Microarray expression profile and functional analysis of circular RNAs in choroidal neovascularization

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Abstract

Choroidal neovascularization (CNV) is a leading cause of visual loss in age-related macular degeneration (AMD). However, the molecular mechanism for CNV progression is still unclear. This study aimed to identify CNV-related circular RNAs (circRNAs), a novel class of non-coding RNAs with diverse functions. A total of 117 circRNAs were differentially expressed in the murine CNV model by microarrays. Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed to identify the functions of selected circRNAs. The host genes of these circRNAs were predicted to be targeted to neurogenesis (ontology: biological process), proteinaceous extracellular matrix (ECM) (ontology: cellular component), and binding (ontology: molecular function). Differentially expressed circRNAs-mediated regulatory networks were enriched in ECM receptor interaction. Most of the dysregulated circRNAs could potentially bind to five different miRNAs by TargetScan and miRanda. Specifically, circ_15752 was identified in this circRNAs pool which may facilitate vascular endothelial cell proliferation, migration, and tube formation, suggesting a critical role in endothelial angiogenesis. Our work suggests that dysregulated circRNAs may be involved in CNV pathogenesis and serve as potential biomarkers for CNV.

Keywords: circRNAs, microarray, choroidal neovascularization

Introduction

Age-related macular degeneration (AMD) is a leading cause of the irreversible visual loss in the elderly over the age of 60 years[1]. AMD can be categorized into two forms: atrophic form (dry form) and exudative form (wet form). In contrast to dry form, wet form usually causes severe and rapid visual loss[2]. The wet form is characterized by aberrant angiogenesis within the Bruch's membrane, retinal

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pigment epithelium (RPE), as well as the subretinal space, referred to as choroidal neovascularization (CNV) within the retina. The abnormal blood vessels can leak fluid and bleed. Oxidative stress, inflammation, and angiogenesis are important factors in the pathogenesis of CNV. CNV can be treated using recombinant anti-vascular endothelial growth factor (VEGF) antibody fragments. However, anti-VEGF-based treatments for CNV require frequent injections, which are burdensome to patients. Despite intensive basic and clinical research, the precise pathogenesis of CNV is still unclear.

Circular RNAs (circRNAs) are a novel class of non-coding RNAs, unlike the commonly known linear RNA, characterized by covalently closed loop structures. A multitude of circRNAs are highly conservative in different species, often exhibiting tissue-specific and developmental stage-specific expression profiles. CircRNAs are involved in a multitude of biological processes and play crucial roles as miRNA (microRNA) sponges, RNA-binding protein sequestering agents, or regulators of nuclear transcription.

Given the critical role of circRNAs in gene regulatory networks, it is not surprising that aberrant circRNA expression may become the potential molecular pathogenesis of human diseases. Several studies have revealed that circRNAs are unconventionally expressed in vascular diseases. CircRNAs are involved in the VEGF signaling pathway. Additionally, circRNAs regulate retinal endothelial cell function and vascular dysfunction. However, the role of circRNAs in CNV remains to be clarified.

In this study, we performed circRNA microarray to investigate the differential expression profile of circRNAs between the choroid of the laser-induced group (CNV model) and the control group in mice. We identified 117 differentially expressed circRNAs, including 58 up-regulated and 59 down-regulated circRNAs. Possible miRNA targets and functions of circRNAs were predicted. We then characterized one of the circRNAs derived from the Nyap2 gene locus (circ_15752), which was significantly upregulated in the CNV group. Functional assays revealed that circ_15752 could regulate vascular endothelial cell function.

Materials and methods

Animals

Animal experiments were performed in agreement with the guidelines of the Association for Research in Vision and Ophthalmology Statement for the use of animals in ophthalmic and vision research, and the study was approved by the Animal Care and Use Committee of Nanjing Medical University. All procedures were conformed to the Guide for the Care and Use of laboratory animals. Male C57BL/6J mice at age of 8–10 weeks were obtained from Model Animal Research Center, Nanjing University and were fed on a normal diet. All mice were housed in an AAALAC accredited SPF animal facility with a 12-hour light/dark cycle and had ad libitum access to water and standard mouse chow diet.

Laser-induced model of CNV

CNV was induced by laser in C57BL/6J mice. Briefly, photocoagulation was performed in mice with dilated pupils (with 1% tropicamide, Santen, China) using a laser photocoagulator (532 nm wavelength, ZEISS, Germany). The parameters were as follows: spot size, 50 μm; duration, 50 ms and power, 200 mW. Four spots were applied to each eye of mice between the major retinal vessels around the optic disc at approximately 2 optic disc diameters from the optic nerve head. A bubble was created at each laser spot. In all experiments, laser burns were conducted on day 0.

Fluorescein fundus angiography (FFA)

FFA was performed with the Micron III retinal imaging microscope and digital imaging hardware as previously described. Initially on day 14, 25% fluorescein light (250 mg/mL; HUB Pharmaceuticals, USA), diluted with sterile 1×DPBS was administered by injection (50 μL) into the tail vein of anesthetized mice. FFA was performed on day 14, digitizing each eye at 1 minute (early), 3 minutes (middle) and 5 minutes (late).

Flat mount choroidal staining for CNV lesions in mice

Choroidal flat mounts were prepared as described previously and then blocked with 5% albumin from bovine serum (BSA)/0.3% Triton X-100 in 1×PBS at room temperature for 1 hour. Isolecitin B4 derived from Griffonia (Bandeiraea) simplicifolia agglutinin (1:100; Invitrogen-Molecular Probes, USA) was incubated overnight at 4°C. Nuclei were counterstained with DAPI (Vector Laboratories, USA). Images were collected by Leica SP8 microscope (Leica, Germany).

Paraffin section

The eyes were enucleated immediately after sacrifice and were fixed overnight in 4% paraformaldehyde (PFA) (Sigma-Aldrich, USA). Then, they were dehydrated in a graded ethanol series and embedded in paraffin wax. Serial sectioning of the
eyecup was performed at 5-μm thickness using a microtome (Leica). The sections were deparaffinized in xylene, rehydrated through graded ethanol series, and stained with eosin and hematoxylin (H&E). Finally, they were examined by a microscope (Leica).

RNA extraction

The eyes were enucleated immediately after sacrifice on day 14. Total RNAs were extracted and purified using Trizol reagent (Ambion, USA) following the manufacturer's instructions. The RNAs for microarray analysis were checked for RNA integrity using Agilent Bioanalyzer 2100 (Agilent Technologies, USA). The purity and quantity of other RNAs were determined using the NanoDrop ND-2000 (NanoDrop, USA). The integrity of total RNAs was assessed by 1% agarose gel electrophoresis.

CircRNA microarray hybridization

SBC Mouse (4×180 K) circular RNA Microarray (Shanghai Biotechnology, China) with a total of 37,920 probes were applied to identify CNV-related circRNAs. Total RNAs were treated with RNase R to remove linear RNA to enrich circRNAs. The enriched circRNAs were amplified and transcribed into fluorescent cRNA, and then were purified using an RNA extraction kit (QIAGEN, Germany). Each slide was hybridized with 1.65 μg Cy3-labeled cRNA using Gene Expression Hybridization Kit (Agilent Technologies) in hybridization oven (Agilent Technologies), according to the manufacturer's instructions. After 17 hours of hybridization, slides were washed in staining dishes (Thermo Shandon, USA) with Gene Expression Wash Buffer Kit (Agilent Technologies) following the manufacturer's recommended protocol. The microarray was then scanned by Agilent microarray scanner (Agilent Technologies).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Complementary DNA was synthesized with the PrimeScrip RT reagent Kit (TaKaRa, Japan) according to the manufacturer's instructions. qRT-PCR assays were performed using SYBR Premix Ex Taq (Takara) in the ABI Prism ABI 7500 detection system (Applied Biosystems, USA). Relative circRNA levels were normalized to β-actin mRNA levels. RNA integrity and genomic DNA contamination were assessed by denaturing agarose gel electrophoresis. The primer sequences are shown in Supplementary Table 1 (available online).

Bioinformatics analysis

The circRNA/miRNA interaction was predicted using Arraystar's software based on TargetScan and miRanda[17], and the differentially expressed circRNAs within all the comparisons were annotated in detail with the circRNA/miRNA interaction information. The analysis of the circRNA functions was conducted according to the host gene of circRNA. The pathway investigation was carried out by the following databases. Gene ontology (GO) analysis was applied to predict cellular components and molecular functions and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) was applied to explore the pathways related to host genes.

Cells culture and transfection

The bEnd.3 cell line was purchased from the American Type Culture Collection (ATCC, USA) and maintained in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% fetal bovine serum at 37 °C in 5% CO₂ and 95% humidity. Small interfering RNAs targeting circ_15752 was purchased from Shanghai GenePharma (China; sense, 5'-CACAGCUCAGUGUCGCCATT-3', and antisense, 5'-UGGCGAGACACUGAGCGUGTTT-3'). For transient transfection, the cells were transfected with the siRNA using the lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions.

EdU incorporation assay

Cell proliferation was examined using EdU (5-ethynyl-2'-deoxyuridine) DNA cell proliferation kit (RiboBio, China). bEnd.3 cells were incubated with 50 mmol/L EdU for 2 hours. They were then fixed with 4% PFA and incubated with Apollo dye solution for labeling the proliferating cells. Cell nuclei were stained by Hoechst 33342 (Life Technology, USA).

Transwell migration assay

Cell migration was evaluated using 24-well Transwell units with 8-μm-porosity polycarbonate filters. A 200 μL aliquot of serum-starved cells (5×10⁵ cells/mL) was added to the upper polycarbonate membrane insert, and 600 μL of cultured medium with 10% fetal bovine serum was added to the lower chamber. After 24 hours of incubation, the membranes were fixed with 4% PFA in PBS for 10 minutes and then stained with 0.5% toluidine blue for 2 hours. The number of migratory cells was counted in five random fields under a microscope.

Tubulogenesis assay

Matrigel (10 mg/mL, BD Biosciences, USA) was plated into 48-well culture plates and incubated at 37 °C
for 30 minutes to allow the matrigel to polymerize. After transfection, cells were seeded on the matrigel in a 48-well plate. After 24-hour culture, the tube-like networks were observed. Tube-like networks were counted by counting the number of branch nodes in images.

**Statistical analysis**

Statistical analyses were performed using SPSS version 13.0 (SPSS Inc, USA). Circular RNA expression data were expressed as mean±SEM. The significant difference was determined by Student t-test (when two groups were compared) or one-way ANOVA to test the effect of group (when >2 groups were compared). A P value <0.05 was considered statistically significant.

**Results**

**CircRNAs were differentially expressed in CNV group**

To make a murine CNV model, we administered targeted laser injury by photocoagulation. The burn cores in fundus photo (black circles in Supplementary Fig. 1A, available online), fluorescein expanding in FFA images (Supplementary Fig. 1C–E, available online), typical feature of laser spot in retinal histology (Supplementary Fig. 1L, available online), and immunohistochemical staining of isolecitin B4 in choroid flat mounts (Supplementary Fig. 2, available online) indicated that the CNV model was successfully constructed. To study the altered circRNA expression in CNV, we used a high-throughput circRNA microarray to detect circRNA expression profiles (3 control choroids and 3 laser-induced choroids). The box plot showed the distribution of circRNA expression profiles. After normalization, the distributions of log2 ratios between laser-induced and control choroids were presented in the box plot (Fig. 1A). The different samples had the same median expression level and scale of the boxes, indicating that the intensity values in the different samples are comparable. A volcano plot was utilized to identify the differentially expressed circRNAs with statistical significance (Fig. 1B). We set a threshold as fold change ≥2.0, P<0.05, and identified 117 differentially expressed circRNAs, including 58 up-regulated circRNAs and 59 down-regulated circRNAs (Supplementary Table 2, available online). To visualize the changed pattern, we presented the differentiated circRNAs in the heatmap (Fig. 1C), which distinctively separated the laser-induced group from the control group.

To confirm the microarray data, 20 dysregulated circRNAs were randomly selected for expression verification by qRT-PCR, including 10 up-regulated circRNAs and 10 down-regulated circRNAs (Fig. 1D). Except for the expression of two circRNAs, expression of all other circRNAs was consistent between qRT-PCR and microarray, suggesting that our microarray data have high accuracy and reliability.

**GO analysis and pathway analysis of the host genes of differentially expressed circRNAs**

CircRNAs are primarily generated from exons or introns of their parental genes. The expression of host genes can be regulated by the circRNAs[11]. To predict the role of circRNAs, we performed GO analysis and pathway analysis of their host genes. We illustrated that the significantly enriched GO term in the biological process was neurogenesis (Fig. 2A). The binding was the most significantly enriched GO term in molecular function (Fig. 2B). The proteinaceous ECM was the most significantly enriched GO term in cellular component (Fig. 2C). Pathway analysis indicated that 10 signaling pathways were potentially involved in the circRNA-mediated regulatory network. Among them, the ECM-receptor interaction pathway was the most significantly enriched GO term in signaling pathways (Fig. 2D), which was also an important pathway in the angiogenic process[19]. We speculated that the circRNA-regulated ECM-receptor interaction pathway could affect the pathogenic process of CNV.

**microRNA response element prediction of dysregulated circRNAs in CNV**

As we know, miRNAs play a pivotal role in CNV[19]. CircRNA could act as miRNA sponges to regulate mRNA expression[20]. To determine whether the changed circRNA could bind to miRNAs, we selected the top 10 up-regulated circRNAs and the top 10 down-regulated circRNAs to determine whether they could bind to miRNAs. The circRNA/miRNA interaction was predicted. We found that 14 circRNAs could potentially bind to five different miRNAs (Supplementary Table 3, available online), implying that most of the dysregulated circRNAs are involved in CNV pathogenesis by acting as miRNA sponges.

**circ_15752 is involved in CNV pathogenesis via regulating vascular endothelial cell function**

The dysfunction of the vascular endothelium is an important step for many vascular diseases, like
Fig. 1  Identification of differentially expressed circRNAs in the laser-induced group and the control group. A: The box plot showed the circRNA expression distribution for the six compared samples which were nearly the same after normalization. B: Volcano plots showed differentially expressed circRNAs between the two groups. The black vertical line represents 2-fold changes, while the horizontal green line marks a P-value of 0.05. C: Hierarchical clustering for differentially expressed circRNAs of the control group (C1–C3) and laser-induced group (L1–L3). The two-way hierarchical cluster heat map showed all dysregulated circRNAs: each column exhibited the relative expression level of a single circRNA; each row demonstrated the expression level of a single sample. Red: high expression; green: low expression; black: no differentiation. D: Twenty differentially expressed circRNAs were validated by qRT-PCR (10 up-regulated circRNAs and 10 down-regulated circRNAs, n=3, *P<0.05, **P<0.01 by Student's t-test). C: control group; L: laser-induced group; mmu: Mus musculus; circ: circRNA.

Fig. 2  GO enrichment and pathway analysis of the host genes of dysregulated circRNAs. A–C: GO enrichment analysis described the differentially expressed circRNAs. The ontology covers three parts: biologic process (A), cellular component (B), and molecular function (C). P<0.05 is recommended. D: The bar plot exhibited the result of pathway analysis, which showed the top 10 signaling pathways potentially related to the circRNA-mediated regulatory network. KEGG: Kyoto Encyclopedia of Genes and Genomes.
We chose circ_15752, a top upregulated circRNAs with high abundance in CNV choroid, to further explore its role in vascular endothelial cells. Transfection of siRNAs targeting circ_15752 significantly downregulated its expression (Fig. 3A).

To evaluate the role of circ_15752 in the function of vascular endothelial cells, we measured cell proliferation, migration, and tube formation in a mouse vascular endothelial cell line (bEnd.3). EdU incorporation assays showed a significantly reduced percentage of EdU positive cells (green) after circ_15752 silencing (Fig. 3B), indicating decreased endothelial proliferation. To assess the migration of vascular endothelial cells, we performed transwell assays. circ_15752 knockdown substantially decreased the number of migrated cells (Fig. 3C). Moreover, silencing of circ_15752 significantly inhibited tube formation of vascular endothelial cells (Fig. 3D).

Taken together, we found the silencing of circ_15752 impaired proliferation, migration, and tube formation of vascular endothelial cells, suggesting that circ_15752 was potentially involved in CNV pathogenesis via regulating vascular endothelial cell function.

Fig. 3  circ_15752 regulated endothelial cell function in vitro. A: bEnd.3 cells were transfected with scrambled siRNA (Scr siRNA), circ_15752 siRNA, or left control for 48 hours. qRT-PCR was conducted to detect circ_15752 expression (n=3, *P<0.05). B: Cell proliferation were detected using EdU detection (n=4, *P<0.05). Scale bar: 50 μm. C: Transwell assay and quantification analysis were conducted to detect cell migration (n=4, *P<0.05). Scale bar: 100 μm. D: Cells were seeded on the matrigel matrix. The tube-like structures were observed 24 hours after cell seeding. The average length of tube formation for each field was statistically analyzed (n=3, *P<0.05). Scale bar: 100 μm. The significant difference was evaluated by one-way ANOVA followed by post hoc Bonferroni’s test.
Discussion

Ninety percent of legal blindness from age-related macular degeneration is caused by choroid endothelial originating neovascularization [22]. Although great progress has been made, the understanding of pathogenesis and therapy of CNV is still limited. We compared circRNA expression differences between control and laser-induced choroid by microarray analysis and identified a total of 117 differentially expressed circRNAs in CNV choroid in a laser-induced CNV mouse model. Our study revealed that the CNV was associated with a significant change in a wide array of circRNAs expression, implicating the potential role of circRNAs in CNV.

To our knowledge, the role of circRNAs in CNV is largely unknown. CircRNA microarray facilitates the analysis of circRNAs. Herein, 117 differentially expressed circRNAs were found in a laser-induced CNV mice model by microarray. It would provide a novel insight into the pathogenesis of CNV. CircRNAs are a novel, abundant type of noncoding RNAs with stable closed loop structure and a conserved class of RNA molecules [23]. CircRNAs are aberrantly expressed in vascular diseases, neurological disorders, and cancers [12,24-25]. They have biological functions in endothelium [26]. Additionally, circRNAs play important roles in ocular vascular diseases [14,27]. Thus, it is not surprising that circRNAs are involved in CNV.

Gene-annotation enrichment analysis indicated that the biological modules are correlated with neurogenesis (ontology: biological process), proteinaceous extracellular matrix (ontology: cellular component), and binding (ontology: molecular function). Signaling pathway analysis provides knowledge about genomes and their relationships to biological systems. The analysis of pathway shows that the ECM-receptor interaction pathway is the most involved in the circRNA-mediated regulatory network. CircRNAs regulate the synthesis of ECM [28-29]. The ECM maintains the histological structure of the vessel wall in shape, provides informational cues to the vascular cells, and regulates the proliferation and differentiation of vascular cells [30]. Additionally, ECM expression is changed in a spatial and temporal pattern during the formation of blood vessels [36-31]. In the rodent eye, the transient expression of ECM components including fibronectin and matricellular proteins plays a vital role in the development of retinal vasculature. Thus, we speculate that circRNAs may alter ECM expression in the pathogenic process of CNV.

CircRNAs can function as miRNA sponges [29]. The roles of miRNAs in ocular neovascular diseases have been widely known [19,32]. Bioinformatic analysis showed that most of the dysregulated circRNAs in our study contain different miRNA binding sites. miRNAs play important roles in a wide range of biological processes, including differentiation, growth, invasion, and migration [33]. Moreover, the miRNA-mediated regulatory network has multiple targets. CircRNAs could act as competing endogenous RNAs to compete with endogenous miRNAs, regulating the level of miRNA targets. Therefore, we speculate that circRNA/miRNA/mRNA network could be involved in the regulation of CNV-related pathological process.

In CNV, angiogenic processes cause immature choroidal vessels to break through the RPE into the subretinal space [34]. Vascular leakage is the main cause of severe manifestations in CNV [1]. Endothelium dysfunction has been recognized as an important step for CNV [21]. In our study, circ_15752 silencing decreases the capacity of endothelial cell proliferation, migration, and tube formation. Phenotypes like cell proliferation, migration, and tube formation are associated with the angiogenic effects of vascular endothelial cells [35]. It suggested that circRNAs play a critical role in CNV by regulating endothelial angiogenic function.

In conclusion, we identify 117 differentially expressed circRNAs between the control choroid and the laser-induced choroid. The identification of the novel changing expression of circRNAs is important for understanding the role of circRNAs in CNV. In the future, in vivo and in vitro studies should be performed to elucidate the mechanism of circRNAs-mediated CNV pathogenesis and evaluate their potential role in prognosis, diagnosis, and treatment of CNV. The current study presents novel evidence showing potential role of circRNAs in CNV, which sheds new light for the study of CNV pathogenesis.

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