Extracellular miRNAs in the serum and feces of mice exposed to high-dose radiation

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Abstract. Exposure to high-dose radiation causes life-threatening intestinal damage. Histopathology is the most accurate method of judging the extent of intestinal damage following death. However, it is difficult to predict the extent of intestinal damage. The present study investigated extracellular microRNAs (miRNAs or miRs) in serum and feces using a radiation-induced intestinal injury mouse model. A peak of 25-200 nucleotide small RNAs was detected in mouse serum and feces by bioanalyzer, indicating the presence of miRNAs. Microarray analysis detected four miRNAs expressed in the small intestine and increased by >2-fold in serum and 19 in feces following 10 Gy radiation exposure. Increased miR-375-3p in both serum and feces suggests leakage due to radiation-induced intestinal injury and may be a candidate for high-dose radiation biomarkers.

Introduction

Nuclear terrorism or power plant accidents risk serious radiation damage (1). An emergency radiation medical system capable of responding to such accidents must be established. Humans and rodents exposed to high-dose ionizing radiation (IR) develop acute radiation syndrome (ARS). In humans, hematopoietic ARS occurs at radiation doses >1 Gy and the severity of the syndrome is directly proportional to the dose of absorbed radiation (2-4). The white blood cell count decreases, predisposing people to infection. When humans are exposed to 6-8 Gy radiation, gastrointestinal ARS induces intestinal mucosa disintegration, along with hematopoietic ARS (3). In 1999, three patients experienced ARS caused by a critical nuclear accident at the JCO nuclear fuel processing facility in Tokai-mura, Japan. Two of these patients died of intestinal injury (5-7). Predicting risk of lethal intestinal injury is essential when high-dose exposure is suspected. Intestinal tissue removal is necessary to assess damage to intestinal epithelial cells cau2sed by radiation. Hence, it has been difficult to assess intestinal damage in a noninvasive manner. Therefore, a less invasive method that can objectively evaluate the degree of intestinal injury is required.

MicroRNAs (miRNAs or miRs) are endogenous, small non-coding RNAs that regulate cellular processes such as proliferation and growth, differentiation, programmed cell death, cell cycle progression and tissue development (8). Studies have detected miRNAs in body fluids such as serum, plasma and urine (9-11); these miRNAs in body fluids have been proposed as biomarkers for various physiological responses and pathological stages of cancers and neurodegenerative disease. For example, miR-122 is specific to the liver and appears in blood in large amounts in liver cancer. Therefore, miR-122 has attracted attention as a biomarker for liver cancer (12).

Intestinal epithelial cells are radiosensitive; when humans or mice are exposed to IR doses >10 Gy, cell death is induced (13-15). IR-induced cell death forms extracellular vesicles called apoptotic bodies or induces extracellular leakage of various intracellular components such as proteins and enzymes (16). Our previous study demonstrated that the release of miR-375-3p from pancreatic β cells increases following high-dose IR exposure (17). Similarly, miRNAs in intestinal epithelial cells are released following IR exposure and may infiltrate blood and feces; to the best of our knowledge, however, no previous study has confirmed this. Therefore, the present study aimed to identify miRNAs excreted in serum or feces as high-dose IR biomarker candidates using an IR-induced intestinal injury mouse model.

Materials and methods

Mice. A total of eight male 7-week-old C57BL/6NJcl (body weight: 23.2±1.0 g) mice were obtained from CLEA Japan. All mice were given access to a solid diet CE-2 (CLEA Japan) and water *ad libitum* and were housed in a conventional animal room with 12/12-h light/dark cycles at room temperature and humidity 40-50%. Up to five mice were housed/cage and

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bedding, feed and water were changed weekly. Mice were observed 2-3 times/day for monitoring; no abnormalities in mouse health or behavior were observed. Mice were allowed to acclimatize for 1 week before irradiation. Blood and small intestine samples from all mice were collected under anesthesia with isoflurane (Pfizer, Inc.). Small animal anesthesia machines (Muromachi Kikai Co., Ltd.) were used to anesthetize the mice. Isoflurane at 4-5% was used for induction and maintained at 2-3%. After anesthesia, 0.5-1.0 ml blood was drawn from the heart and mice were promptly cervically dislocated for euthanasia. The time-lapse from the start of the anesthesia to the end of blood collection was <10 min/animal. Death was confirmed by respiratory and cardiac arrest. Small intestine samples were collected after the death of the mice. Blood samples were placed in a BD MicroTainer® SST (Becton, Dickinson and Company) and the coagulated blood was centrifuged at 6,000 x g for 3 min at room temperature for serum separation. Feces samples were directly collected in tubes. The Hirosaki University Ethics Committee for Animal Experiments approved the experiments (approval no. G12003), which were conducted under the Hirosaki University Guidelines for Animal Experiments.

X-ray irradiation. Following 1 week acclimatization, mice were exposed to X-rays (MBR-1520R-3 X-ray machine; Hitachi Ltd.) at 1.0 Gy/min (150 kVp, 20 mA, 0.5 mm aluminum and 0.3 mm copper filters). Mice were fixed in a circular mouse holder (Natsume Seisakusho Co., Ltd.) and irradiated uniformly with X-rays while rotating the holder. The mice in the irradiated group were irradiated with 10 Gy, while those in the non-irradiated group were not irradiated. Blood, feces and small intestine samples were collected 3 days after exposure to X-rays.

TUNEL assay. TUNEL assay was performed to confirm tissue damage in the small intestine caused by exposure to 10 Gy X-rays. For tissue analyses, the small intestine was fixed with 4% paraformaldehyde solution in Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline [D-PBS (-)]at pH 7.2 for 2 days at room temperature. The fixed small intestine was embedded in paraffin. Sections were cut to 4 μm and placed on glass slides. Paraffin-embedded sections were deparaffinized with xylene and ethanol after which they were washed with D-PBS (-). Cell death analysis was performed using the DeadEnd™ Fluorometric TUNEL System (Promega Corporation) according to the manufacturer's instructions. To label fragmented DNA with fluorescein-12-dUTP, small intestinal tissue was incubated with the reaction solution for 1 h at 37˚C. ProLong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (Thermo Fisher Scientific, Inc.) was used for nuclear staining and mounting at room temperature. The stained tissues were examined using a confocal laser scanning microscope LSM710 (Carl Zeiss GmbH). At least five fields of view per sample were observed.

Total RNA extraction. Total RNA from the small intestine, serum and feces were extracted using the Isogen II reagent and ethachinmate (both Nippon Gene Co., Ltd.) according to the manufacturer's instructions. Total RNA was extracted from drinking water and feed used as controls. RNA concentrations from the small intestine were assessed using NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). RNA samples had 260/280 nm absorbance ratios of 1.8-2.0. RNA concentration of serum and feces was measured using Quant-iT RiboGreen RNA Reagent and Fluoroskan Ascent (both Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The quality of total RNAs was confirmed using the Agilent 2100 Bioanalyzer and Agilent RNA 6000 Pico kit (both Agilent Technologies, Inc.), according to the manufacturer's instructions.

Microarray analysis. Cyanine 3 (Cy3)-labeled miRNAs were synthesized from total RNAs of irradiated and non-irradiated samples (small intestine, serum and feces; all n=4) using the miRNA Complete Labeling Reagent and Hyb kit (cat. no. 5190-0456; Agilent Technologies, Inc.). SurePrint G3 mouse miRNA microarray slides (8x60 K, Ver.21.0; cat. no. G4872A; Agilent Technologies, Inc.) were hybridized with Cy3-labeled miRNA in hybridization solution prepared with Gene Expression Hybridization kit (Agilent Technologies, Inc.), according to the manufacturer's instructions. Cy3 fluorescence signals were obtained using the SureScan microarray scanner and processed using Feature Extraction version 10.7 software (both Agilent Technologies, Inc.) according to the manufacturer's instructions. The expression data obtained were processed using GeneSpring GX14.5 software (Agilent Technologies, Inc.) to normalize all values to the 90% shift on the respective microarrays, followed by the normalization of the median expression of all samples. miRNAs with expression change >2.0-fold were selected. miRNA accession numbers were confirmed in miRbase (mirbase.org/). The obtained microarray data were registered with Gene Expression Omnibus (ncbi.nlm.nih.gov/geo/) (accession no. GSE247876). To predict target genes of miRNAs and pathways, TargetScan Mouse (targetscan.org/mmu_72/) and WikiPathways (wikipathways.org/) analyses were performed using the GeneSpring 14.5 software (Agilent Technologies, Inc.). Pathway data of *Mus musculus* were downloaded from WikiPathways (/data.wikipathways.org/current/gpml/).

Statistical analysis. Target genes of miRNAs were searched by TargetScan and pathway predictions related to the target genes were searched on WikiPathways in GeneSpring 14.5 software with cut-off value of $P<0.05$.

Results

Small intestine damage in mice exposed to 10 Gy X-rays. Small intestinal damage following 10 Gy X-ray irradiation was confirmed by TUNEL assay. At 72 h after radiation exposure, green fluorescent TUNEL labeling increased and showed positive signals, especially in small intestinal pit sites rich in small intestinal epithelial stem cells (Fig. 1). This indicated that cell death was induced in the small intestines of mice exposed to 10 Gy X-rays.

Small RNAs are detected in mouse serum or feces. Total RNAs were extracted from the small intestine, serum and feces. Peaks of 18 and 28 S ribosomal RNAs were detected in total RNAs from the small intestines (Fig. 2A), whereas

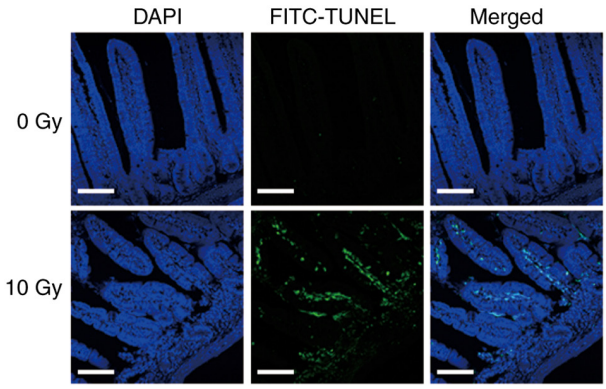

Figure 1. Effects of 10 Gy X-ray exposure in the small intestine. TUNEL assay of small intestine following radiation exposure. Scale bar, 100 μm.

these peaks were not detected in serum and feces. However, a peak of small RNAs of 25-200 nucleotides was detected in serum and feces (Fig. 2B and C). Almost no RNA peak was detected in the drinking water and animal feed used as a control (Fig. 2D and E). This demonstrated the presence of small RNAs in mouse serum and feces.

miRNA expression in the serum or feces of mice exposed to 10 Gy X-rays. Microarray analysis was performed to examine miRNAs expressed in serum and feces. In serum, 21 and 73 miRNAs were up- and downregulated >2.0-fold in the 10 Gy irradiation compared with the non-irradiated group, respectively (Fig. 3A; Table SI). In feces, 119 and 226 miRNAs increased and decreased more than 2.0-fold in the 10 Gy irradiation compared with the non-irradiated group, respectively (Fig. 3B; Table SII). Venn diagram of these miRNAs is presented in Fig. 3C and a breakdown of these miRNAs is shown in Table I.

Using TargetScan, 646 genes were predicted as a targets of up- and 1,306 of downregulated miRNAs in serum (data not shown). In feces, 926 and 499 genes were predicted as target genes of up- and downregulated miRNAs, respectively (data not shown).

Using a threshold P-value of 0.05, the WikiPathways analysis detected 107 and 140 pathways using 646 and 1,306 target genes predicted for the 21 and 73 miRNAs in serum, respectively. In addition, 128 and 91 pathways were detected by pathway analysis performed using 926 and 499 predicted target genes of the 119 and 226 miRNAs in feces, respectively. Tables II and III present the top 20 pathways involving predicted target genes of increased miRNAs in serum or feces; serum and feces shared 14 pathways.

There were four upregulated miRNAs with signal intensity >100 in the small intestine in serum and 19 in feces (Table IV). mir-375-3p was detected in both serum and feces. Therefore, these miRNAs in serum and/or feces may be derived from the small intestine.

Discussion

The present study identified four miRNAs in serum and 19 in feces derived from the small intestine that represent novel high-dose radiation exposure candidate biomarkers. In particular, miR-375-3p was upregulated in serum and feces after 10 Gy X-ray exposure and may be a candidate biomarker to estimate intestinal injury.

Large amounts of small RNAs were present in supernatant of cultured cells and serum, which is consistent with previous results (17-19). Feed and drinking water contained little RNA. Therefore, most miRNAs detected in serum and feces were derived from murine tissue. Our previous study demonstrated that miR-375-3p is abundant in the digestive tract (including the small intestine) (17). Because feces are in direct contact with the digestive tract (including the small and large intestine), it was hypothesized that radiation-induced injury of digestive tract cells would result the leakage of miRNAs, including miR-375-3p (which is abundant in the digestive tract (17)) into the feces. Therefore, it was hypothesized that upregulated miRNAs, including miR-375-3p, in feces originated from the gastrointestinal tract.

Here, 10 Gy irradiation increased the expression of 21 miRNAs in serum and 119 miRNAs in feces >2.0-fold. miR-375-3p was increased in both serum and feces after 10 Gy exposure. In our previous study, serum miR-375-3p was highly expressed in the pancreas, small intestine, and colon and the expression of this miRNA increased following exposure to 7 Gy in mice (17). This suggests that upregulated miR-375-3p in serum is derived from the pancreas and small intestine. Therefore, in the present study, following 10 Gy exposure, upregulated miR-375-3p in the serum was likely derived from the leakage from the pancreas and small intestine.

miR-375-3p is primarily expressed in β-cell islets of the pancreas and plays an important role in the complex regulatory network of pancreatic development and insulin secretion (20-23). Here, the insulin signaling pathway was one of the top 20 pathways associated with predicted targeted genes of upregulated miRNAs in serum and feces. The increase in miR-375-3p in serum may be due to pancreatic or small intestinal damage. Hence, impairment of the insulin signaling pathway is also expected to occur. Fendler *et al* (24) reported that a combination of three serum miRNAs (miR-133b, miR-215 and miR-375) predicts radiation-induced fatality in mice and macaques. With upregulated miR-375-3p in feces suggesting intestinal injury and increased miR-375-3p in serum, this miRNA may be a biomarker capable of predicting the lethal dose of radiation exposure in humans.

Fecal calprotectin has been reported as a biomarker of radiation exposure (25). Fecal calprotectin is used for adjuvant diagnosis of ulcerative colitis and Crohn's disease and it is measured via fluorescence enzyme immunoassay (26-28). Calprotectin is a calcium- and zinc-binding heterodimer of 36.5 kDa that belongs to the S100 family and is present on the surface of monocytes and macrophages, facilitating recruitment to the site of inflammation. The synthesis of calprotectin is increased during the inflammatory process (27). Calprotectin has been reported as a biomarker of acute radiation enteritis caused by radiation treatment of prostate cancer (29,30). To evaluate sensitivity and specificity, comparative analyses of the usefulness of fecal calprotectin and the 19 miRNAs identified in the present study (including miR-375-3p) will be necessary.

The present study compared irradiated and non-irradiated mice. Although 10 Gy irradiation caused intestinal damage,

Table I. Up- and downregulated miRs in serum and feces of mice exposed to 10 Gy X-ray irradiation.

Serum expression	Fecal expression	miR
Upregulated	Upregulated	miR-375-3p, miR-574-3p
Upregulated	Downregulated	miR-500-3p, miR-3076-5p
Downregulated	Upregulated	let-7i-5p, miR-25-3p, miR-27b-3p, miR-29c-3p, miR-129-1-3p, miR-468-3p, miR-486a-5p, miR-669n, miR-7016-5p
Downregulated	Downregulated	miR-140-3p, miR-181a-5p, miR-223-3p, miR-361-5p, miR-758-5p, miR-2916-5p, miR-3091-5p, miR-3113-5p, miR-6899-5p, miR-6938-5p, miR-6946-5p, miR-6961-5p, miR-7066-5p, miR-7687-5p

miR, microRNA.

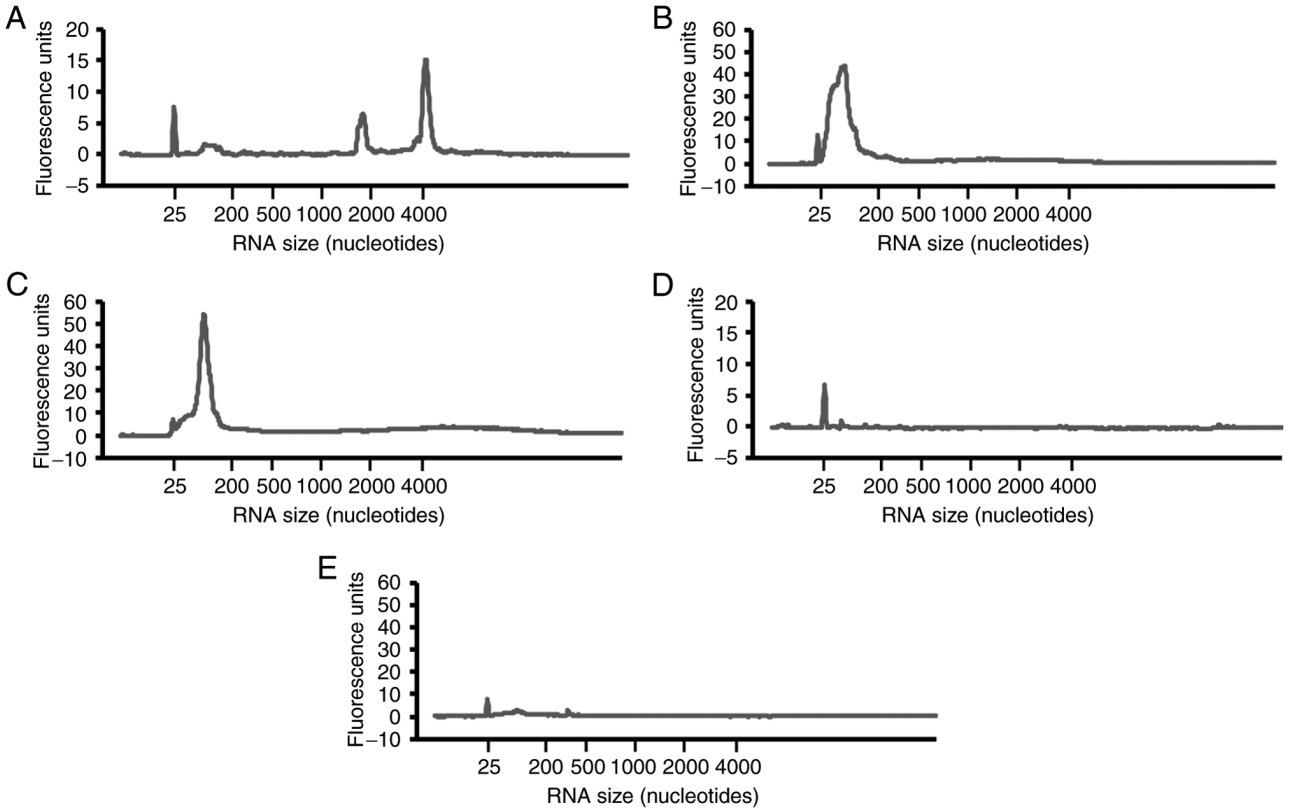

Figure 2. Detection of total RNAs from biological samples in mice. Total RNAs were detected in (A) small intestine, (B) serum, (C) feces, (D) drinking water, and (E) animal feed using the Agilent 2100 bioanalyzer.

the present study did not compare the degree of intestinal damage and miRNA expression in the small intestine following exposure to various doses of radiation. Serum miRNAs may reflect damage at various tissue sites, while fecal miRNAs are thought to be primarily of intestinal origin. Future studies should compare intestinal damage caused by various radiation doses and miRNA expression levels. The tissue specificity of the miRNAs detected in this study should also be examined to determine if they are biomarkers specific for intestinal damage.

miRNAs released by radiation exposure may be degraded in body fluids. miRNAs bound to miRNA-binding proteins and/or miRNAs internalized in extracellular vesicles are not degraded and remain in body fluids (31). miRNAs in extracellular vesicles may be transferred to other cells and cause damage and/or alter their function (32).

The detection of small RNA was not performed in irradiated samples. Our previous study examined changes in serum RNA at lethal doses of 7 Gy irradiation at 0, 24, 48 and 72 h after irradiation and found no increase in overall RNA levels (17). Similarly, there should be no change in total yield in feces; this should be investigated in future.

When humans are exposed to high doses of radiation, treatments such as hematopoietic factors and stem cell transplantation are effective in restoring bone marrow (33). However, if intestinal damage is severe, recovery is difficult and an indicator to assess degree of intestinal damage is needed to triage patients. Future studies should investigate the

Table II. Top 20 pathways involving predicted target genes of upregulated microRNAs in serum.

Pathway name	Pathway ID	P-value	Target gene count
Mm_Non-odorant_GPCRs	WP1396_69993	$3.58x10^{29}$	14
Mm_Focal_Adhesion-PI3K-Akt-mTOR-signaling_pathway	WP2841_94308	$3.58x10^{29}$	14
Mm_MAPK_signaling_pathway	WP493_78412	$4.58x10^{23}$	11
Mm_mRNA_processing	WP310_78419	$4.96x10^{21}$	10
Mm_PluriNetWork	WP1763_89515	$5.35x10^{19}$	9
Mm_EGFR1_Signaling_Pathway	WP572_82883	$5.35x10^{19}$	9
Mm_IL-3_Signaling_Pathway	WP373_69196	$6.22x10^{15}$	7
Mm_Chemokine_signaling_pathway	WP2292_97515	$6.22x10^{15}$	7
Mm_Regulation_of_Actin_Cytoskeleton	WP523_71326	$6.22x10^{15}$	7
Mm_MicroRNAs_in_Cardiomyocyte_Hypertrophy	WP1560_70037	$6.22x10^{15}$	7
Mm_GPCRs,_Class_A_Rhodopsin-like	WP189_79710	$6.22x10^{15}$	7
Mm_Focal_Adhesion	WP85_94410	$6.69x10^{13}$	6
Mm_IL-2_Signaling_Pathway	WP450_89849	$6.69x10^{13}$	6
Mm_ESC_Pluripotency_Pathways	WP339_94309	$6.69x10^{13}$	6
Mm_Myometrial_Relaxation_and_Contraction_Pathways	WP385_95806	$6.69x10^{13}$	6
Mm_Purine_metabolism	WP2185_101822	$6.69x10^{13}$	6
Mm_Odorant_GPCRs	WP1397_82866	$7.18x10^{11}$	5
Mm_Insulin_Signaling	WP65_88446	$7.18x10^{11}$	5
Mm_Alpha6-Beta4_Integrin_Signaling_Pathway	WP488_72049	$7.18x10^{11}$	5
Mm_Apoptosis	WP1254_95784	$7.18x10^{11}$	5

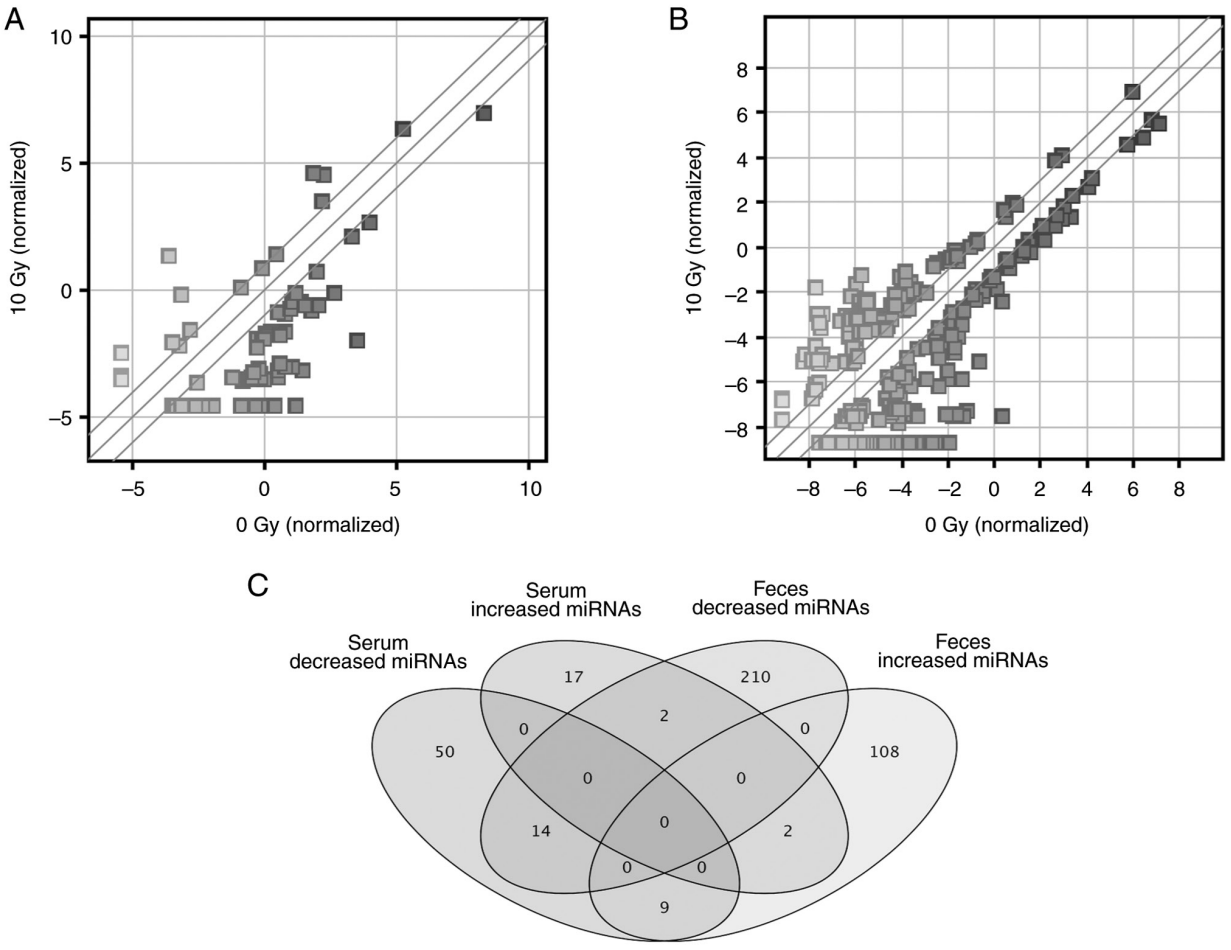

Figure 3. miRNA expression in serum or feces of mice exposed to 10 Gy X-ray irradiation. Scatter plot of (A) serum and (B) fecal miRNAs in mice exposed to 10 Gy X-ray irradiation. (C) Venn diagram of serum and fecal miRNAs in mice exposed to 10 Gy X-rays. miRNA, microRNA.

Table III. Top 20 pathways involving predicted target genes of upregulated microRNAs in feces.

Pathway name	Pathway ID	P-value	Target gene count
Mm_mRNA_processing	WP310_78419	<0.001	27
Mm_Focal_Adhesion	WP85_94410	$1.77x10^{34}$	18
Mm_Focal_Adhesion-PI3K-Akt-mTOR-signaling_pathway	WP2841_94308	$1.34x10^{32}$	17
Mm_PluriNetWork	WP1763_89515	$5.79x10^{27}$	14
Mm_Non-odorant_GPCRs	WP1396_69993	$3.28x10^{23}$	12
Mm_EGFR1_Signaling_Pathway	WP572_82883	$3.28x10^{23}$	12
Mm_GPCRs,_Class_A_Rhodopsin-like	WP189_79710	$2.47x10^{21}$	11
Mm_Insulin_Signaling	WP65_88446	$1.85x10^{19}$	10
Mm_Delta-Notch_Signaling_Pathway	WP265_69189	$1.85x10^{19}$	10
Mm_MicroRNAs_in_Cardiomyocyte_Hypertrophy	WP1560_70037	$1.85x10^{19}$	10
Mm_Myometrial_Relaxation_and_Contraction_Pathways	WP385_95806	$1.39x10^{17}$	9
Mm_Integrin-mediated_Cell_Adhesion	WP6_97547	$1.39x10^{17}$	9
Mm_Odorant_GPCRs	WP1397_82866	$1.39x10^{17}$	9
Mm_Chemokine_signaling_pathway	WP2292_97515	$1.39x10^{17}$	9
Mm_Metapathway_biotransformation	WP1251_94721	$1.04x10^{15}$	8
Mm_Kit_Receptor_Signaling_Pathway	WP407_69079	$1.04x10^{15}$	8
Mm_Spinal_Cord_Injury	WP2432_102465	$1.04x10^{15}$	8
Mm_MAPK_signaling_pathway	WP493_78412	$7.81x10^{14}$	7
Mm_Regulation_of_Actin_Cytoskeleton	WP523_71326	$7.81x10^{14}$	7
Mm_IL-3_Signaling_Pathway	WP373_69196	$7.81x10^{14}$	7

Table IV. Candidate upregulated miRs in serum and feces derived from small intestine of mice exposed to 10 Gy X-ray irradiation.

Sample	miRs in small intestine (raw signal >100)
Serum	miR-23b-3p, miR-24-3p, miR-27a-3p, miR-375-3p
Feces	let-7i-5p, miR-103-3p, miR-107-3p, miR-148a-3p, miR-19b-3p, miR-200b-3p, miR-200c-3p, miR-25-3p, miR-27b-3p, miR-29a-3p, miR-29c-3p, miR-30c-5p, miR-3473a, miR-3473b, miR-375-3p, miR-3968, miR-494-3p, miR-690, miR-8110

miR, microRNA.

potential role of such indicators as a biomarker for early detection of intestinal disorder.

Here, miR-375-3p levels were increased in serum and feces samples by high-dose radiation exposure. Future studies should compare miR-375-3p with existing markers of intestinal damage and confirm the present results using specimens from patients with colorectal cancer undergoing radiotherapy to determine whether miR-375-3p may be a biomarker of early intestinal damage.

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Availability of data and materials

The data generated in the present study may be found in the Gene Expression Omnibus under accession number GSE247876 or at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE247876.

Authors' contributions

MC performed experiments and wrote the manuscript. HU, HK and IN performed experiments. All authors have read and approved the final manuscript. MC and HU confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All experiments were performed in accordance with the Guidelines for Animal Experimentation of Hirosaki University. The procedures were approved and monitored

by the Animal Research Committee of Hirosaki University (approval no. G12003).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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