Detection of Novel Human MiRNAs Responding to X-ray Irradiation

Nan DING¹,²,³, Xin WU¹,³, Jinpeng HE¹, Lei CHANG¹,³, Wentao HU¹,³, Wenjian LI¹, Jufang WANG¹, Tieshan WANG² and Guangming ZHOU¹*

microRNA/Ionizing radiation/Solexa/qRT-PCR.

Up to now, more than 1048 human miRNAs have been identified. However, the recognition of new human miRNAs is becoming more and more difficult. Based on the hypothesis that the expression of some miRNAs can be induced by ionizing radiation, total RNAs of HeLa cells were isolated 1 h after exposure to 2 Gy of X-rays, and total small RNAs were enriched and sequenced by PAGE and Solexa technology, respectively. As a result, 421 kinds of known miRNAs and 337 kinds of unknown sequences were identified, among which 10 novel miRNAs were characterized by bioinformatic approach and verified by qRT-PCR. Finally, putative targets of these miRNAs were predicted by TargetScan software and compared with known proteins down-regulated by radiation. It was confirmed that some of the targets of these novel miRNAs were radiation-related proteins. These results imply that these 10 novel miRNAs are radiation-related miRNAs. This study reveals a new way to find novel miRNAs.

INTRODUCTION

MicroRNAs (miRNAs) are single-stranded, ~22 nt long endogenous noncoding RNAs and function in interfering the expression of their target genes by binding to the 3'-untranslated region (UTR) of the mRNAs.¹⁻³ They play an important role in molecular functions of multicellular eukaryotes.¹⁻³ In animals, miRNAs are found participating in regulation of signaling pathways, cell proliferation, apoptosis, metabolism, haematopoiesis organogenesis, and developmental timing.³⁻⁴

lin-4 was the first miRNA discovered in C. elegans in 1993.⁵ The discovery of novel miRNAs was accelerated after 2001 when M. Lagos-Quintana’s group invented a method to clone small RNAs by adding adapters to both ends of small RNAs, building cDNA library by reverse transcription, and sequencing the library at last.⁶ Quintana et al. found 37 new miRNAs from C. elegans by following this protocol.⁶ Lau et al. cloned 55 novel miRNAs from C. elegans by using a modified method in the same year.⁶ So far, there are more than 15 thousands of miRNAs cloned from 142 different species (Sep. 2010, miRBase 16.0), among which 1048 are from human being. Computational predictions basing on genome sequences indicate that there are 1,000 to 10,000 human miRNA genes.⁸⁻⁹ That means there should be more human miRNAs waiting to be discovered. However, the identification of human novel miRNA becomes more and more difficult with routine methods. In 2009, no more than 30 human miRNAs have been found (Sep. 2008, miRBase Release 12.0, Jan. 2010, miRBase Release 14.0). Low expression level is assumed to be the main reason since the successful cloning of a candidate sequence depends on its abundance in one sample. Although advanced technologies such as microarray, deep sequencing, and Ago2 immunoprecipitation are well developed, it is very hard to detect a miRNA expressed at low expression level.¹⁰⁻¹²

MiRNA expression profile varies in different stages of development and in different tissues.³⁻¹³⁻¹⁸ Abnormal expressions of miRNAs have been observed in cancer cells.¹⁶⁻¹⁹⁻²¹ Some miRNAs function as tumor suppressors and express at a high level in normal tissues while others function as oncogenes and express at a high level in tumor tissues. Recently, many reports indicate that miRNA expression level changes in response to cellular stresses, such as hypoxia, drought, cold stress, oxidative stress, radiation, and so on.²²⁻³⁷ Excitingly, several novel miRNAs were found in plants by administration with cold, dehydration, salinity, and abscisic acid (ABA).³⁸ However, reports on the observations of novel miRNAs in response to ionizing radiation are still rare so far. Ionizing radiation, such as X-rays, γ-rays, and heavy ion beams, causes cellular damages, signal transduction, DNA repair, cell cycle checkpoints, and apoptosis, in which many miRNAs take part.³⁵⁻³⁷ Therefore, we hypoth-
esize that some miRNAs which rarely express under normal condition may increase their expression upon irradiation. Up to date, there is no report on novel miRNA findings by using ionizing radiation as an exogenous stress yet.

In this study, HeLa cells were exposed to X-rays and small RNAs were isolated and sequenced via Solexa technology.39,40 After computational analysis and elimination of disturbing small RNAs, such as rRNA, tRNA, snRNA, and known miRNAs, several radiation-induced novel miRNA candidates were obtained. Finally, 10 novel miRNAs were confirmed by qRT-PCR.

**MATERIALS AND METHODS**

**Cell Culture and irradiation**

HeLa cells were maintained in RPMI-1640 media ( Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah, USA) at 37°C and 5% CO2. Cells were plated in 25 cm2 culture flask. Exponentially growing cells were irradiated with a single dose of 2 Gy X-rays (Radiation Therapy Section, General Hospital of Lanzhou Military Area), and a sham control was prepared by following the same procedure except irradiation.

**RNA isolation and sequencing**

Total RNA was extracted with TRizol Reagent (Invitrogen, Carlsbad, CA, USA) 1 h after exposure. 40 μg of total RNA was subjected to Solexa sequencing (BGI, Shenzhen, China). Briefly, RNA was purified by using polyacrylamide gel electrophoresis (PAGE) to enrich small RNA molecules (under 30 bases), and ligated with Solexa adapters to the 5’ and 3’ termini of the RNA for cDNA synthesis. The cDNA was amplified using the adaptor primers, and then the DNA was used directly for cluster generation and sequencing analysis by the Illumina Genome Analyzer (Illumina, San Diego, CA, USA).

Sequence reads with Solexa 3’ adapter (the read length being 36 nt) were picked for miRNA mapping (the same adapter was used for each sequencing run). Each sequence read was passed through a number of quality control filters. miRNAs with reads less than 4, length less than 10 nt, or more than 10 consecutive repetitive nucleotides were also removed. Then, the reads were compared with databases, sequences were annotated, and novel miRNA candidates were selected after computational analysis. At last, the remaining reads of each sample was normalized to 106 and normalized reads of each kind of miRNA, no matter of known miRNA or novel miRNA candidates, were obtained with following formula, normalized reads of a certain miRNA = detected reads of this miRNA / total reads of remained miRNAs × 106.

Data resources used in this paper were as following:

- Rfam sequences: Rfam 9.0.

**Confirmation of novel miRNAs**

C. DNA was synthesized from total RNA using miRNA-specific stem-loop primers obtained from commercial service (Takara, Dalian, China). All of the stem-loop RT primers and gene-specific primers are listed in supplemental Table 1 (See electric appendix). Reverse transcriptase reactions contained 20 ng of RNA samples, 50 nM stem-loop RT primer, 1 × RT buffer, 0.25 mM each of dNTPs (Promega, Madison, WI, USA), 0.01 M DTT (Invitrogen), 5 U/μL SuperScript™ II reverse transcriptase (Invitrogen), and 0.25 U/μL RNase Inhibitor (Promega). The 15 μL reactions were incubated in a Biometra T1 Thermalcycler in a 96-well plate at 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and then 4°C for subsequent process. The cDNAs were 1:15 diluted for performing PCR for confirmation and additional real-time PCR for expression analysis. PCR mixture contains 1 μL cDNA, 0.5 μM forward and reverse primers, 1 × PCR buffer, 1.75 mM Mg2+, 0.25 mM each of dNTPs (Promega) and 1.25 U Taq polymerase (Fermentas, Burlington, ON, Canada). The 20 μL PCR reactions were performed using Biometra T1 Thermalcycler in 200 μL micro-tubes at 95°C for 5 min, followed by 40 cycles of 15 s at 95°C, 30 s at 53°C to 60°C, and 30 s at 72°C. PCR products were detected by ethidium bromide and photographed under UV light.

**Target Analysis**

The putative target genes of all candidate miRNAs were figured out using TargetScan software, release 5.1 (www.targetscan.org).41 For these miRNAs are unreported sequences, only 2nd–8th nucleotides of each miRNA sequence were used. We further compared these targets proteins with reported radiation-related proteins.42–44 Because the expression levels of miRNAs we detected were all increased after irradiation, we only considered the down-regulated proteins.

**RESULTS**

**Solexa sequencing**

In this study, we chose X-ray radiation as exogenous stress to increase the expression level of those miRNAs rarely expressing in normal condition but up-regulated in responding to ionizing radiation, and then sequenced all small molecular RNAs using Solexa technology.39,40 Finally, 13,560,379 high quality reads were obtained from...
Novel microRNAs

control sample and 11,358,032 reads from irradiated sample. Length distribution peaked at 22 nucleotides (control: 37.31%, irradiated: 43.99%) and the reads of 19–24 nt increased 6.85% after irradiation (control: 78.29%, irradiated: 85.14%), which was consistent with the common size of miRNAs (Fig. 1). After removing the reads of low quality and masking adaptor sequences, we obtained 12,141,394 clean reads (from 18 to 30 bases) from control cells and 9,128,526 reads from irradiated cells (Table 1). Next, all reads were aligned against the homo sapience genome, NCBI Genebank non-coding sequences, Rfam sequences, known miRNA sequences, repeat-associated small RNAs, and mRNA fractions (Table 1). Finally, we obtained 421 kinds of known miRNAs, among which 397 miRNAs (5,640,350 reads) were from control and 380 (4,495,622 reads) miRNAs from irradiated sample (See electric appendix, sup. Table 2). The reads of known miRNAs from control were 46.46% and those from irradiated samples were 49.25% of total reads, which confirms a relative enrichment of miRNAs in the small RNA pool of HeLa cells after exposed to X-rays. There were 356 known miRNAs detected from both samples, among which 166 kinds of miRNAs were up-regulated while 190 down-regulated (Fig. 2). Among these changes, 20 miRNAs were up-regulated while 20 were down-regulated more than two folds (Fig. 2). Additionally, among the 421 known miRNAs, there were 41 kinds of miRNA only detected in non-irradiated sample and 24 miRNAs only in irradiated sample. All together, there were 190 kinds of miRNA up-regulated and 231 miRNAs down-regulated after X-ray irradiation.

**Prediction of novel miRNA candidates**

There were also many unknown sequences cloned from total small RNA pools. Novel miRNA candidates were identified by aligning the unknown sequences in homo sapience genome, folding the flanking genome sequences, and analyzing their structure features. Their pre-miRNA structures were gained by software called Mireap. Altogether, we obtained 337 kinds of candidate sequences, among which 198 candidates (978 reads) were from control and 208 (1,235 reads) from irradiated sample. Table 3 shows the hairpin constructions predicted by mfold software.

**Confirmation of novel miRNAs**

In order to confirm that these candidates were novel...
miRNAs indeed, we designed stem-loop reverse transcription primers (See electric appendix, sup. Table 1) as described by Yu et al. and performed qRT-PCR. As showed in Fig. 3, 10 candidate sequences were successfully confirmed by qRT-PCR in irradiated human cancer cells.

Each PCR experiment was performed no less than 3 times independently, and the relative expression level was calculated and shown in the same figure. Results show that all these miRNAs were expressed at a relatively higher level in irradiated sample than in sham control.

### Table 2. Details of novel human miRNAs.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Sequence(5'-3')</th>
<th>Size (nt)</th>
<th>Strand</th>
<th>Localization (hairpin)</th>
<th>Mfe (kcal/mol)</th>
<th>Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-mir-5087</td>
<td>GGGTTTGTAGTTTGTGGCAGATG</td>
<td>23</td>
<td>5'-arm</td>
<td>chr1 (146273227–146273302)</td>
<td>–19.02</td>
<td>10</td>
</tr>
<tr>
<td>hsa-mir-5088</td>
<td>CAGGGCTCAGGATGATGAG</td>
<td>23</td>
<td>3'-arm</td>
<td>chr19 (54877140–54877223)</td>
<td>–32.8</td>
<td>10</td>
</tr>
<tr>
<td>hsa-mir-5089</td>
<td>GTGGGATTCTGATGATCGCTC</td>
<td>21</td>
<td>5'-arm</td>
<td>chr17 (42405382–42405465)</td>
<td>–63.5</td>
<td>10</td>
</tr>
<tr>
<td>hsa-mir-5090</td>
<td>CCGGGCAGATTGTGTTGGGTTG</td>
<td>23</td>
<td>5'-arm</td>
<td>chr7 (101893194–101893278)</td>
<td>–35.3</td>
<td>11</td>
</tr>
<tr>
<td>hsa-mir-5091</td>
<td>ACGGAGACGACAAGACTTGAGC</td>
<td>23</td>
<td>3'-arm</td>
<td>chr4 (13238587–13238679)</td>
<td>–29.29</td>
<td>14</td>
</tr>
<tr>
<td>hsa-mir-5092</td>
<td>AATCCAGCCTGAGCTGCTG</td>
<td>22</td>
<td>3'-arm</td>
<td>chr3 (126352999–126353086)</td>
<td>–48.01</td>
<td>14</td>
</tr>
<tr>
<td>hsa-mir-5093</td>
<td>AGGAAATGAGGTGGCATGAGC</td>
<td>23</td>
<td>5'-arm</td>
<td>chr15 (88194873–88194957)</td>
<td>–24.7</td>
<td>55</td>
</tr>
<tr>
<td>hsa-mir-5094</td>
<td>ACAACGTGAGCTGCTTGGAATG</td>
<td>23</td>
<td>5'-arm</td>
<td>chr15 (637983641–637987325)</td>
<td>–53.5</td>
<td>27</td>
</tr>
<tr>
<td>hsa-mir-5095</td>
<td>AACCCAGAGTTGGCTGGAAATG</td>
<td>22</td>
<td>5'-arm</td>
<td>chr10 (106018879–106018156)</td>
<td>–29.7</td>
<td>48</td>
</tr>
<tr>
<td>hsa-mir-5096</td>
<td>AATCCAGTGAATGCTGCACTG</td>
<td>22</td>
<td>5'-arm</td>
<td>chr10 (88194873–88194957)</td>
<td>–24.7</td>
<td>55</td>
</tr>
</tbody>
</table>

Fig. 2. Expression of known miRNAs detected in both samples. A: Fold changes of all detected known miRNAs in both samples after reads normalization; B: Normalized reads of detected known miRNAs which changed more than two folds.
### Table 3. Predicted secondary structures of the candidate novel human miRNA precursors.

<table>
<thead>
<tr>
<th>Name</th>
<th>Stem-Loop Structure of Putative miRNA Precursors</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-mir-5087</td>
<td><img src="image1" alt="Secondary Structure" /></td>
</tr>
<tr>
<td>hsa-mir-5088</td>
<td><img src="image2" alt="Secondary Structure" /></td>
</tr>
<tr>
<td>hsa-mir-5089</td>
<td><img src="image3" alt="Secondary Structure" /></td>
</tr>
<tr>
<td>hsa-mir-5090</td>
<td><img src="image4" alt="Secondary Structure" /></td>
</tr>
<tr>
<td>hsa-mir-5091</td>
<td><img src="image5" alt="Secondary Structure" /></td>
</tr>
<tr>
<td>hsa-mir-5092</td>
<td><img src="image6" alt="Secondary Structure" /></td>
</tr>
<tr>
<td>hsa-mir-5093</td>
<td><img src="image7" alt="Secondary Structure" /></td>
</tr>
<tr>
<td>hsa-mir-5094</td>
<td><img src="image8" alt="Secondary Structure" /></td>
</tr>
<tr>
<td>hsa-mir-5095</td>
<td><img src="image9" alt="Secondary Structure" /></td>
</tr>
</tbody>
</table>

Human genomic sequences upstream and downstream of the novel miRNAs were folded with the computer program mfold. Areas with bold and underline represent the cloned miRNA.
Putative Target Prediction of novel miRNAs

All putative targets of novel miRNAs were predicted by TargetScan, which can be used to predict the targets of unreported sequences through their 2–8 nucleotides as seed sequence. After comparison, all putative targets with lower expression level after radiations according to other reports were shown in Table 4.42–44) These genes might be the targets of the novel miRNAs that we discovered since they are down-regulated by ionizing radiation while these novel miRNAs reported here are up-regulated by radiation.

DISCUSSION

Up to date, 1048 human miRNAs have been cloned (Sep. 2010, miRBase 16.0), but there are still more unknown sequences waiting to be identified.8,9) As the probability of a miRNA to be detected is dependent on its abundance, it becomes more and more difficult to find novel human miRNAs. Abiotic cellular stress is a feasible way to change the expression level of related miRNAs, thus, we assume that ionizing radiation up-regulates the expression of some radiation-related miRNAs and make them easier to be detected. To confirm this, we treated HeLa cells with X-ray irradiation, and sequenced all small RNAs using Solexa method.39,40) Finally, we obtained 421 kinds of known miRNAs from both samples, 397 known miRNAs (5,640,350 reads) from sham control and 380 (4,495,622 reads) miRNAs from irradiated sample (See electric appendix, sup. Table 2), among which there were 190 kinds of miRNA up-regulated and 231 miRNAs down-regulated after X-ray irradiation (sup. Table 2).

In all these 421 miRNAs, miR-34a is a well known tumor suppressor gene, it is induced by DNA damage through p53-miR-34 path way and it can induce cell-cycle progression, apoptosis and DNA repair.35) The increased expression of miR-34a after X-ray radiation was reported by many papers,35) our data also confirmed it is a radiation responsive miRNA as it was up-regulated more than two fold after irradiation (sup. Table 2).

Include miR-34a, we have 22 miRNA expression changes consistent with earlier reports, some miRNAs were up-regulated upon X-ray radiation, such as let-7a, let-7b, let-7c, let-7d*, let-7f, mir-137, mir-142-3p, mir-142-5p, mir-206, mir-324-3p, and mir-346, while some others were down-regulated, such as let-7g, let-7, mir-15a, mir-16, mir-18a, mir-93, mir-143, mir-221, mir-181a and mir-181b (sup. Table 2).32,35,37,48,49)

Table 4. Predicted targets of novel miRNA.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Putative Target Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-mir-5087</td>
<td>ABCC4, BTG1, KIAA0240, NR2F2, PSME3, TLN2, YWHAE</td>
</tr>
<tr>
<td>hsa-mir-5088</td>
<td>DHDDS, KIAA0240, MKL2, SFRS1, SOX5, TEAD1, TLN2</td>
</tr>
<tr>
<td>hsa-mir-5089</td>
<td>B3GAT1, DRYK2, EIF4B, RHOBTB1, SFXN3, SFRS1, Tyro3, TNPO1, ZNF395</td>
</tr>
<tr>
<td>hsa-mir-5090</td>
<td>MARK4</td>
</tr>
<tr>
<td>hsa-mir-5091</td>
<td>WHSC1</td>
</tr>
<tr>
<td>hsa-mir-5092</td>
<td>ARL4A, CuGbp2, PSD3</td>
</tr>
<tr>
<td>hsa-mir-5093</td>
<td>Ell2, SNTB2, WDR76, WDR68, WHSC1</td>
</tr>
<tr>
<td>hsa-mir-5094</td>
<td>BSN, Ccnd2, Drap1, FBXO9, Gsor1, MAF, NSFL1C, QKI, Tyro3, Txnip, YWHAE, ZNF623</td>
</tr>
<tr>
<td>hsa-mir-5095</td>
<td>CDK6, PBX1, MKRN1, NLGN2, ZCCHC2</td>
</tr>
<tr>
<td>hsa-mir-5096</td>
<td>CALD1, DDAH1, RHOBTB1, STAT5B, YWHAB</td>
</tr>
</tbody>
</table>
198 sequences (978 reads) obtained from control and 208 (1,235 reads) from irradiated sample were found to be novel miRNA candidates, among which there were 69 overlapping sequences. We chose radiation-specific candidates with high reads for further studies. After confirmation with qRT-PCR, 10 sequences were successfully verified as shown in Fig. 3. Most of these sequences were cloned in both samples though, higher expression levels were observed in irradiated sample (Fig. 3). This result indicates that it was the X-ray irradiation that up-regulated the expression level of these candidates and made them easier to be detected.

There are four acknowledged criteria to identify novel miRNAs. A, its expression should be confirmed by hybridization to a size-fractionated RNA sample. B, the small RNA sequence should be present in one arm of its hairpin precursor, which lacks large internal loops or bulges. C, the small RNA sequences should be phylogenetically conserved. D, the evidence can be strengthened if the precursor accumulates in the presence of reduced Dicer function. Typically, the first criterion (expression) is more important to annotate a new miRNA and the first criterion (expression) plus the second criterion (structure), or the first criterion (expression) plus the third criterion (conservation), are regarded as adequate.

The expression of the 10 miRNAs that we identified was confirmed by Solexa sequencing (Fig. 1) and qRT-PCR (Fig. 3). The higher miRNAs expression level in irradiated sample compared with control indicated that these miRNAs were induced by X-ray irradiation (Fig. 3). Their localizations in human genome (Table 2) were clarified and their secondary hairpin structures (Table 3) were simulated by using mfold software. Thus, the first criterion (expression) and the second criterion (structure) were well met so that we affirmed that these 10 candidates were novel human miRNAs. Furthermore, we predicted putative targets of these candidate miRNAs with TargetScan (data not shown), some of which are radiation-related (Table 4), so that the novel miRNAs that we reported here are radiation-induced miRNAs. Conclusively, our hypothesis on finding novel miRNAs from human cells by increasing their abundance with ionizing radiation was proved by identifying 10 novel human miRNAs which rarely express in normal conditions but are up-regulated by X-rays. It might be expanded to other stresses and other species for discovering unknown miRNAs. Up to date, there is only one paper reporting novel miRNA by using exogenous stimuli. Their initial aim was to reveal known miRNAs responding to exogenous stresses in Oryza sativa L. Basing on our knowledge, this work is the first one using ionizing radiation as a cellular stress to increase the expression level of rarely expressing miRNA for efficient cloning and sequencing.

There were plenty of early works focusing on radiation-related proteins, from which we selected down-regulated proteins and compared them with the predicted target genes of the novel miRNAs we found. Luckily, for all novel miRNAs we found, there are some overlaps between known radiation-downregulated proteins and TargetScan-predicted target genes. The correlation between each novel miRNA and their predicted target gene remains to be further confirmed, however, it is confirmative that these novel miRNAs reported here might take part in cellular responses to irradiation.

In summary, we recognized 10 novel human miRNAs which responded to ionizing radiation. Furthermore, we confirmed that the expression profiles of miRNA in human cells can be changed by ionizing radiation, which provides a feasible way for enriching and identifying rarely expressing miRNAs.

ACKNOWLEDGEMENTS

We would like to thank Dr. Sha Li and her crew of Radiation Therapy Section, General Hospital of Lanzhou Military Area for sample treatment with X-rays. This work was supported by the Hundred Talent Program of the Chinese Academy of Sciences (0760140BRO), Scientific Innovation Program of Chinese Academy of Sciences (No. KJCF2-YW-N34-2), the Major State Basic Research Development Program of China (973 Program, No. 2010CB834201), and National Natural Science Foundation of China (No. 10979062).

REFERENCES


Received on October 28, 2010
Revision received on January 7, 2011
Accepted on February 7, 2011