Analysis of Circular RNA Expression Profile in HEK 293T Cells Exposed to Ionizing Radiation

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Abstract

Radiation therapy is one of the most common cancer treatments. It is important to understand how cells respond to ionizing radiation (IR) to improve therapeutic efficacy. Circular RNAs (circRNAs) recently have been found to regulate a variety of cellular processes. However, it is poorly defined that their expression pattern and their identity in cells following IR exposure. Here, we performed high-throughput sequencing and comprehensive analysis of circRNA expression in human embryonic kidney (HEK) 293T cells before and after irradiation. We identified totally 5592 circRNAs and discovered 1038 new circRNAs. We found 158 circRNAs with significantly differential expression after IR exposure. Among them, there were 61 upregulated and 97 downregulated circRNAs. Using Gene Ontology, Kyoto Encyclopedia of Genes and Genomes pathway, and circRNA-microRNA-messenger RNA network analyses, we found the differentially expressed circRNAs might be involved in the signal pathways of oxidative phosphorylation, epithelial growth factor receptor (EGFR) tyrosine kinase inhibitor resistance, and mammalian target of rapamycin (mTOR) signaling.

Keywords
circular RNA, ionizing radiation, HEK 293T cells, high-throughput sequencing

Introduction

Circular RNAs (circRNAs) are a unique class of noncoding RNAs widely expressed in metazoans, plants, and fungi.¹ Unlike traditional linear RNAs that contain 5' and 3' ends, circRNAs form covalently closed loops without 5'-3' polarity. The resistance to RNA exonucleases renders circRNAs higher stability.² Most circRNAs derive from exons of protein-coding genes and may include one or multiple exons, while some circRNAs originate from introns, intergenic regions, and other portions of the genome.³ Circular RNAs can regulate gene expression through multiple mechanisms, mainly acting as microRNAs (miRNAs) sponges.⁴ MicroRNAs are short RNAs that block gene expression by binding to target messenger RNAs (mRNAs) and preventing mRNAs translation.⁵ The circRNA specifically binds to the miRNA, which releases the cognate mRNA for translation, thus resulting in upregulation of the encoded protein. In addition, circRNAs can associate with RNA polymerase II to promote transcription⁶ and even can be translated into proteins.⁷

The human embryonic kidney (HEK) 293T cell line is an important variant of HEK 293 cells. Both of these cells have been widely used in cell biology research and biotechnology for a long time due to their reliable growth and high transfectability. 293T cells express the SV40 T antigen, which enables the amplification of transfected plasmids containing the SV40 origin of replication and thus considerably increases the expression levels of desired gene products.⁸ Exposure to ionizing radiation (IR) leads to a profound alteration of cellular processes, such as gene expression, posttranslational protein modification, and chromatin structure. However, how IR affects circRNAs expression is still poorly understood.

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investigated. To elucidate the underlying roles of circRNAs in cellular response to IR, here we used high-throughput RNA sequencing to examine circRNA expression profiles in IR-treated 293T cells compared to unirradiated 293T cells. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to analyze the differentially expressed circRNAs to explore the potential functions of related circRNAs. The comprehensive analysis of IR-responsive circRNAs may provide insight for understanding the functions of these circRNAs in the cellular radiation response.

Materials and Methods

Cell Culture

Human embryonic kidney cell line 293T (ATCC CRL-3216) was purchased from the American Type Culture Collection (Manassas, Virginia). The cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37°C in a humidified chamber with 5% CO₂.

Irradiation

Human embryonic kidney 293T cells were divided into 2 groups: the control group and the irradiated group. The irradiation group was exposed to IR in a Gammacell-40 ³⁷Cesium γ-ray irradiator (Atomic Energy of Canada Ltd, Chalk River, Ontario, Canada) for a total dose of 8 Gy. The control group underwent the same condition as the irradiated group except for exposure to IR.

Extraction and Separation of Sample RNAs

RNA was isolated from the control group and the irradiation group using TRizol reagent (Invitrogen, Carlsbad, California) according to the manufacturer’s instructions. Each of the RNA sample concentration was measured using a NanoDrop ND-2000 instrument (Thermo, Waltham, Massachusetts). Besides, denaturing agarose gel electrophoresis was used to assess the integrity of RNA.

High-Throughput Sequencing

High-throughput transcriptome sequencing and bioinformatic analysis were done by Cloud-Seq Biotech (Shanghai, China). Briefly, the total RNAs were treated with Ribo-Zero rRNA Removal kits (Illumina, San Diego, California) to remove the ribosomal RNAs (rRNAs). RNA libraries were constructed by using rRNA-depleted RNAs with TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, California) according to the manufacturer’s instructions. Libraries were controlled for quality and quantified using the BioAnalyzer 2100 system (Agilent Technologies). Ten pM libraries were denatured as single-stranded DNA molecules, captured on Illumina flow cells, amplified in situ as clusters and finally sequenced for 150 cycles on Illumina HiSeq Sequencer according to the manufacturer’s instructions.

Identification and Quantification of circRNAs

Paired-end reads were harvested from Illumina HiSeq 4000 sequencer and were quality controlled by Q30. After 3’ adaptor-trimming and low-quality reads removing by Cutadapt software (version 1.9.3), the high-quality reads were aligned to the reference genome/transcriptome with STAR software (version 2.5.1b) and circRNAs were detected and identified with DCC software (version 0.4.4). Briefly, high-quality reads are primarily screened by Cutadapt software, and the remaining unmodified reads were ignored. The STAR comparison software was used to plot. In the next step, some nucleotide sequences derived from reads were chosen to be as anchors and then entered the results into DCC. DCC will eventually perform comparisons between junction reads and nonjunction reads to find possible circRNAs. EdgeR software (version 3.16.5) was used to normalize the data and performed differentially expressed circRNA analysis.

Analysis of Differential circRNAs

A differential expression of the circRNA between 2 sets of samples is calculated by using a standardized number of reads. Any circRNA exhibiting the fold change ≥ 2.0, P value ≤ 0.05 can be considered as significantly differential expression.

Functional Analysis of Differential circRNAs

Gene Ontology functional analysis and KEGG pathway analysis were used to analyze differentially expressed circRNA-associated genes. Gene Ontology functions were classified into 3 subcategories: molecular function (MF), biological process (BP), and cellular component (CC). The 10 most enriched GO terms were ranked by P value. The KEGG pathway analysis was performed to explore the biological pathways which differentially expressed circRNAs might be involved in.

Quantitative Real-Time Polymerase Chain Reaction of circRNAs

The reliability of high-throughput RNA sequencing was validated by quantitative real-time polymerase chain reaction (qRT-PCR). Among all of the circRNAs identified, 5 upregulated and 5 downregulated circRNAs were selected for validation. And we chose Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to be a reference for normalization. The primers of all selected circRNAs are shown in Table 1. Total RNA was reverse-transcribed into complementary DNA using a PrimeScript RT Reagent Kit (Perfect Real Time; TaKaRa, Osaka, Japan) and subjected to qRT-PCR on an Applied Biosystems 7500 Fast Real-Time PCR System. Three independent assays were performed for all samples. The expression
was determined by using the threshold cycle, and relative expression levels were calculated via the $2^{-\Delta \Delta Ct}$ method.

**Analysis of circRNA-miRNA-mRNA Network and Related Prediction**

The binding sites of disease-associated miRNAs on selected circRNAs were predicted using proprietary software based on miRanda (version 3.3a) and TargetScan (version 7.0). The same software was also used to predict the binding sites of the disease-associated miRNAs on the 3'UTR of the target mRNAs. Based on the predicted results, a circRNA-miRNA-mRNA network map was built using Cytoscape software (version 3.1.0).

**Results**

**Overview of circRNA Expression in HEK 293T Cells**

To investigate the cellular effects of IR, HEK 293T cells were treated with a single dose of 8 Gy $\gamma$-ray and high-throughput sequencing was performed to determine the expression profile of circRNAs in irradiated and unirradiated cells. Each group was performed with 3 independent biological repeats. A total of 5592 circRNAs were identified in both groups of 293T cells, among which 1038 candidates were newly discovered circRNAs (Figure 1A). All of these circRNAs are widely distributed across all human chromosomes. Most circRNAs locate on chromosomes 1-22. Chromosome X contains 161 circRNAs, while chromosome Y and mitochondrial chromosomes contain less than 20 circRNAs (Figure 1B). There are a variety of catalogs of circRNAs, and most of them are exonic circRNAs (Figure 1C). There are 4787 exonic circRNAs, which ranged in size from 84 nucleotides (nt) to more than 2000 nt (96.2%) and most (26.4%) are 201- to 400-nt-long (Figure 1D). The overall average length is 772 nt.

**Identification of Differentially Expressed circRNAs After $\gamma$-Radiation Treatment**

Among the total 5592 circRNAs, 2205 circRNAs were detected in the control group, 1026 circRNAs were detected in the IR group, and 2361 circRNAs were detected in both groups (Figure 2A). In the process of differential analysis of circRNAs, under the criteria of fold change $\geq 2$, $P$ value $\leq 0.05$, there were 61 circRNAs with upregulated expression in the IR group compared to the control group. Ninety-seven circRNAs were downregulated in the IR group, of which the type of circRNAs was mostly exonic. The hierarchical clustering showed a distinguishable circRNA expression profiling between the control and IR-exposed groups (Figure 2B).

**Validation of the circRNA Expression**

To validate the high-throughput sequencing results, the expression levels of 5 upregulated and 5 downregulated circRNAs were confirmed by qRT-PCR. The results of the qRT-PCR were shown in Figure 3. The relative expression levels of these 10 circRNAs determined by qRT-PCR were consistent with the high-throughput sequencing data. The primers used in qRT-PCR are shown in Table 1.
Figure 1. Expression pattern of circRNAs in 293T cells. A, The proportion of newly discovered circRNAs in all identified circRNAs. B, Distribution of circRNAs on human chromosomes. C, Genomic origin of the detected circRNAs. D, The length distribution of exonic circRNAs. circRNA indicates circular RNAs.

Figure 2. Identification of differentially expressed circRNAs after γ radiation treatment. A, Venn diagram of differentially expressed circRNAs. Control represents nontreated group and IR represents irradiation group. 2361 circRNAs were found in both groups. B, Clustered heatmap. Hierarchical clustering displayed the circRNA expression profile of the irradiation group \( (n = 3) \) versus the control group \( (n = 3) \). circRNA indicates circular RNAs; IR, ionizing radiation.
To explore the functional roles of the differentially expressed circRNAs, GO analysis was performed in terms of BP, CC, and MF. The top 10 (ranked by Enrichment Score) BP terms, CC terms, and MF terms are shown in Figure 4. In this study, upregulated and downregulated circRNAs were analyzed separately. The identified BP terms corresponding to the upregulated circRNAs were insulin-like growth factor receptor signaling genes, and those involved in histone H3-K36 methylation paraxial mesoderm morphogenesis and DNA repair (Figure 4A). The identified CC terms were related to intracellular part and nucleoplasm part (Figure 4B). The identified MF terms were translation initiation factor activity, RNA binding, and RNA polymerase II transcription cofactor activity (Figure 4C). Kyoto Encyclopedia of Genes and Genomes analysis showed that there were 5 pathways related to the upregulated circRNAs, including ubiquitin-mediated proteolysis and oxidative phosphorylation (Figure 4D).

On the other hand, the downregulated circRNAs were analyzed in the same way. The identified BP terms corresponding to the downregulated circRNAs were positive regulation of GTPase activity and protein localization (Figure 5A). The
identified CC terms were related to intracellular part and organelle (Figure 5B). The identified MF terms were small molecule binding and nucleotide binding (Figure 5C). The primary identified KEGG pathways were epithelial growth factor receptor (EGFR) tyrosine kinase inhibitor resistance and mammalian target of rapamycin (mTOR) signaling pathway (Figure 5D). Notably, the MAPK1 gene was found in both EGFR tyrosine kinase inhibitor resistance and mTOR signaling pathway.

**Functional Prediction and Biological Analysis of circRNA**

To further explore the potential biological functions of the differentially expressed circRNAs, we utilized the 10 qPCR-validated circRNAs to construct a predicted circRNA-miRNA-mRNA interaction network (Figure 6). The network also involved 20 miRNAs and 37 mRNAs. In this network, 1 circRNA usually interacts with 2 miRNAs and 1 miRNA may regulate 3 mRNAs, indicating a multi-targeting regulatory role of each of the circRNAs. The network may help to predict the potential connections of circRNAs and target genes.

**Discussion**

Ionizing radiation includes energetic particles or electromagnetic waves with high energy, such as X-rays and γ-rays, which have a very strong penetrating power. When the human body is exposed to them, X-rays and γ-rays can enter the interior of the human body and ionize biomolecules in cells. The ions generated by ionization disrupt complex organic molecules such as proteins, nucleic acids, and lipids, all of which constitute living tissues. Furthermore, X-rays and γ-rays also cause the ionization of water molecules to produce free radicals, which are so reactive that disturb the normal functions of biomolecules. Despite of the beneficial effects of radiotherapy, exposure to radiation hurts normal cells and tissues at the same time. Thus it is important to understand the different cellular response to IR between normal cells and tumor cells. Yu et al have reported the circRNA expression profile in radiation-treated HeLa cells, indicating circRNA expression has been altered upon radiation.

In this study, we aimed to explore differential circRNA expression between nonirradiated and irradiation-treated HEK 293T cell. An 8 Gy γ-ray was applied to 293T cells and we harvested cells for high-throughput sequencing 24 hours...
A total of 5592 circRNAs were identified and there were 1038 novel circRNAs. Among the 5592 circRNAs, some were verified to be associated with diseases, such as Alzheimer disease\textsuperscript{16} and B-cell chronic lymphocytic leukemia.\textsuperscript{17} A total of 5592 circRNAs were located in all the chromosomes, including the mitochondrial chromosomes. These circRNAs had a variety of regions, including exonic, intronic, antisense, and sense overlapping regions.

One hundred fifty-eight circRNAs were differentially expressed ($P < 0.05$, fold change $\geq 2$). We selected 5 upregulated and 5 downregulated circRNAs for qRT-PCR validation and the results are in accordance with the high-throughput sequencing data, suggesting the reliability of high-throughput sequencing.

The target genes of circRNAs which were differentially expressed were further investigated by conducting GO and KEGG analyses to determine the differential expression of mRNAs in the 2 study groups. The GO analysis results indicated that the high expression of target genes might be involved in gene activation and protein binding.\textsuperscript{18} While other study in HeLa cells showed that radiation led to the alteration of circRNAs expression related to cell migration\textsuperscript{15} and angiogenesis.\textsuperscript{19} Other studies have shown that several circRNA molecules can be involved in gene transcriptional and translational regulations.\textsuperscript{20} Perhaps radiation can induce certain circRNAs to promote transcription and translation, which are related to some diseases.

Kyoto Encyclopedia of Genes and Genomes signaling pathway was complex, which revealed the relationship of circRNA-target mRNAs. In our study, these differentially expressed circRNAs may be relevant to oxidative phosphorylation, ubiquitin-mediated proteolysis, EGFR tyrosine kinase inhibitor

![Figure 6. CircRNA-miRNA-target gene network. The circRNAs marked red, the miRNAs marked yellow, and the mRNAs marked blue. Six upregulated circRNAs were hsa_circ_0001050, hsa_circ_0000408, hsa_circ_0002717, hsa_circ_0007548, hsa_circ_0007386, hsa_circ_0005347, and 5 downregulated circRNAs were hsa_circ_0008319, hsa_circ_0006898, hsa_circ_0097977, hsa_circ_0026497, hsa_circ_0000734. The network was built by using Cytoscape software. circRNA indicates circular RNAs; mRNA, messenger RN; miRNA, microRNA.](image-url)
resistance, and mTOR signaling pathway. Above all, ubiquitination-mediated proteolysis abnormalities were associated with cancer, which was the key to autophagy and apoptosis. When 293T cells were irradiated, the mTOR signaling pathway was activated, promoting autophagy and apoptosis. mTOR signaling pathway also affects cell proliferation and protein synthesis, making it a new target for anti-tumor therapy. Indeed, we can see that high-dose irradiation directly induces cancerous genes and activates pathways involved in tumor formation and insulin resistance.

The circRNA-miRNA-target gene network was built to further study the relationship between circRNAs and their target genes. One circRNA can interact with many miRNAs, and one miRNA can also inhibit the related target genes. For example, hsa_circ_0000734 can control hsa-miR-432-5p and hsa-miR-124-5p, which can further regulate the expression of target genes. In the network, the expression of hsa_circ_0000734 was downregulated after radiation, which caused the low expression of RNF168. While the reduced protein expression of RNF168 can inhibit the damage repair ability of DNA and increase the incidence of tumors. Furthermore, one single target gene may be controlled by multiple miRNAs. We hope that the network may provide some novel ideas for the subsequent experimental research.

At present, the incidence of malignant tumors is increasing year by year, with 4.2 million new cases of cancer each year. Due to more precise radiotherapy, it also causes a certain degree of secondary damage to patients. The occurrence of many cancers is also related to the accumulation of mutant genes. Some circRNAs play a crucial role in various signaling pathways and gene regulation, and almost all of them involve the expression of circRNAs in the pathogenesis of all diseases, including the initiation of the apoptotic pathway and various signaling pathways. Therefore, some circRNAs with different expression levels after radiation exposure need our attention and analysis, and they are likely to be important causes of related diseases.

In conclusion, the circRNA expression pattern has been changed in HEK 293T cells upon IR. Our studies mainly focused on the circRNAs that differentially expressed after normal cells treated with γ radiation. Only a small part of the circRNAs in the detected circRNAs were involved in radiation-induced DNA damage and repair. Our findings presented here may provide insight into the regulatory roles of circRNAs in cellular response to γ radiation.

Authors’ Note
Ningning He, Yuxiao Sun and Mengmeng Yang equally contributed to this work.

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