Research Article

Identification of Differential Gene Expression Pattern in Lens Epithelial Cells Derived from Cataractous and Noncataractous Lenses of Shumiya Cataract Rat

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Received 25 June 2020; Revised 10 September 2020; Accepted 16 October 2020; Published 2 November 2020

Academic Editor: Marco Fichera

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The Shumiya cataract rat (SCR) is a model for hereditary cataract. Two-thirds of these rats develop lens opacity within 10-11 weeks. Onset of cataract is attributed to the synergetic effect of lanosterol synthase (Lss) and farnesyl-diphosphate farnesyltransferase 1 (Fdft1) mutant alleles that lead to cholesterol deficiency in the lenses, which in turn adversely affects lens biology including the growth and differentiation of lens epithelial cells (LECs). Nevertheless, the molecular events and changes in gene expression associated with the onset of lens opacity in SCR are poorly understood. In the present study, a microarray-based approach was employed to analyze comparative gene expression changes in LECs isolated from the precataractous and cataractous stages of lenses of 5-week-old SCRs. The changes in gene expression observed in microarray results in the LECs were further validated using real-time reverse transcribed quantitative PCR (RT-qPCR) in 5-, 8-, and 10-week-old SCRs. A mild posterior and cortical opacity was observed in 5-week-old rats. Expressions of approximately 100 genes, including the major intrinsic protein of the lens fiber (Mip and Aquaporin 0), deoxyribonuclease II beta (Dnase2B), heat shock protein B1 (HspB1), and crystallin γ (γCry) B, C, and F, were found to be significantly downregulated (0.07-0.5-fold) in rat LECs derived from cataract lenses compared to that in noncataractous lenses (control). Thus, our study was aimed at identifying the gene expression patterns during cataract formation in SCRs, which may be responsible for cataractogenesis in SCR. We proposed that cataracts in SCR are associated with reduced expression of these lens genes that have been reported to be related with lens fiber differentiation. Our findings may have wider implications in understanding the effect of cholesterol deficiency and the role of cholesterol-lowering therapeutics on cataractogenesis.

1. Introduction

Age-related eye disease is a serious public health issue, and age-related cataract is the leading cause of blindness worldwide [1]. Currently, surgery is the only treatment for cataract. It has been reported that if the progression of cataract is delayed by 10 years, the huge expense associated with surgical intervention can be reduced [2]. Cataracts are caused by the degeneration of the lens protein called crystallin. Lenses have almost no protein turnover and are therefore susceptible to ultraviolet rays, oxidative stress, and glycative stress. These stressors damage protein integrity and function leading to denaturation and aggregation of lens protein, which in turn results in lens opacification [3, 4]. Recent studies have shown that oxidative stress controls various cellular processes associated with cell survival, such as cell proliferation, differentiation, aging, and cell death. It promotes cell apoptosis and senescence and is also associated with many diseases [5, 6]. Oxidative stress and reactive oxygen species (ROS) are a major cause of age-related eye diseases, and diets rich in
2. Materials and Methods

2.1. Animals. All animal experiments were approved by the Committee of Animal Research at Kanazawa Medical University (Permission no. 2017-107) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals implemented by the National Institutes of Health, the recommendations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the Institutional Guidelines for Laboratory Animals of Kanazawa Medical University. SCRs (SCR/Scrc: NBRP Rat No: 0823) were obtained from the National BioResource Project-Rat, Kyoto University (Kyoto, Japan).

We used 5-, 8- and 10-week-old SCRs in this study. Since mature cataracts develop in SCR after 11 weeks of age, rats with precataracts and mild to moderate cataracts were selected (5-10 weeks old). All rats were provided ad libitum access to regular or experimental chow (Sankyo Labo Service, Tokyo, Japan). The animals were sacrificed by administration of a lethal dose of CO2. Cataract onset in SCR was uniquely regulated by a specific combination of different mutant (Lss") and polymorphic alleles (Lss") on the Lss locus [16]. The prerequisite for cataract onset was the Lss"/Lss" genotype, which reduced Lss activity below the threshold that is about 12% of normal [16]. Lens opacity in SCR appears spontaneously at 9-11 weeks of age in 2/3 of animals (Lss"/Lss"). In 1/3 of SCR (Lss"/Lss'), no cataractous changes appeared even after 11 weeks of age. SCRs having Lss"/Lss' are embryonically lethal. Therefore, the rats were divided into two groups, i.e., control (Lss"/Lss'; Cat-) and cataractous (Lss"/Lss'; Cat+). Cat+ and Cat- SCRs were distinguished via PCR using genomic DNA isolated from the tails of 4-week-old rats. The amplified products were then separated on a 15% gel via polyacrylamide gel electrophoresis (PAGE) to detect Lss mutation (Lss"/Lss' or Lss"/Lss") as reported in a previous study of ours [20]. The sequences of primers used to detect Lss mutation were as follows: 5'-GCACACTGGACTGTGGCTGG-3' and 5'-GCCCAGCATTTGAGTGCGCT-3'.

2.2. RNA Extraction. Total RNA from each LEC sample obtained from SCR was extracted using the miRNeasy Micro Kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. Since RNA is extremely susceptible to degradation due to the ubiquitous presence of RNAses in the environment, purity and integrity of RNA were examined and validated as previously described [21]. Quality of total RNA was analyzed by evaluating the RNA integrity number (RIN) using Bioanalyzer RNA analysis (Agilent Technologies Japan Ltd., Tokyo, Japan). All RNA samples showed RIN > 9.0. In this study, we used LECs from SCR, because quality of total RNA obtained from whole lens in Cat+ SCR was poor.

2.3. Microarray Analysis. Cataracts as well as Lss-mutation-related genes were screened by microarray analysis using LEC samples from SCR as follows. Total RNAs from each LEC sample obtained from 2 eyes of 5-week-old Cat+ SCR with Lss"/Lss' or Cat- SCRs with Lss"/Lss" as the control were used for the microarray analysis (n = 1, each). All samples...
were processed for the microarray analysis as follows: for RNA labeling and hybridization, GeneChip™ WT Pico Reagent Kit (Applied Biosystems, Thermo Fisher Scientific, Tokyo, Japan) and GeneChip® Rat gene 2.0 ST array (Affymetrix, Thermo Fisher Scientific) were used according to the manufacturer’s protocol. Washing, scanning of the arrays, and analysis of scanned images were performed according to manufacturer’s instructions. Each chip was normalized by dividing the measurement of each gene by the measurement of the specific control or by average intensity in the single array. Normalized data were exported for subsequent analysis. Genes with a normalized ratio > 2.0-fold or <0.5-fold were selected as significant genes using the GeneSpring software package version 14.9 (Agilent). A Gene Ontology (GO) analysis was conducted using the DAVID database (https://david.ncifcrf.gov/) for the 110 downregulated genes in LECs of SCR Cat+.

2.4. Real-Time Reverse Transcriptase-Quantitative PCR (RT-qPCR). Total RNAs from each LEC sample obtained from 5-, 8-, and 10-week-old Cat+ SCR with Lss'/Lss' and Cat- SCRs with Lss'/Lss as the control were used for the microarray analysis (n = 3, each). To measure the expression of rat deoxyribonuclease II beta (Dnase2B), heat shock protein B1 (HspB1), major intrinsic protein of lens (Mip), crystallin, gamma D (CryyD), heat shock transcription factor 4 (Hsf4), lensin (Lgsn), and tudor domain containing 7 (Tdrd7) mRNAs, we conducted a relative quantification of mRNA using Prism 7300 (Applied Biosystems, Thermo Fisher Scientific). The comparative Ct method was used for relative quantification of mRNA expression. The PCR amplification was performed with the TaqMan Universal PCR Master Mix. The probe mix containing the primers for Dnase2B, Hspb1, γCry, and Mip was obtained from Thermo Fisher Scientific. All reactions were performed in triplicates. Differential expression for each gene was calculated using the comparative CT method using a predeveloped TaqMan Ribosomal RNA Control Reagent VIC probe as an endogenous control (Thermo Fisher Scientific).

2.5. Statistical Analysis. The statistical analysis was performed for all experiments using Student’s t-test and/or one-way analysis of variance (ANOVA), as applicable. The data were presented as the mean ± standard deviation (SD). A significant difference between the control and treatment group was defined as a p value < 0.05 for two or more independent experiments.

3. Results

3.1. Lens Morphology in 5- and 10-Week-Old SCRs. In Cat-SCR, the lens was clear, at 5 and 10 weeks of age, but in the case of Cat + SCR, a mild posterior and cortical opacity was observed at 5 weeks of age, and severe cortical and nuclear opacity was observed at age 10 weeks (Figure 1).

3.2. Analysis of Gene Expression Profile. As described in Materials and Methods, LECs from, 5-week-old Cat+ and Cat-SCR were used for microarray analysis (n = 1 in each group) to screen genes associated with cholesterol deficiency and cataracts. The data for the microarray analysis was deposited to the Gene Expression Omnibus (GEO) database (Accession number: GSE152616). First, with 28,407 genes on the array, 110 genes were detected that showed a fold change < 0.5 in the Cat+ group compared to those in Cat-, as shown in Figure 2. In the scatter plot, many genes were distributed in the lower part, and specifically, genes whose expression was downregulated could be detected using the scatter plot (Figure 2). Fold changes (<0.5-fold) were observed in 20 top-ranked genes (Table 1) in the microarray analysis including Dnase2B, Hspb1, γCry, Mip, beaded filament structural protein 1 (Bfsp1), and Lgsn that have been reported to be associated with cataract and lens development [22–27]. Additionally, Hsf4, Tdrd7, gap junction protein epsilon 1 (Gje1), beaded filament structural protein 2 (Bfsp2), and lens intrinsic membrane protein 2 (Lim2) which are also significantly reduced in the SCR lens (<0.5-fold) were linked to human or animal cataracts (Supplement 1) [27–33]. Furthermore, the expressions of six genes were upregulated (2.1- to 2.5-fold) in rat LECs from Cat+ compared to those from Cat-. Of the six genes, five genes were unidentifed, and the other was Schlafen 4. However, the function of Schlafen 4 in the lens is not clear.

GO analysis revealed 110 downregulations of genes related to lens development, cellular water homeostasis, negative regulation of apoptotic process, etc. in LECs of Cat+ SCR (Table 2).

3.3. Validation of Gene Expression Data Using RT-qPCR. The data from the microarray experiment shown above revealed the downregulation of various genes during the progression of cataracts. Therefore, we selected the following four genes: Dnase2B, Mip, Hspb1, γCry, Hsf4, Lgsn, and Tdrd7 that showed significant changes in expression according to the microarray data, and accordingly, we validated these results using RT-qPCR.

Data from the RT-qPCR showed that Dnase2B and Mip mRNA showed a significant downregulation in 5-, 8-, and 10-week-old Cat+ SCRs compared to Cat- SCRs (Figures 3(a) and 3(b)). The expressions of Hspb1, Hsf4, Lgsn, and Tdrd7 mRNA significantly downregulated in 5-
and 10-week-old Cat+ SCRs compared to Cat- SCRs (Figures 3(c), 3(e), 3(f), and 3(g)). However, the expression of CryγD mRNA was significantly downregulated only in 5-week-old Cat+ SCRs (Figure 3(d)).

4. Discussion

In this study, we comprehensively analyzed gene expression changes in the lens depending on the presence or absence of Lss deficiency in SCRs of different ages. SCRs with Lss deficiency gradually develop cataracts and show mature cataracts at 11 weeks of age. Lens opacity is slightly observed in 5-week-old SCRs with Lss mutations. In microarray analysis, 110 genes were downregulated with 0.5-fold change in Cat+ SCR at 5 weeks of age. It is speculated that carrying the Lss mutation alters many gene expressions in the lens and induces lens opacity. In LECs of Cat+ SCRs with Lss mutations, the expressions of many genes reported to be associated with cataract development such as Dnase2B, HspB1, γCry, Mip, Hsf4, Tdrd7, Lim2, Gje1, Bfsp1, Bfsp2, and Lgsn were downregulated. In this study, we analyzed Dnase2B, HspB1, γCry, and Mip genes using RT-qPCR. We confirmed that expressions of Dnase2B, HspB1, and Mip mRNAs were significantly downregulated in the LECs of Cat+ SCRs with Lss mutations.
Lss mutation before and after cataract onset. Furthermore, significant downregulation in the expression of γCry mRNA was observed in the LECs of Cat+ SCRs with Lss/Lssl only before the onset of the disease.

In this study, Hsf4 and Tdrd7 are the transcription factors (TF) that are found to be reduced in LECs of Cat+ SCR. It has been reported that Hsf4 mutation causes human congenital and age-related cataracts [28, 29]. Furthermore, the mutations of Tdrd7 also cause human congenital and age-related cataracts [30, 31]. Hsf4- knockout mice (Hsf4<sup>-/-</sup>) and Tdrd7-homozygous KO mice (Tdrd7<sup>-/-</sup>) also cause cataracts [32, 33]. Among 111 genes whose expression was decreased by LECs of Cat+ SCR, genes whose expression was also changed by the lens of Hsf4-conditional knockout mice (Hsf4-CKO) or Tdrd7-homzygous KO mice (Tdrd7<sup>-/-</sup>) were analyzed using the iSyTE database (PMID: 29036527 and PMID: 32420594, respectively). Genes whose expressions were commonly reduced in LEC of Cat+ SCR and the lens of Hsf4-CKO were HspB1, Sh3bg1, Hmx1, Atp8a2, Pbld1, Bfsp1, Tdrd7, and Dnase2B (fold change < 0.5) (Supplement 2). A gene whose expression was commonly reduced in LEC of Cat+ SCR and lenses of Tdrd7<sup>-/-</sup> was HspB1 (fold change < 0.5). Thus, HspB1 may induce the reduction of Hsf4 and/or Tdrd7 as TF in Cat+ SCR. Reduction of HspB1, Sh3bg1, Hmx1, Atp8a2, Pbld1, Bfsp1, and Dnase2B genes may be due to the downstream change by the Hsf4 regulation in Cat+ SCR.

HspB1 also known as heat shock protein 27 (Hsp27) is a protein that is encoded by the HspB1 gene in humans. HspB1 is an ATP-independent molecular chaperone with a conserved αβ-crystalline domain in the C-terminus region [34]. Heat shock proteins (Hsp) play a central role in maintaining cellular homeostasis and altering protein folding, thereby protecting αA- and other crystalline proteins [35]. Further investigation of Hsp27 revealed that the protein responds to cellular oxidative and chemical stress conditions other than heat shock [35]. In the presence of oxidative stress, Hsp27 plays a role as an antioxidant, decreasing the ROS by raising levels of intracellular glutathione (GSH) [36, 37]. It has been reported that mutation in HspB1 and/or αβ-crystallin is responsible for the development of cataract [38] and is considered as major targets for the development of anticataract drugs [39]. We have previously reported that expression of Prdx6, an antioxidant protein, is decreased resulting in an increase in ROS in the LECs of Cat+ SCRs [7]. Thus, low activity of Lss may be involved in the decreased expression of antioxidant genes, causing oxidative stress and inducing cataracts in SCRs.

In mice, DNase II-like acid DNase (DLAD; Dnase2B) has been identified as a DNA-degrading enzyme that functions during lens enucleation [25]. Since the optimum pH for DLAD is acidic, it was suggested that the nucleus could be engulfed by lysosomes through autophagy and denucleated by the action of DLAD [25]. The lens consists of LECs and differentiated lens fiber cells. In the process of fiber differentiation, intracellular structures such as the nucleus, mitochondria, and endoplasmic reticulum disappear and the lens becomes transparent. In the process of enucleation, the DNA that encodes the genetic information is degraded. For this enucleation process, DLAD plays an important role. In Dlad knockout mice, the eye lens seemed to have developed normally; however, undegraded DNA was observed in the lens fiber that contributed to lens opacity. Thus, DLAD is necessary to maintain lens transparency and normal fiber differentiation. Lss mutation downregulates the expression of Dlad (Dnase2B), which could be attributed to cataract development in SCRs.

MIP is a lens fiber major intrinsic protein, also known as Aquaporin 0, which is a water channel in lens fiber cells, facilitating the movement of water, gap junction channels, and solute transporters [40]. MIP plays a crucial role in regulating the osmolarity and homeostasis of the lens and stabilizing cell junctions in the lens nucleus. Currently, 12 mutations in MIP have been linked to autosomal-dominant cataracts in humans [26]. The decreased expression of Mip in LEC of

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**Table 2: GO analysis that showed changes of smaller than 0.5-fold in LECs from 5-week-old SCR Cat+ compared to SCR Cat-**

| GO accession | GO term | p value |
|--------------|---------|---------|
| GO:000208    | Lens development in camera-type eye | 6.18E-06 |
| GO:007030    | Lens fiber cell development         | 5.05E-04 |
| GO:0034220   | Ion transmembrane transport         | 0.003999 |
| GO:0007601   | Visual perception                    | 0.005318 |
| GO:0001654   | Eye development                      | 0.005798 |
| GO:0043010   | Camera-type eye development          | 0.021266 |
| GO:0008104   | Protein localization                 | 0.030793 |
| GO:0015793   | Glycerol transport                   | 0.041241 |
| GO:0009992   | Cellular water homeostasis            | 0.044906 |
| GO:0043627   | Response to estrogen                  | 0.051878 |
| GO:0006833   | Water transport                       | 0.063030 |
| GO:0009966   | Regulation of signal transduction     | 0.073742 |
| GO:0043066   | Negative regulation of apoptotic process | 0.078634 |
| GO:0010976   | Positive regulation of neuron projection development | 0.083444 |
| GO:2001234   | Negative regulation of apoptotic signaling pathway | 0.091330 |
Figure 3: Continued.
Cat+ SCRs may disrupt cellular water homeostasis and induce lens fiber swelling and vacuole formation observed in SCR lenses.

The nuclear region of the eye lens is particularly rich in the γCry protein, which is necessary to maintain structural and functional properties in the lens. γCry mRNA was down-regulated in the LECs of Cat+ SCRs before the onset of lens opacity compared to Cat- rats. However, there was no significant change in expression of this gene after the onset of cataracts. It is not clear why γCry mRNA expression did not decrease in cataractous lenses in SCRs. Further studies are required to understand this phenomenon.

Sterol, such as cholesterol in animals, is a compound that is important as a biosynthetic raw material for steroid hormones, vitamin D, and bile acids, in addition to its role in regulating membrane fluidity as a component of eukaryotic biological membranes [41]. Cholesterol synthesis is carried out through a process of approximately 30 enzymatic reactions using acetyl-CoA as a starting substrate. Lanosterol is the first sterol in the cholesterol synthesis pathway and LSS is an essential rate-limiting enzyme that functions as a downstream element in the lanosterol biosynthetic pathway, catalyzing the cyclization of the linear 2,3-monoepoxysqualene to cyclic lanosterol [42]. Congenital cataracts with homozygous Lss mutations have been reported to affect the catalysing functions of Lss [18, 19]. Furthermore, the polymorphism rs2968 of the Lss gene was associated with nuclear type of age-related cataract (ARC) risk in the Chinese population [43]. Consequently, it has been reported that the mRNA expression of Lss was significantly lower in LECs of all subtypes of the ARC group than the control group [43]. Epidemiological studies have shown that individuals receiving statins, which are cholesterol synthesis inhibitors, have an increased risk of being diagnosed with cataracts [44, 45]. Previous studies have reported that Lss might play a significant role in oxidative stress and maintenance of lens transparency [46]. These results indicate that Lss deficiency may be a risk factor of ARC. Additionally, it has also been reported that lanosterol plays a protective role in cataract formation, inhibiting lens opacity and reversing crystalline aggregation [18]. Additionally, intravitreal injection of lanosterol nanoparticles has been reported to rescue the early stage of lens damage in SCRs [8]. Thus, synthesis of cholesterol by LSS is important to maintain lens transparency.

5. Conclusions
In conclusion, our study demonstrated that Lss mutations in SCRs result in reduction of Hsf4 and Tdrd7 inducing the downregulation of several genes associated with maintaining lens transparency and identified their relationship with cholesterol deficiency. Cholesterol and Lss in lenses may be important to maintain normal lens homeostasis such as lens fiber differentiation, oxidative and heat stresses, and regulation of lens osmolarity to maintain lens transparency.

Data Availability
All data generated or analyzed during this study are included in this published article. More details are available from the corresponding author upon reasonable request.

Conflicts of Interest
There was no conflict of interest.

Acknowledgments
We are thankful to the National BioResource Project-Rat (http://www.anim.med.kyoto-u.ac.jp/NBR/) for providing the rat strains (SCR/Sscr: NBRP Rat No. 0823). We would like to thank Editage (http://www.editage.com) for English language editing. This work was supported by grants from the Japan Society for the Promotion of Science (JSPS KAKENHI, Grant Number JP 17K11470 to EK) and National Eye Institute, National Institute of Health (NIH) (EY024589) to (DPS).
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