Biochemical and Biophysical Research Communications 377 (2008) 41–45

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Altered microRNA expression patterns in irradiated hematopoietic tissues suggest a sex-specific protective mechanism

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ARTICLE INFO

Article history:
Received 3 September 2008
Available online 26 September 2008

Keywords:
microRNA
Radiation
Genome stability
LSH
Methylation

ABSTRACT

To investigate involvement of miRNAs in radiation responses we used microRNAome profiling to analyze the sex-specific response of radiation sensitive hematopoietic lymphoid tissues. We show that radiation exposure resulted in a significant and sex-specific deregulation of microRNA expression in murine spleen and thymus tissues. Among the regulated miRNAs, we found that changes in expression of miR-34a and miR-7 may be involved in important protective mechanisms counteracting radiation cytotoxicity. We observed a significant increase in the expression of tumor-suppressor miR-34a, paralleled by **a** decrease in the expression of its target oncogenes NOTCH1, MYC, E2F3 and cyclin D1. Additionally, we show that miR-7 targets the lymphoid–specific helicase LSH, a pivotal regulator of DNA methylation and genome stability. While miR-7 was significantly down-regulated LSH was significantly up-regulated. These cellular changes may constitute an attempt to counteract radiation-induced hypomethylation. Tissue specificity of miRNA responses and possible regulation of miRNA expression upon irradiation are discussed.

Ionizing radiation (IR) is an important diagnostic and treatment modality. Conversely, it is a potent DNA damaging and cancer-inducing agent [1]. Lymphoid and myeloid hematopoietic tissues are the main targets for radiation carcinogenesis in both humans [2,3] and animal models [4,5]. IR-induced leukemia in humans was first reported early in the 20th century [1]. Since, numerous studies of atomic bomb survivors, people working or living near nuclear power plants, radiologists, and radiotherapy patients have proved that IR exposure results in a high risk of leukemia and lymphoma [2–8]. However, the exact mechanisms by which IR exposure may result in a predisposition to the development of IR-induced leukemia and lymphoma remains to be determined.

In recent years, the role of epigenetics in the etiology of disease, including cancer, has been increasingly recognized [6,7]. Epigenetic changes are stable alterations in gene expression that include DNA methylation, histone modification, and small RNA-mediated effects.

Amongst the small RNAs in mammals, microRNA (miRNAs) are the most abundant and well understood. MiRNAs are small non-coding regulatory RNAs that act as translational inhibitors to down-regulate the expression of a wide variety of genes [8]. MiRNAs originate from various areas of the genome, including repetitive regions, transposons, and the intronic sequences of coding genes. To regulate the expression of target mRNAs, mature miRNAs associate with the RNA-induced silencing complex (RISC) proteins to bind to target mRNAs in a sequence-specific manner [8]. In doing so, the RISC associated miRNAs can impact various processes such as cellular differentiation, proliferation, and apoptosis, therefore potentially affecting an individuals predisposition to malignancy [8]. Indeed, aberrant levels of miRNAs have been reported in a variety of human cancers [8], including leukemia and lymphoma [9–11].

In carcinogenesis, miRNAs can act as tumor-suppressors or oncogenes by regulating other tumour-suppressor or oncogene mRNAs. For example, over-expression of the oncogenic miR-17-92 cluster facilitates the development of small-cell lung cancer and chronic myeloid leukemia [12]. Mir-34a is a potential tumor suppressor that induces apoptosis in neuroblastoma cells [13]. Similarly, tumor-suppressors miR-15 and miR-16 induce apoptosis by targeting the anti-apoptotic B cell lymphoma 2 (BCL2) transcript and have been found to be either absent or down-regulated in the majority of chronic lymphocytic leukemia (CLL) cases [11].

Despite the growing evidence of the importance of miRNAs in the development of hematopoietic tissue malignancies, fairly little is known about the radiation-induced miRNA response in these tissues. Further, as IR is a potent inducer of these types of cancer, dissecting microRNAome changes in IR-exposed hematopoietic tissues may help us in the identification of the initial, pre-malignant radiation induced changes that may further lead to carcinogenesis.

With this in mind, we analyzed the IR-induced changes to the microRNAome in the radiation-sensitive lymphatic tissues of the hematopoietic system in a well-established murine model. Here we show that whole body IR exposure resulted in a significant and sex-specific deregulation of microRNA expression in mouse spleen and thymus tissue. MicroRNAome changes were paralleled by altered expression of microRNA target proteins.

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doi:10.1016/j.bbrc.2008.09.080

Materials and methods

Model. In this study, we utilized an *in vivo* mouse model to study microRNAome changes in the thymus and spleen of male and female animals following IR exposure. The C57BL/6 mouse model is widely used, well characterized, and generally accepted for studies of IR-induced changes in hematopoietic lymphoid tissue [5]. Handling and care of animals was in accordance with the recommendations of the Canadian Council for Animal Care and Use.

Radiation exposure. Fifty-day-old C57BL/6 mice (10 males and 10 females) were randomly assigned to different treatment groups. All animals had comparable body weights, were kept in a virus-free facility, and given food and water *ad libitum*.

Five male and five female mice received 2.5 Gy of X-ray exposure to the entire body (90 kV, 5 mA). Ten animals (5 male and 5 female mice) were sham treated and served as controls. No signs of pain or distress were observed during the experiment. Animals were humanly sacrificed 6 h after exposure. Thymus and spleen tissues were harvested immediately following sacrifice, divided into two equal parts, frozen in liquid nitrogen, and stored at −80 °C for further analysis. This experiment was independently replicated once according to the same experimental scheme using a cohort of 12 C57/BL6 mice each time (3 animals per group/per sex). Both experiments yielded congruent data.

miRNA microarray expression analysis: Total RNA was extracted from mouse spleen and thymus tissues using TRIzol Reagent (Invitrogen, Burlington, Ontario) according to the manufacturer's instructions. Microarray analysis was conducted using the tissue from control and exposed animals. Tissue from 3 animals per group per sex was used for the analysis. The miRNA microarray analysis was performed by LC Sciences (Houston, TX), as previously described [14]. Normalization was performed with a cyclic LOWESS (locally weighted regression) method to remove system-related variations, as previously described [14]. The *t*-test analysis was conducted between the control, whole-body exposed and bystander sample groups. MicroRNAs with *p*-values <0.05 were selected for cluster analysis.

Quantitative real-time PCR (qRT-PCR) analysis. Quantitative real-time PCRs (qRT-PCRs) were performed by using SuperTaq Polymerase (Ambion) and a mirVana qRT-PCR miRNA Detection Kit (Ambion) following the manufacturer's instructions. Reactions contained mirVana qRT-PCR primer sets specific for miR-34a. The 5S rRNA served as a positive control. Quantitative real-time PCR was performed on a SmartCycler (Cepheid, Sunnyvale, CA).

Western immunoblotting. Western immunoblotting was conducted using spleen tissue of both groups using a well-established protocol [15]. Membranes were incubated with antibodies against NOTCH1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), cyclin D1 (1:2000; Cell Signalling), MYC (1:500; Santa Cruz Biotechnology), LSH (1:1000, Abcam, Cambridge, MA), E2F3 (1:500, Lab Vision) and actin (1:2000; Abcam). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) and the ECL Plus immunoblotting detection system (GE Healthcare). Chemiluminescence was detected by Biomax MR films (Eastman Kodak, New Haven, CT). Unaltered PVDF membranes were stained with Coomassie Blue (Bio-Rad, Hercules) and the intensity of the Mr 50,000 protein band was assessed as a loading control. Signals were quantified using NIH ImageJ 1.63 Software and normalized to both actin and the Mr 50,000 protein band, which gave consistent results (values relative to Mr 50,000 are plotted). The protein levels in the tissues of exposed animals were related to controls. Two technical replicates were used for each immunoblotting.

Luciferase reporter assay for targeting LSH-3′-UTR. For the luciferase reporter experiments, a 3′-UTR segment of LSH gene corresponding to a region of 366 nucleotides (nts) (from 2642 nt through 3008 nt of the total transcript) for LSH (Acc. No. NM_008234) was amplified by PCR from mouse genomic DNA and cloned into the pGL3-control vector (Promega, Madison, WI). The HEK293 cells were transfected with the firefly luciferase UTR-report vector, control *Renilla* luciferase pRL-TK vector (Promega), transfection controls and precursor miR-7 using lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA), as previously described [16]. Twenty-four hours after transfection, cells were lysed with a 1× passive lysis buffer and the activity of both *Renilla* and firefly luciferases was assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions, as previously described [16].

Statistical analysis. Statistical difference between the group's means was determined by Student's *t*-test using JMP 5.0 and Excel software (Microsoft Corporation, Redmond, WA).

Results & discussion

In this study, we utilized an *in vivo* murine model to analyze and compare microRNAome changes induced by exposure to X-rays in the lymphoid hematopoietic spleen and thymus tissue of male and female mice. Equal cohorts of male and female experimental animals were divided in two experimental groups, 'exposed' animals (2.5 Gy of whole-body X-ray irradiation) and sham treated 'control' animals.

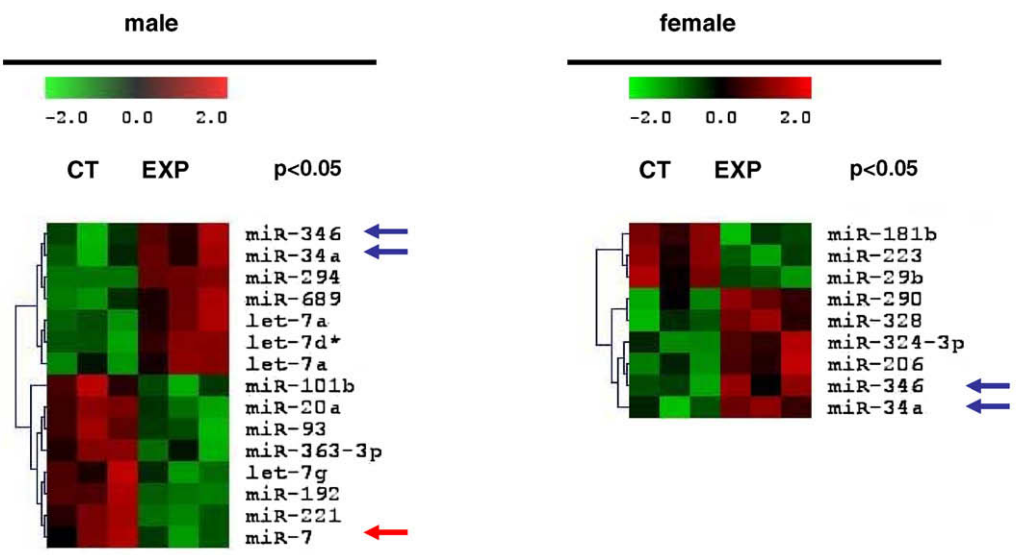
Analysis of the murine spleen microRNAome revealed a number of intriguing patterns. In male spleen samples, 15 miRNA were significantly regulated ($p < 0.05$; 7 up- and 8 down-regulated; Fig. 1A), and in female spleen samples 9 miRNAs were significantly regulated ($p < 0.05$; 6 up- and 3 down-regulated; Fig. 1A). Interestingly, between these groups, only two miRNAs were similarly regulated in male and female samples, miR-346 and miR-34a. These miRNAs were 4.82- and 2.34-fold up-regulated in females and 3.07- and 1.99-fold up-regulated in males, respectively. The up-regulation of miR-34a was independently confirmed by qRT-PCR (Fig. 1B).

MiR-34a is a known tumor-suppressor miRNA, as it targets the Myc [17], cyclin D1 [18] and Notch1 [19] oncogenes, as well as the potent transcriptional inducer of cell-cycle progression E2F3 [13]. Indeed, we observed a significant down-regulation of MYC, cyclin D1, NOTCH1 and E2F3 in the spleen tissue of exposed male and female mice (Fig. 1B), suggesting that the radiation-induced up-regulation of miR-34a may be viewed as a protective mechanism aimed to suppress oncogene overexpression after IR exposure. Further, miR-34a up-regulation and concurrent E2F3 down-regulation were recently shown to lead to increased apoptosis [13].

Overall, miRNAs 34a and 346 were the only miRNAs to have similarly changed in response to irradiation in both male and female spleen, suggesting that these miRNAs are radiation responsive regardless of sex. The miR-34 family is highly conserved among animals and invertebrates and has been previously shown to be regulated via the p53 transcription factor [20]. These data correspond with our previously reports of p53 and miR-34 up-regulation of in the spleen of male and female mice exposed to X-rays [21,22]. Interestingly, it has previously been shown that the radioresistant model of C57BL/6 has an earlier p53 response than other radio-sensitive strains, resulting in a twofold higher apoptotic response in the spleen of irradiated animals [23]. Indeed, this may be a similar mechanism as seen with miR-34 up-regulating apoptosis neuroblastoma cells [13], and further asserts miR-34s role as a protective miRNA in response to radiation.

MiR-346 was previously shown to be significantly up-regulated in follicular thyroid carcinoma, where *in vitro* overexpression of miR-346 caused increases in cellular proliferation [24]. Similarly

A



B

MicroRNAlevel, Fold change from control	male spleen		female spleen	
	array	qRT-PCR	array	qRT-PCR
miR-34a	2.0	3.7	2.4	3.3

Level of target protein/ Fold change from control	male spleen	female spleen
NOTCH1	-1.5	-1.4
cyclin D1	-1.5	-1.5
MYC	-1.3	-1.2
E2F3	-1.6	-1.5

Fig. 1. Differentially expressed miRNAs in the spleen tissue of male and female mice subjected to whole-body exposure to 2.5 Gy of X-rays. (A) Hierarchical clusters of differentially expressed miRNA genes in the spleen of exposed male and female mice (as determined by *t*-test). Each miRNA listed is differentially expressed between control and exposed groups ($p < 0.05$). Arrows denote microRNAs discussed in the text. (B) Fold changes of miR-34a as measured by both microarray and qRT-PCR. These changes in miRNA expression correlate to protein level changes found in their targeted oncogenes NOTCH1, cyclin D1, MYC and E2F3.

to leukemia's and lymphoma, thyroid carcinomas are frequently induced by radiation exposure [25]. Therefore, the analysis of functional consequences of miR-346 activation in the spleen of irradiated mice deserves future attention.

Activation of both of miR-34a and miR-346 in a similar manner suggests their like regulation. As it has been previously established that miR-34a is regulated by p53 [20], we searched the area surrounding miR-346 for putative p53 binding elements (RRRCWWGYYY RRRCWWGYYY); however, no strong p53 binding sites could be found within 30 kB of the miRNA. miR-346 is an intronic miRNA, and, as such, could be under the transcriptional regulation of its host gene glutamate receptor, ionotropic, delta 1 (GRID1). Unfortunately, p53 binding elements could not be found within 30 kB of the GRID1 transcription start site.

Yet, when the 12 kB regions surrounding miR-34a and miR-346/GRID1 putative promoters were analyzed *in silico* for transcription factor binding sites using Match™, 26 and 17 consensus sequences were found for the miR-34a and GRID1 promoter, respectively, with 11 and 15 different transcription factor (TF) binding sites in each. Interestingly, 5 common TF binding site types were found between the two putative promoters: Pax-4, Oct-1, MyoD, HNF-4, and Elk-1. Of particular interest, Oct-1 is a well known DNA damage-induced response element [26]. Future studies are needed to dissect the details of the IR-induced transcriptional regulation of these microRNA loci, the biological repercussions of their up-regulation, and their roles, if any, in the predisposition to IR-induced genome instability and carcinogenesis.

Amongst the other miRNAs, miR-7 was significantly down-regulated in the spleen tissue of exposed males. Computational analysis (TargetScan 4.2) revealed that miR-7 targets murine lymphoid-specific helicase (LSH), with conserved binding sites in the human, rat, and dog LSH UTRs. This prediction was further confirmed by the miRGen software. LSH belongs to the SNF2 family of chromatin-remodeling ATPases and is required for the proper establishment and maintenance of DNA methylation in mammals [27,28]. DNA methylation was shown to be profoundly affected in radiation-exposed hematopoietic tissue [29], and recent data suggest the involvement of LSH-induced DNA hypomethylation in the development of erythroleukemia [30]. Therefore, to examine whether or not LSH is indeed functionally targeted by miR-7, the segment of LSH-3′-UTR containing the miR-7 complementary site was cloned into the 3′-UTR of a luciferase reporter system (Fig. 2A). The resulting reporter vector was transfected into the HEK293 cells together with transfection controls and miR-7. The luciferase reporter construct that did not contain the LSH-UTR was used as a negative control. Fig. 2B shows that miR-7 inhibited the luciferase activity from the construct with the LSH-3′-UTR segment in a concentration-dependent manner (Fig. 2B). There was no change in the luciferase reporter activity when the cells were co-transfected with negative control (scrambled oligonucleotides). No luciferase expression changes were seen when the cells were transfected with the plasmid lacking the LSH-3′-UTR fragment (data not shown).

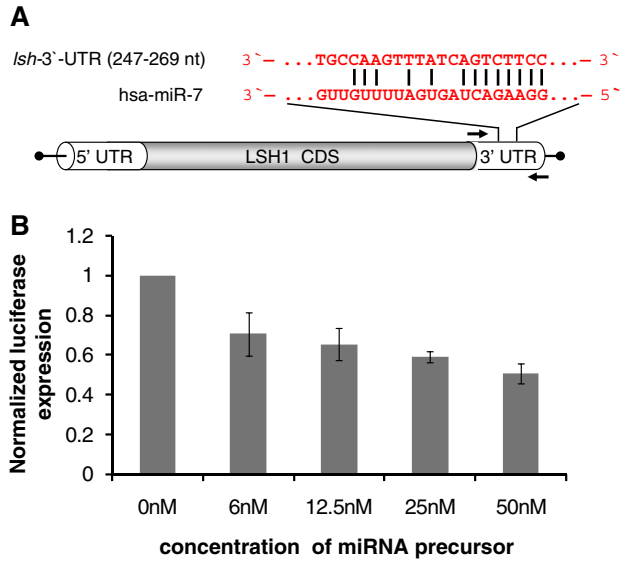


Fig. 2. Mir-7 directly targets LSH. (A) Complementary site for miR-7 in the 3′-UTR of LSH. (B) Dose-dependent inhibition of LSH expression in the luciferase assay after transfection of the HEK293 cells with miR-7 or a negative control.

In order to further confirm that miR-7 indeed affects LSH *in vivo*, we analyzed LSH protein levels in the spleen tissue of control and exposed animals. We observed a negative correlation between levels of miR-7 and LSH. While the expression of miR-7 was decreased in the splenic tissue of the exposed male mice, the levels LSH protein increased (Fig. 3). Furthermore, no LSH changes were seen in the spleen of exposed female mice, where miR-7 levels remained unchanged.

The male-specific up-regulation of LSH through miR-7 in response to ionizing radiation constitutes an intriguing find. LSH is essential for correct DNA methylation patterning, as it has been shown that loss-of-function mutations in the murine LSH gene result in dramatic hypomethylation of the murine genome and reactivation of transposable elements [31]. LSH overexpression, contrarily, leads to re-establishment of methylation marks and is viewed as a genome stabilizing event [32]. Radiation is well-known to induce a significant loss of DNA methylation, which is much more pronounced in the male spleen tissue when compared to female spleen tissue [33]. Therefore, one could reason that the down-regulation of miR-7 with the consequent LSH up-regulation in male spleen may constitute a cellular attempt to counteract the radiation-induced hypomethylation. Interestingly, in female spleen, where the IR-induced hypomethylation was far less pronounced than in males, no miR-7 down-regulation and LSH activation was seen. The mechanism of miR-7 expression and the roles of miR-7 and LSH in genotoxic stress responses need to be further dissected in the future.

Another interesting outcome of this study is the fact that IR exposure-induced microRNAome changes in the spleen that were distinct from those in the thymus. Changes in the thymus microRNAome were much less pronounced, especially in the female thymus where only one microRNA was significantly changed upon irradiation (Fig. 4). In male thymus, 1 miRNA was upregulated, and 6 miRNAs were down-regulated (Fig. 4). Both the spleen and thymus are radiation target organs, and are important components of lymphoid hematopoietic tissue; yet, their physiological roles in the organism are distinct. The spleen is involved in the destruction of redundant erythrocytes, and holds a reservoir of red blood cells and B-lymphocytes. The thymus holds a large number of apoptotic lymphocytes, and is involved in T-lymphocyte generation and maturation. Therefore, it is quite logical that these highly specialized parts of the hematopoietic system exhibit distinct microRNAome responses upon irradiation. In the future the molecular mechanism and physiological consequences of radiation response of the spleen and thymus of male and female animals needs to be further addressed. This will help in further understanding the sex-specific changes to hematopoietic tissue under genotoxic stress conditions.

Overall, future studies are needed to understand the transcriptional regulation of microRNA loci upon irradiation, and other genotoic stresses. Additionally, the changes to DNA methylation patterns under the influence of radiation may itself lead to the changes in the expression of a number of miRNA species, further contributing to aberrant miRNA expression patterns [34]. The link between DNA methylation and microRNAome expression upon genotoxic stress exposure needs to be established and studied in a tissue- and sex-specific context. This analysis is especially important given that approximately half of all known miRNA gene promoters are associated with CpG islands [35], and about 10% of known miRNAs are regulated by DNA methylation [36].

Our microRNAome datasets may provide a roadmap for further analysis of the role of the microRNAome in radiation responses of hematopoietic tissues of males and females. The radiation-induced microRNAs identified in our study may further be explored as sex-specific biomarkers of radiation exposure in hematopoietic tissues.

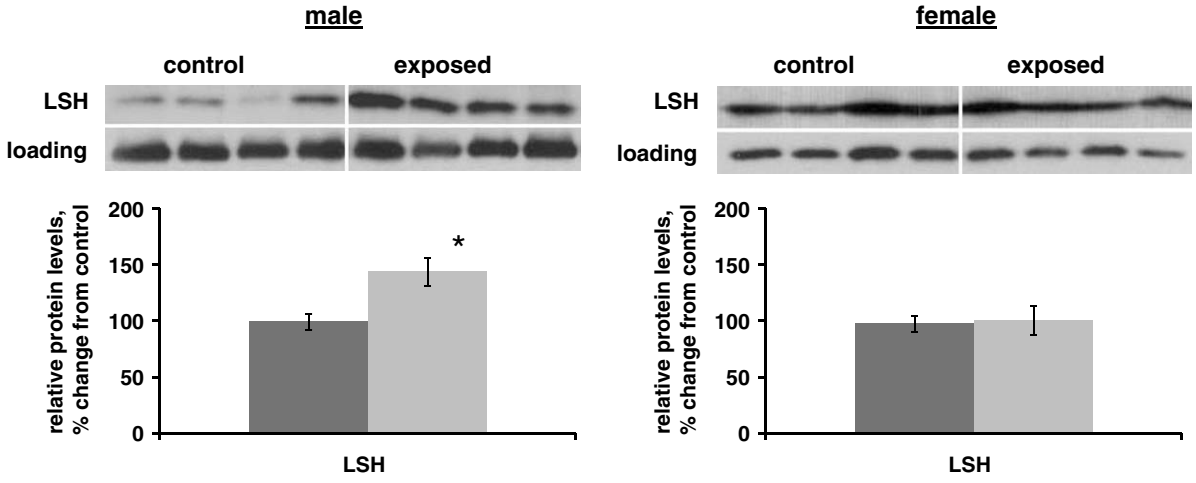


Fig. 3. Altered expression of LSH in murine spleen after IR exposure. Protein levels relative to those of control animals are shown as the means ± SD, *significant, 95% confidence limit, $p < 0.05$, Student's *t*-test. Representative western blots from among three independent technical repeats of the experiments are shown.

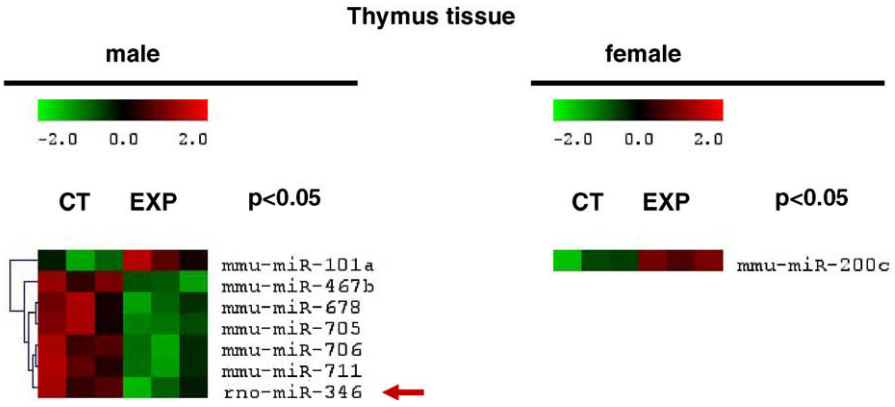


Fig. 4. Differentially expressed miRNAs in the thymus tissue of male and female mice subjected to whole-body exposure to 2.5 Gy of X-rays. (A) Hierarchical clusters of differentially expressed miRNA genes in thymus of exposed male and female mice. Each miRNA listed is differentially expressed between control and exposed groups ($p < 0.05$).

Acknowledgments

We are grateful to Karen Dow-Cazal and Charlotte Holmes for animal care. We appreciate the help of Dr. Valentina Titova in proofreading this manuscript. This research was supported by ACB and CIHR operating grants to Olga Kovalchuk. Igor Koturbash is the recipient of the Dr. Cyril Kay Alberta Cancer Board (ACB) Graduate Scholarship. Olga Kovalchuk is a CIHR Research Chair in Gender and Health.

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