Identification of specific microRNAs responding to low and high dose γ-irradiation in the human lymphoblast line IM9

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Abstract. MicroRNAs (miRNAs) are short single-stranded RNA molecules that regulate the stability or translational efficiency of target messenger RNAs. Specific miRNAs are required for strict tissue- and developmental stage-specific expression. These miRNAs have roles in many human tumor malignancies and their expression is specifically regulated on each stage of oncogenic process. Therefore, miRNA expression profiling can be used as a new class of biomarker that indicates the development of cancer. Many recent studies indicated that cell exposure to ionizing radiation also induces various physiological responses including DNA repair, cell cycle arrest, cell death and differentiation. In addition, some studies suggest that exposure to low dose radiation induces a favorable effect on cells. However, the functions of miRNAs related to the response of irradiated cells have not been well studied, especially after low dose radiation. In this study, expression profiles of miRNAs isolated from irradiated cells at low and high dose radiation were analyzed with microarrays, and these data were validated using quantitative RT-PCR. Here, we describe specific miRNAs that are expressed in a dose-dependent manner that serve as new markers of irradiated immune cells.

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Introduction

MicroRNAs (miRNAs) are small single-stranded RNAs of 21-25 nucleotides in length that are generated by the endonuclease Dicer from endogenous hairpin-shaped transcripts (1-3). One of the strands cleaved by Dicer is incorporated into the RNA-induced silencing complex (RISC) where it induces post-transcriptional gene silencing by complementary binding to target mRNAs (4-6). Recent studies have revealed that hundreds of miRNAs are found in the human genome and are responsible for diverse cellular processes including the control of developmental timing, cell proliferation, apoptosis and tumorigenesis (2,7-9). Regulation and expression of miRNAs and their target mRNAs are complicated. Some miRNAs are widely expressed, whereas others are exhibited at only limited developmental stages in a tissue- and cell type-specific manner (10-14). A single miRNA can regulate many different target mRNAs, and several miRNAs can repress a single target gene in a coordinated fashion (15,16). In addition, miRNAs are one of the largest regulatory gene families, and they could have coordinated roles for total gene expression at the cellular level (3,8,16). These miRNAs are also involved in the development of many human malignant tumors and their expression is specifically regulated depending on each oncogenic stage. This suggests that miRNA expression profiling may provide a new class of biomarkers for cancer assessment (17-19). The production of mature miRNA is induced by transcriptional activators such as p53 and NF-κB (20,21). Some miRNAs as direct and conserved target genes of tumor suppressor p53 presumably mediate induction of apoptosis, cell cycle arrest, and senescence under DNA damaging conditions (22,23). DNA damage is caused by multiple stresses including ionizing radiation, reactive oxygen species, UV exposure, and many DNA damaging chemicals such as doxorubicin and camptothecin (24-26). Radiation is widely used in medicine, academics, and industry, as well as for generating electricity. As the use of radiation is expanded in diverse fields, concerns about radiation exposure in everyday surroundings have also increased. Exposure to high dose radiation causes ionization in the molecules of living cells, causing DNA damage and promoting malignant tumor

formation (27-31). However, low dose radiation induces some beneficial effects on organisms, called hormesis (32). For example, after low dose irradiation, cell proliferation was increased in normal human lung fibroblasts, and the lifespan of fruit flies and mice was extended (33,34). The biological effects of low dose radiation exposure, usually found in industrial workers or people near power plants has not been extensively studied. In the present study, we describe several specific miRNAs that responded to ionizing radiation in a dose-specific manner, and analyze the importance of miRNAs in cellular responses. We also expect that these miRNAs can be used in characterization the cellular mechanism in response to low and high doses of radiation.

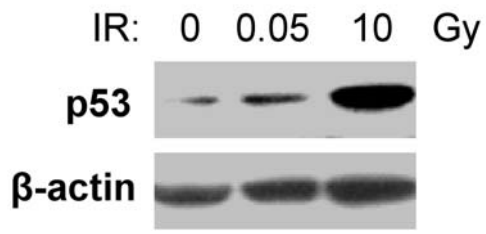

Figure 1. The level of p53 protein in the irradiated IM9 human B lymphoblastic cells at low and high dose. Whole-cell lysates from IM9 human B lymphoblastic cells were prepared at 8 h after exposure to ionizing radiation (0, 0.05 and 10 Gy), and expression levels of p53 protein were examined by Western blot analysis.

Materials and methods

Cell culture and irradiation. Human B lymphoblast cell line IM9 was grown in RPMI-1640 media (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum at 37˚C in a humidified incubator containing 5% CO_2. A total of $1x10^6$ cells were seeded and cultivated for 24 h. These cells were irradiated with 0, 0.05, or 10 Gy using a Gammacell 3000 Elan irradiator (^{137}Cs γ-ray source; MDS Nordin, ON, Canada). Before analysis, irradiated cells were incubated for 8 h.

Western blotting. Irradiated cells were collected by centrifugation. Total cell lysates were extracted in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate) and boiled for 3 min with 4X SDS sample buffer. Twenty micrograms of lysates were resolved on a 9% SDS-polyacrylamide gel. The proteins on this gel were electrically transferred to a nitrocellulose membrane and subject to Western blotting. Antibodies against p53 (Santa Cruz, CA, USA) and ß-actin (Sigma, MO, USA) were diluted in 5% skim milk solution dissolved in PBS-T (0.5% Tween-20 in PBS). Protein bands were detected with the ECL Western blot detection reagent (Pierce, CA, USA)

RNA isolation and miRNA microarray analysis. To purify the miRNAs for microarray and qRT-PCR, total RNAs were isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. The purity of the extracted RNA was determined by the ratio of absorbance at 260/280 nm, and degradation was determined by ratio of 28s and 18s rRNAs (28s/18s) with the Bioanalyzer 2100 (Agilent, CA, USA). miRNAs were then isolated from the qualified total RNAs using the PureLink miRNA Isolation kit (Invitrogen). Two micrograms of miRNAs were fluorescently labeled with Cy3 and Cy5 dyes and hybridized for 12 h at 42˚C with miRNA array slides, mirVana miRNA Probe Set (Ambion, TX, USA). The microarrays were washed and dried as recommended by the manufacturer and scanned using an Axon GenePix 4000B fluorescent scanner.

miRNA real-time PCR analysis. miRNA expression was also analyzed using the mirVana qRT-PCR miRNA primer set (Ambion). Briefly, cDNA was made from 50 μg of miRNA using a specific primer for each miRNA in reverse transcription at 42˚C for 10 min. qRT-PCR was carried out with Line Gene K real-time PCR instrument (Bioer, China) using SuperTaq Polymerase (Ambion). Expression profiling of miRNAs was detected with SYBR green (Invitrogen). Cycle parameters for the PCR reaction were 95˚C for 3 min for initial denaturing, followed by 30 cycles at 95˚C for 15 sec and an annealing and extension step at 60˚C for 30 sec. All reactions were run in triplicate. The PCR products were normalized with the mirVana qRT-PCR miRNA primer set for Normalization (U6) (Ambion).

Results and Discussion

p53 expression after low and high dose radiation in IM9 cells. The p53 tumor suppressor gene is a transcriptional factor that accumulates in cells upon DNA damage (24,35). To evaluate DNA damage caused by irradiation, p53 protein levels were verified with Western blotting. IM9 human B lymphoblast cells were irradiated at each dose of 0 (control), 0.05 (low dose) and 10 Gy (high dose). After incubation for 8 h, whole cell lysates were extracted and subjected to SDS-PAGE. p53 protein levels were detected with specific antibodies. p53 protein levels were virtually unchanged when IM9 cells were exposed to low dose radiation (0.05 Gy) compared to control (Fig. 1). However, high dose radiation (10 Gy) induced significant p53 protein accumulation. This means that DNA damage did not occur after low dose radiation (0.05 Gy) and the high dose irradiation of 10 Gy increased the p53 protein levels in response to the DNA damage in IM9 cells. This result showed that the physiological response and regulation could be quite different in low and high dose irradiated cells.

miRNA expression profiling in response to ionizing radiation in IM9 cells. Recently, miRNAs were shown to play an important role as a key molecule in the development of diseases (14,36-38) and their expression is different in tissue development lineages and in the differentiation states of various human malignant cancers (38-40). Profiling miRNA expression may uncover a promising new class of cancer markers. Based on this idea, we attempted to find specific miRNAs responding to low and high dose irradiation. To determine the change in miRNA expression induced by irradiation, microarrays were performed with miRNAs extracted from cells irradiated at different doses. miRNAs were isolated from the irradiated IM9 cells at 8 h after irradiation. Non-irradiated control miRNAs were labeled with

Table I. The expression profiles of miRNAs in IM9 human B lymphoblastic cells in response to low and high doses of ionizing radiation.

	Number of miRNA	miRNA name	Fold changes		Known functions
			0.05 Gy	10 Gy	
Only 10 Gy					
Increase	5	hsa-miR-324-3p	1.87	4.08	-
		hsa-miR-328	1.87	3.70	Regulates expression of mouse ß-amyloid precursor protein converting enzyme 1 and targets to CD44; down-regulated by prion-induced neurodegeneration
		hsa-miR-187	1.61	2.79	Significant expression level change between thyroid tumor and normal tissues
		hsa-miR-99b	1.07	2.32	Down-regulated in adenocarcinoma tissues
		hsa-miR-326	1.57	2.24	Regulates expression of smo to suppress cell growth
Only 0.05 Gy					
Decrease	13	hsa-let-7f-2	-2.13[a]	-1.46	Significantly up-regulated in bladder cancers
		hsa-miR-19a	-2.14	-1.42	Targets to ATXN1
		hsa-miR-106b	-2.40	-1.43	Down-regulated during human mega-karyocytopoiesis
		hsa-miR-376a	-2.44	-1.34	Highly expressed in human islet beta- and non-beta cells
		hsa-miR-16-1	-2.48	-1.33	-
		hsa-miR-23a	-2.97	-1.59	Targets to Hes1 during retinoic acid-induced neuronal differentiation
		hsa-miR-18	-2.98	-1.44	Down-regulates the glucocorticoid receptor in brain
		hsa-miR-23b	-3.00	-1.53	Targets to Hes1 during retinoic acid-induced neuronal differentiation
		hsa-miR-155	-3.95	-1.23	Regulated by TGF beta/Smad pathway and targets to RhoA; highly expressed in various tumors
		hsa-miR-106a	-5.49	-1.43	Down-regulated during human mega-karyocytopoiesis
		hsa-miR-17-5p	-5.92	-1.50	Targets to AIB1; key regulator of cell cycle
		hsa-miR-21	-6.49	-1.30	Over-expressed in various tumor cells; targets to PTEN and TPM1
		hsa-miR-20	-11.82	-1.43	Down-regulated during human mega-karyocytopoiesis; over-expressed in megakaryoblastic leukemic cells; targets to c-myc and E2F; up-regulated by E2F
0.05 and 10 Gy					
Increase	3	hsa-miR-199a-2	2.36	4.25	Down-regulated in the lungs of F344 rats chronically treated with a tobacco-specific carcinogen NNK
		hsa-miR-197	2.22	5.34	Over-expressed in follicular thyroid carcinoma; causes over-growth and inhibition of growth arrest in cells
		hsa-miR-207	2.02	5.24	-
Decrease	1	hsa-miR-220	-2.00	-2.09	-

[a]The minus symbol (-) in the numeral indicates a down-regulated value.

Table II. Specifically decreased miRNAs at low dose irradiation in IM9 cells.

miRNA name	0.05 Gy	
	Microarray	qRT-PCR
hsa-miR-20	-11.82[a]	-1.84
hsa-miR-21	-6.49	-1.93

[a]The minus symbol (-) indicates a down-regulated value.

Cy3, and miRNAs from irradiated cells were labeled with Cy5.

Microrarray analyses showed that miRNA expression profiles were changed more significantly in irradiated cells compared to non-irradiated cells. We identified differentially expressed miRNAs in irradiated IM9 cells. Of 210 probe sets on the mirVana miRNA Probe Set Chip, 203 spots for each irradiated cell at 0.05 and 10 Gy were detectable for each comparison of miRNAs between non-irradiated cells and irradiated cells. A total of 22 probe sets were changed more than 2-fold in miRNAs from irradiated cells (Table I). Among them, five miRNAs (hsa-miR-324-3p, -328, -187, -99b, and -326) were increased in the high dose cells (10 Gy) and 13 miRNAs (hsa-let-7f-2, hsa-miR-19a, -106b, -376a, -16-1, -23a, -18, -23b, -155, -106a, -17-5p, -21, and -20) were decreased in the low dose cells (0.05 Gy). We also found that the expression of four miRNAs (hsa-miR-199a-2, -197, -207, and -220) had similar patterns in both low and high dose irradiated cells. These data show that exposure to ionizing radiation induced a change in specific miRNA expression in a dose-dependent manner, suggesting the possibility that a specific response to low dose radiation mediates these specific miRNA molecules.

Validation of miRNA profiling by microarray using qRT-PCR. To verify the accuracy of microarray data for miRNA expression profiling, qRT-PCR was performed with several miRNAs that showed expression changes. The miRNAs were isolated and subject to qRT-PCR with a mirVana qRT-PCR miRNA primer set. Four expression profiles, miR-20 and -21 at low dose radiation and miR-197 and -199a at high dose radiation, were repeatedly in accordance with microarray data. Expression of miR-20 and -21 from the low dose irradiated cells were decreased in both experimental assays (Table II). The expression pattern of miR-197 and -199a in the microarrays were similar to the qRT-PCR data only at high dose irradiation. Although low and high dose irradiation increased expression of both miRNAs in the microarrays, they were not significantly changed in low dose irradiation in qRT-PCR (Table III). The increased or decreased patterns of four selected miRNAs were consistent between the microarray and qRT-PCR. However, the analysis of the fold changes for the miRNAs did not show good concordance between the two platforms. The disagreement seems to result from the different sensitivities between fluorescent labeling with Cy3/5 and SYBR green (41).

miR-20 that was decreased in low dose irradiated cells is included in miR-17-92 cluster on chromosome 13, which finely regulates the expression of E2F-1 together with c-Myc on both a transcriptional and translational level (42,43). E2F-1 and c-Myc are involved in cell proliferation. Activated E2F-1 induces p53-dependent apoptosis, blocking carcinogenesis through the regulation of c-Myc expression (44,45). In addition, E2F-1 activated the expression of miR-20a, and miR-20 repressed the translation of E2F-1 as part of a negative feedback circuit (43). Therefore, miR-20a could be a key inhibitor that represses many cancers by regulating E2F-1 in cell proliferation and apoptosis. miR-20a expression was also decreased during differentiation from a $CD34^+$ hematopoietic precursor cells to megakaryocytes, and was increased in megakaryoblastic leukemia cell lines (46). These studies also proposed that miR-20a is increased in tumorigenesis as a suppressor.

miR-21 suppressed translation of tumor suppressor PTEN, which is induced upon malignant transformation of normal cells by the accumulation of Akt (47). The tumor suppressor gene tropomyosin 1 (TPM1) is another target of miR-21 that is involved in tumor suppression (48). This miRNA, the so-called onco-miRNA, is highly expressed in various solid tumors such as breast and brain cancers (49,50).

Taken together, our results provide evidence that low dose radiation could suppress the progression of malignant cancer by controlling miRNA expression. miR-197 expression was decreased in follicular thyroid carcinoma cells compared to follicular adenoma, although the pathogenesis and its relation to follicular adenoma remain unclear (51). Injection of this miRNA into a cell induced proliferation and growth inhibition that are typical of carcinogenesis. Together with our data, these findings indicate that high dose radiation stimulates the expression of miR-197 and the progression of tumorigenesis. The other miRNA (miR-199) that was increased at high dose irradiation has unknown cellular function and expression

Table III. Comparison of the expression levels of miR-197a and -199 between microarray and qRT-PCR.

miRNA name	0.05 Gy		10 Gy	
	Microarray	qRT-PCR	Microarray	qRT-PCR
hsa-miR-197a	2.22	-1.30[a]	5.34	4.05
hsa-miR-199	2.36	-1.55	4.25	4.64

[a]The minus symbol (-) indicates a down-regulated value.

level. However, its expression profile could be also used to reveal physiological mechanisms in the irradiated immune cells.

Our data presented several characteristic miRNAs with changing expression levels depending on the irradiation dose, suggesting possible key roles in the regulation of cellular response on exposure to radiation, and their mechanisms should be further studied.

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