of the small intestine to small doses of ionizing radiation (8 Gy or lower); the treatment caused visibly apoptosis in the intestinal crypts, followed by a shortening of the villi length within 5–7 days. The mice subsequently achieved full recovery with low or no persistent injury to intestinal stem cells. However, doses above 15 Gy of total body irradiation led to complete death of mice within two weeks, with complete damage of most intestinal crypts and severe loss of the epithelium [6–8].

\textsuperscript{1} These authors contributed equally to this work.

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The cellular exposure to ionizing radiation can directly disrupt the atomic structures through the direct energy deposition onto biomolecules or indirectly via generating the reactive oxygen species (ROS), this causes damage to the nucleic acids, proteins, and lipids [9]. Heat shock proteins (HSPs) family, particularly Hsp70 and Hsp90, displays a cytoprotective role against the pathological conditions such as hyperthermia, oxidative stress, ischemia, and bacterial infection via enabling the cellular protection against protein denaturation, accelerating protein folding, and degradation of misfolded proteins [10]. HSPs are overexpressed after the cellular exposure to ionizing radiation as a response to ROS production, thus suggesting their role in the regulation of cell cycle, DNA repair, and apoptotic cascades [11].

Metabolomics is a promising emerging technology that can analyze small metabolites (50–150 Da) in biological specimens and that is being applied to various pathological cases and therapies [12]. Superior to transcriptomics and proteomics, metabolomics registers the end-response to therapy in terms of changes at the gene and protein expression levels, providing insight into the ongoing biological processes after radiation exposure [13]. Metabolic profiling in plasma suggests potential new radiation injury metabolites related to tryptophan and pyrimidine pathways that might be utilized to diagnose whole-body radiation injury [14].

Another mouse total body irradiation model was established to determine the metabolites that were expressed in the jejunum and circulating plasma after the exposure to sub-lethal and lethal radiation dose. Several amino acids were obviously elevated in both jejunum and plasma at 24 h post-radiation exposure indicating prospective insight into the initial inflammatory reaction during Radiation-induced small bowel disease [15]. Putative gastrointestinal markers were reported after acute exposure to low doses of radiation. Tryptophan was detected upregulated while Glutamate, Taurine, and Cysteine-Glycine dipeptide and methionine were also found to be upregulated in the jejunum and circulating plasma at 24 h post-radiation exposure as a model was established to determine the metabolites that were expressed in the jejunum and circulating plasma after the exposure to sub-lethal and lethal radiation dose. Several amino acids were obviously elevated in both jejunum and plasma at 24 h post-radiation exposure indicating prospective insight into the initial inflammatory reaction during Radiation-induced small bowel disease [15]. Putative gastrointestinal markers were reported after acute exposure to low doses of radiation. Tryptophan was detected upregulated while Glutamate, Taurine, and Cysteine-Glycine dipeptide and methionine were also found to be upregulated in the jejunum and circulating plasma at 24 h post-radiation exposure as a response to ROS production, thus suggesting their role in the regulation of cell cycle, DNA repair, and apoptotic cascades [11].

### Table 1

| No. | Metabolite | ANOVA f value | ANOVA p-value | Tukey-Kramer HSD | 0 Gy vs 2 Gy | 0 Gy vs 20 Gy | 2 Gy vs 20 Gy | Class |
|-----|------------|---------------|---------------|------------------|-------------|---------------|--------------|-------|
| 1   | l-Serine   | 27.018        | 0.001         | **< 0.05        |            |               |              | Amino acid |
| 2   | l-Isoleucine | 26.475       | 0.001         | **< 0.05        |            |               |              | Amino acid |
| 3   | l-Leucine  | 23.095        | 0.001         | **< 0.05        |            |               |              | Amino acid |
| 4   | l-Methionine | 20.784       | 0.001         | **< 0.05        |            |               |              | Amino acid |
| 5   | l-Valine   | 20.731        | 0.001         | **< 0.05        |            |               |              | Amino acid |
| 6   | l-Threonine | 12.533        | 0.003         | *< 0.05         |            |               |              | Amino acid |
| 7   | Glycine    | 10.116        | 0.006         | *< 0.05         |            |               |              | Amino acid |
| 8   | l-Alanine  | 9.222         | 0.008         | *< 0.05         |            |               |              | Amino acid |
| 9   | Cadaverine | 8.310         | 0.011         | *< 0.05         |            |               |              | Biogenic amine |
| 10  | l-Proline  | 8.043         | 0.012         | *< 0.05         |            |               |              | Amino acid |
| 11  | l-Phenylalanine | 6.119   | 0.024       |               |            |               |              | Amino acid |
| 12  | Glycerol   | 5.511         | 0.031         |            |            |               |              | Lipid metabolite |
| 13  | Creatinine | 5.382         | 0.033         |            |            |               |              | Protein waste product |
| 14  | Pyroglutamic acid | 5.234    | 0.035       |               |            |               |              | Amino acid |
| 15  | DL-β-Hydroxybutyric acid | 5.074    | 0.038       |               |            |               |              | Ketone body |
| 16  | l-Aspartic acid | 4.999      | 0.039       |               |            |               |              | Amino acid |
| 17  | GABA       | 4.263         | 0.055         |            |            |               |              | Protein intermediate |
| 18  | Stearic acid | 4.128        | 0.059         |            |            |               |              | Fatty acid |
| 19  | Adenosine  | 4.031         | 0.062         |            |            |               |              | Nucleoside |
| 20  | Oxalic acid | 3.764         | 0.070         |            |            |               |              | Carbohydrate intermediate |
| 21  | 2,3-Bisphospho-glyceric acid | 3.707    | 0.073       |               |            |               |              | Carbohydrate intermediate |
| 22  | 2-Arabinose 5-phosphate | 3.689    | 0.073       |               |            |               |              | Carbohydrate intermediate |
| 23  | Pyruvic acid | 3.570        | 0.078         |            |            |               |              | Carbohydrate intermediate |
| 24  | Citric acid | 3.549         | 0.079         |            |            |               |              | Carbohydrate intermediate |
| 25  | Ethanolamine | 3.039        | 0.104         |            |            |               |              | Lipid intermediate |
| 26  | α-Malonic acid | 2.927       | 0.111        |            |            |               |              | Carbohydrate intermediate |
| 27  | l-Sorbose | 2.779         | 0.121         |            |            |               |              | Monosaccharide |
| 28  | Taurine    | 2.223         | 0.171         |            |            |               |              | Protein intermediate |
| 29  | Urea       | 2.018         | 0.195         |            |            |               |              | Protein waste product |
| 30  | D-Glucaric acid | 1.505    | 0.279       |               |            |               |              | Carbohydrate intermediate |
| 31  | Succinic acid | 1.453        | 0.289         |            |            |               |              | Carbohydrate intermediate |
| 32  | α-Phosphoenolpyruvate | 1.391   | 0.303       |               |            |               |              | Lipid intermediate |
| 33  | Ascorbic acid | 1.259        | 0.335         |            |            |               |              | Vitamin |
| 34  | D-Ribose  | 1.202         | 0.350         |            |            |               |              | Monosaccharide |
| 35  | Glyceric acid | 1.082        | 0.384         |            |            |               |              | Carbohydrate intermediate |
| 36  | Hypotaurine | 1.008         | 0.407         |            |            |               |              | Protein intermediate |
| 37  | Inositol   | 0.892         | 0.447         |            |            |               |              | Vitamin |
| 38  | D-Glucose | 0.725         | 0.514         |            |            |               |              | Monosaccharide |
| 39  | D-Galactose | 0.724         | 0.514         |            |            |               |              | Monosaccharide |
| 40  | Fumaric acid | 0.722        | 0.515         |            |            |               |              | Carbohydrate intermediate |
| 41  | Inosine    | 0.578         | 0.583         |            |            |               |              | Nucleoside |
| 42  | Pyrophosphoric acid | 0.523    | 0.612       |               |            |               |              | Energy carrier product |
| 43  | D-GLutamic acid | 0.223       | 0.805        |               |            |               |              | Amino acid |
| 44  | l-Lactic acid | 0.176        | 0.842         |               |            |               |              | Carbohydrate waste product |

HSD = Honestly Significant Difference. *p < 0.05 and **p < 0.01 (one-way ANOVA followed by post-hoc Tukey-Kramer HSD test).
pelvic malignancies.
The primary goal of the present study is to identify the intestinal-specific metabolic markers during abdominal exposure to conventional (2 Gy) or potentially destructive (20 Gy) doses of ionizing radiation. That will be beneficial to find out a means to inform on injury status, expect injury outcome, and potential for expediting medical countermeasure development. We showed that radiation exposure induces distinct changes in the metabolic profile of the intestine, using a murine model.

2. Materials and methods

2.1. Experimental animals

Animals were handled under the guidance of the Committee of the Ethics of Animal Experiments of Kobe University (Permission number: P160403). All surgical approaches were performed under isoflurane anesthesia and every effort was made to minimize suffering. The mice were 10-week-old adult male C57BL/6J mice weighing 20–25 g. They were housed in standard cages at a temperature of 20 ± 5 °C under a 12-h day/night cycle in pathogen-free conditions and were allowed access to food and water ad libitum. Mice were randomly assigned to treatment groups.

2.2. X-ray irradiation

X-ray irradiation was carried out using a MBR-1505R2 instrument (Hitachi, Tokyo, Japan) at a voltage of 150 kV and a current of 5 mA with a 1-mm-thick aluminum filter (0.5 Gy/min at the target) for in vivo studies. The anesthetic Somnopentyl (0.1 mg/g body weight) was intraperitoneally administrated prior to radiation exposure. A lead shield covering the head and chest of each mouse was used prior to x-ray irradiation [21].

2.3. Sample collection and preparation

After 24 h of irradiation treatment, 6 cm of mouse intestinal tissue, starting from the lowest part of the stomach, was excised and collected. The weight of each sample was approximately 10 mg. Intestinal samples were frozen in liquid nitrogen and stored at −80 °C for comparative metabolomics. Low molecular weight metabolites were extracted from intestinal tissue according to previously mentioned methods [22-24]. Intestinal samples were mixed with 1.0 mL of a solvent mixture (MeOH: H2O:CHCl3 = 2.5:1:1). 10 μL of 0.5 mg/mL 2-isopropylmalic acid dissolved in distilled water was added as an internal standard, followed by sonication for 20 s. The solution was incubated for 30 min at 37 °C and centrifuged for 3 min at 4 °C at 15,000 rpm. 500 μL of CHCl3 was poured to 1.0 mL of the supernatant followed by lyophilisation using a freeze dryer. The lyophilized samples were dissolved in 40 μL of 20 mg/mL methoxyamine in pyridine and incubated for 90 min at 30 °C. The samples were derivatized with 20 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) for 30 min at 37 °C.

2.4. Gas chromatography-mass spectrometry

The gas chromatography-mass spectrometry (GC-MS) analysis of the intestinal samples was implemented using a GCMS-QP2010 Ultra (Shimadzu Co., Kyoto, Japan) according to previously described research [22,25]. A fused silica capillary column (CP-SIL 8 CB low bleed/MS; 30 m 0.25 mm inner diameter, film thickness: 0.25 mm; Agilent Co., Palo Alto, CA, USA) was utilized. The temperature of the column was kept at 80 °C for 2 min, then elevated at 15 °C/min to 330 °C. The temperatures of the transfer line and ion source were 250 °C and 200 °C, respectively. An 85–500 m/z mass was exposed to 20 scans per second using the Advanced Scanning Speed Protocol (ASSP, Shimadzu Co., Kyoto, Japan). Data were analyzed using MS-DIAL software [22,26] and normalized to the tissue weight.

2.5. ROS measurement

Dihydroethidium (DHE) was measured in the intestinal tissue as previously described [21]. In brief, DHE was dissolved in dimethyl sulfoxide and diluted with PBS immediately before use. One hour prior to irradiation, 200 μL DHE (30 mg/kg) was injected intraperitoneally. Intestinal tissue was collected 24 h after irradiation and rapidly frozen at −80 °C. Frozen sections were prepared and ROS was assessed by BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

2.6. Immunohistochemistry, and immunofluorescence

The intestinal tissue was removed and cut into sections in 5 mm thickness, and immediately fixed in 4% paraformaldehyde in PBS. 5 μm sections were cut and stained with hematoxylin and eosin (HE) for histological examination. For immunohistochemistry, sections were stained using the peroxidase-labeled, peroxidase, anti-peroxidase (PAP) antibody method (Dako REAL peroxidase blocking solution S2023, Glostrup, Denmark) with an anti-PCNA antibody (1:100, Santa Cruz Biotechnology, INC, sc-56), anti-HSP70 (1:100, Cell Signaling, #4872), and anti-HSP90 (1:100, Santa Cruz Biotechnology, INC, sc-7947). Mayer’s hematoxylin stain was used for nuclei staining (Muto Pure Chemicals Co., Tokyo, Japan). For immunofluorescence, Anti-caspase-3 (1:100, Cell Signaling, #9664) was purchased. Stained slides were assessed using BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

2.7. Data processing and statistical analysis

MetaboAnalyst 4.0 (http://www.metaboanalyst.ca) was used for metabolite analysis [27–29]. Principal component analysis (PCA) and Hierarchical clustering analysis were applied to efficiently demonstrate
the variance between irradiated and non-irradiated groups. Data were analyzed statistically using multiple comparison one-way ANOVA with Tukey-Kramer as a post-hoc. \( p \)-values less than 0.05 were considered statistically significant.

3. Results

To examine the metabolic profile associated with radiation exposure, we used a comprehensive metabolome analysis with GC-MS to analyze the relative abundance of metabolites in the intestines. We detected 44 metabolites overall in both control and radiation groups. These metabolites were classified into carbohydrate, lipid, and protein intermediates (Table 1 and Table S1). PCA segregated the data into three groups according to the principal component 1 (PC1) value (35.0% of variance; Fig. 1), suggesting that PC1 captured radiation dose-dependent variance in the data. Hierarchical clustering for each treatment group demonstrated distinct clustering of the three groups (Fig. 2). The key differentiating metabolites were amino acids. We identified nine amino acids that significantly differed between the control and radiation groups, with a \( p \)-value < 0.05 (Fig. 3 and Table S1). The levels of serine, isoleucine, leucine, methionine, valine, threonine, alanine, and proline were significantly increased in a dose-dependent manner. The level of glycine was significantly elevated in response to the low dose of radiation. These results suggest that radiation exposure of the intestines affects amino acid metabolism.

We further investigated the effect of low (2 Gy) and high (20 Gy) radiation dose on ROS-induced intestinal injury. We analyzed DHE, a ROS marker, and caspase-3, an apoptotic marker, 24 h post-radiation exposure. ROS-generating cells as well as apoptotic cells were widely detected after the exposure to the high radiation dose that localized specifically throughout the crypts and lamina propria (Fig. 4A). Histopathological analysis showed markedly shortening in the intestinal villi length after the exposure to radiation, as a dose-dependent response. High radiation dose (20 Gy) showed severe destruction in the intestinal villi and crypts (Fig. 4B). In addition, the intestinal proliferating cells, detected by proliferating cell nuclear antigen (PCNA), were markedly decreased in the crypts after the exposure to low radiation dose, whereas no proliferating cells were detected after the exposure to the toxic dose (20 Gy, Fig. 4B). Finally, we checked HSP 70 and HSP 90, stress markers, in the intestinal tissue as a response to the ROS-induced radiation. Both HSP 70 and HSP 90 expression was visibly increased after radiation exposure, as a dose dependent response. HSP 90 was partially expressed in the non-irradiated intestinal villi and its expression was extensively elevated and distributed in both the crypt and villi as a response to the different radiation doses (Fig. 4B), indicating the role of HSPs in protecting the intestinal tissue from the radiation damage.
Fig. 3. Barplot visualization of 44 metabolites relative abundance in the intestinal tissue after radiation exposure. Nine amino acids showed a significant difference between the control and radiation groups. X-axis shows radiation dose. Y-axis shows relative abundance of metabolites. Statistical significance was determined by one way ANOVA, *p < 0.05 and **p < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
4. Discussion

The present study identified a gradual increase in multiple amino acids in the intestinal tissue including, serine, isoleucine, leucine, methionine, valine, threonine, alanine, and proline, which may be associated with oxidative stress, as a dose-dependent response to radiation. In contrast, the upregulation of glycine level was associated with a low dose of radiation. Interestingly, glycine and L-alanine were upregulated most in the intestinal tissue, concerning other previously mentioned amino acids. Glycine represents 11.5% of the total amino acids.
acid content in the human body and 20% of the total amino acid nitrogen in body proteins is derived from glycine. Additionally, glycine plays a key role in the protection of the intestine from radiation injury and mesenteric ischemia, immune response, and survival of living beings [30]. Similar to glycine, alanine constitutes a high percentage of the amino acids in the total proteins and other branched amino acids such as valine, leucine, and isoleucine can yield alanine during amino acidings [30]. Similar to glycine, alanine constitutes a high percentage of the nitrogen in body proteins is derived from glycine. Additionally, glycine acid content in the human body and 20% of the total amino acid ni-

Thus, it is possible that radiation activates autophagy, which helps eliminating oxidative stress damage.

Previous work showed that amino acids, including alanine and branched-chain amino acids (isoleucine, leucine, and valine) suppressed the oxidative stress–induced inflammatory response in intestinal epithelial cells [40]. The branched-chain amino acids in particular have potential as inducers of antioxidant enzymes, leading to active glutathione-S-transferase and catalase [41]. Another study indicated that an upregulated concentration of serine mitigated oxidative stress by mediating glutathione and methionine biosynthesis. Serine in particular combines with homocysteine to create cysteine, furnishing one carbon for homocysteine demethylation [42]. Glycine exerts an antioxidative effect to protect intestinal cells from oxidative stress by preserving intracellular glutathione levels and promoting GLYTI expression, as well as reducing the activation of ERK, JNK, and p38 in the MAPK signaling pathway [43,44]. However, the molecular mechanisms and signaling pathways remain unclear; thus, further investigations are needed.

5. Conclusions

From this metabolomic profiles we concluded that exposure of intestinal tissue to 0, 2, and 20 Gy irradiation doses shows a gradual increase in the level of amino acids in a dose-dependent response to radiation. Ionizing radiation–induced oxidative damage may activate the autophagy pathway, resulting in the degradation of cellular proteins and production of excessive amino acids. These amino acids may suppress the oxidative stress–induced inflammatory response in intestinal epithelial cells suggested that amino acids level may be a potential marker of oxidative stress-related gastrointestinal toxicity due to radiation exposure.

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Authors’ contributions

All authors participated in the conception and design the study. Mohammed Salah, Saki Osuga, Makiko Nakahana, and Yasuyuki Shinizu are involved in vivo study. Mohammed Salah, Saki Osuga, Yasuhiro Irino, Masakazu Shinohara, Ai Nakaoaka, Kenji Yoshida, Yoshiaki Okamoto, and Ryohei Sasaki analyzed the data, Mohammed Salah, Naritoshi Mukumoto, Hiroaki Akasaka, Daisuke Miyawaki, and Takeaki Ishihara shared in the interpretation of this study. All authors read, revised, and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100789.

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Appendix A. Supplementary data

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