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MicroRNA Dysregulation in Human Thyroid Cells Following Exposure to Ionizing Radiation

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Background: Ionizing radiation is a well-known mutagen and a risk factor for thyroid cancer. MicroRNAs (miRNAs) play an important role in the regulation of gene expression on post-transcriptional level and are dysregulated in thyroid cancer. The goal of this study was to investigate the effects of acute exposure to 1 and 10 Gy of γ-irradiation on miRNA expression in normal human thyroid cells.
Methods: Expression of 319 miRNAs was studied in primary cultures of normal human thyroid cells 4 and 24 hours postirradiation using a miRNA expression array with further confirmation of miRNAs expression by reverse transcription-polymerase chain reaction.
Results: We identified 30 miRNAs that were unregulated or downregulated more than twofold after irradiation as compared to nonirradiated thyroid cells, with no significant difference found between 1 and 10 Gy of radiation. Four distinct patterns of miRNA expression change were observed: miRNAs downregulated at 4 hours and returned to normal levels at 24 hours, miRNAs upregulated at 4 hours and returned to normal levels at 24 hours, and miRNAs either upregulated or downregulated at both time points. No dysregulation of miRNAs known to occur in thyroid cancer was observed.
Conclusions: Acute exposure of thyroid cells to γ-radiation results in several specific patterns of miRNA response. It appears that alteration in miRNA expression seen 4–24 hours after irradiation has no direct association with carcinogenesis. However, it is likely to affect other cell functions, such as DNA repair.

Introduction

Ionizing radiation is a well-established risk factor for various types of cancer, including thyroid cancer. Numerous reports have documented an increased incidence of thyroid tumors in populations exposed to ionizing radiation, including survivors of atomic bomb explosions in Japan in 1945 (1,2), residents of the Marshall Islands exposed to radioactive fallout in 1954 (3,4), patients exposed to therapeutic irradiation (5,6), and children exposed to radiation after the Chernobyl nuclear power accident (7,8). In all of these populations, thyroid papillary carcinoma has been identified as a type of thyroid cancer associated with radiation exposure.

Ionizing radiation induces damage to various cell components, whereas damage to cellular DNA is primarily responsible for mutagenesis and carcinogenesis, and double-strand breaks are likely to be the initial events in the generation of specific carcinogenic mutations (9,10). However, it remains unclear whether these mutations are formed immediately after radiation exposure or later. Thyroid papillary carcinoma developed after radiation exposure at Chernobyl and in atomic bomb survivors in Japan is characterized by a very high frequency of *RET/PTC* chromosomal rearrangement, which was found to directly correlate with radiation dose in some studies (11–13).

To repair radiation damage, a complex cellular response is initiated, including alteration in gene expression, especially in genes involved in stress response, cell cycle control, and DNA synthesis/repair (14–16). It has been recently discovered that microRNAs (miRNAs) play an important role in the regulation of gene expression on post-transcriptional level (17,18). However, the role of miRNAs in cell response to radiation damage in human cells remains largely unknown.

miRNAs are small endogenous noncoding RNAs that act as negative regulators of the protein-coding gene expression through the complementary binding to 3′ untranslated region (UTR) of target mRNA, which lead to translational repression and inhibition of protein synthesis. miRNA expression is deregulated in many types of human cancers, including thyroid cancer. Several studies have demonstrated that normal thyroid cells have a unique profile of miRNA expression and many miRNAs are dysregulated in thyroid cancer cells. A subset of these miRNAs, including miR-221, miR-222, miR-146b, miR-155, and miR-187, have been consistently found to

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be upregulated in thyroid papillary carcinoma (19–21). Moreover, strong correlation was found between miRNA expression and somatic mutations found in this tumor type. Specifically, upregulation of miR-187, miR-146b, and miR-155 was found to be significantly more pronounced in papillary carcinomas carrying *RET/PTC* rearrangements (20), a genetic event characteristically found in radiation-induced thyroid tumors.

Several studies have reported the association between miRNAs and radiation exposure. He *et al.* found upregulation of miR-34 miRNA family (miR-34a, −34b, and −34c) in a variety of mouse tissues after exposure to ionizing radiation (22). Interestingly, they showed that these miRNAs were transcriptionally activated by TP53 in response to DNA damage, and their upregulation, in turn, led to downregulation of their target genes (i.e., *CDK4* and *MET*) and to cell cycle arrest. Weidhaas *et al.* showed the dysregulation of let-7 family miRNAs in response to radiation in lung cancer cells and demonstrated that overexpression of some of these miRNAs altered radiosensitivity of these cells (23). In another study, dysregulation of miR-521 and miR-34c was observed in prostate cancer cells subjected to external beam radiation (24). However, according to our knowledge, no studies of miRNA expression in normal human thyroid cells exposure to ionizing radiation have been reported to date. In this observation, we report the results of our analysis of miRNA expression in human normal thyroid cells subjected to different doses of γ-irradiation and followed for the first 24 hours after irradiation.

Materials and Methods

Cell culture and irradiation

Normal thyroid tissue from surgically removed thyroid samples was collected at the Department of Pathology, University of Cincinnati, after the University of Cincinnati Institutional Review Board (IRB) approval. Primary cell cultures were established from the two freshly collected normal thyroid tissue samples as previously described (25). In both cases, the tissue was obtained from an opposite lobe of total thyroidectomy specimens containing a discrete nodule diagnosed pathologically as either an encapsulated papillary carcinoma or a hyperplastic nodule. Cells were cultured for 3–4 days at 37°C in 95% humidity and 5% CO_2 in RPMI-1640 medium containing 10% of fetal bovine serum. The presence of epithelial thyroid cells was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) detection of thyroglobulin expression performed as previously described (26). Confluent cell cultures were exposed to a single dose of γ-irradiation from a cesium-137 source at a dose rate of 1.7 Gy/min. Cells were exposed to 1 or 10 Gy γ-irradiation in triplicate for each dose and collected at 4 and 24 hours after exposure.

RNA isolation

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) as previously described (27). RNA yield was determined using the NanoDrop 1000 spectrophotometer (ThermoScientific, Wilmington, DE). RNA integrity was assessed with the Agilent 6000 NANO kit for the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to manufacture's protocol.

miRNA expression array

Quantitation of expression of 319 mature miRNA was performed using FlexmiR™ human microRNA pool, version 8 (Exiqon, Vedbaek, Denmark), and analyzed on Luminex 200 (Luminex Corporation, Austin, TX) according to the manufacturer's instructions. Specifically, 2.5 μg of total RNA was labeled with biotin using the FlexmiR™ MicroRNA Labeling Kit (Luminex) followed by hybridization to beads coated with locked nucleic acids probes complementary to mature miRNA sequences. All samples were assayed in duplicate. The system calibration was performed using the xMAP™ calibration control reagents (Luminex). A blank control set of beads was used to normalize the background value for every individual miRNA. Five synthetic controls and four small nucleolar RNA (snoRNA) normalization controls were used to adjust mean fluorescence intensities between samples and between runs using the average correction factor for these controls as recommended by the manufacturer. Finally, the miRNA expression in the irradiated cells was quantitated relatively to nonirradiated cells using Luminex IS™ software v.2.3 (Luminex).

miRNA real-time RT-PCR

Expression of individual miRNAs was detected by real-time RT-PCR using miRNA sequence-specific primers (Applied Biosystems, Inc., Foster City, CA). Briefly, 10 ng of total RNA was reverse transcribed using High-Capacity cDNA Archive kit (Applied Biosystems, Inc.) followed by amplification on ABI 7500 Real-Time PCR System (Applied Biosystems, Inc.). All RT-PCRs were performed in triplicate. Small nucleolar RNA RNU44 was used as endogenous control for the normalization of RNA input. miRNA expression levels were calculated by relative quantitation using the ABI 7500 Real-Time PCR SDS 1.2 software (Applied Biosystems, Inc.) and the fold change of expression was determined by $2^{-\Delta\Delta CT}$ method (28). No template reaction was used as a negative control.

Statistical analysis

One-way analysis of variance was initially used to identify a subset of miRNAs significantly altered after 1 and 10 Gy of γ-irradiation. Student's *t*-test was used to determine statistical significance of differentially expressed miRNAs between irradiated and nonirradiated cells and for comparison of miRNA expression between different doses of irradiation. Agglomerative hierarchical clustering analysis was performed using Cluster software and TreeView software (http://genome-www5.stanford.edu/resources/restech.shtml) (29).

Search for target genes

Putative miRNA target genes were identified using miRBase (http://microrna.sanger.ac.uk), TargetScan (http://genes.mit.edu/targetscan) (30), and PicTar (http://pictar.bio.nyu.edu) (31) target prediction programs.

Results

First, primary cultures of normal human thyroid cells exposed to 1 and 10 Gy of γ-irradiation were collected 4 and 24

hours postirradiation and studied for expression of 319 human mature miRNAs by miRNA expression array. The analysis revealed a significant number of miRNAs that were dysregulated after radiation exposure as compared to nonirradiated thyroid cells. Of those, 30 miRNAs were upregulated or downregulated more than twofold either after 1 or 10 Gy of irradiation at each time point. These 30 miRNAs were further studied by real-time RT-PCR to confirm the results and determine the expression levels with higher degree of precision. Some of these miRNAs were up to 24-fold upregulated and other were up to 50-fold downregulated at different time intervals after cell irradiation (Table 1).

Next, the unsupervised hierarchical clustering analysis of miRNA expression was performed. The analysis grouped samples by time of collection after radiation (4 hours vs. 24 hours), but not by radiation dose (Fig. 1). It revealed four distinct groups of miRNAs: (i) miRNAs downregulated at 4 hours and returned to normal or to upregulated level at 24 hours; (ii) miRNAs upregulated at 4 hours and returned to normal or downregulated level at 24 hours; (iii) miRNAs upregulated at both time points; and (iv) miRNAs downregulated at both time points (Fig. 1).

Table 1. Differentially Expressed MicroRNAs in Normal Human Thyroid Cells After 1 and 10 Gy of γ-Radiation

	Irradiated cells, 1 Gy		*Irradiated cells, 10 Gy*	
miRNA	*4 hours*	*24 hours*	*4 hours*	*24 hours*
hsa-miR-409-5p	17.3	0.3	6.8	0.5
has-miR-520a	13.3	11.5	6.3	8.6
has-let-7d	12.4	13.5	24.4	16.1
has-miR-489	10.1	13.7	11.3	9.1
has-miR-193a	6.8	4.0	6.2	3.9
has-let-7g	5.7	0.5	9.4	0.8
has-miR-188	5.5	7.5	1.7	7.2
has-miR-122a	4.3	2.9	2.2	2.6
has-let-7c	2.2	2.1	2.3	1.2#
has-miR-146a	2.0	0.5	2.9#	0.2
has-miR-96	2.0	0.9#	1.5	0.8#
has-miR-203	2.0	2.1	2.3	2.3
has-miR-365	1.7#	0.9	1.6#	0.9#
has-miR-34b	1.6#	3.2	1.8	3.3
has-miR-34a	1.4#	0.3	1.8	0.7#
has-miR-452*	0.7#	4.5	0.4	4.4
has-miR-527	0.6#	1.6#	0.6#	1.0#
has-miR-377	0.5	1.1#	0.2	1.2#
has-miR-526b	0.5	1.7	0.1	1.3
has-miR-522	0.4	0.3	0.4	0.5
has-miR-181c	0.3	0.1	0.2	0.2
has-miR-326	0.3	0.3	0.4	0.2
has-miR-186	0.2	0.5	0.1	0.1
has-miR-384	0.2	0.4	0.3	0.4
has-miR-453	0.1	11.1	0.5	9.8
has-miR-520e	0.1	5.2	0.1	1.7#
has-miR-1	0.1	0.2	0.02	0.1
has-miR-93	0.1	0.3	0.1	0.2
hsa-miR-338	0.1	0.2	0.2	0.1
hsa-let-7f	0.1	0.2	0.1	0.3

Values represent fold change relative to nonirradiated cells as detected by reverse transcription-polymerase chain reaction; all values have a significant difference as compared to nonirradiated cells ($p < 0.05$) except those labeled with #.

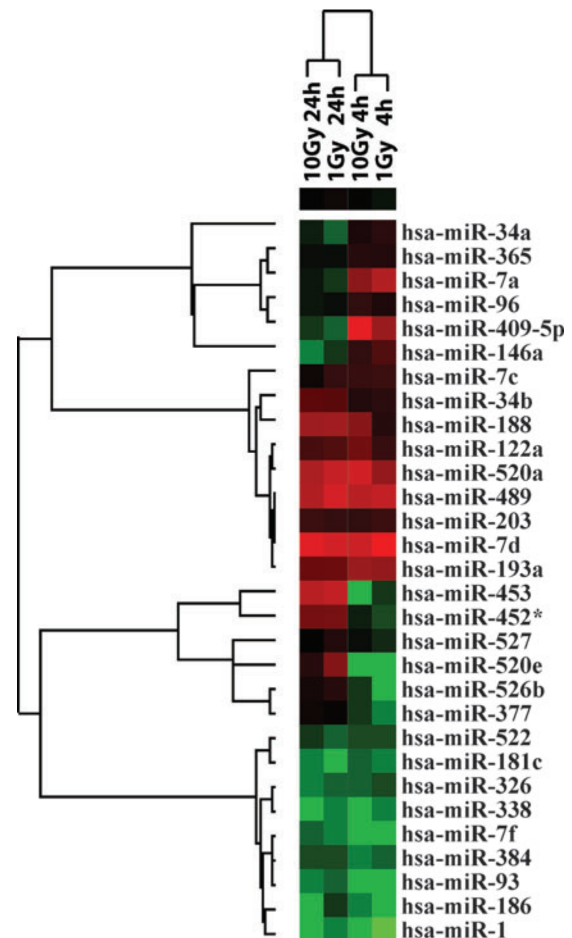

FIG. 1. Hierarchical cluster analysis of microRNA (miRNA) expression detected 4 and 24 hours after 1 and 10 Gy of γ-radiation. The upregulated miRNAs are depicted in shades of red, and downregulated miRNAs are depicted in shades of green. Color images available online at www.liebertonline.com/thy.

Six miRNAs constituted the first group: mir-377, miR-452*, miR-453, miR-520e, miR-526b, and miR-527. They showed more than twofold downregulation at 4 hours postexposure as compared to nonirradiated cells. At 24 hours, their expression was either at normal levels or became elevated (Fig. 2A). The patterns of expression were similar between 1 and 10 Gy doses of radiation. The second group also contained six miRNAs (miR-409-5p, let-7g, miR-146a, miR-96, miR-365, and miR-34a), which were more than twofold upregulated early after irradiation and returned to their normal levels of expression or became slightly downregulated at 24 hours postirradiation with 1 and 10 Gy (Fig. 2B). Two other groups showed consistent upregulation after 4 and 24 hours postirradiation with 1 or 10 Gy (miR-520a, let-7d, miR-489, miR-193a, miR-188, miR-122a, let-7c, miR-203, and miR-34b) or downregulation (miR-1, miR-93, let-7f, miR-338, miR-186, miR-384, miR-181c, miR-326, and miR-522) as compared to nonirradiated cells (Fig. 2C).

Among miRNAs found to be significantly dysregulated after the acute exposure to radiation, there was no miRNAs known to be strongly upregulated in thyroid papillary carcinomas, such as miR-221, miR-222, miR-146b, miR-155, and

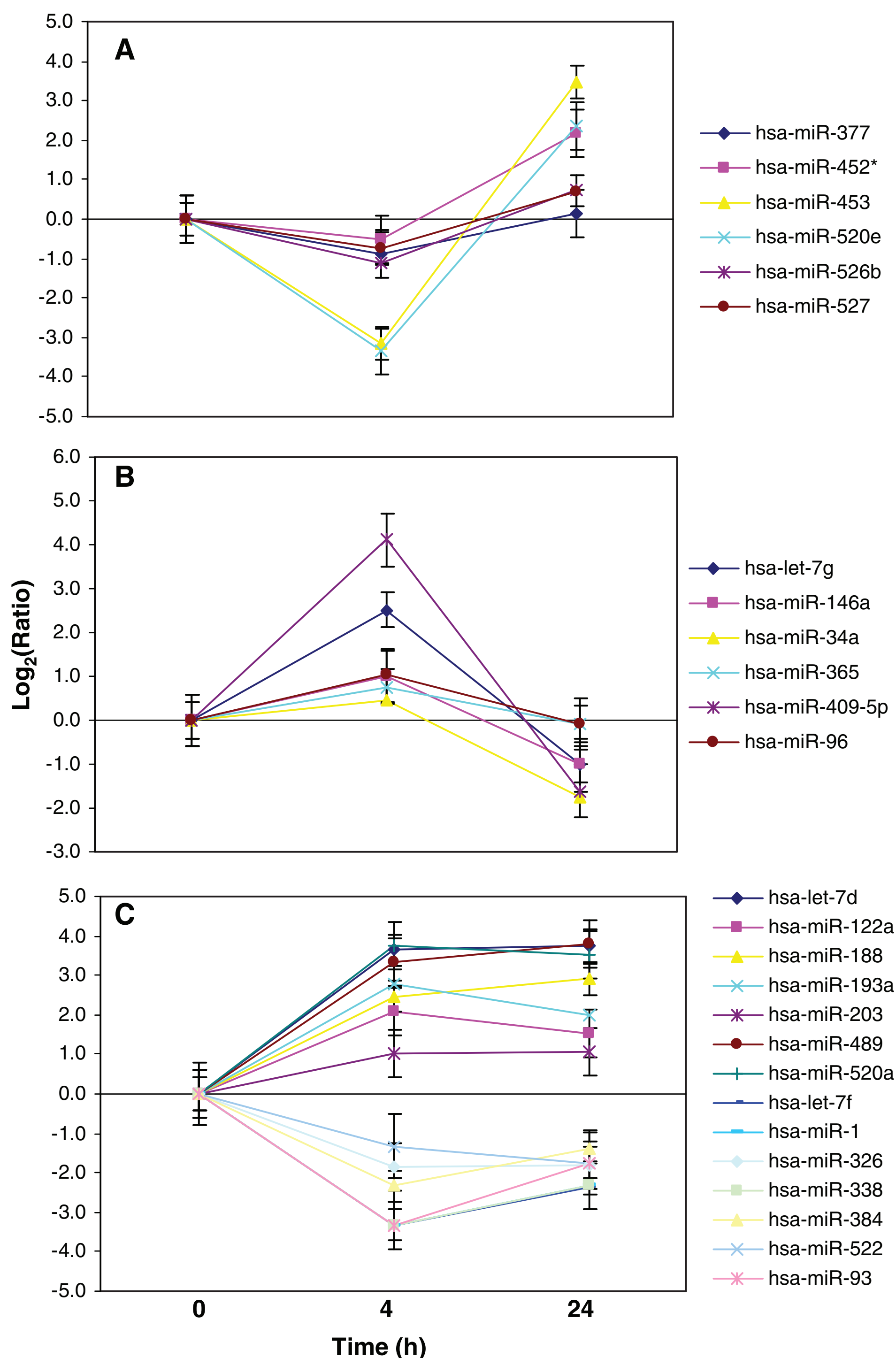

FIG. 2. Temporal patterns of miRNA expression after 1 Gy of γ-radiation. **(A)** miRNAs downregulated at 4 hours and returned to normal or to upregulated level at 24 hours; **(B)** miRNAs upregulated at 4 hours and returned to normal or downregulated level at 24 hours; **(C)** miRNAs either upregulated or downregulated at both time points. Bars, mean ± standard error. Color images available online at www.liebertonline.com/thy.

miR-187. The latter three of those are particularly frequently dysregulated in tumors carrying a radiation-related *RET/PTC* rearrangement.

Discussion

In this study, we investigated the expression levels of miRNAs after acute exposure of human normal thyroid cells to 1 and 10 Gy of γ-radiation. These doses of radiation were in general comparable to those received by individuals who subsequently developed thyroid cancer in human populations. For example, thyroid doses in those affected by the Chernobyl accident were in the range of 0.05–4 Gy and thyroid cancer patients previously exposed to therapeutic or environmental radiation received 0.09–12.5 Gy (5). We found a significant overall change in the levels of miRNA expression at both 4 and 24 hours time intervals after exposure. Similar to our findings, early changes in miRNA expression after irradiation were found in human T-cell and B-cell lymphoblast cell lines (32), human prostate cell lines (24), and lung cancer

cells (23), which showed miRNAs dysregulation at 0.5 to 4 hours postexposure.

We observed several specific patterns of miRNA response to γ-radiation, including some miRNAs that were consistently upregulated or downregulated at both time intervals. However, other miRNAs showed a time-dependant response, with specific miRNAs being downregulated or upregulated at 4 hours, the time of most active DNA repair, with the levels of expression returning to their baseline levels by 24 hours. Similar patterns in miRNA dysregulation were observed in lung epithelial cells after exposure to 2.5 Gy of radiation (23) and in human fibroblasts after low-linear energy transfer radiation (33), suggesting that these time-dependent patterns represent a common paradigm of miRNA response of various types of normal human cells to radiation exposure. The difference in miRNA expression patterns points to the complexity of cellular response to radiation. However, the exact function of miRNAs in the cell response to radiation remains unclear. If miRNA plays an active role in this process, the downregulated miRNAs may act to increase expression of genes responsible for DNA repair, whereas the upregulated miRNAs may aim to decrease the expression levels of pro-apoptotic genes to prevent cell death. On the other hand, it is possible that after radiation exposure miRNAs react on the changed levels of the coding gene expression aiming to return those to the baseline levels. In this case, they would be expected to have a limited biological role, but still can be exploited as biomarkers of radiation exposure and DNA damage repair.

Several miRNAs found to be dysregulated in thyroid cells in this study, such as let-7 family, miR-34, and miR-520, have been previously found to be dysregulated after radiation exposure in other cell types, suggesting that they participate in radiation response across the different cell types (23,34). Other miRNAs found to be dysregulated in this study (such as miR-409-5b, miR-452, miR-489, and miR-203) have not been previously reported in association with radiation exposure and may be unique for thyroid cells. It is important to note that this study was based on the analysis of 319 mature miRNAs and more recently a significant number of additional miRNAs has been discovered (35). Therefore, additional miRNAs whose expression is altered in thyroid cells after exposure to ionizing radiation may be discovered in the future.

Many upregulated and downregulated miRNAs found in this study are predicted to target genes involved in DNA repair. In fact, using three prediction programs, miRBase, TargetScan, and PicTar, the dysregulated miRNAs may target several key components of this process such as *ATM*, *ATR*, *RAD51*, and *HRCC4* genes. Interestingly, miR-203 is predicted to target several genes, *RAD51, HRCC4*, and *ATM,* following the basic principle of miRNA action where no perfect complementarity between the miRNA sequence and 3′-UTR of a gene is required for inducing the translational repression, and, therefore, a single miRNA molecule can regulate expression of multiple protein coding genes. On the other hand, many individual coding genes have at their 3′-UTRs the predicted target sites for multiple miRNAs, indicating the likelihood of a combinatory action of several miRNAs on gene activity. In our study, 6 dysregulated miRNAs, miR-520a, miR-520e, miR-526b, miR-203, miR-186, and miR-96, are predicted to target the *RAD51* gene.

This study also explored the possibility that miRNAs may directly participate in radiation-induced carcinogenesis by influencing the generation of cancer-specific chromosomal rearrangements such as *RET/PTC*. This genetic abnormality is a common feature of radiation-induced thyroid cancer, and evidence exists suggesting that it may be formed as a direct consequence of radiation-induced DNA breaks (36). Importantly, the presence of this chromosomal rearrangement in thyroid cancer cells correlates with strong upregulation of miR-187, miR-146b, and miR-155 (20). If upregulation of those miRNA was found in thyroid cells after radiation exposure, it would suggest that miRNAs play a direct role in the generation of carcinogenic chromosomal rearrangements in human cells. However, none of those miRNAs were found to be upregulated either after 4 or 24 hours after exposure. This indicates that miRNAs are unlikely to participate directly in the generation of carcinogenic chromosomal rearrangements after radiation exposure, but more likely to play a role in the cellular response to radiation by modulating the expression of DNA repair genes.

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Disclosure Statement

The authors declare no conflict of interest related to this study.

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