Identification of up-regulated and down-regulated cis-natural antisense transcripts in the human B lymphoblastic cell line IM-9 after X-ray irradiation

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Abstract. Ionizing radiation (IR) causes DNA injury and induces multiple signal mechanisms, including the regulation of DNA repair, the cell cycle and gene expression through the activation of p53-related pathways. Cis-natural antisense transcripts (cis-NATs), which are transcribed from the DNA strand opposite to that for mRNA of the gene, are recognized as important regulators of gene expression in eukaryotic cells, but the effects on cis-NAT expression by IR are unknown to date. Therefore, we investigated the effects of X-ray irradiation on cis-NAT expression together with mRNA expression using a human B lymphoblast cell line (IM-9), a custom-microarray and strand-specific RT-qPCR. Eighteen and 106 mRNAs were demonstrated to be differentially expressed in IM-9 cells after 1, 2 and 4 Gy irradiation, respectively, as compared to 0 Gy by microarray analysis (fold change, FC >2.0). On the other hand, 10, 22 and 43 NATs were demonstrated to be differentially expressed in IM-9 cells after 1, 2 and 4 Gy irradiation, respectively, as compared to 0 Gy by microarray analysis (FC >2.0). Among these mRNAs/NATs, the IR dose-dependent up-regulation of mRNAs and cis-NATs of MDM2 and CDKN1A were confirmed by strand-specific RT-qPCR. Additionally, the cis-NATs of MDM2 were indicated to be localized in the cytoplasm, while cis-NATs of CDKN1A were located in the nucleus and cytoplasm. In conclusion, the radiation-responsive cis-NATs in conjunction with mRNAs were identified for the first time in the present study. It is possible that these cis-NATs regulate the gene expression in a post-transcriptional fashion. The IR dose-dependent up- and down-regulation of these mRNAs/cis-NATs may be a marker for ionizing radiation.

Introduction

Ionizing radiation (IR) is effectively used for the diagnosis and treatment of various types of cancer in the medical field. However, recently, radiation exposure accidents in radiation therapy and at nuclear power plants have occurred. Therefore, it is important to study the effect of IR on living organisms to minimize the influence of accidental exposures.

IR exposure is shown to cause two distinct phenomena: one is DNA injury and the other is non-DNA injury, such as cell membrane disorder and lipid peroxidation. As a DNA injury response, poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase repair complex (DNA-PK) are activated by double-strand breaks of DNA to engage in DNA repair (1,2). In addition, ATM, which is known as the causative gene, when affected, of Ataxia telangiectasia, is activated in response to DNA injury in order to initiate phosphorylation of p53. p53 was found to up-regulate mRNA expression of GADD45, MDM2, CDKN1A (p21) and BAX to control the cell cycle, DNA repair and apoptosis (3-5). As a non-DNA injury phenomena, cell membrane disorder and lipid peroxidation have been observed, which induce activation of signal transduction pathways, such as those with protein kinase C (PKC), c-Jun NH2-terminal kinase (JNK), ceramide and/or mitogen-activated protein kinase (MAPK) (3). Recent studies have revealed that IR generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells, which leads to DNA injury at random sites as well as the non-DNA injury phenomena (3,6).

Cis-natural antisense transcripts (cis-NATs), which are transcribed from the DNA strand opposite to the sense strand, have been discovered by full-length cDNA sequencing analysis in the human and mouse (7,8). In a recent study, cis-NATs were demonstrated to be involved in the control of gene expression in a eukaryote (9). For example, it has been shown that the cis-NATs of the BACE1 gene are involved in the stability of the...
**Table I. Primer sequences for strand-specific RT-qPCR.**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Size (mer)</th>
<th>PCR products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDM2-forward</td>
<td>5'-AGACAACCAATATCAATGTGATTCGTG-3'</td>
<td>25</td>
<td>60</td>
</tr>
<tr>
<td>MDM2-reverse</td>
<td>5'-CTCTTATTAGACGGCTACTAGG-3'</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>CDKN1A-forward</td>
<td>5'-TTGATTAGCGACGGGAAACAGG-3'</td>
<td>21</td>
<td>60</td>
</tr>
<tr>
<td>CDKN1A-reverse</td>
<td>5'-TCCATAGCCCTACTTGCAA-3'</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>ACTB-forward</td>
<td>5'-CCAACCCGCGAGAAGATGA-3'</td>
<td>18</td>
<td>97</td>
</tr>
<tr>
<td>ACTB-reverse</td>
<td>5'-CCAGAGGCGTGACGGGATAG-3'</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>EGFP-forward</td>
<td>5'-CAGCAGAAACACCCCCATC-3'</td>
<td>18</td>
<td>120</td>
</tr>
<tr>
<td>EGFP-reverse</td>
<td>5'-GAACCTCAGCGAACCAGATG-3'</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

**BACE1** mRNA by forming RNA duplex with **BACE1** mRNA (10), and that the **cis-NATs** of the **BCMA** gene are involved in the control of gene expression through translational inhibition of **BCMA** mRNA (11). Furthermore, it has been shown that the **cis-NATs** of the **CDKN2B** gene are involved in the control of epigenetic gene expression of the **CDKN2B** gene through heterochromatin formation in leukemia cells (12). In addition, differential **cis-NAT** expression patterns were observed between cancerous tissues and their corresponding non-cancerous tissues obtained from colorectal and breast cancers (13,14). However, the effects on **cis-NAT** expression by IR are unknown to date.

In the present study, the **cis-NATs**, up- and down-regulated by X-ray irradiation, were investigated using the human B lymphoblastic cell line IM-9 having a high sensitivity to IR, and using a custom-microarray containing human sense/antisense probes for approximately 21,000 genes.

**Materials and methods**

**Cell line and cell culture.** The human B lymphoblastic cell line IM-9 (JCRB0024) was purchased from the Health Science Research Resources Bank (Osaka, Japan). The IM-9 cells were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C in an atmosphere of 5% CO₂. The IM-9 cells were seeded onto 6-well plates at a concentration of 2x10⁴ cells/well for X-ray irradiation experiments.

**X-ray irradiation.** The IM-9 cells plated on the wells were cultured as described above for 24 h prior to X-ray irradiation. Irradiation (150 kVp, 20 mA, 0.5-mm aluminium and 0.3-mm copper filters) was performed using an X-ray generator (MBR-1520R-3; Hitachi Medical Corporation, Tokyo, Japan) at a distance of 45 cm between the focus and the target. The dose rate was 80 cGy/min. The dose was monitored with a thimble ionization chamber that was placed next to the sample during the irradiation. The irradiated cells were further cultured for 24 h under the same conditions, and then collected by centrifugation. The cells thus collected were frozen in liquid nitrogen and stocked at -80°C until being used for RNA extraction.

**RNA extraction.** For microarray analysis, total RNAs were extracted from the cells using ISOGEN II (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The qualities and concentrations of the RNAs extracted were examined using the NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) according to the manufacturer's instructions. In addition, the integrities of the RNAs were examined using Agilent Bioanalyzer (Agilent Technologies, CA, USA), according to the manufacturer's instructions. The isolation of nuclear RNA and cytoplasmic RNA separately from the cells was performed using PARIS kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

**Microarray analysis.** Cyanine 3 (Cy3)-labeled cDNA was synthesized from 10 µg total RNA of irradiated or non-irradiated samples using a LabelStar Array kit (Qiagen, Valencia, CA, USA), Cy3-dUTP (GE Healthcare, Buckinghamshire, UK) and random nonamer primer. The sense and antisense sequences of 60 nt custom-designed were arranged in an Agilent 44K x 4 system [20882 ORFs: Agilent eArray Design ID = 19052 produced by Tsukuba GeneTech Laboratory (Ibaraki, Japan)] (13). Agilent 44K x 4 human sense/antisense custom-microarray slides were hybridized with the Cy3-labeled cDNA (2 µg) in a hybridization solution prepared with a GE Hybridization kit (Agilent Technologies). The Cy3 fluorescence signal images on the slides were obtained by a DNA microarray scanner (Agilent Technologies) and processed using the Feature Extraction version 8.1 software based on the instructions from Agilent Technologies.

The expression data thus obtained were processed using GeneSpring GX ver. 10 software (Agilent Technologies) in order to carry out a normalization to the 75 percentile of all values on the respective microarrays, followed by normalization of the median expression levels of all samples. The expression profiles of the mRNAs and **cis-NATs** were compared, based on the fold-change of the values (>100) of the respective genes. The up- and down-regulated transcripts of >2-fold at 1, 2 and 4 Gy IR against 0 Gy were selected.

**Strand-specific RT-qPCR.** Total RNAs from irradiated and non-irradiated IM-9 cells were used for strand-specific RT-qPCR of mRNAs and **cis-NATs**. An aliquot of each RNA sample was mixed with an amount of RNA fragment (218 nucleotides) synthesized from the pEGFP-C1 vector (Invitrogen, CA, USA) to attain a final amount of 5x10⁻⁵ pmol/10 µg total RNA. These
RNAs were subjected to synthesis of the first-strand cDNA only from mRNA or cis-NATs using reverse or forward primer (Table I), and AMV Reverse Transcriptase (Promega, Madison, WI, USA) together with ACTB reverse primer as the internal control and EGFP forward primer as the external control, according to the procedure recommended by Promega (15). The mixtures were then incubated at 50˚C for 60 min. The resulting cDNAs were incubated at 99˚C for 5 min and at 37˚C for 60 min with RNase A to digest RNA. The first-strand cDNAs were first subjected to ordinary PCR using the primer sets described in Table I in order to confirm whether the fragments amplified in the PCR were the ones derived from the target transcripts based on fragment size in 4% agarose gel electrophoresis.

The first-strand cDNAs derived from mRNAs or cis-NATs were used as a template for quantitative PCR (qPCR) using Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) and primer pair sets described in Table I. The qPCRs were performed using an Applied Biosystems StepOne Plus Real-Time PCR system (Applied Biosystems) under the conditions of 7 min at 95˚C followed by 40 cycles each of 95˚C for 15 sec and 60˚C for 60 sec, following the procedure recommended by the manufacturer. In order to compare the PCR results, the values for mRNAs and cis-NATs were normalized based on the values of the control.

Strand-specific RT-qPCR data thus obtained were statistically examined using one-way analysis of variance (one-way ANOVA). Within each data set, post hoc multiple comparisons were run with Tukey's Honestly Significant Difference test to determine the statistical significance for differences between each pair of means. Differences were considered significant at a value of p<0.05.

**Strand-specific RT-PCR for the intracellular localization of transcripts.** To know the intracellular localization of mRNAs and cis-NATs, RT-PCR was performed using strand-specific cDNAs synthesized from total RNA (250 ng), cytoplasmic RNA (200 ng) and nuclear RNA (50 ng). These cDNAs were subjected to PCR in a 45 µl reaction mixture containing 1x buffer, 1.5 mM MgCl₂, 0.1 mM of each dNTP, 0.025 U/µl of Biotaq HS DNA polymerase (Nippon Genetics, Tokyo, Japan), 0.5 µM of the primer pairs (Table I) and the cDNA template. The PCRs were performed using the Veriti 96-Well Thermal Cycler (Applied Biosystems) under the conditions of 7 min at 95˚C, followed by 35 cycles each of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec and a final elongation step at 72˚C for 5 min. The PCR products were examined by electrophoresis using a 4% agarose gel.

**Results**

**Gene expression profiling of mRNAs and NATs in IM-9 cells irradiated with X-ray.** A large number of studies have been performed to assess the difference in the expression of genes to obtain a clue for understanding DNA repair mechanisms and signal transduction mechanisms by IR. However, most of these studies are studies with mRNAs and microRNAs (16-18). The effects on NAT expression by IR are unknown to date. In the present study, in order to identify radiation-responsive mRNAs and NATs in IM-9 cells, we investigated mRNA and NAT expression profiles by microarray analysis using total RNA from the IM-9 cell line irradiated with X-ray doses ranging from 0 to 4 Gy. Total RNA extraction and microarray analysis were performed as described in Materials and methods.

Eighteen, 33 and 106 mRNAs were found to be expressed differentially in IM-9 cells after 1, 2 and 4 Gy irradiation, respectively, with a magnitude of >2-fold change as compared to 0 Gy. On the other hand, 10, 22 and 43 NATs were found to be expressed differentially in IM-9 cells after 1, 2 and 4 Gy irradiation, respectively, with a magnitude of >2-fold change as compared to 0 Gy.

Of the 18 mRNAs expressed after 1 Gy irradiation, 12 were up-regulated and 6 were down-regulated. Of the 33 mRNAs expressed after 2 Gy irradiation, 26 were up-regulated and 7 were down-regulated. Of the 106 mRNAs expressed after 4 Gy irradiation, 97 were up-regulated and 9 were down-regulated (data not shown). Some of the mRNAs described above have already been reported to be up-/down-regulated by IR (16,19), indicating that the results of the microarray analysis in the present study were essentially consistent with those reported in earlier studies.

Concerning the NATs, 3 of the 10 NATs expressed after 1 Gy irradiation were up-regulated and 7 were down-regulated. Of the 22 NATs expressed after 2 Gy irradiation, 7 were up-regulated and 15 were down-regulated. Of the 43 NATs expressed after 4 Gy irradiation, 32 were up-regulated and 11 were down-regulated (Tables II and III). We identified the up-regulated and down-regulated NATs caused by radiation exposure for the first time.

When it was examined whether common genes were present in the mRNAs and NATs described above, 6 genes, MDM2, CDKN1A, LSP1, PSAP, TM7SF3 and MS4A1, exhibited up-regulated mRNAs and cis-NATs with irradiation.

**Strand-specific RT-qPCR analysis of MDM2 and CDKN1A mRNAs/cis-NATs after X-ray irradiation.** In order to confirm the expression levels detected by microarray analysis, strand-specific RT-qPCR of mRNAs and cis-NATs was carried out as described in Materials and methods. The two genes, MDM2 and CDKN1A, which are shown to be involved in p53-ubiquitination and cell cycle arrest, were selected for the strand-specific RT-qPCR analysis. Since cis-NATs were found to have various initiation sites and termination sites along the genes (15,20), we designed primer pairs inside the microarray probe region for strand-specific RT-qPCR (Table I).

As shown in Fig. 1, when strand-specific RT-qPCR was performed using cDNAs from mRNAs, the expression levels of MDM2 and CDKN1A mRNAs were significantly up-regulated with X-ray dose. The up-regulation was consistent with the results of the microarray (data not shown).

Likewise, when strand-specific RT-qPCR was performed using cDNAs from cis-NATs, the expression levels of MDM2 and CDKN1A cis-NATs were significantly up-regulated with X-ray dose (Fig. 1). The up-regulation was consistent with the results of the microarray (Table II). In addition, the microarray results and strand-specific RT-qPCR results were reported to show a good agreement in the analysis of colorectal cancer using the microarray as in the present study (13). Although, the comparisons of the present microarray system and the strand-specific RT-qPCR performed to date
were still limited, it is strongly indicated that the results of the microarray represented the real expression profiles in IM-9 cells.

Analysis of the intracellular localization of MDM2 and CDKN1A cis-NATs. In order to obtain additional features of cis-NATs to understand their function, we selected MDM2 and
CDKN1A cis-NATs as an example, and examined the intracellular localization of these cis-NATs in IM-9 cells.

Total RNA, cytoplasmic RNA and nuclear RNA fractions were prepared from IM-9 cells. As shown in Fig. 2A, 18 and 28 S ribosomal RNAs were detected in the cytoplasmic fraction, but not in the nuclear fraction, indicating that preparation of RNA from the cytoplasm and nucleus functioned well. The RNA fractions thus obtained were examined to ascertain whether they contain the cis-NATs. As shown in Fig. 2B, PCR using cDNAs from mRNAs demonstrated that the MDM2 and CDKN1A mRNAs were detected in the cytoplasmic fraction. Concerning cis-NATs, the MDM2 cis-NATs were detected in the cytoplasmic fraction, and the CDKN1A cis-NATs was detected in both the nuclear and cytoplasmic fraction. These results suggest that cis-NATs of MDM2 and CDKN1A have a different function in IM-9 cells.

Discussion

Ionizing radiation is frequently used for the diagnosis and treatment of various types of cancer in the medical field, but accidental exposure may result. In the present study, we examined the effect of ionizing radiation on cells through the analysis of expression profiles of mRNAs/cis-NATs in IM-9 cells irradiated by X-ray, using a custom microarray. Eighteen, 33 and
106 mRNAs were demonstrated to be differentially expressed in the IM-9 cells after 1, 2 and 4 Gy irradiation, respectively, as compared to 0 Gy by microarray analysis (FC >2.0). On the other hand, 10, 22 and 43 cis-NATS were demonstrated to be differentially expressed in the IM-9 cells after 1, 2 and 4 Gy irradiation, respectively, as compared to 0 Gy by microarray analysis (FC >2.0).

MDM2 and CDKN1A, which are involved in the regulation of the cell cycle through p53, were selected as probes for verification of the results of the microarray analysis using strand-specific RT-qPCR. The PCR provided supportive evidence for the results of the microarray. In addition, the mRNA expression levels elevated by X-ray exposure in the present study were found to include those such as MDM2 and CDKN1A as reported earlier (16,19). Collectively, these findings strongly indicate that the expression profiles obtained in the present study represent the expression of transcripts in the cells.

Morris et al demonstrated that the cis-NAT of CDKN1A induces trimethylation of Lys27 of Histone 3 (H3K27me3) to reduce the expression levels of CDKN1A mRNA (21). This function of CDKN1A cis-NAT is considered to be restricted to the nucleus. However, since the cis-NAT of CDKN1A was

Figure 1. Relative expression of MDM2 and CDKN1A mRNAs/cis-NATS after X-ray irradiation in IM-9 cells. Total RNAs were isolated from irradiated and non-irradiated IM-9 cells as described in Materials and methods. The mRNA and cis-NAT expression levels were determined using strand-specific RT-qPCR analysis. The values for mRNAs and cis-NATS were normalized based on values of the ACTB mRNA. *Statistical significance, compared to 0 Gy (p<0.05).

Figure 2. Intracellular localization of MDM2 and CDKN1A mRNAs/cis-NATS in IM-9 cells. (A) Isolation of the cytoplasmic and nuclear fraction of RNA. Total RNA, cytoplasmic RNA and nuclear RNA were isolated using PARIS kit. (B) Strand-specific RT-PCR for intracellular localization of mRNAs and cis-NATS. Strand-specific cDNAs derived from mRNAs or cis-NATS were synthesized using reverse or forward primer (Table I), and PCRs were performed as described in Materials and methods. The amplified fragments were electrophoresed on 4% agarose gel.
found not only in the nucleus, but also in the cytoplasm, the *cis*-NAT should have additional functions in the cytoplasm. As for the *cis*-NAT of MDM2, the *cis*-NAT was found only in the cytoplasm, indicating that its function is different from that of the CDKN1A *cis*-NAT.

In conclusion, the radiation-responsive *cis*-NATs in conjunction with mRNAs were identified for the first time in the present study. It is possible that these *cis*-NATs regulate the gene expression in a post-transcriptional fashion. Currently, the function of *cis*-NAT in ionizing radiation has been unknown. However, the expression profiles of mRNAs/*cis*-NATs may be used as markers for the amount of ionizing radiation which cells receive. In order to clarify the function of *cis*-NATs in X-ray exposure, the products of the respective genes should first be examined in terms of their amounts in future studies.

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References