

Alteration of miRNA profiles by ionizing radiation in A549 human non-small cell lung cancer cells

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Abstract. Ionizing radiation (IR) is widely used in cancer treatment and in biological studies. It disrupts cellular homeostasis through multiple mechanisms including changes of the expression profile of genes. Although microRNAs (miRNAs) have recently been recognized as important post-transcriptional regulators and are involved in various biological processes, whether miRNAs play any roles in the cellular response to IR, is not well examined. We investigated the profile of miRNA expression following IR in the human lung carcinoma cell line A549, and the expression profiles of IR-responsive miRNAs were confirmed by qRT-PCR. The target mRNAs of IR-responsive miRNAs were predicted with a target prediction tool. Microarray analysis identified 12 and 18 miRNAs in 20- and 40 Gy-exposed A549 cells, respectively, that exhibited more than 2-fold changes in their expression levels. Of these, four were changed in only 20-Gy-treated cells, ten only in 40-Gy-treated cells, and eight miRNAs were found to change after both treatments. qRT-PCR analysis of a subset of the miRNAs showed patterns of regulation as the microarray data, although the magnitude of the changes differed in the two data sets. Target prediction for IR-responsive miRNAs suggests that they target genes related to apoptosis, regulation of cell cycle, and DNA damage and repair. Taken together, these data suggest that miRNA expression is affected by radiation, and they may be involved in the regulation of radiation responses.

Introduction

MicroRNAs (miRNAs), non-coding RNAs of about 22 nucleotides in length, are critical regulators of the translation of specific target mRNAs (1-4). miRNAs usually direct the

miRNA-induced silencing complex to target mRNAs through partial sequence complementarity to the 3' UTRs of target miRNAs, resulting in the suppression of their translation (1,5-7). However, alternative functions of miRNAs, such as the activation of gene expression through interactions with complementary sequences in a gene promoter or the 5' UTR of mRNAs (8,9), have also begun to emerge. The primary miRNA is initially transcribed by RNA polymerase II and processed to a stem-loop-containing precursor miRNA (pre-miRNA) by the microprocessor complex in the nucleus (10-12). Finally, the pre-miRNA is exported to the cytoplasm where it is cleaved by Dicer to form the mature miRNA (5,13-15).

As regulators of mRNA translation, miRNAs are involved in many biological processes including developmental timing, stem cell function, cellular differentiation, proliferation, apoptosis, cell cycle, and diseases such as cancer (16-20). It is known that a single miRNA can target multiple genes and a given gene may also be targeted by multiple miRNAs (21-24). Interestingly, miRNAs may be useful as biomarkers for the identification classification and tissue origin identification of various tumors (25).

Ionizing radiation (IR) is broadly used in cancer treatment and in biological studies. It induces oxidative molecules that cause damage to DNA, proteins, and lipids in the cells (26). The cellular radiation response, which may include DNA repair, cell cycle arrest, and apoptosis, is regulated by numerous genes, such as ataxia-telangiectasia mutated (ATM), tumor suppressor protein p53 (TP53) and all its downstream targets, and DNA damage checkpoint-related genes including cyclin-dependent kinases (CDKs) and CDC25 family proteins (27-30). The radiation sensitivity of a cell is affected by many factors including cell growth rate, DNA ploidy, and the presence of inhibitors of DNA repair or histone deacetylation (31-34). Furthermore, the activity of genes such as Akt/protein kinase B (PKB) influences the radiosensitivity of non-small cell lung cancer cell lines (35,36).

This study was designed to test the hypothesis that miRNA are involved in the cellular responses to IR. To test this hypothesis, we first conducted a miRNA microarray experiment with which we identified radiation-responsive miRNAs. We

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then confirmed the expression pattern of a subset of these miRNAs by qRT-PCR. Our data indicate that several miRNAs exhibit radiation-responsive changes in expression, suggesting that miRNAs are involved in the cellular responses to IR.

Materials and methods

Cell culture. The human lung carcinoma cell line A549 was cultured at 37°C in RPMI-1640 containing 10% fetal bovine serum (FBS) and antibiotics at in a humidified chamber supplemented with 5% CO₂. At one day before irradiating, 5×10⁵ cells were seeded on 60-mm culture dishes.

Irradiation and RNA preparation. Following 24 h in culture, the cells were exposed to either 20 or 40 Gy of gamma IR with a Gammacell® 3000 Elan irradiator (¹³⁷Cs γ-ray source; MDS Nordin, ON, Canada). The irradiated cells were then maintained for 24 h before extracting total RNA with TRIzol® reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. For the microarray studies, the quality and concentration of RNA samples were determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and an Ultrospec 3300 Pro UV/Visible Spectrophotometer (Amersham Biosciences, NJ, USA). The recommended RNA quality parameters for the microarray analysis are an OD 260/280 ratio in the range of 1.8-2.0, an OD 260/230 ratio >1.8, a 18s/28s rRNA ratio in the range of 1.8-2.1, and an RNA integrity number (RIN) >8.0.

Microarray analysis of miRNA profiles. The human miRNA microarray ver. 2 kit (Agilent Technologies), which contains probes for 723 human and 76 human viral miRNAs was adapted to analyze the expression profiles of miRNAs. Before hybridizing the miRNA to the microarray, the total RNA (100 ng) including miRNAs was dephosphorylated with calf intestine alkaline phosphatase and denatured by adding DMSO and heating. The dephosphorylated RNA was labeled with pCp-Cy3 by T4 RNA ligase, and the labeled RNA was purified with a Micro Bio-Spin P-6 column (Bio-Rad Laboratories, CA, USA). The purified RNA was denatured and hybridized to the microarray at 55°C and 20 rpm in an Agilent Microarray Hybridization Chamber (Agilent Technologies) for 20 h. Then the microarray slide was washed with wash buffers and scanned with an Agilent scanner to obtain the microarray image. The numerical data for the miRNA profiles were extracted from the images with the Feature Extraction program (Agilent Technologies). These data were analyzed with GeneSpring GX software version 7.3 (Agilent Technologies).

qRT-PCR analysis of miRNAs. qRT-PCR was used to confirm the miRNA expression profiles obtained from the microarray data. The template miRNA cDNA was constructed from total RNA by reverse transcriptase with RT primers from miScript PCR Systems (Qiagen, Hilden, Germany). The PCR was conducted with a Line gene K Real-Time PCR machine (BIOER Technology Co., China). The SYBR and U6 gene were used for detecting the gene amplification and normalizing the each sample, respectively. Relative quantification of the miRNAs was calculated with

Pfaffl's model (37) after determining the PCR efficiency by the method of Rasmussen (38).

Target prediction of miRNAs. Candidate miRNAs, which changed more than 2-fold following radiation exposure, were picked for target prediction and analyzed with miRBase Targets Version 5 on the Enright Lab (Wellcome Trust Sanger Institute) website (<http://microrna.sanger.ac.uk/targets/v5/>). Human genes with experimentally verified functions related to apoptosis, cell cycle arrest, or DNA damage and repair, were selected from the Gene Ontology website (<http://www.geneontology.org/>). Finally, the genes predicted as targets by candidate miRNAs and those selected on the basis of gene ontology were aligned by their gene names, and genes appearing in both lists were chosen and listed.

Results and Discussion

We first wished to determine which miRNAs showed changes in expression in response to IR. To monitor the expression profiles of miRNAs following IR treatment, we employed a microarray containing probes to 723 human miRNAs. In our preliminary study, we found that A549 was less sensitive to radiation than other cell types (data not shown); therefore, we chose relatively high radiation doses (20 and 40 Gy) for this study. At 24 h after IR exposure, total RNA was extracted from the cells and was used to probe the microarray. A miRNA was flagged as 'Present' if it yielded sufficiently high quality data in experimental conditions. In total, 202 miRNAs were flagged as Present, and their expression profiles following each radiation dose are shown in Fig. 1A. Although the expression levels for all miRNAs showed some variability, most miRNAs did not show strong response in expression following IR.

To identify the miRNAs with robust changes in expression following IR treatment, we carried out fold change analysis on the microarray data. In this analysis, 12 and 18 miRNAs in 20- and 40-Gy-treated cells, respectively, showed >2-fold changes compared to control cells (Fig. 1B). Interestingly, the fraction of up- versus down-regulated miRNAs was very different between the 20- and 40-Gy-treated cells. Among IR-responsive miRNAs, eight miRNAs were found to be changed following both the 20- and 40-Gy treatments. In contrast, four miRNAs were unique to the 20-Gy-treated cells and ten miRNAs showed expression changes only in the 40-Gy-treated cells (Fig. 1C). The IR-responsive miRNAs and their expression values are listed in Table I. Although we found that the fraction of strongly up- and down-regulated miRNAs differed between the two radiation doses, we noted that for each of the eight miRNAs showing expression changes following both doses of IR, the direction, but not necessarily the magnitude, of the expression change was the same for both radiation doses (Table I). However, the miRNAs such as miR-139-3p, -34b*, -22* and -34a were included only either side of radiation, they were showing near the 2-fold changes in the other radiation dose.

We next used qRT-PCR to confirm the expression profiles of five of the IR-responsive miRNAs identified in the microarray analysis. qRT-PCR analysis of miRNAs hsa-miR-16-2*, -106a, -139-3p, -345, and -516a-5p is shown in Fig. 2. For each miRNA, the expression levels following 20- and 40-Gy

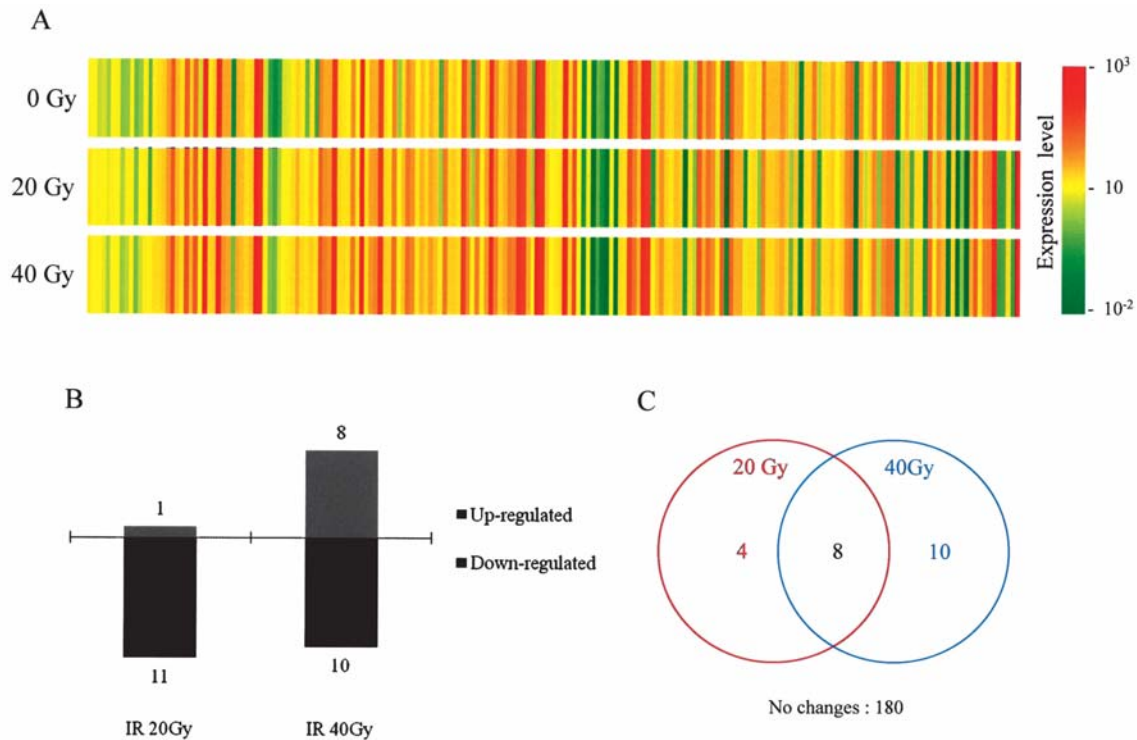


Figure 1. miRNA profiles associated with the radiation doses. After microarray analysis, the expression profiles of miRNAs flagged as Present in the A549 cells were described by color ranges (A). Among the Present-flagged miRNAs, miRNAs that changed 2-fold were selected, and their regulated patterns are summarized according to the number of miRNAs up- or down-regulated by radiation (B). The miRNAs are presented in a Venn diagram depending on the radiation dosage (C).

Table I. miRNAs changed more than 2-fold by at least one of the two radiation doses.

Inclusion	No. of miRNAs	miRNA name	Fold changes ^a	
			20 Gy	40 Gy
Only 20 Gy	4	hsa-miR-636	-25.2	-1.1
		hsa-miR-593	-13.5	-1.4
		hsa-miR-760	-2.9	-1.2
		hsa-miR-139-3p	-2.6	-1.9
20 and 40 Gy	8	hsa-miR-345	-39.1	-58.3
		hsa-miR-885-3p	-8.1	-9.5
		hsa-miR-206	-3.9	-2.0
		hsa-miR-516a-5p	-3.2	-3.0
		hsa-miR-16-2*	-2.6	-2.2
		hsa-miR-106a	-2.2	-2.2
		hsa-miR-548c-3p	-2.1	-25.4
		hsa-miR-127-3p	2.0	2.7
		hsa-miR-1228*	-1.2	-5.2
		hsa-miR-30b*	-1.3	-3.1
Only 40 Gy	10	hsa-miR-376a	1.4	2.0
		hsa-miR-34b*	1.8	2.1
		hsa-miR-215	1.5	2.2
		hsa-miR-183	1.4	2.2
		hsa-miR-22*	1.8	2.2
		hsa-miR-34a	1.9	2.4
		hsa-miR-192	1.7	2.4
		hsa-miR-30c-1*	1.3	15.6

^aThe minus symbol (-) in the numeral indicates a down-regulated value.

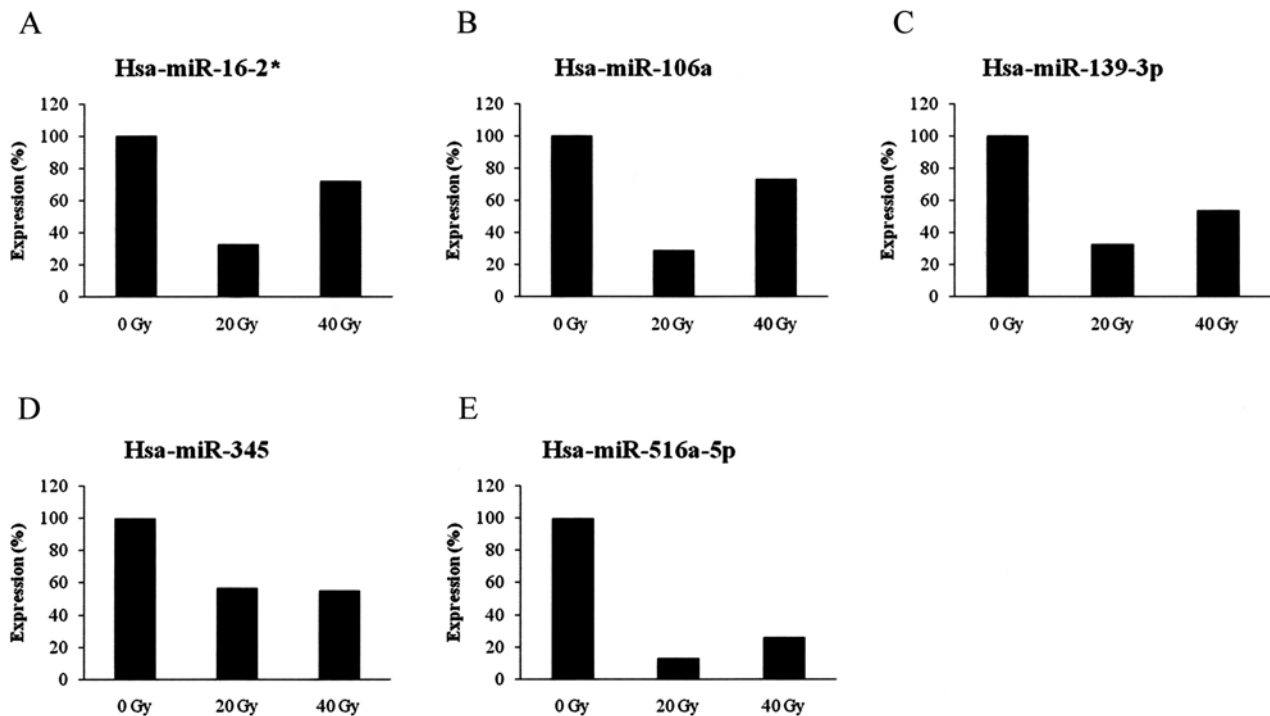


Figure 2. Quantification and confirmation of miRNA expression levels by qRT-PCR. Among the miRNAs that changed more than 2-fold, five miRNAs, hsa-miR-16-2* (A), hsa-miR-106a (B), hsa-miR-139-3p (C), hsa-miR-345 (D), and hsa-miR-516a-5p (E), were selected, and their expression levels were confirmed by qRT-PCR. The expression levels were compared with those in 0 Gy-irradiated cells (controls normalized as 100).

IR treatments were compared to those of control cells, the value of which was set to 100. For all five miRNAs, the qRT-PCR experiment confirmed their down-regulation by IR. However, the magnitude of the changes in the expression levels was found to be different between the microarray and qRT-PCR analyses. In particular, miR-345 showed the largest discrepancy between the microarray and qRT-PCR data. Whereas the microarray data indicated a 50-fold decrease in mir-345 expression after IR, the qRT-PCR analysis revealed only a 2-fold change.

To begin to identify potential targets of the IR-responsive miRNAs identified in the fold-change analysis, we conducted a bioinformatical analysis directed at identifying potential targets having functions related to apoptosis, cell cycle control, and DNA damage and repair. We chose genes with these functions, since these represent the main responses observed in IR-treated cells. First, the targets of the IR-responsive miRNAs were predicted with a miRNA target prediction tool, miRBase Targets Version 5. For each miRNA, approximately 1,000 potential target genes were predicted (data not shown). However, no data was available in the target prediction tool for miR-1228*. Next, we used the Gene Ontology website to produce lists of genes having experimentally confirmed functions related to apoptosis, cell cycle regulation, or DNA damage and repair. These lists contained 172, 76 and 82 genes, respectively. Finally, genes from miRNA target prediction were compared with those from the gene ontology analysis, and the overlaps between these sets of genes are listed in Table II. We found that more of the predicted targets had functions related to apoptosis compared to cell cycle control and DNA repair. Some of the genes from this analysis were predicted to be targets of more than one miRNA. As reported

by John *et al* (39), one miRNA has the potential to target many mRNAs, and a single mRNA could be regulated by several miRNAs.

Among the IR-responsive miRNAs with potential roles in apoptosis and cell cycle arrest that we have identified, miR-34a is the best characterized (40-45): it is directly trans-activated by p53 and is involved in the regulation of apoptosis, cell cycle arrest, and senescence-like growth arrest; it also functions by targeting many genes, including SIRT1, CCND1, CDK6, and by modulating the activity of pathways such as androgen receptor (AR)-dependent p53-induced apoptosis and the E2F pathway. Although these genes were not among those predicted by miRBase, another prediction program, TargetScan 4.2, predicted them as targets of miR-34a (data not shown). This may indicate that target predictions vary greatly between different programs, and that researchers must carefully analyze potential targets. In addition, miR-34a also has tumor suppressor activity through the targeting of oncogenes such as MYCN, and aberrant CpG methylation or deletion of 1p36, the chromosomal location of miR-34a, is observed in many types of cancer (46-48).

In this study, we have demonstrated that a subset of human miRNAs show robust expression changes in response to IR. Given the broad use of radiation in cancer therapy and biological studies, it is important to note that miRNAs may have unidentified roles in the cellular response irradiation. Although we have not proved that the potential miRNA targets are directly regulated by the miRNAs identified in this study, we have highlighted the possibility of miRNA-mediated regulation of the radiation response. Future studies will be required to identify the precise mechanisms underlying this regulation.

Table II. Prediction of apoptosis-, cell cycle control-, or DNA damage and repair-related targets of miRNAs from Table I.

miRNA name	Functions of target genes		
	Apoptosis	Cell cycle and arrest	DNA damage and repair
hsa-miR-16-2*	CADM1, GTF2H3, HSPA5, NRG1	BTG3, KNTC1	EXO1, MSH2, PCNA, POLD4, SETX
hsa-miR-22*	AIFM3, CASP8, CIDEA, IL6, RTKN, TP53BP2, TRADD	ZW10	EXO1, HMGB1, WRN
hsa-miR-30c-1*	CARD8, CASP8, DFFB, ERCC3, TNFRSF10B, TRADD	CCND3, GMNN	POLD4, POLL, ERCC3
hsa-miR-30b*	AGT, BCL3, DDB2, ERCC3, PML, RTKN, TP53	PML	ERCC1, ERCC3, GTF2H4, PML, POLD4, RPA1, TP53
hsa-miR-34a	APOH, BAX, BID, BNP1, DEDD2, FAS, GRIK2, PCBP4, RYR2, TGFB1	CD28, DBC1, PCBP4, TCF3, TGFB1	PCBP4, POLD1
hsa-miR-34b*	BAX, BNP1, BRE, FAS, NLRP2, SCG2	ATR, DBC1, SPHK1, TCF3	ATR, ERCC5, RFC5
hsa-miR-106a	BAX, BNP1, HBXIP, NUP62, P2RX4, PPP3R1, TNFRSF10B	BTG3	GTF2H3, MSH3, POLH
hsa-miR-127-3p	AIFM3, BAD, BCL6, NLRP1, PPP3CC	CCND3, RBM38	NEIL1, RBM38, RPA1
hsa-miR-139-3p	ADAMTSL4, AKT1, BAD, BRE, CASP10, CDC2, DNABP6, MAEA, NLRP1	BRK1, GTPBP4, TCF3	POLA1, POLE
hsa-miR-183	CASP10, DFFB, GRIK2, HIP1, IL12B, LCK, PPP3CC, SRA1, TCF7L2	BTG3, CD28, CDKN3, HEXIM2, IL12B, NUSAP1	EXO1
hsa-miR-192	ALB, ANGPTL4, ERCC3, FURIN, TCF7L2	SPHK1	CDK7, ERCC3, ERCC4, XPA
hsa-miR-206	CIDEA, HSPD1, RASA1, SCG2, TBX3, TCF7L2	CDKN3, SPHK1, TBX3	-
hsa-miR-215	ALB, ERCC3, FURIN, P2RX4, TCF7L2	SPHK1	CDK7, ERCC3, ERCC4, TDG, XPA
hsa-miR-345	ANGPTL4, DHCR24, KNTC1, PCBP4	ZW10	LIG1, POLD3, POLL, RFC1, RPA1
hsa-miR-376a	CADM1, CASP8, FAS, HBXIP, IL6, PPP3CC, TERF1	ATR, BTG3, CDT1, GMNN, NEK11	ATR, MNAT1, NEK11
hsa-miR-516a-5p	ADAMTSL4, BRCA1, CASP8, CD27, CIDEA, FURIN, MAEA, TNF	BMP4	ASF1A, BRCA1, GTF2H4, MBD4
hsa-miR-548c-3p	AGT, CD70, DFFB, FAS	AFAP1L2, EGF, GMNN	CDK7, GTF2H1, HMGB2, MBD4, MPG, MSH2, PCNA, PMS2
hsa-miR-593	CD27, DDB2, DYNLL1, RYR2, STK4	-	-
hsa-miR-636	AGT, BFAR, COL4A3, P2RX4, TRAF2	DBC1	ERCC4, ERCC5, OGG1
hsa-miR-760	ACIN1, BRCA1, BRE, CASP10, CIDEA, HTATIP2, IL6, LGALS12	CDT1, SPHK1	BRCA1
hsa-miR-885-3p	AIFM3, AKT1, BNP1, CDKN2A, CDKN2C, INHA, SFN, TCF7L2	CDKN2A, CDKN2C, EDN3, ZW10	DDB1, POLD1, PRKCG, RFC5
hsa-miR-1228*	N/A	N/A	N/A

N/A, not applicable.

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