Published in final edited form as:

Radiat Res. 2017 December ; 188(6): 626–635. doi:10.1667/RR14828.1.

Urinary miRNAs as Biomarkers for Noninvasive Evaluation of Radiation-Induced Renal Tubular Injury

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Abstract

Radiation nephropathy is one of the common late effects in cancer survivors who received radiotherapy as well as in victims of radiation accidents. The clinical manifestations of radiation nephropathy occur months after exposure. To date, there are no known early biomarkers to predict the future development of radiation nephropathy. This study focuses on the development of urinary biomarkers providing readout of acute responses in renal tubular epithelial cells. An amplification-free hybridization-based nCounter assay was used to detect changes in mouse urinary miRNAs after irradiation. After a single LD50 of total-body irradiation (TBI) or clinically relevant fractionated doses (2 Gy twice daily for 3 days), changes in urinary levels of microRNAs followed either an early pattern, peaking at 6–8 h postirradiation and gradually declining, or later pattern, peaking from 24 h to 7 days. Of 600 miRNAs compared, 12 urinary miRNAs showed the acute response and seven showed the late response, common to both irradiation protocols. miR-1224 and miR-21 were of particular interest, since they were the most robust acute and late responders, respectively. The early responding miR-1224 also exhibited good dose response after 2, 4, 6 and 8 Gy TBI, indicating its potential use as a biomarker for radiation exposure. *In situ* hybridization of irradiated mouse kidney sections and cultured mouse primary renal tubular cells confirmed the tubular origin of miR-1224. A significant upregulation in hsa-miR-1224–3p expression was also observed in human proximal renal tubular cells after irradiation. Consistent with mouse urine data, a similar expression pattern of hsa-miR-1224–3p and hsa-miR-21 were observed in urine samples collected from human leukemia patients preconditioned with TBI. This proof-of-concept study shows the potential translational utility of urinary miRNA biomarkers for radiation damage in renal tubules with possible prediction of late effects.

INTRODUCTION

Radiation-induced nephropathy is defined as renal injury and pathological loss of renal function resulting from an acute exposure to ionizing radiation. Radiation nephropathy has been reported in over 20% of patients who received total-body irradiation (TBI) as part of a conditioning regimen before hematopoietic stem cell transplantation (1, 2). In addition,

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radiation nephropathy is among the major late effects in victims of radiation accidents. In general, the radiation effects follow a deterministic path, with increase in incidence and intensity of effects and/or decrease in latency with dose. However, the effects can be confounded by chemotherapy, age, genetic makeup and pre-existing conditions. The development of biomarkers providing physiological readout of an individual's response is critical for early detection and treatment. Radiation nephropathy can appear in the first few months after renal exposure, and/or it can manifest as late or chronic nephropathy years after exposure. Radiation nephropathy typically presents as decreased kidney function, proteinuria, hypertension and disproportionately severe anemia occurring many months after significant exposure to the kidneys (3). These clinical symptoms are not detectable by imaging or by urine or blood analysis at early stages. Moreover, currently available urinary biomarkers such as Kim1, NGAL and cystatin C are not capable of providing early detection of tubular injury (4–6). To date, there are no diagnostic molecular biomarkers available for early prediction of radiation nephropathy.

With rapid advances in the area of liquid biopsy, cell-free noncoding RNAs have recently gained enormous interest as diagnostic and predictive biomarkers. miRNAs are noncoding RNAs of 19–22 nucleotides, originally identified as regulators of gene expression (7). miRNAs are reported to be present in body fluids and these “circulating miRNAs” have been proposed as diagnostic and prognostic biomarkers for preclinical studies and in clinical practice (8). Interestingly, extracellular vesicles, such as exosomes, released from the cells have been shown to selectively transfer miRNAs to distant cells during pathological conditions (9). Exosomes are microvesicles of 50–150 nm diameter released by many cell types carrying molecular signature of the cell from which it originated. For example, high levels of miR-195 are found in exosomes secreted from breast cancer cells (10), while plasma exosomes of prostate cancer patients contain higher levels of miR-145 (11). Circulating miRNAs have been found to be more stable when bound to RNA-binding protein (12) and when protected in macrovesicles.

Urine is a source of exosomal or protein-bound miRNAs potentially serving as a platform for evaluation of systemic, as well as organ-specific injury (13), particularly for kidney. Urinary miRNAs can be derived from glomerular ultra-filtrate and/or excreted by the tubules in response to injury (14, 15). The potential of urinary miRNAs as biodosimeters and response markers of radiation injury has not been well explored. Thus, in search of urinary biomarkers of radiation nephropathy, we investigated the dose- and time-dependent response of urinary miRNAs and evaluated the corresponding changes in kidney in a mouse model. A combination of *in vivo* and *in vitro* experiments allowed us to identify and validate candidate miRNA biomarkers with the potential to provide a noninvasive readout of the injury to renal tubular cells. Translational potential of candidate biomarkers was validated using urine samples collected from human leukemia patients who underwent radiation-based conditioning before hematopoietic stem cell transplantation.

MATERIALS AND METHODS

Animal Irradiation Study

The mouse experiments were performed in compliance with the guidelines and protocols approved by the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University (Columbus, OH) under protocol no. 2011A00000029. All irradiations were performed on nonanesthetized C57BL/6 10–12-week-old mice (Jackson Laboratory, Bar Harbor, ME), and nonirradiated animals were used as controls. Multiple TBI regimens were used, with doses of 2, 4, 6 and 8 Gy for evaluation of dose response, 7.7 Gy (LD_{50/30}) for evaluation of time-dependent changes and 2 Gy twice daily (bid) to a total of 12 Gy, which is a more complex yet clinically relevant fractionated regimen. For single-dose TBI, animals were placed in a RadDisk irradiator disk (Braintree Scientific Inc., Braintree, MA) and exposed to various ¹³⁷Cs-gamma-ray doses using Gammacell® 40 Exactor (Best® Theratronics Ltd., Ottawa, Canada) at a dose rate of 94 cGy/min. For fractionated irradiations, the RS2000 Biological Irradiator (Rad Source Technologies Inc., Suwanee, GA) (1 Gy/min, 165 kV X rays) was used. Urine was collected by placing each animal in a clean cage on a stainless-steel screen, where it was allowed to roam freely and spontaneously urinate; urine was then collected. The volume obtained from an individual animal in a 2-h time period varied from 0–300 µl. For each time point, 0.7–1 ml of pooled urine from animals were centrifuged twice at 5,000 rpm (2,300g) for 10 min, refrigerated with urine preservative (Norgen Biotek Corp., Thorold, Canada) until used for RNA isolation.

Human Patient Samples

Urine specimens were obtained from acute lymphoblastic leukemia (ALL)/acute myeloid leukemia (AML) patients under an approved clinical trial (no. 2014C0014) at The Ohio State University James Cancer Hospital. Patients gave written informed consent and urine was collected at baseline (one day prior to radiotherapy), during fractionated radiotherapy (2 Gy bid for 3 days) and after completion of radiotherapy. Urine was transferred to 50-ml tubes (Norgen Biotek) with RNAase inhibitors, centrifuged for 5 min at 4,300 rpm (1,700g), aliquoted frozen at –70°C for RNA downstream analysis.

Urinary miRNA Expression Profiling and miRNA Extraction

The relative abundance of miRNAs present in cell-free urine from control and irradiated animals was compared using an amplification-free hybridization-based direct digital multiplexed nCounter® miRNA expression assay (nanoString Technologies Inc., Seattle, WA) (16). This assay is capable of detecting approximately 600 mouse-specific miRNAs. A mixture of three synthetic spike-in oligonucleotides (Osa-miRNA-414, Ath-miRNA-159a and Cel-miRNA-39) was used for initial volume normalization. Urinary miRNA was extracted by processing a 400-µl urine sample using microRNA Purification Kit (miRNeasy Mini Kit, QIAGEN®, Valencia, CA) and following manufacturer's instructions. Synthetic oligonucleotides (4–20 pg; spike-in-oligos) were added in the initial lysis incubation step. RNA was eluted in 100 µl of RNAse-free water and concentrated to 20 µl (Amicon® Ultra Centrifugal Filters, EMD Millipore, Billerica, MA), of which 3 µl was used for nanoString miRNA expression profiling.

qPCR

qPCR was performed according to manufacturer protocol using the 7900HT System (Applied Biosystems®, Carlsbad, CA). TaqMan® probes (Thermo Fisher Scientific™ Inc., Rockford, IL) were used, as follows: mmu-miR-1224, assay ID: 240985_mat; hsa-miR-1224-3p, assay ID: 002752; hsa-miR-21, assay ID: 000397; IL6, assay ID: Mm00446190_m1; Hs00174131_m1. snoRNAs (snoRNA135, assay ID: 001230 and snoRNA234, assay ID: 001234) were used as endogenous controls in the case of mouse primary tubular epithelial cells (PTEC). RNU38B (assay ID: 001004) and RNU43 (assay ID: 001095) were used as endogenous controls in the case of human renal proximal tubular epithelial cells (HRPTEC).

In Situ Hybridization of Kidney Sections

Mice were euthanized 6 h after 6 Gy TBI. Kidneys were kept in 10% neutral buffered formalin for 24–48 h and then transferred into 70% ethanol. The kidney samples were cut at horizontal and vertical directions to make different slides. To examine target microRNAs in irradiated kidney sections, *in situ* hybridization was performed according to the previously described protocol (17) using locked nucleic acid (LNA™)-5' DIG labeled probes (Exiqon, Vedbaek, Denmark).

Mouse Primary Tubular Epithelial Cells and Human Renal Proximal Tubular Epithelial Cells

Mouse primary tubular epithelial cells were isolated according to the previously described protocol (18). HRPTEC cryovial was purchased from Lonza Group Ltd., Basel, Switzerland.

Immunostainings

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by permeabilization in 0.5% Triton™ X-100 or 0.5% Tween® 20, depending on the localization of antigen, for 10 min at room temperature. Cells were blocked for 30 min in 2% bovine serum albumin (BSA) and incubated overnight at 4°C with diluted antibodies, 1:200, ZO-1 (Thermo Fisher Scientific), 1:200, Aquaporin1 (Abcam®, Cambridge, MA). The following day, cells were washed with PBS and secondary antibodies (Thermo Fisher Scientific) were applied at 1:200 (2% BSA) and incubated for 1 h. Thereafter, cells were washed and were mounted with ProLongt Gold antifade reagent with DAPI (Thermo Fisher Scientific). Cells were visualized under a microscope (Leica Biosystems Inc., Lincolnshire, IL) and 10× and 20× pictures were taken.

Exosome Isolation

Animal urine (2 ml) was collected in preservative-containing tubes and centrifuged at 5,000 rpm (2,300g) to get rid of exfoliated cells and debris. The supernatant was then centrifuged again at 13,600 rpm (16,200g). Supernatant was collected in a fresh tube, where the pellet was resuspended in isolation solution (250 mM sucrose, 10 mM triethanolamine). DTT (200 mg/ml, Roche, Indianapolis, IN) was added to the resuspension solution and incubated at 37°C for 10 min with vortexing every 2 min. The addition of DTT released the exosomes trapped by Tamm-Horsfall protein (THP). The resuspension solution containing DTT was centrifuged at 13,600 rpm (16,200g) for 10 min. The supernatant was pooled with the

previous supernatant and was subsequently filtered through 0.22- μ m filter columns. The filtered supernatant was subjected to ultracentrifugation (Sorvall™ Rotor SW-28) at 100,000g for 1 h at 4°C. The pellet was washed with ice-cold PBS and ultracentrifuged again at 100,000g for 1 h. The pellet was resuspended in 220 μ l of PBS, of which 20 μ l was provided for NanoSight analysis. The remaining 200 μ l was used for miRNA isolation by miRNeasy Mini Kit (QIAGEN).

Urine Creatinine ELISA

Creatinine ELISA was performed from 1:10 diluted urine according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI)

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software (La Jolla, CA). Three independent experiments were carried out for each study. For the urine experiments, urine was pooled from five animals for miRNA profiling by NanoString nCounter, whereas in the exosome purification experiments, urine was pooled from 10 animals. One-way analysis of variance (ANOVA) was applied for more than two groups followed by Dunnett's test. Urine specimens from three TBI patients were used to validate our mouse findings, therefore, statistical requirements were not met for clinical data analysis. The results from *in vitro* experiments are expressed as the mean $\pm$ SE and analyzed with the two-tailed Student's *t* test.

RESULTS

Dose and Time-Dependent Changes in Urinary miRNAs after Irradiation

To study the kinetics of radiation response, nanoString nCounter profiling was performed on cell-free urine miRNAs extracted at 0, 3, 6, 24 and 48 h, and 7 days after single-dose irradiation with 7.7 Gy (LD_{50/30}) (Fig. 1A). Early changes in miRNAs were detected within 3 h postirradiation (urine collection: 2–4 h), peaking at 6 h (urine collection: 5–7 h) and gradually declining to near basal level at later time points. Dose response of several of the urinary miRNAs peaked at 6 h, as made evident by incremental changes in animals irradiated with 2, 4, 6 and 8 Gy (Table 1). Later changes were detected in few miRNAs responding on or after 24 h. To further validate the response in the setting of therapeutic radiation, we compared changes in urinary miRNAs in animals exposed to fractionated doses, a regimen that is frequently used clinically in leukemia patients (Fig. 1B). Similar patterns of urinary miRNAs changes were observed. Interestingly, in the case of single-dose response, 30 urinary miRNAs peaked at 3–6 h postirradiation, qualifying as early responders, and eight urinary miRNAs peaked at 24–48 h postirradiation, qualifying as late responders (Fig. 2A). However, with the fractionated doses, 19 urinary miRNAs peaked early and 16 urinary miRNAs peaked at later time points. Furthermore, we identified 12 urinary miRNAs with significant expression that were acutely responsive to both irradiation protocols, as well as seven urinary miRNAs that peaked at later time points (Fig. 2A and B).

The selection of potential biomarkers was further narrowed down, based on the following criteria: 1. high counts of the miRNA, measured by NanoString nCounter assay; 2.

progressively increasing counts with dose or time; 3. previously shown to be a part of inflammatory or fibrotic pathways; and 4. evolutionarily conserved. Of all the rodent miRNAs identified, miR-1224, conserved in humans, exhibited linearity in dose response from 2–8 Gy (Table 1, left side) and fulfilled every criterion, qualifying for a radiation biosimulator and potential early indicator of kidney response. In addition, at 6 h postirradiation, miR-1224 exhibited progressive-fold increases of 1.5-, >3- and >6-fold (after 2, 4 and 6 Gy, respectively) and >10-fold after 8 Gy (Table 1, right side). In this proof-of-concept study, miR-1224 was chosen for investigation as an early biomarker, and miR-21, which has been shown to be a driver of inflammation and fibrosis (19, 20), was chosen as a control late responder and additional positive control (21, 22).

Early-Responding Urinary miR-1224 is Highly Expressed in Tubular Epithelial Cells

The molecular composition of urine is usually reflective of renal functional changes, correlating with the specific responses by renal cells. Therefore, to compare the expression of urinary miR-1224 at the sub-structural level, an *in situ* hybridization technique was applied. The whole kidney sections from control and 6 Gy irradiated mice (at 6 h postirradiation, which showed the highest response in urine) were used for hybridization with digoxigenin-labeled LNA-modified probes. miR-1224 was found to be expressed in the outer medulla, representing the expression in tubular cells (Fig. 3A), which increased further after irradiation. Since the outer medulla is composed mainly of proximal tubular cells (23), we then isolated kidney PTEC *in vitro* and irradiated them at 6 Gy. As was observed in animal urine after TBI, a highly significant increase (~40 fold) in intracellular miR-1224 levels was detected at 6 h postirradiation (Fig. 3B and C). The PTEC were characterized and validated for aquaporin1 (24), a water channel mainly expressed in apical membrane of proximal tubules, and for epithelial markers such as ZO-1 (Fig. 3B). Primary renal fibroblasts were also isolated to validate the specificity of source of origin, and we found no significant changes in miR-1224 levels after irradiation (data not shown). qPCR for pro-inflammatory cytokine IL6 was also performed to recapitulate the established link between immune response and miR-1224 (25, 26). We observed an increasing trend in IL6 mRNA expression at 6 h postirradiation (Fig. 3D). Overall, the *in vitro* and *in situ* data showed an increase in miR-1224 levels in renal tubular cells after irradiation and this increase in miR-1224 levels could potentially be a readout or a driver of immune response activation, subsequently leading to progressive nephropathy.

Non-Exosomal Secretion of miR-1224

The possible mechanisms of secretion of extracellular miRNAs are either associated with protein such as Ago complexes (27) or high-density lipoproteins (HDLs) (28) or packaged inside the exosomes. All three of these modes of secretion protect extracellular miRNAs from degradation and maintain their stability. We investigated the mechanism of release of miR-1224 by comparing the urinary exosomal versus total urinary miRNAs at different time points after irradiation. One-half of the urine was directly processed for extraction of total miRNAs and the other half was used for exosome purification via ultracentrifugation. The comparison of exosome concentration in urine collected from nonirradiated and irradiated mice by NanoSight technology did not show major differences (Fig. 4A). After purification, the exosomal miRNA content was validated by RNA gel analysis (Fig. 4B) followed by

evaluation of changes in miR-1224. In addition to normalization to exogenous controls, urinary creatinine levels were also measured to eliminate the urine concentration factor (29, 30). We observed no significant change in expression levels of miR-1224 in urine exosomes after irradiation (Fig. 4C), however, we did detect a ~3.5-fold increase of miR-1224 in whole urine RNA at 6 h postirradiation (Fig. 4D). Additionally, we investigated the expression of miR-1224 in exosomes isolated from the conditioned media of mouse PTEC, however, no significant changes were observed after irradiation (data not shown). Taken together, the data indicate that the secretion of miR-1224 is nonexosomal, potentially via other mechanisms which needs to be further defined.

Validation of Candidate Radio-Responsive miRNAs in HRPTEC and Radio-Conditioned Human Patient Urine

To investigate the translational potential of rodent miR-1224 that was found responsive in mouse PTEC and urine after single dose and clinically relevant fractionated doses, we cross-validated the radiation response in HRPTEC. After HRPTEC were irradiated and the time course response of hsa-miR-1224-3p was analyzed by qPCR. Consistent with the data obtained from mouse PTEC, intracellular hsa-miR-1224-3p was found significantly upregulated at 6 h postirradiation (Fig. 5A), indicating a conserved mechanism of radiation response. In agreement with the mouse data, a trend towards the increased IL6 expression was observed, pointing to the link between immune response and the miR-1224-3p (Fig. 5B).

We next extended our studies to urine samples from ALL/AML patients who received TBI as a preparative regimen prior to stem cell transplantation. Urinary miRNA was purified from three patients collected at the following times: 1. prior to TBI; 2. during TBI (two time points during a 3-day period); and 3. after completion of TBI (Fig. 5C). The yield, composition and cell-free nature (absence of large ribosomal RNA) of the isolated urinary RNA were analyzed using a bioanalyzer (Fig. 5D). Consistent with the data obtained from mouse urine, qPCR showed early response of miR-1224-3p, whereas the response of miR-21 persisted (Fig. 5E and F), indicating dose response and translational value of miRNA biomarkers in TBI patients.

DISCUSSION

Nuclear reactor accidents or terrorist attacks could expose victims to substantial doses of ionizing radiation. Depending on the absorbed dose and the geography of exposure, the intensity and onset/latency of acute radiation syndromes (ARS) and late effects will vary. Current approaches for biodosimetry primarily rely on clinical symptoms and sensitivity of bone marrow and blood cells to radiation-induced DNA damage and cell killing (lymphocyte depletion kinetics, and the dicentric chromosome assay) (31, 32). Evaluation of the extent of radiation-induced damage, especially to late-responding organs such as the kidney, continues to be a major challenge. Currently, evaluation of glomerular filtration rate (GFR) is the gold standard for measuring renal function, whereas KIM1 and NGAL are the available tubular injury markers (33). Although these measures undoubtedly have utility in identifying patients with renal disease, it is difficult to predict individual patient outcome from exposure

to radiation. Previously published studies have shown diagnostic and predictive value of urine-based miRNA biomarkers (34, 35), because they carry the molecular signature of the organ of origin that allows noninvasive evaluation response in a fairly controlled manner. For instance, in a cohort study of 62 patients with acute renal transplant rejection, urinary miR-10b and miR-210 were found to be downregulated and miR-10a upregulated in these patients compared to that in controls (36). In another study, a panel of six urinary miRNAs were developed (miR-16, miR-200c, miR-205, miR-21, miR-221 and miR-34a), which were capable of detecting recurrence of urothelial carcinoma of the bladder (37).

In our mouse model study, a number of miRNAs exhibiting dose response were identified, providing proof-of-concept for direct noninvasive evaluation of kidney injury response molecules. After single dose (relevant to biodosimetry for triage in radiation accidents) and fractionated doses (relevant for clinical biodosimetry), urinary miR-1224, miR-804, miR-709, miR-1196, miR-155, miR-540-3p, miR-466, miR-467f, miR-3473, miR-1943, miR-2133 and miR-2132 peaked at early time points (e.g., 6 h after single dose). Response of miR-21, miR-200b, let7d, let7a, miR-1944, miR-16 and miR-22 peaked at 24–48 h postirradiation, while molecules such as miR-378 that are also abundant in urine did not show significant alteration indicating specificity of response. A set of criteria to select miRNAs that hold diagnostic potential led to an identification of miR-1224 as an early responder and miR-21 as a late responder. Early-responding miRNAs likely provide direct readout of injury in a tissue, whereas the late-responding miRNAs are likely reflective of cascade of responsive pathways that are activated as a function of radiation exposure. In an earlier mouse model study performed in the context of bilateral renal ischemia, concurrent elevation of miR-714, miR-1188, miR-1897-3p, miR-877* and miR-1224 in kidneys at 3, 6 and 24 h after acute kidney injury was reported, whereas miR-21 was found upregulated in kidney at 24 h (22). In line with this notion, our *in situ* hybridization and *in vitro* studies have shown proximal tubular origin of miR-1224 and subsequently, high fold increase in urine after irradiation. Conserved expression pattern of hsa-miR-1224-3p and hsa-miR-21 response to radiation in ALL/AML patients demonstrates its translational utility as noninvasive detection of these two miRNAs in urine released from a sensitive subset of tubular epithelial cells in kidney, which is widely considered to be a resistant and late-responding organ.

The large fold change and the early response are not typically associated with DNA damage response and the activation of cell cycle checkpoints that are among the results of radiation injury in the nucleus. On the other hand, such changes could be interpreted as readouts of immune response to tubular injury as a cause or effect and/or contributing to the progressive late changes. miR-1224 is known to be responsive to inflammatory cues, as shown in a study where induction of miR-1224 expression was observed at 6 h after exposure to bacterial endotoxin lipopolysaccharide in mouse macrophage-like RAW264.7 cell lines (26), further supporting it as an immune response marker. It was also shown earlier that miR-1224 can bind to the 3'UTR of SP1 causing its targeted degradation and downregulating the expression of TNF- α at the transcriptional level (26). Thus, radiation-induced increase in intracellular and release in urinary miR-1224 could be a mechanism that modulates acute inflammatory response, potentially via IL6. Absence of significant alternation of exosomal fraction of miR-1224 after irradiation indicates nonexosomal release of miR-1224 by tubular

cells, complexed with either Ago2 or lipoproteins. Moreover, there is accumulating evidence that the majority of miRNAs are rather bound to RNA binding proteins than found inside exosomes. It is also likely that cell membrane-associated receptors or channels allow the specific release of these Ago2-miRNA complexes.

The caveats in using urinary biomarkers for diagnostic studies include the lack of stringent internal controls other than creatinine, which is not a direct measure of renal function, especially after acute inflammation. The short-time frame of response, especially after acute single-dose irradiation, is an impediment since samples need to be collected at the right time point. Moreover, similar kinetics were shown in earlier studies exploring radiation biodosimetry potential of urinary metabolites, where 6–12 h postirradiation was found to be the time frame of highest response (38).

ACKNOWLEDGMENTS

This work was supported in part with the intramural funds from the Department of Radiation Oncology at Arthur G. James Cancer Hospital, The Ohio State University Comprehensive Cancer Center and Defense for Health Affairs through the Peer Reviewed Medical Research Program (award no. W81XWH-15-2-0054). We thank Dr. Jerry Nuovo of Phylogeny, Inc. (Powell, OH) for technical assistance.

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FIG. 1.

Identification of differentially expressed cell-free urinary miRNAs after acute single-dose and fractionated doses in C57BL/6 mice. Panels A and B: Heat map generated from the normalized counts of cell-free urinary miRNAs detected by NanoString nCounter system after acute single dose and fractionated doses, respectively. The schematics of dose time are shown for each irradiation protocol. All time points are in reference to the first fraction. Each miRNA count is normalized to spike-in oligos (Osa-miRNA-414, Ath-miRNA-159a and Cel-miRNA-39). Red indicates high expression of miRNA and green indicates relatively low expression of miRNA. Urine from five animals was pooled.

FIG. 2.

Pattern of urinary miRNAs modulated in both single dose and fractionated doses. Panel A: Venn diagram showing early and late responsive urinary miRNAs with single dose and fractionated doses, respectively. Early-responsive miRNAs are categorized into the 3–6 h time period, whereas late-responsive miRNAs are categorized into the 24–48 h time period. Area of overlap shows the common miRNAs responsive to both irradiation protocols. Panel B: Identification of common urinary miRNAs that are responsive in similar fashion in both irradiation protocols, categorized as early- and late-responsive miRNAs.

FIG. 3. Determination of increased level of intracellular miR-1224 in mouse primary tubular epithelial cells (PTEC) after irradiation. Panel A: *In situ* hybridization for miR-1224 in irradiated kidney sections compared to scrambled control. High-resolution image shows the distribution of miR-1224 in a longitudinal section of kidney from an irradiated mouse. Panel B: Characterization of mouse PTEC, by immunostaining with aquaporin-1 (AQP1) and ZO-1, original magnification, 20 $\times$ and 10 $\times$, respectively. Scale: 50 μ m. Panels C and D: Representative qPCR of miR-1224 and IL6 performed at 6 h after 6 Gy irradiated PTEC normalized to endogenous controls (snoRNA135 and snoRNA234 in case of miR-1224) and (GPADH and HPRT in case of IL6); n = 3 (independent experiments). * $P < 0.05$.

FIG. 4. Nonexosomal release of miR-1224 in urine. Panel A: NanoSight data indicating the size distribution and the concentration of urinary exosomes from nonirradiated and irradiated mice, respectively. Panel B: Small-RNA gel analysis for validating presence of exosomal miRNA isolated from urine. Panel C: qPCR data showing miR-1224 fold change in exosomes; $n = 3$ (independent experiments). Panel D: qPCR data showing miR-1224 fold change in urine at different time points after TBI; urine pooled from 10 animals, $n = 3$ (independent experiments). * $P < 0.05$.

FIG. 5.

Radiation response of hsa-miR-1224-3p and hsa-miR-21 in human tubular epithelial cells and in a cohort of TBI patients. Panel A: qPCR validation of miR-1224-3p at 6 h after 6 Gy irradiated human renal proximal tubular epithelial cells (HRPTEC) normalized to endogenous controls RNU38 and RNU43; $n = 3$, (independent experiments), $**P < 0.005$. Panel B: Representative qPCR of proinflammatory cytokine IL6 at 6 h after 6 Gy irradiated HRPTEC normalized to GAPDH and HRPT. Panel C: Schematic representation of TBI given to ALL/AML, followed by bone marrow transplant (BMT) in high-risk patients. Panel D: Pico gel showing the quality of miRNA isolated from urine. Panel E: qPCR data of miR-1224-3p from urine of TBI patients, collected at different times postirradiation; $n = 3$ for TBI. Panel F: qPCR data of miR-21 from urine of TBI patients, collected at different times postirradiation; $n = 3$ for TBI.

TABLE 1

Dose Response of Urinary miRNAs with Biodosimetry Potential

miR	NanoString counts in 60 µl mouse urine ^a					Fold change ^b		
	Control	2 Gy	4 Gy	6 Gy	8 Gy	2 Gy	4 Gy	6 Gy
miR-1224 ^c	1,425	1,943	5,140	10,601	17,851	1.36	3.61	7.44
miR-804	225	376	1,399	3,982	6,226	1.67	6.22	17.70
miR-2132	172	255	1,001	2,614	5,176	1.48	5.82	15.20
miR-378 ^c	2,978	1,878	7,241	6,366	4,405	0.63	2.43	2.14
miR-2133	223	283	937	1,895	3,545	1.27	4.20	8.50
miR-2137	299	330	369	1,339	2,265	1.10	1.23	4.48
miR-709	206	173	656	1,030	1,460	0.84	3.18	5.00
miR-2146	16	115	211	580	1,241	7.19	13.19	36.25
miR-762 ^c	304	325	1,165	1,213	922	1.07	3.83	3.99
miR-714	78	73	293	483	848	0.94	3.76	6.19
miR-466g ^c	172	149	422	748	800	0.87	2.45	4.35
miR-467f ^c	31	79	252	553	615	2.55	8.13	17.84
miR-2183	125	197	334	589	575	1.58	2.67	4.71
miR-1196	110	100	252	515	552	0.91	2.29	4.68
miR-2861 ^c	82	222	363	468	521	2.71	4.43	5.71
miR-1937a+b	210	131	615	171	507	0.62	2.93	0.81
miR-486 ^c	22	28	59	283	378	1.27	2.68	12.86
miR-540-3p	45	84	193	344	319	1.87	4.29	7.64
miR-3473	49	40	123	247	282	0.82	2.51	5.04
let-7 ^c	101	75	363	147	276	0.74	3.59	1.46
miR-489 ^c	43	163	105	233	273	3.79	2.44	5.42
miR-297 ^c	206	145	351	336	253	0.70	1.70	1.63
miR-1198	45	372	100	215	250	8.27	2.22	4.78
miR-16 ^c	74	96	158	174	216	1.30	2.14	2.35

miR	NanoString counts in 60 µl mouse urine ^a					Fold change ^b		
	Control	2 Gy	4 Gy	6 Gy	8 Gy	2 Gy	4 Gy	8 Gy
miR-22 ^c	123	105	100	100	196	0.85	0.81	1.59
miR-338-5p ^c	96	93	287	180	174	0.97	2.99	1.88
let-7a ^c	47	66	164	174	171	1.40	3.49	3.64
let-7b ^c	78	58	100	144	168	0.74	1.28	1.85
miR-710	42	342	117	150	159	8.14	2.79	3.57
miR-200b ^c	34	66	82	71	134	1.94	2.41	2.09
miR-30b ^c	76	87	117	127	134	1.14	1.54	1.67
let-7d ^c	29	61	199	141	125	2.10	6.86	4.86
miR-21 ^c	36	58	111	74	114	1.61	3.08	2.06
miR-689	42	143	100	80	114	3.40	2.38	1.90
miR-376a ^c	76	63	234	174	111	0.83	3.08	2.29
miR-145 ^c	94	73	105	100	74	0.78	1.12	1.06

^aList of dose-dependent changes in urinary miRNAs ranked in order of abundance in counts as well as in their progressive incremental increase 6 h after 2, 4, 6 and 8 Gy irradiation.

^bRepresentation of counts as a fold change in miRNAs in reference to controls 6 h postirradiation.

^cConserved (similar ID) in humans.