High-throughput sequencing reveals novel lincRNA in age-related cataract

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Abstract. Age-related cataract (ARC) is a major cause of blindness. Long non-coding RNAs (lncRNAs) are a heterogeneous class of RNAs that are non-protein-coding transcripts >200 nucleotides in length. LncRNAs are involved in various critical biological processes, such as chromatin remodeling, gene transcription, and protein transport and trafficking. Furthermore, the dysregulation of lncRNAs causes a number of complex human diseases, including coronary artery diseases, autoimmune diseases, neurological disorders and various cancers. However, the role of lncRNA in cataract remains unclear. Therefore, in the present study, lens anterior capsular membrane was collected from normal subjects and patients with ARC and total RNA was extracted. High-throughput sequencing was applied to detect differentially expressed lncRNAs and mRNAs. The analysis identified a total of 42,556 candidate differentially expressed mRNAs (27,447 + 15,109) and a total of 7,041 candidate differentially expressed lncRNAs (4,146 + 2,895). Through bioinformatics analysis, the significant differential expression of novel lincRNA was observed and its possible molecular mechanism was explored. Reverse transcription-quantitative polymerase chain reaction was used to validate the different expression levels of selected lncRNAs. These findings may lead to the development of novel strategies for genetic diagnosis and gene therapy.

Introduction

Age-related cataract (ARC) is one of the most common chronic diseases and a main cause of blindness worldwide (1). At present, surgery is the most effective method of treating cataracts. However, surgery is an economic burden to society, and has associated risks and complications (2). On the basis of the location of the opacity in the lens, ARC is classified as cortical, nuclear or posterior sub-capsular cataract (3,4). In the present study, the main focus was on nuclear cataract (S2) and posterior capsule cataract (S3). ARC is associated with a variety of factors, including age, sex, radiation (visible light, ultraviolet and X-ray), oxidation, trauma, diet and drugs (5). ARC may also be associated with the immune response and growth factors (6,7).

With the emergence of high-throughput sequencing technology, long non-coding RNA (lncRNA) has emerged as an alternative to microRNA (miRNA) as a research topic of great interest. LncRNAs are transcripts that are >200 nucleotides in length, and are similar in structure to mRNA but have little or no protein-coding potential (8,9). According to their genomic locations, lncRNAs may be divided into several types, one of which is intronic lncRNAs (lincRNA), which refers to lncRNA located within an intergenic region of the genome (10). LncRNAs regulate gene expression at the transcriptional, epigenetic or translational level, and thereby alter cellular responses to various stresses (11). Aberrant lncRNA expression is implicated in several human diseases, including tumorigenesis, neurological diseases and cardiovascular diseases (12-14). Certain lncRNAs have been demonstrated to serve important roles in the development of the eye and ocular diseases. Ventral anterior homeobox 2, opposite strand (Vax2os), retinal non-coding RNA 2 (RNCR2), six3 opposite strand (Six3OS) and taurine upregulated 1 (Tug1) have been indicated to have an association with eye development: Vax2os1 controls the cell cycle progression of photoreceptor progenitors in the mouse retina, whereas RNCR2, Six3OS and Tug1 play key roles in the management of retinal cell-specific differentiation (9,15). In addition, metastasis associated lung adenocarcinoma transcript 1 (MALAT1) has been identified to have significantly increased expression in the retinal tissue and aqueous humor of diabetic mice, and in the vascular fiber membranes of diabetic patients (16,17). However, the role of lncRNA in the lens is unclear. Therefore, the present study was designed to explore the possible regulation mechanism of lncRNAs in ARC.

Materials and methods

Clinical sample collection. The lens samples for the lncRNA microarray were collected from postmortem donors (free from ocular diseases) (S1) and ARC patients (9 patients, free of other
ocular diseases; these were divided into 2 groups: the S2 group included 3 patients with nuclear cataract, and the S3 group included 6 patients with posterior capsule cataract) at the Second Affiliated Hospital of Harbin Medical University (Harbin, China) from November, 2014 to January, 2015. All samples were obtained from male donors. The average age of the normal control group was 36, and the average age of the cataract patients was 65. The present study was approved by the Human Ethics Review Board of the Second Affiliated Hospital of Harbin Medical University (Harbin, China) and informed consent was provided by all 9 cataract patients. All lens samples were obtained by intact continuous curvilinear capsulorhexis, without vascular contacting or damage to the iris or any other intraocular structures. Since the RNA of a single lens was not sufficient for microarray analysis, 3 cataract lenses were pooled together as a biological repeat to obtain enough RNA. The degree of lenticular opacification was determined using the Lens Opacities Classification System II (18).

**Tissue collection and RNA extraction.** The collected samples were stored in liquid nitrogen. Total RNAs were extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). The yield of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). LncRNA high throughput sequencing analysis, including labelling, hybridization, scanning, normalization and data analysis, was performed by Annoroad Geomics (Beijing, China) (16,17).

**RNA sequencing.** A quality check of the input total RNA was performed to confirm its integrity by running an aliquot on an Agilent Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA), and the concentration of the RNA was measured using an ultra-micro spectrophotometer. A 3-µg quantity of RNA was selected as the initial amount of each sample from which to construct a lncRNA library, following removal of the ribosomal RNA (rRNA) in the sample using Ribo-Zero<sup>TM</sup> Gold kits (human/mouse/rat) (Epicentre, Madison, WI, USA). Different index tags were selected according to the NEB Next Ultra database direct operating protocol (NEB Next Ultra Directional RNA Library Prep kit for Illumina;NEB Ipswich, MA, USA; all reagents used here were provided in the kit). The specific steps of library construction were as follows: Firstly, the Ribo-Zero<sup>TM</sup> Gold kit was used to remove rRNA. Then, fragmentation buffer was added to the reaction system to fragment RNA into short segments, and the short fragments were used as templates for first cDNA chain synthesis using six base random primers (random hexamers). This was followed by second-strand cDNA synthesis using DNA polymerase I and RNaseH. The cDNA fragments were then subjected to an end-repair process, the addition of a single ‘A’ base, and ligation of the Illumina multiplexing adapters. The products were purified and enriched using polymerase chain reaction (PCR) to create the final cDNA sequencing library, as previously described (19-21). For PCR, the thermocycling conditions were as follows: step 1, 65°C for 15 min; step 2, 30°C for 10 min, 42-50°C for 45 min and 95°C for 5 min.

**Data processing.** The raw data was filtered to provide high-quality, cleaned reads, and a follow-up analysis was then conducted. The data processing steps were as follows: i) Removal of contamination reads, ii) removal of low-quality reads and iii) removal of the reads for bases having a nitrogen content >5% (5,22).

**LncRNA analysis.** Only lncRNA was analyzed in the present study. According to the characteristics of the lncRNA, a series of strict filters were used. The filter conditions were a length of ≥200 bp, exon number of ≥2 and minimum coverage of ≥3 transcription reads. Known mRNA transcripts, known non-coding RNA transcripts, homologous transcripts and transcripts with protein-coding capability were removed.

**Read comparison.** In this study, we used the TopHat software version 2.0.12 and selected the default parameters, and compared the reference sequence to hg19 (31).

**Differential expression analysis.** The cataract samples and normal samples in each group were subjected to differential expression analysis using DESeq2. Differential expression analysis was then carried out following standardization and variance estimation. The standards for screening the expression differences of mRNA and lncRNA were P<0.001, a false discovery rate (FDR) of <0.001 and a log2, ratiol >2 (21).

**Gene set enrichment analysis.** Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the DAVID functional annotation web server (http://david.abcc.ncifcrf.gov) using default parameters. Enriched GO terms (FDR <0.05) and KEGG pathways (P<0.1) were obtained (21-24).

**Screening target genes.** Cis target prediction was based on the distance between the lncRNA its linked protein-coding gene and to forecast the targets. Trans target prediction was according to the sequence of lncRNA and mRNA sequence, and the evaluation of the combination of free energy, which is a combination of the stability (25).

During the construction of all of the networks, Cytoscape software was used. Cytoscape is an open source software platform for visualizing molecular interaction networks and biological pathways and integrating these networks with annotations, gene expression profiles and other state data (http://www.cytoscape.org/).

**Conservation analysis.** The conservation of the corresponding sequence and site of the identified novel lncRNA was analyzed in order to evaluate the extent of its variation. Conservation scores (mammalian phastCons scores) were used to carry out the conservation analysis, as previously described (26).

**Reverse transcription-quantitative PCR (RT-qPCR) validation of differentially expressed lncRNAs.** Total RNA was extracted for use in microarray experiments from cataract patients and postmortem eyes as described above. Agarose gel electrophoresis was used to determine the integrity of the RNA, and its concentration was determined using a UV spectrophotometer. Quantification was performed with a
Results

Sequencing data analysis. The base distribution was analyzed, in order to investigate the presence of the AT and GC separation phenomenon; this phenomenon may arise during the sequencing or building of the library, and will affect the subsequent quantitative analysis. It was found that the frequencies of AT and CG was almost the same, which confirmed the reliability of the obtained data (Fig. 1A and B).

Read comparison. TopHat software was used to compare the filtered samples with the reference genome, and to locate the sequence within the genome. When the reference genome selection is appropriate and assembly is complete, in samples without exogenous species contamination, the matching rate of the sample is usually >80% (28-30) (Table I). The total number of reads in S2 was 126,905,134, including 92% mapped reads and 4% multiMap reads. The total number of reads in S3 was 102,287,578, including 96% mapped reads and 7% multiMap reads. The number and proportion of matching sequences of three functional components (exon, intron and intergenic) were confirmed. The majority of the matched sequences in the three samples were in the exon (Fig. 1C). In general, if the species of annotation information comprehensive, the majority of the sequence should match with the exon region (31,32).

Estimation of mRNA expression. Gene expression levels are generally measured on the basis of the mRNA transcription number of the gene. The units reads per kilobase million mapped reads (RPKM) were used as a measure of gene expression. It was observed that the number of differentially expressed genes accounted for only a small proportion of all genes, and so these genes are likely to have little impact on the overall distribution of genes in the samples, and all samples should have a similar distribution of expression (Fig. 1D) (33).

Characteristics of the novel lincRNA. In order to better define the characteristics of the novel lincRNA, its length (Fig. 2A), the number of exons (Fig. 2B) and expression quantity (Fig. 2C) were analyzed. The distribution of lincRNA expression was calculated according to the gene expression in all groups, and it was observed that all samples were similar in expression. The differentially expressed genes account for only a small fraction of the overall gene (34).

Differential expression and functional analysis of mRNA. DEseq was used to conduct a differential expression analysis between S2 and S1, and between S3 and S1 using q<0.05 (35). The analysis identified 27,447 and 15,019 candidate differentially expressed mRNAs, respectively, in these two comparisons. Hierarchical cluster analysis was conducted for the classification of candidate mRNAs in each group. Then, fold change values were used to classify the candidate mRNAs into upregulated mRNAs (fold change ≥2) and downregulated mRNAs (fold change ≤0.05). For S2 vs. S1 and S3 vs. S1, 24,947 and 10,478 upregulated mRNAs and 2,500 and 4,631 downregulated mRNAs, respectively, were identified (Table II). Further analysis of the differential expression is presented in Fig. 3. Intersections of the differentially expressed mRNAs were detected, and it was found that there were 11,348 differentially expressed mRNAs, 9,261 upregulated mRNAs and 2,087 downregulated mRNAs (Fig. 3C). Furthermore, an enrichment analysis was performed to investigate the functions of these upregulated and downregulated mRNAs. These mRNAs, whether upregulated or downregulated, were enriched in several basic biological or nerve-related terms and pathways, such as the phenylalanine metabolism pathway (Fig. 3A and B). Notable GO terms included cytoskeletal protein binding and actin binding. The phenylalanine metabolism pathway and cytoskeletal protein binding and actin binding are the biological processes of the marked enrichment of all data after processing (data not shown). In particular, the mRNAs were significantly rich in GO terms associated with...
structural constituents of the eye lens, which was related to the function and nerves of eyes. Some researchers considered ARC to be a structural disease (36). The lens is composed of >90% crystalline protein, with the remainder comprising skeletal, membrane, connective and water channel proteins (37). A large number of studies have indicated that the occurrence of cataract is directly associated with the apoptosis of lens epithelial cells induced by the modification of lens proteins, such as changes in the ratio of proteins or protein structure (38-40). The results of the present study revealed various differentially expressed mRNAs that were strongly associated with the function of the eyes. In the KEGG pathway analysis (data not shown), downregulated mRNA expression was particularly enriched in the tyrosine metabolism pathway, which is closely associated with cataract. The abnormal metabolism of tyrosine in the lens results in the production of quinones, which may be oxidized and thereby damage the protein of the lens, causing lens opacity and ultimately the occurrence of cataract (40-42). In addition, tyrosine is able to bind to lens proteins through phosphorylation, which may also have an association with cataract (43).

**Differential expression and functional analysis of lncRNA.**

Due to the low expression feature of IncRNAs, q<1.1 was used to identify the differentially expressed IncRNAs from the novel lincRNAs between S2 and S1, and between S3 and S1. This revealed that there were 4,146 and 2,895 differentially expressed IncRNAs, respectively (Table III). Fold-change values were used to classify the candidate IncRNAs into upregulated IncRNAs (fold change ≥2) and downregulated...
lncRNAs (fold change ≤0.05), and 3,477 and 2,051 upregulated lncRNAs and 669 and 844 downregulated lncRNAs were identified, respectively. Intersections of the lncRNAs were also obtained for each group. There were found to be 2,269 common differentially expressed lncRNAs, 1,701 upregulated lncRNAs and 568 downregulated lncRNAs (Fig. 3D).

Hierarchical cluster analysis was also performed, to classify the candidate lncRNAs for each group.

**LncRNA-mRNA network.** The 12,097 and 2,332 differential mRNAs and lncRNAs were further analyzed by integrating all differential mRNAs and lncRNAs for each group using intersection sets. Firstly, comprehensive mRNA-lncRNA interactions were obtained by integrating cis-targets and trans-targets. The interactions were also classified into upregulated and downregulated interactions using the dysregulated mRNAs. The functions of the dysregulated lncRNAs were analyzed on the basis of their target mRNAs (Figs. 4 and 5). It was observed that these dysregulated lncRNAs were enriched in certain basic pathways and GO terms, including regulation of the actin cytoskeleton pathway and protein binding terms. The result was coincident with the dysregulated mRNAs. Genes such as paired box 6 and chaperone-like activity of αA-crystallin (CRYAA), have similar functions. The lncRNA target gene major intrinsic protein of lens...
Fiber (MIP, ENSG00000135517) encodes the most abundant junctional membrane protein in the mature lens and this protein serves a critical role in the maintenance of the normal structure and internal circulation of the lens (44,45). In ARC, CRYAA is considered to be critical for the maintenance of lens transparency (46,47). In the GO analysis, it was found that ENSG00000160202 and CRYAA were linked nodes (Fig. 4C), indicating that they have similar biological functions (48). ENSG00000166147 and fibrillin-1 (FBN1) were also linked nodes (Fig. 4B). FBN1 is the most common pathogenic gene for Marfan syndrome, and encodes the fibrillin-1 protein. Eye diseases including lens dislocation or subluxation, and cataract, are observed in typical Marfan syndrome patients. Studies have suggested that fibrillin-1 is not a component of the normal lens, but may serve as a connector between latent-transforming growth factor β-binding protein 1 and the extracellular matrix (ECM) in capsular opacification. In addition, FBN1 is an important gene in eye development (49,50).

Furthermore, a human protein-protein interaction (PPI) network based on the HPRD data in the present study was constructed. The largest sub-network of the upregulated and downregulated mRNAs was then obtained from the PPI network. During the construction of all of the networks, the software Cytoscape was used. The upregulated network included 359 nodes and 395 edges, and the downregulated network included 305 nodes and 346 edges. The two networks approximated the scale-free network topology of dysregulated networks. The connectivity and betweenness of nodes were also analyzed. Some other networks also have similar topological features (51,52) (data not shown).
Conservation analysis. The PhastCons score of each chromosome was downloaded, which contains all loci on each gene from the University of California, Santa Cruz database, in order to analyze the conservative of IncRNA. The conservation scores of the majority of the IncRNAs in the present study (Fig. 2D) were at a low level (53).

RT-qPCR validation. To validate the results of sequencing, 30 differentially expressed IncRNA transcripts were selected for validation using RT-qPCR. The RT-qPCR results were consistent with the sequencing results for 28 of the IncRNA transcripts, showing the same trends of differential expression (P<0.05). Of note, due to individual differences or other reasons, two were
inconsistent with the sequencing results (Table IV). The specific mechanisms need to be further explored.

Discussion

Cataract is the leading cause of blindness globally, and its incidence increases yearly (1). To date, the pathogenesis of cataract has mainly been investigated at the cellular and molecular levels, including the post-translational modification, physical and chemical factors of lens proteins. miRNA has tissue and cell specificity in the eye. It serves an important regulatory role in the regulation of cell proliferation, differentiation and apoptosis processes (54).

With the rapid development of high-throughput sequencing and bioinformatics technology, IncRNAs have been identi-
High-throughput promoter and functional components are highly conservative. Their sequences are less conservative than those of mRNAs, the unknown. The study of lncRNA has revealed that although in mammalian genomes; however, their functions are mostly classified as a class of non-encoding RNAs abundantly expressed.

The majority of these events are considered to be influenced by dynamic reorganization of the cellular actin cytoskeleton. These signaling pathways including tyrosine metabolism, phenylalanine metabolism and regulation of the actin cytoskeleton. These signaling pathways are associated with numerous pathological processes. Thus, it appears that lncRNAs and the differentially expressed gene network are involved in the regulation of multiple cellular processes. Therefore, they may be a novel target for the treatment of cataract and associated diseases, which may have potential clinical significance.

It is worthy of note that the calcium signaling pathway, and regulation of the actin cytoskeleton, are closely associated with the underlying pathological processes of a number of diseases. The calcium ion is the most common signal transduction factor in the human body, and plays an important role in cell division, growth and death. Calcium ions act as the second messenger in the regulation of cell apoptosis, which is known to be one of the main causes of ARC (61,62).

It may be speculated that the involvement of the lncRNAs in the pathogenesis of ARC involves regulation of the calcium signaling pathway, which provides new opportunities for the diagnosis and treatment of ARC. The lens is a fascinating and unique transparent tissue that grows continuously throughout life. During the process of differentiation into fiber cells, lens epithelial cells undergo marked morphological changes, membrane remodeling, polarization, transcriptional activation and the elimination of cellular organelles, including nuclei, concomitant with migration towards the lens interior (63,64). The majority of these events are considered to be influenced by dynamic reorganization of the cellular actin cytoskeleton.
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