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14. ABSTRACT

The study during the reporting period has used blood samples from leukemia patients who received therapeutic radiation for discovery of biomarkers. This could be used for exposure estimation in soldiers and emergency responders involved in nuclear war. The method for discovery and validation of biomarkers was optimized using samples collected from patients enrolled in two clinical trials. The utility of selected biomarkers identified from our earlier animal models has been validated and identified as responders in human samples. Biomarkers identified and/or validated include miRNA-150 and miRNA-574-5p that alters as a function of dose and time. The levels of circulating miR-150 was found reduced in a dose and time dependent manner, while that of miRNA-574 was found increasing after radiation in dose and time dependent manner. Additional putative biomarkers exhibiting dose-time response have been identified, which need validation. The findings will likely have significant impact on Nation's Radiation Biodosimetry Program and the preparedness for emergency responses in radiological events.

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1. INTRODUCTION

The topic of the study is "**Illnesses Related to Radiation Exposure".** The focus of the work is to develop circulating miRNAs as radiation biodosimeters for rapid triage in radiological events. The warfighters and military personnel deployed to the field as emergency responders in the aftermath of radiological events will need to be screened for radiation exposure, potentially leading to Acute Radiation Syndromes (ARS) and Delayed Effects of Acute Radiation Exposure (DEARE). The scope of the project is to discover and validate blood microRNAs as biomarkers of radiation injury, specific to major affected organs such as bone marrow and lung, with dose and time response. The study uses blood from human patients who received radiation therapy and rodent models to evaluate the exposure level and the extent of radiation injury using minimally invasive assay. The outcome of the study has significant immediate impact on Nation's Radiation Biodosimetry Program and the preparedness for emergency diagnosis in radiological events. The scope of the study further extends to the area of therapeutic radiation oncology, where the treatment associated normal tissue toxicity is significant concern and the biomarkers developed from the current study could help therapy guiding as overdosing and under dosing are of major concern because of underlying conditions and inherent variations.

2. KEYWORDS

Radiation Biodosimetry, Radiation Biomarkers, microRNA, Acute Radiation Syndromes, Delayed Effects of Acute Radiation Exposure.

3. ACCOMPLISHMENTS

What were the major goals of the project?

Goal 1: Profiling circulating miRNAs in patients who received total body irradiation and chemotherapy based myeloablation

Goal 2: Investigate the clinical utility of miRNA Biodosimetry -early time points from TBI pts

Milestone 1: Assay for the discovery and development of circulating miRNAs as biomarkers/dosimeters optimized in human patient serum samples- Completed (07/2016)

Milestone 2: Discovery and validation of 5-10 conserved miRNAs altered as a function of radiation dose and time in pts sera –60% completed.

Milestone 3: Biodosimetry utility of miR-150 as a readout of bone marrow depression and reconstitution validated in human pts samples- 80% completed

Goad 3: Test biomarkers in tissue vs plasma in GT3 treated animals after Total Body Irradiation (TBI) and Partial Body irradiation (PBI) - Planned for Year 2-3

Goal 4: Evaluate dose and time effect of miRNAs in UBI, LBI, WTLI and RHLI mice irradiation models - Planned for year 2-3

What was accomplished under these goals?

Major activities:

1. Regulatory approval for using human specimen and animal subjects for research
2. Discovery of circulating miRNAs that are altered as a function of radiation dose and time using body fluids collected from patients enrolled in two clinical trials

Specific Objectives

1. Procurement of bio-specimens from two clinical trials.
2. Optimization of miRNA assays in body fluids
3. Discovery of biomarkers by NanoString nCounter assays and validation by qRT-PCR

Regulatory Protocol and Activity Status:

Protocol [HRPO Assigned Number]: A-18889
Title:" *miRNA biomarkers of acute and delayed radiation toxicities*
Date of Approval: 01/16/2016

Protocol [ACURO Assigned Number]: PR140885.01
Title: Minimally Invasive Radiation Biodosimetry and Evaluation of Organ Responses
Date of Approval: 11/25/2016

Protocol [ACURO Assigned Number]: *PR140885.02*
Title: "Understanding Radiation Health Effects and Developing Countermeasures (Renewal 1)
Date of Approval: 02/01/2016

Significant results

1. Optimized the assays for discovery of biomarkers (circulating miRNAs) exhibiting progressive changes in human patients after myeloablation and bone marrow transplant.

We have optimized a method for microRNA profiling in serum and plasma collected from human patients. After initial optimization in serum from mouse and non-human primates, we have analyzed serum samples from 5 patients collected at 7 time points under approved protocol. In the initial experiments 200ul serum was used for extraction using Qiagen miRNeasy kit. After lysis, three synthetic oligonucleotides (spike-in oligos) were added for volume normalization. Purified RNA was eluted in 100µL water and concentrated to 20µL Amicon column (3kDa cut off). The digital multiplexed NanoString nCounter miRNA expression assay was performed with RNA present in 3µL (10–20ng cell free RNA). An amplification-free, hybridization based direct digital counting method developed by NanoString Technologies was used for the evaluation of changes in circulating miRNAs level. Since the counts detected for several of the conserved miRNAs were low in total RNA isolated and concentrated from 200ul of serum or plasma, we have scaled up the sample volume to 400ul. In order to achieve better sensitivity, multiple extractions were pooled and samples were enriched by concentrating to 20ul prior to Nanostring assay and validation by qPCR assay. These changes in isolation protocol have significantly improved the detection. We continue working on improving the sensitivity of detection by improving the sample processing procedures.

For discovery, the human miRNA nCounter chip containing probes for 800 miRNAs was used and over 43 serum detectable markers were identified as major responders (Table 1) (two way ANOVA) to radiation and compared for the time dependent changes in miRNAs between baseline and 4 wk, 14 wk and 24 wk after the completion of 2x6 = 12Gy radiation treatment. Selected responders with acceptable p-Value (0.05) on at least one of the time points tested during the reconstitution of marrow (with reference to baseline are highlighted).

Table 1:

Serum miRNA	Wk-4 vs baseline		Wk-14 vs baseline		24 Wk vs baseline	
	Ratio	P-value	Ratio	P-value	Ratio	P-value
hsa-let-7b-5p	1.49	0.52	1.41	0.58	1.31	0.67
hsa-miR-17-5p	1.13	0.88	0.74	0.71	1.47	0.63
hsa-miR-122-5p	2.05	0.31	2.42	0.21	1.03	0.97
hsa-miR-1246	2.53	0.14	1.75	0.36	1.97	0.27
hsa-miR-126-3p	2.16	0.45	1.42	0.73	0.48	0.47
hsa-miR-1283	0.94	0.96	0.17	0.12	0.82	0.86
hsa-miR-1285-5p	2.77	0.2	0.98	0.98	2.05	0.37
hsa-miR-1305	3.69	0.04	0.69	0.54	4.66	0.02
hsa-miR-130a-3p	1.8	0.42	0.93	0.92	0.44	0.26
hsa-miR-142-3p	4.02	0.12	2.11	0.4	0.79	0.79
hsa-miR-143-3p	3.83	0.05	1.87	0.34	3.13	0.09
hsa-miR-144-3p	1.02	0.98	1.34	0.73	0.98	0.98
hsa-miR-15b-5p	2.83	0.24	0.78	0.78	1.02	0.99
hsa-miR-16-5p	3.48	0.23	0.85	0.87	0.88	0.9
hsa-miR-192-5p	1.15	0.81	1.24	0.72	1.7	0.37
hsa-miR-1976	1.89	0.28	0.98	0.97	0.68	0.51
hsa-miR-19b-3p	1.09	0.93	1.03	0.97	0.7	0.7
hsa-miR-20a-5p+	0.76	0.79	0.72	0.75	0.35	0.31
hsa-miR-21-5p	1.53	0.49	0.65	0.49	0.95	0.94
hsa-miR-22-3p	1.21	0.82	0.74	0.72	0.43	0.31
hsa-miR-223-3p	1.97	0.1	1.9	0.12	1.57	0.27
hsa-miR-23a-3p	1.58	0.28	1.57	0.29	1.11	0.8
hsa-miR-25-3p	1.11	0.92	1.3	0.8	0.37	0.35
hsa-miR-26a-5p	1.56	0.54	0.56	0.43	0.45	0.28
hsa-miR-302d-3p	1.48	0.63	1.52	0.6	1.75	0.49
hsa-miR-3065-5p	0.91	0.66	0.89	0.56	0.95	0.81
hsa-miR-30d-5p	1.27	0.78	0.86	0.86	0.94	0.94
hsa-miR-30e-5p	1.65	0.55	0.74	0.72	0.35	0.21
hsa-miR-320e	1	0.99	1.1	0.81	1.14	0.73
hsa-miR-33a-5p	0.88	0.22	1.03	0.81	1	0.99
hsa-miR-363-3p	4.15	0.05	3.94	0.06	2.66	0.18
hsa-miR-378e	2.66	0.3	8.71	0.03	5.72	0.07
hsa-miR-423-5p	2.05	0.43	0.4	0.32	0.37	0.28
hsa-miR-4443	1.89	0.41	0.92	0.91	2.52	0.23
hsa-miR-4454+	1.38	0.29	1.21	0.52	1.28	0.41
hsa-miR-4516	0.85	0.76	1.12	0.83	2.18	0.15
hsa-miR-451a	1.41	0.59	1.17	0.8	0.66	0.53
hsa-miR-494-3p	1.48	0.71	1.54	0.68	0.51	0.52
hsa-miR-520f-3p	1.05	0.63	0.92	0.37	0.93	0.44
hsa-miR-556-5p	3.32	0.13	1.15	0.85	0.7	0.65
hsa-miR-574-5p	4.74	0.08	0.89	0.89	1.56	0.6
hsa-miR-579-3p	4	0.18	3.47	0.23	13.27	0.02
hsa-miR-93-5p	1.28	0.78	0.44	0.36	0.67	0.66
hsa-miR-93-5p	0.58	0.49	0.83	0.82	0.34	0.19

Responders warrant additional studies, include miR-378e and miR-574-5p, miR-579-3p. On the basal counts for biomarkers such as miR-150-5p and miR-23a (internal normalizer) identified from our earlier animal models showed a lower count. This could be because of inherently low level of expression for those markers in human serum or because of the differences between the two cartridges used for assay (human vs mouse miRNA assay). This prompted us to used additional experiments using a quantitative PCR assay after a pre-amplification of the template.

Screening using early time points was unsuccessful in NanoString assay as the count was either very low or undetectable. The lower count could be correlated with depletion of overall blood molecules following myeloablation (W0) as major fraction of the molecules in blood originate from blood forming cells. Thus, a qPCR based approach was considered when early time point samples were processed (Figure 1).

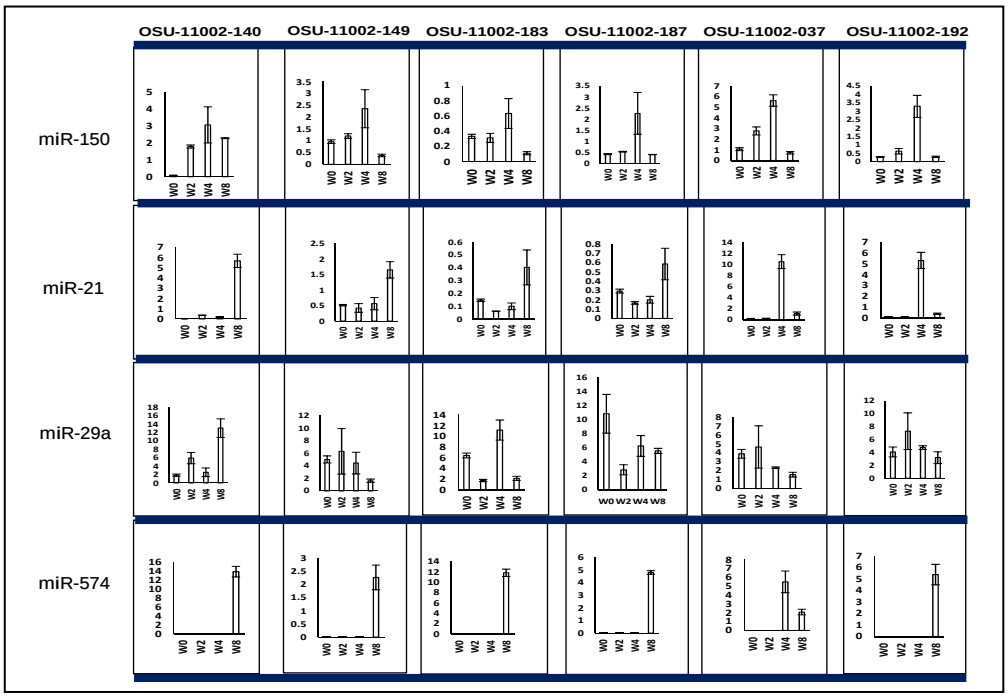
2. PCR based comparison of candidate miRNA biomarkers from human patients received radiation versus chemo based myeloablation

In this experiment we have compared progressive changes in miRNAs purified from human patients who received radiation vs chemo-based myeloablation followed by bone marrow transplantation. A total of 48 samples collected at four time points, Week 0 (day of transplant), Week 2, Week 4 and Week 8 were compared for changes in candidate markers. The assay used a sensitive qRT-PCR based method with pre-amplification of the template to enhance the sensitivity. MicroRNA sample purified from patient' serum were subjected to cDNA synthesis using 'TaqMan Advanced miRNA cDNA synthesis kit' (A28007; Thermo Fischer). The method is based on universal reverse transcription chemistry and uses 3' poly-A tailing and 5' ligation of an adaptor sequence to extend the mature miRNAs present in the sample on each end prior to reverse transcription. Universal RT primers recognized the universal sequences present on both the 5' and 3' extended ends of the mature miRNAs. All mature miRNAs in the sample were reverse transcribed to a full length cDNA. Template thus obtained was further amplified by pre-Amp cycles to achieve the greater sensitivity of the final product. cDNA obtained in this method was used to check the level of candidate molecules such as hsa-miR-150, hsa-miR-21, hsa-miR-29a, hsa-miR-378e, hsa-miR-579 and hsa-miR-574 by TaqMan Advanced miR probes in triplicates on ABI-7900HT system. Threshold cycles were normalized to Ct values of spike-in controls; osa-miR-414 and ath-miR-159a. The expression was calculated using $\Delta\Delta$Ct method.

miR-150-5p, miR-21 and miR-29a exhibited an increasing pattern with time in both Chemo and TBI serum samples. miR-574-5p has shown a different expression tendency between the treatment groups. The expression was undetectable at earlier time points in chemo group and appears after 8 weeks while in TBI group, it was detected at all time points with no noticeable difference in expression. The level of miR-579 and miR-378e was undetectable in both groups at all time points.

We conclude that majority of miRNAs are originated from blood cells and, radiation or chemo based myeloablation dramatically decreases the miRNA signals in early time points. Nonetheless, an increase in miR-150-5p was observed as a function of marrow reconstitution, further supporting the finding that miR-150-5p is a biomarker capable of biodosimetry and could provide a direct readout of the level of functional marrow, and hence could predict hematological ARS. The inclusion of samples collected at later time points also allowed us to identify additional markers potentially connected with multi-organ dysfunction.

Figure 1 (A)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OSU-11002-183
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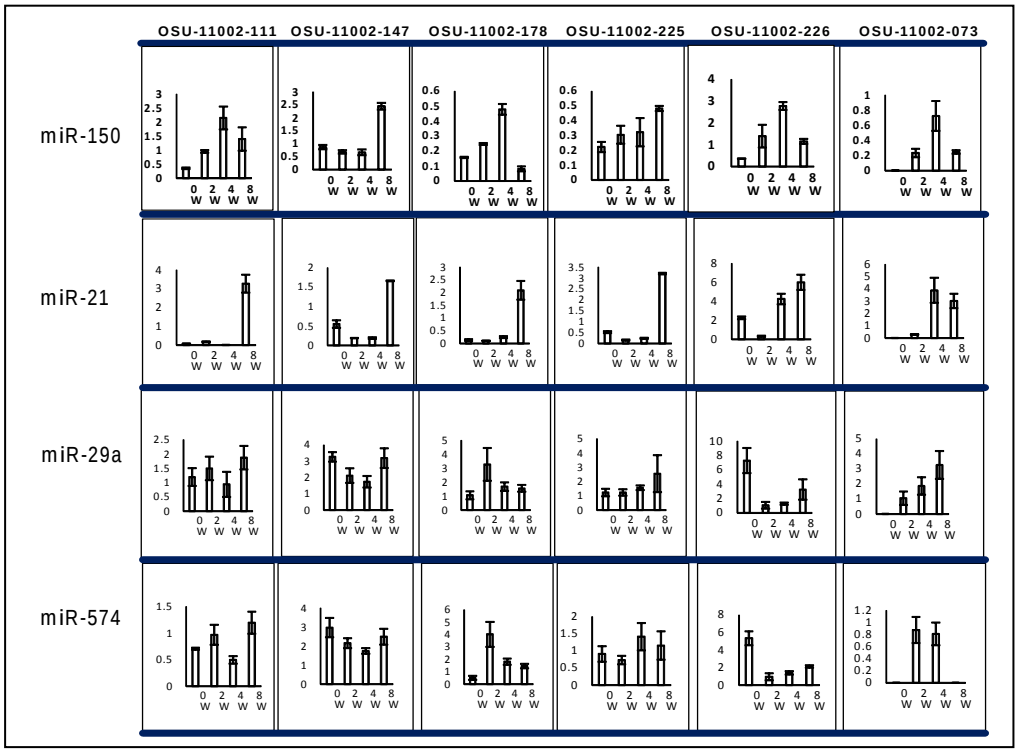
miR-29a
miR-574
W0 W2 W4 W8

(B)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OSU-11002-178
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miR-150
miR-21
miR-29a
miR-574
0 W 2 W 4 W 8 W

(C)

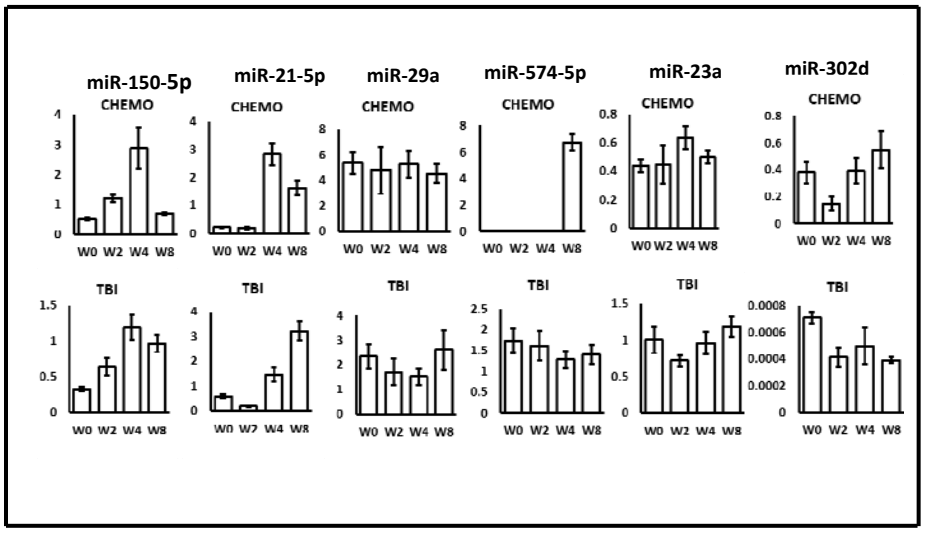

Figure 1: Relative expression of miRs in **(A)** Chemo (N=6) and **(B)** TBI (N=6) patients serum collected at different time intervals (W0, W2, W4 and W8). **(C)** Overall Average of 6 patient's samples in each group. Bar represent Mean ±SD of 6 samples

3. Investigated the dose and time dependent changes in circulating miRNAs at early time points after total body irradiation.

In order to discover and validate biomarkers with biodosimetry potential, serum miRNA purified from five patients collected at baseline, Day- 4 (4 Gy), Day-3 (8 Gy) and Day-1 (12 Gy) were compared by NanoString profiling. The data thus obtained from comparative analysis of miRNAs showed high level expression in serum (Table 2). The acute radio responsive biomarkers identified in human serum exhibited statistically significant changes are miR-574-5p, miR-30d and miR-630. miR-574-5p exhibited a 7.55 fold increase at Day -3 (after completion of 8 Gy) and 6.19 fold increase a day after completion of 12 Gy fractionated radiation. miR-30d and miR-630 expression declined to 0.21 and 0.14 fold respectively at day-1 (12Gy) compared to baseline.

Table 2:

miRNA	D-4 vs baseline		D-3 vs baseline		D-1 vs baseline	
	Ratio	P-value	Ratio	P-value	Ratio	P-value
hsa-let-7b-5p	0.84	0.75	1.6	0.4	0.53	0.26
hsa-miR-106a-5p+hsa-miR-17-5p	0.66	0.56	1.05	0.94	0.49	0.32
hsa-miR-122-5p	1.28	0.69	1.22	0.75	0.4	0.15
hsa-miR-1246	1.04	0.94	0.9	0.84	1.56	0.41
hsa-miR-126-3p	0.47	0.41	0.33	0.24	0.18	0.07
hsa-miR-1283	0.4	0.36	0.81	0.83	0.38	0.33
hsa-miR-1285-5p	2.34	0.23	8.07	0.01	1.86	0.38
hsa-miR-1305	0.71	0.53	1.33	0.59	0.77	0.63
hsa-miR-130a-3p	0.66	0.52	0.65	0.51	0.65	0.51
hsa-miR-142-3p	0.91	0.9	0.6	0.51	1.08	0.92
hsa-miR-143-3p	1.23	0.72	0.85	0.79	1.75	0.33
hsa-miR-144-3p	0.75	0.7	0.55	0.41	0.69	0.61
hsa-miR-15b-5p	1.22	0.8	1.19	0.82	0.79	0.76
hsa-miR-16-5p	0.39	0.31	0.27	0.15	0.28	0.17
hsa-miR-192-5p	0.85	0.76	0.59	0.32	2.38	0.1
hsa-miR-1976	0.85	0.76	0.52	0.21	0.66	0.42
hsa-miR-19b-3p	0.5	0.4	0.84	0.83	0.41	0.29
hsa-miR-20a-5p+	0.23	0.12	0.47	0.41	0.37	0.28

hsa-miR-21-5p	0.68	0.48	0.66	0.46	0.48	0.19
hsa-miR-22-3p	0.25	0.07	0.66	0.58	0.24	0.06
hsa-miR-223-3p	0.92	0.82	1.19	0.63	0.48	0.05
hsa-miR-23a-3p	0.72	0.38	0.88	0.73	0.45	0.04
hsa-miR-25-3p	0.38	0.3	0.45	0.39	0.21	0.1
hsa-miR-26a-5p	0.84	0.79	0.93	0.91	0.39	0.16
hsa-miR-302d-3p	0.75	0.69	0.98	0.98	1.71	0.45
hsa-miR-3065-5p	1.06	0.74	1.29	0.18	1.09	0.64
hsa-miR-30d-5p	0.24	0.07	1.27	0.76	**0.21**	**0.05**
hsa-miR-30e-5p	0.33	0.15	1.56	0.55	0.48	0.33
hsa-miR-320e	0.6	0.15	0.81	0.55	0.26	0
hsa-miR-33a-5p	0.97	0.71	0.97	0.75	1.07	0.49
hsa-miR-363-3p	1.43	0.57	1.97	0.29	2.36	0.18
hsa-miR-378e	0.67	0.62	0.66	0.62	1.58	0.58
hsa-miR-423-5p	0.3	0.14	0.74	0.71	0.32	0.17
hsa-miR-4443	0.83	0.78	1.5	0.55	1.06	0.94
hsa-miR-4454+hsa-miR-7975	0.66	0.12	0.58	0.05	0.42	0
hsa-miR-4516	1.11	0.82	1.61	0.31	0.66	0.37
hsa-miR-451a	0.59	0.35	1	1	0.68	0.5
hsa-miR-494-3p	0.56	0.53	0.55	0.51	0.89	0.9
hsa-miR-520f-3p	1.03	0.75	1	0.99	0.91	0.27
hsa-miR-556-5p	1.76	0.42	0.77	0.71	0.7	0.61
hsa-miR-574-5p	**2.9**	**0.17**	**7.55**	**0.01**	**6.19**	**0.02**
hsa-miR-579-3p	1.15	0.87	1.12	0.89	2.71	0.27
hsa-miR-612	0.96	0.95	1.3	0.7	1.41	0.61
hsa-miR-630	0.46	0.39	0.97	0.97	**0.14**	**0.04**
hsa-miR-6721-5p	0.84	0.8	2.49	0.18	2.05	0.29
hsa-miR-873-3p	0.83	0.77	0.68	0.55	1.33	0.66
hsa-miR-93-5p	0.58	0.49	0.83	0.82	0.34	0.19

4. Validation of miRNAs in an additional samples at early time points

In order to validate the response, we have used samples from a new set of patient samples, including additional time points as shown in figure 4 and the responses were longitudinally followed for individual human patient. Here miR-150 was selected as biodosimetry that showed a dose and time dependent decline in expression level in serum. The expression revives after 30 days of marrow transplantation. This downregulation in miRs level following irradiation is due to the myeloablation of marrow cells which then replenishes once the marrow is reconstituted. We are further validating these samples for new candidates using additional more sensitive assays with a pre-amplification step and in parallel using endogenous controls such as miR-23a and miR-302d and spike-in oligonucleotide based normalization.

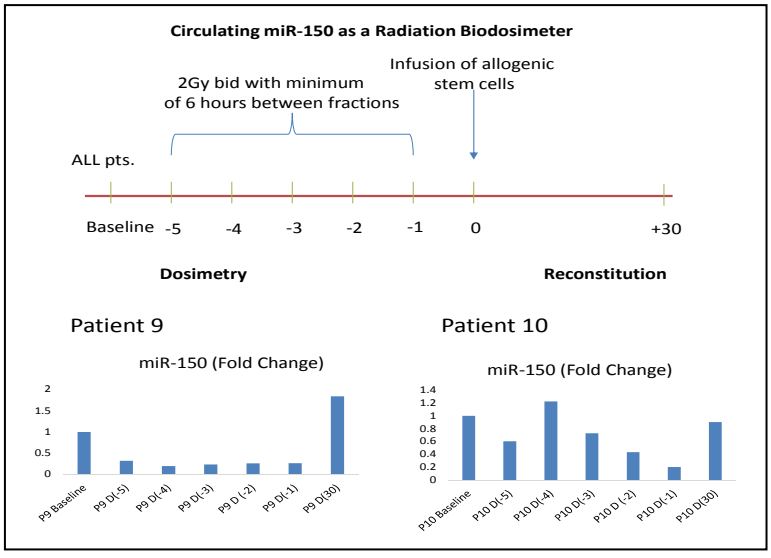

Figure 4: Circulating miR-150 as a biodosimetry. Serum samples were collected at baseline, d-5 to d+30 from clinical trial patients and processed for qPCR for miR-150 detection. Fold change calculated with reference to baseline

Summary: Several candidate biomarkers were identified using a combination of assays involving NanoString based initial screening, validation by direct qRT-PCR or pre-amplification followed by qRT-PCR. miR-150 was identified as a most consistent responder, with a clear dose and time dependent decrease after radiation and increase during the reconstitution of marrow. miR-574-5p was identified as another candidate biomarker that exhibit a several fold upregulation in serum after radiation but express only in later time points after chemotherapy. Currently, we are further testing the expression in relation to those serum markers that do not alter after radiation (such as miR-23a and miR-302d; Figure 1C). We have also tested the potential of several molecules as a normalizer and internal controls but the data so far suggested miR-23a as a potential internal control than miR-302d. Further, the changes due to hemolysis is being evaluated using a ratio of miR-451 and miR-23a. The putative novel biomarkers such as miR-630, miR-579 and miR-30d need additional validation.

5. Validation of selected samples in serum from non-human primates

While radiation is given in fractions to the patient, and samples thus collected are from cancer patients, which is unlikely be the case in nuclear accidents, we did profiling of biomarkers response to radiation doses in non-human primates (NHP). Here we desired to obtain the injury kinetics and immune response that could match to human response. We compared the changes in circulating miRNA response in NHP exposed to total body irradiation at a low (1 Gy), sub-lethal (3 Gy) and a potentially lethal dose (6.5 Gy), evaluated at day 1, 3 and 7, the time points most relevant to triage in a radiological events. The data shows a conserved pattern in changes in miRNAs as found in rodents and human patients after whole body exposure. Circulating miR-150-5p was identified as the most sensitive biomarker for radiation biodosimetry. Also, miR-574-5p showed a robust increase after radiation detectable at 24 hrs after radiation. These data underscore the significance of the results for its potential use as a biodosimetry after radiation accidents (Menon et al, 2016 PLOS One-in revision)

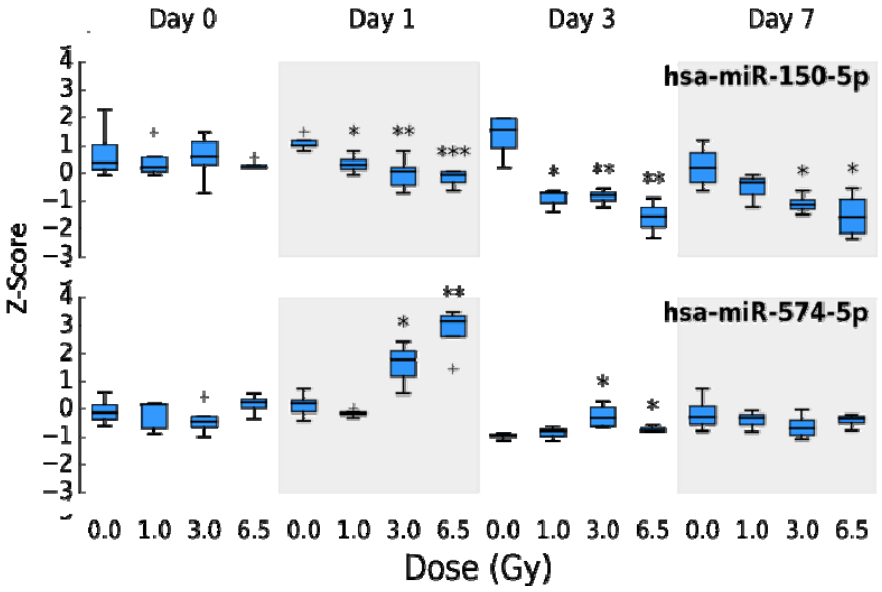

Figure 6: (A) Dose and time dependence of miRNA levels that significantly change (p-value < 0.05) after exposure to ionizing radiation at any dose or time point. From this data we observe that hsa-miR-574-5p and hsa-miR-150-5p show significant dose response to WBI. * = p-value < 0.05, ** = p-value < 0.005, and *** = p-value < 0.0005.

Other achievements:

6. Breeding C57LJ mice for lung irradiation studies: We have generated a cohort of C57LJ mice (in-house breeding). This strain was chosen as it is particularly useful for studying radiation response in lung, as the kinetics of response has been shown to be similar to that in humans. C57LJ strain is recommended for evaluation of mechanisms of action of radiation and efficacy testing of agents that fall under Animal Rule of the FDA (21 CFR Parts 314 and 601). The breeding pairs were purchased from commercial vendor and maintained in-house. The breeding of these animals was extremely challenging as the females of C57LJ mice were hard to get pregnant and mothers were also found not feeding the pubs. To tackle these issues we implemented adding food supplement such as love mash in cages which improved the breeding potential, litter size and the nursing. Thus, so far we have generated over 100 new mice by this strategy. In order to mimic the response comparable to middle aged human, we plan to age animals until 6-9 months prior to irradiation. Nevertheless, we also have optimized partial body irradiation protocol in SARRP for C57L/J mice and C57BL/6 (reference strains).

7. Optimization of partial body irradiation in Cone Beam CT Guided Small Animal Radiation Research Platform (SARRP): A next generation micro irradiator, SARRP (Small Animal Radiation Research Platform) X-ray with real time CT imaging was used for organ targeted irradiation and initial experiments were conducted to optimize the dosimetry and partial body irradiation (Goal 3 and 4). SARRP is a novel state-of-the-art Image Guided Micro-Irradiation (IGM™) system that is capable of delivering multi-directional (focal), kilo-voltage radiation fields to target organs in small animals. It works under robotic control using high accuracy cone-beam CT (CBCT) imaging system and a high dose delivery therapeutic X-ray source in a single platform. CBCT allows excellent accuracy through volumetric imaging and computational registration to reference templates. The treatment plate sits on a motorized three axis platform that can make shifts to a far greater accuracy (~15μm) compared to other image registration. SARRP machine was purchased via NIH S10 instrumentation grant and was commissioned early 2016. So far, we have optimized the conditions for hemi-lung irradiation and whole thorax lung irradiation.

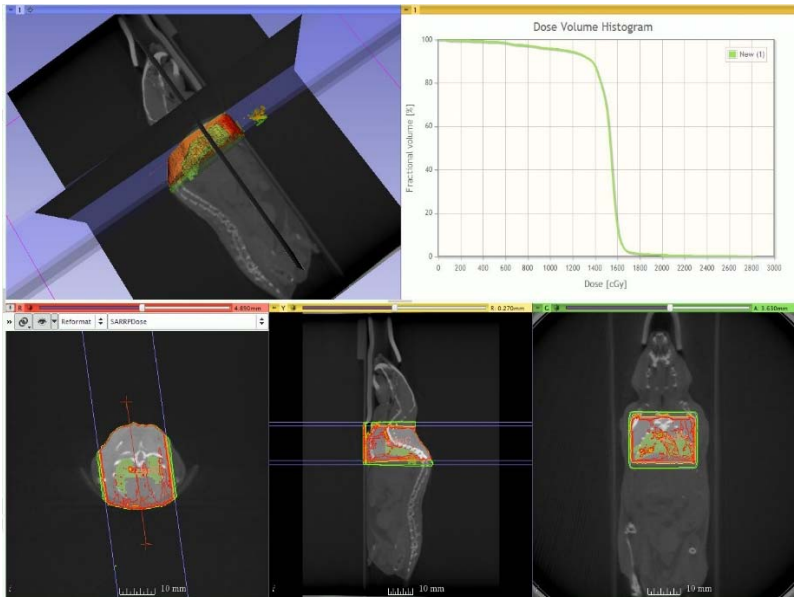

Figure 5: Imaging of small animal radiation research platform (SARRP) based irradiation technique to deliver radiaiton specifically to lungs, dose volume histogram indicating that 90% of the lung tissue can absorb up to 16 Gy.

What opportunities for training and professional development has the project provided?

"Nothing to report"

How were the results disseminated to communities of interest?

"Nothing to report"

What do you plan to do during the next reporting period to accomplish the goals?

Completion of Goal 1 and 2: Based on the data obtained from the training set, we plan to validate the response in additional samples collected weekly from chemo and TBI patients. The available clinical samples will be sorted out and outcome will be categorized in to multiple groups based on the toxicity response. The qualitative and quantitative differences in miRNAs will be compared as
1) Patients with toxicity in lung and GI
2) No toxicity in lung and GI
3) Diseases recurrence,
4) No apparent toxicity.

The comparison of circulating miRNA response along with toxicity biomarkers at 6-8 time points will allow discovery of biomarkers associated with acute radiation syndromes and delayed effects of acute radiation exposure. We expect to identify the circulating miRNA biomarkers as early predictors of late effects of the therapy through these studies.

Goal 3: The planned experiments are to
1) Analyze qualitative and quantitative differences in circulating miRNAs altered as a function of prophylactic GT3 administration in mice exposed to 4 and 7 Gy TBI.
2) Determine miRNA profiles and corresponding tissue responses in mice after partial body radiation directed to gut and lungs in the range 12 and 16 Gy and evaluate the effects of GT3.

4. IMPACT

What was the impact on the development of the principal discipline of the project?

The principle discipline under "illness associated with radiation injury" is to 'develop biomarkers for acute radiation syndromes" and delayed effects of acute radiation sickness". Utilizing patient samples, we have identified novel biomarkers with potential to develop as radiation biodosimeters and biomarkers of radiation injury.

Project Bio shield anticipates and considers exposure of the general population to a radiation/nuclear (RAD-NUC) terrorist event a major national threat. Radiation injury is directly proportional to the dose absorbed by specific tissue in the body as well as the individual's sensitivity to ionizing radiation. The current study has identified several biomarkers that are potentially connected with radiation. As a result, the complexity of health problems ranges from ARS, that could lead to morbidity within days/weeks, to Delayed Effects of Acute Radiation

Exposure (DEARE), that result in chronic conditions in lung, cardiovascular and kidneys over months. In order to manage and effectively treat soldier and civilians during a mass casualty RAD-NUC event when resources are severely strained, a point-of-care (POC) tissue-specific biodosimeter that addresses specific risk to tissue associated with radiation is urgently needed. Circulating miRNA-based radiation biodosimetry holds significant promise as the assay is relatively straightforward and it requires only small volumes of blood obtainable without a phlebotomist, with the aid of commercially available lancets. Radiation-sensitive biomarkers identified would facilitate the treatment of mass casualty victims. miRNA-based assays are capable of taking into account inter-individual variations as the actual dose effect could vary depending on pre-existing conditions, inherent differences in immune responses and other confounders. Here we used recent advances in liquid biopsy, coupled with our current studies in identifying specific biomarkers altered as function of WBI dose, will help in the development of an effective radiation biodosimeter that will be minimally invasive. A multi-marker assay that include specific as well as systemic responders will allow post exposure dose estimation based on individuals' own physiological response, thus reducing the effects due to several confounders. Circulating miRNA biomarkers also could help monitor victims in the recovery stage and be an early assay to predict the onset and progression of delayed effects from exposure to radiation.

Consistent with our previous report using rodent model of WBI, miR-150-5p exhibited the most robust dose and time dependent response. miR-150-5p is abundant in bone marrow and circulating lymphocytes, some of the cell-free circulating miR-150-5p are likely originated from bone marrow and the decrease in circulating miR-150-5p could be partly connected to the depletion in lymphocytes. As bone marrow is highly sensitive to radiation, the levels of circulating miR-150-5p will allow gauging the radiation dose in a range relevant to triage in radiological events. The levels of miR-150-5p in the circulating system could provide a direct readout of the level of functional marrow, and hence also provide a functional readout for hematological ARS.

Another major outcome from the current study is the discovery of miR-574-5p as a novel early response biomarker, with clear dose response. A similar increase in circulating miR-574-5p abundance was observed in NHPs exposed to bacterial endotoxin LPS as well (Menon et al 2016), suggesting that the plasma response here was associated with acute inflammatory response as a cause or effect. Significant levels of miR-574-5p are expressed in lung, heart, and liver tissue, and radiation/LPS could also be stimulating the releases of extracellular vesicles from those organs, contributing to the observed increase in serum at early time points.

Key outcomes from Goal 1 and 2:

1. We have identified miR-150 as a radiation biodosimeter using patient samples. The data is consistent addition to confirming the radiation biodosimetry potential of miR-150. The potential use was identified in multiple animal models and in human patients.

2. MiR-574-5p has shown to be an evolutionarily and functionally conserved biomarker. With good linearity and response in humans and in monkeys.

3. Four additional putative biomarkers (miR-378e, miR-579, miR-30d and miR-630) with potential to develop as biomarkers were identified.

What was the impact on other disciplines?

Biomarkers of normal tissue toxicity identified in this study will likely have impact in therapeutic radiation oncology as well. Whole body irradiation is commonly used for myeloablation in leukemia patients prior to bone marrow transplantation and there is significant concern of overdosing and/or under dosing because of underlying conditions and inherent variations in radiation sensitivity. Circulating biomarkers capable of providing early readout of, systemic as well as organ specific responses will allow tailoring treatment based on individuals' own physiological responses. Further, the comparison of samples from patients with known outcome (pneumonitis, cancer recurrence, GvHD and healthy) early will allow developing predictive dosimetry

What was the impact on technology transfer? "Nothing to report"

What was the impact on society beyond science and technology? "Nothing to report"

5. CHANGES/PROBLEMS

Nothing to report"

6. PRODUCTS

Publications/Presentation in Major Scientific Meetings.

Journal Publications.

Menon N, Rogers CJ, Lukaszewicz A, Axtelle J, Yadav M, Song F, Chakravarti A, Jacob NK: Detection of Acute Radiation Sickness: A Feasibility Study in Non-human Primates Circulating miRNAs for triage in Radiological Events. PLOS One- in (minor) Revision-resubmitted.

Other Publications, Conference Papers, and Presentations:

Yadav M, Chakravarti A and Jacob NK: Phytochemical Orientin as a countermeasure for radiation induced thrombocytopenia: Abstract accepted for poster presentation in Radiation Research Society Meeting, Hawaii, October 16-20, 2016

Bhayana S, Song F and Jacob NK: "Urinary microRNAs as biomarkers for early prediction of radiation nephropathy" Abstract accepted for poster presentation in American Society of Nephrology Meeting, Chicago, November 15-20, 2016. November 15-20 in Chicago, IL

Website(s) or other Internet site(s): "Nothing to report"

Technologies or techniques: "Nothing to report"

Inventions, patent applications, and/or licenses: "Nothing to report"

Other Products: "Nothing to report"

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name: *Naduparambil K. Jacob PhD*
Project Role: *Project Director/PI*
Researcher Identifier (e.g. ORCID ID): *05151436*
Nearest person month worked: *1*
Contribution to Project: *Dr. Jacob directed the project, trained key personnel, wrote protocols, optimized the methods, and wrote report.*

Name: *Sagar Bhayana PhD*
Project Role: *Postdoctoral Researcher*
Researcher Identifier (e.g. ORCID ID): *500170524*
Nearest person month worked: *3*
Contribution to Project: *Dr. Bhayana is replacement of Feifei Song from May 2016 He assisted in irradiation and sample processing*

Name: *Marshleen Yadav PhD*
Project Role: *Postdoctoral Researcher*
Researcher Identifier (e.g. ORCID ID): *500015731*
Nearest person month worked: *3*
Contribution to Project: *Dr. Yadav performed optimization of the research protocols and Human sample processing and assays*

Name: *Prasanthi Kumchala*
Project Role: *Research Associate*
Researcher Identifier (e.g. ORCID ID): *200329416*
Nearest person month worked: *1*
Contribution to Project: *Ms. Kumchala assisted in human sample procurement.*

Name: *Meng Xu Welliver MD PhD*
Project Role: *Co-Investigator*
Researcher Identifier (e.g. ORCID ID): 200221791
Nearest person month worked: *1*
Contribution to Project: *Dr. Welliver assisted with the human subject protocol.*

Name: *Xiaokui Mo, MAS PhD*
Project Role: *Biostatistician*
Researcher Identifier (e.g. ORCID ID): 96066577
Nearest person month worked: *1*
Contribution to Project: *Help in statistical analysis.*

Has there been a change in the active other support of the PD/PI or senior key personnel since last reporting period?

"Nothing to report"

What other organizations were involved as partners?

Organization Name:
Henry M. Jackson Foundation for the Advancement of Military Medicine

Location of Organization:
Armed Forces Radiobiology Research Institute
4301 Jones Bridge Road
Bethesda, MA

Partner's contribution to the project:
No significant contribution in the reporting period. The subcontract has been imitated. Experiments are planned for year 2 and 3 only.

8. SPECIAL REPORTING REQUIRMENTS

Collaborative Awards" Not applicable"

Quad Charts" See Attached"

9. APPENDICES

Minimally Invasive Radiation Biodosimetry and Evaluation of Organ Responses
W81XWH-15-2-0054
Insert Award Number Here

PI: Jacob, Naduparambil K. **Org:** The Ohio State University **Award Amount: $1,741,023**

Study Aims

- Identify and validate distinct sets of miRNAs as biomarkers of acute radiation syndromes (ARS) using human patient samples.
- Investigate translational potential of circulating miRNAs in radiation countermeasure development and pre-clinical testing.
- Develop multi-marker biodosimetry panel evaluating kinetics of hematopoietic, gastrointestinal and lung injury response markers.

Approach

- miRNA profiling in serum samples from human patients with leukemia who received partial of complete myeloablative radiation or chemotherapy and compare dose/time responses.
- Evaluate the kinetics of circulating miRNAs and tissue responses in radiation countermeasure, gamma-tocotrenol (GT3) treated versus control mice after lung and gut irradiation.
- Evaluate miRNAs after upper versus lower body exposure in male and female adult mice with varying sensitivity to radiation.

miR-Rad Development Plan

Discovery: human clinical trial samples

↓

Evaluate: miRNA changes after targeted radiation-mice

↓

Test: miRNA response with countermeasure GT3

↓

Validate: partial body irradiation models

↓

Multi-marker biodosimetry panel *for dose estimation in soldiers potentially get exposed to radiation in nuclear war/disaster*

Accomplishment: Method for RNA purification and detection of miRNA biomarkers in cell-free body fluids from animals has been optimized.

Timeline and Cost

Activities / CY	15	16	17	18
Assay optimization/discovery				
Test biodosimetry potential in pts				
Test utility in mouse models				
Develop multi-marker panel				
Estimated Budget ($K)	**$200**	**$500**	**$600**	**$441**

Updated: 09/30/2016

Goals/Milestones

CY15 Goal –Assay optimization

☑ Develop blood miRNA assays for discovery and validation

CY16 Goals – Discovery and validation of biomarkers

☑ Discovery of miRNA biomarkers in patients' sera (in progress)

☐ Investigate dosimetric potential-dose/time response

CY17 Goals – Test organ responses and translational utility

☐ Investigate dose-time effect after gut and lung irradiation in mice

☐ Evaluate the use of miRNAs in testing the effect of GT3

CY18 Goal – Develop/test the multi-marker biodosimetry panel

☐ Investigate the utility in partial body radiation exposure scenario

Comments/Challenges/Issues/Concerns

- Sensitivity inter-individual variation ,sample quality, confounders
- Distinguish radiation and organ specific injury markers

Budget Expenditure to Date

Projected Expenditure: $544,924

Actual Expenditure: **$376,662**