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Exome Sequencing and Epigenetic Analysis of Twins Who Are Discordant for Congenital Cataract

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Purpose: To further understand genetic factors that contribute to congenital cataracts, we sought to identify early post-twinning mutational and epigenetic events that may account for the discordant phenotypes of a twin pair. *Methods:* A patient with a congenital cataract and her twin sister were assessed for genetic factors that might contribute to their discordant phenotypes by mutation screening of 11 candidate genes (CRYGC, CRYGD, CRYAA, CRYAB, CRYBA1, CRYBB1, CRYBB2, MIP, HSF4, GJA3, and GJA8), exome analysis followed by Sanger sequencing of 10 additional candidate genes (PLEKHO2, FRYL, RBP3, P2RX2, GSR, TRAM1, VEGFA, NARS2, CADPS, and TEKT4), and promoter methylation analysis of five representative genes (TRAM1, CRYAA, HSF4, VEGFA, GJA3, DCT) plus one additional candidate gene (FTL). *Results:* Mutation screening revealed no gene mutation differences between the patient and her twin sister for the 11 candidate genes. Exome sequencing analysis revealed variations between the twins in 442 genes, 10 of which are expressed in the eye. However, these differential variants could not be confirmed by Sanger sequencing. Furthermore, epigenetic discordance was not detected in the twin pair. *Conclusions:* The genomic DNA mutational and epigenetic events assessed in this study could not explain the discordance in the development of phenotypic differences between the twin pair, suggesting the possible involvement of somatic mutations or environmental factors. Identification of possible causes requires further research.

■ **Keywords:** twins, congenital cataract, exome sequencing, methylation sensitive restriction enzyme-polymerase chain reaction, MSRE-PCR

Cataracts can be defined as complete or partial lens opacification caused by either congenital or acquired factors. On a global scale, cataracts are the leading cause of blindness, accounting for approximately 48% of all blindness (Lau et al., 2004). Although there are multiple factors involved in the etiology and development of cataracts, accumulating evidence indicates that the genetic background plays an important role in the overall process. Childhood cataracts are also a clinically and genetically heterogeneous disorder in which the phenotype varies considerably between and within families (Ionides et al., 1999). Autosomal dominant inheritance is commonly observed among hereditary cataracts, while autosomal recessive and X-linked patterns have also been reported (Yang et al., 2014). From an etiological point of view, genetic mutations might be the most common cause, especially for bilateral cataracts.

Research on hereditary congenital cataracts led to the identification of several classes of candidate genes (Kannabiran & Balasubramanian, 2000). To date, scientists have identified more than 35 loci, including over 20 genes, that are associated with congenital cataracts (Shiels et al., 2010), and this number is constantly increasing. Among the causative congenital cataract mutations discovered thus far, approximately half are in crystalline genes (αA-crystallin [CRYAA], αB-crystallin [CRYAB], CRYBA3/A1, βA4- crystallin [CRYBA4], βB1-crystallin [CRYBB1], βB2-crystallin [CRYBB2], γC-crystallin [CRYGC], and γD-crystallin [CRYGD]; Johnson et al., 2013); approximately one quarter are in connexin genes (Connexin 46 [GJA3] and Connexin 50 [GJA8]; Guleria et al., 2007; Vanita et al., 2008); and the remainder are divided among the genes for heat shock transcription factor-4 (HSF4; Bu et al., 2002), aquaporin-0 (AQP0, MIP; Ding et al., 2014; Jiang et al., 2009), paired-like homeodomain 3 (PITX3; Finzi et al., 2005), chromatin modifying protein 4B (CHMP4B; Shiels

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et al., 2007), and EPH receptor A2 (EPHA2; Shiels et al., 2007; 2008).

Twins provide the ideal model to study and quantify the relative importance of genetic and environmental factors. The search for differences in genetic constitutions within discordant monozygotic (MZ) twin pairs has been suggested as a promising method for gene identification (Zwijnenburg et al., 2010): very early post-twinning mutational events can cause discordance in MZ twin pairs (Helderman-van den Enden et al., 1999; Kondo et al., 2002; Kruyer et al., 1994; Taylor et al., 2008). The investigation of discordant MZ twin pairs may be applicable not only to monogenic disorders but also to multifactorial disorders, including congenital malformations. In the present study, we applied this strategy for the first time to the investigation of congenital cataracts. We hypothesized that gene mutation affecting coding regions that occur after twinning might contribute to the discordance in MZ twin pairs with congenital cataracts. To test this hypothesis, we investigated a discordant MZ twin pair using mutation screening for the candidate genes, exome sequencing and promoter methylation analysis.

Materials and Methods

Case Report

In this study, a three-generation family with one twin pair was contacted and recruited through Zhongnan Hospital of Wuhan University, Wuhan, China. Ethics approval for this study was obtained from the Medical Ethics Committee of Zhongnan Hospital of Wuhan University. Informed written consent was obtained from all adult individuals and the parents of the twins prior to study enrolment.

The patient was the older sister of the twin pair. She was diagnosed with a congenital pulverulent nuclear cataract, which was present at birth and developed during childhood. The congenital bilateral cataract was observed in this study at age 7, at which time blood samples were obtained from both twins. There was no record of prematurity, intrauterine infections, or any systemic diseases or trauma in the twins' case histories. The status of the affected twin was verified by ophthalmologic examination, which included visual acuity, slit lamp, and fundus examination with a dilated pupil.

Genomic DNA Extraction

Genomic DNA was directly extracted from peripheral blood samples using a standard proteinase K/phenol method (Soetens et al., 2008). DNA samples were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher) at 260 nm and assessed for purity by electrophoresis and the ratio of absorbance at 260 nm and 280 nm. Purified DNA was stored at −20 degree Celsius until use.

Mutation Screening

The purified DNA was amplified by polymerase chain reaction (PCR) of the exons (and their flanking regions) for CRYGC(MIM:123680), CRYGD(MIM:123690), and CRYAA (MIM:123580); CRYAB(MIM:123590); and CRYBA1 (MIM:123610), CRYBB1(MIM: 600929), CRYBB2(MIM:123620), MIP(MIM:154050), HSF4(MIM: 602438), GJA3(MIM: 121015), and GJA8 (MIM: 600897). Mutation screening of the 11 candidate genes was performed by direct DNA sequencing of the PCR amplicons. The sets of primer pairs used in the PCR amplification and sequencing are listed in Table S1. The PCR conditions were as follows: denaturation at 96 degree Celsius for 5 min; 35 cycles of denaturation at 94 degree Celsius for 60 s, annealing at different temperatures (listed in Table S1) for 60 s, and extension at 72 degree Celsius for 60 s; and a final extension for 10 min at 72 degree Celsius. Each reaction mix (50 μL total) contained genomic DNA, PCR buffer, 5 pmol each of sense and antisense primers, and 2.5 U of Taq DNA polymerase (Fermentas, Hanover, MD, USA). Thermal cycling was performed using a C1000TM 48 well thermal cycler (Bio-Rad, Hercules, and CA). PCR products were isolated by electrophoresis on 2% agarose gels, purified and sequenced using an ABI Genetic Analyzer 3730 (Invitrogen Ltd, Shanghai, China). The sequencing results were analyzed using Chromas 2.3 and compared against known sequences in the NCBI database by BLAST analysis.

Exome and Sanger Sequencing

The genomic DNA samples of the twins were sent to BGI-Shenzhen (Shenzhen, China) for exome capture and sequencing. Using an ultrasonoscope (Covaris, Massachusetts, USA), the genomic DNA samples were randomly fragmented with a target size distribution of 150–200 bp and subjected to library preparation according to the manufacturer's protocol (Illumina, Inc., San Diego, CA). Exome enrichment was performed for the shotgun libraries using an Agilent SureSelect Human All Exon v2 kit (50 Mb) (Agilent Technologies, Inc. Santa Clara, CA). The enriched shotgun libraries were sequenced using the Illumina Hiseq2000 platform, and 90 bp paired-end reads were generated. Raw image data and base calling were processed by Illumina Pipeline software v1.7 using the default parameters.

SNP detection was performed as follows: (1) SOAP aligner (v2.21; Li et al., 2009) was used to align the high-quality reads to the human reference genome (hg19); (2) for paired-end reads with duplicated start and end sites, only one copy with the highest quality read was retained; (3) the reads with alignment length less than 75 bp were removed; (4) SOAP snp (v1.05) (Li et al., 2009) was used to assemble the consensus sequences and call the genotypes.

A series of exclusion steps were followed to reveal the causative mutation (Table 1). The SNPs within intergenic, intronic, and UTR regions, synonymous mutations, or variants present on four public genetic variant databases (dbSNP129, 1,000 Genomes, HapMap, and YH; see URLs in Supplemental Web Resources) and SNPs with a minor

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TABLE 1
Variation Prioritization Pipeline After Exome Sequencing

Filters	Variants
Different between the case and control (1+2)	344 NSs + 65 Indel
Filtered dbSNP129, 1000 Genomes, HapMap and YH inhouse database	106 NSs + 6 Indel
Expressed in eyes	10 NSs

allele frequency of more than 0.5% (except for YH) were all excluded. Quality control of reads was performed as before. The detected variants were annotated based on four databases: NCBI CCDS, RefSeq, Ensembl, and Encode. The variants predicted to be differential in the twins and expressed on the eyes were prioritized for validation by Sanger sequencing.

The primers flanking exons of candidate genes were designed by Primer 3.0 (listed in Table S2).

Promoter Methylation Analysis

Methylation sensitive restriction enzyme digestion (MSRE)-PCR includes extensive digestion of genomic DNA, requires limited amount of starting material and can identify methylation in a heterogeneous mix containing less than 2% of cells with methylated fragments. The CpG island fragments on promoters of the six candidate genes, including TRAM1, CRYAA, HSF4, VEGFA, GJA3, and FTL, were detected by MSRE-PCR.

Restriction enzyme digestion was performed with Hin6I (recognition site GCGC; Fermentas, Hanover, MD, USA). The total volume of reaction system was 50 μL with 1 × buffer, 50 U of enzyme, and 200 ng of genomic DNA samples from twins. The reaction mixture was incubated at 37 degree Celsius for 72 hours under a layer of mineral oil.

Genomic fragments located within corresponding CpG islands and containing at least two Hin6I recognition sites were selected for amplification. Primers were designed with Primer 3.0 (listed in Table S3). A C1000 TM 48 well thermal cycler (Bio-Rad, Hercules, CA) was programmed for PCR: denaturation at 95 degree Celsius for 5 min followed by 35 cycles of denaturation at 94 degree Celsius for 60 s, annealing at 57 degree Celsius for 60 s, and extension at 72 degree Celsius for 60 s, with a final extension for 10 min at 72 degree Celsius. Finally, 5 μL of the product were loaded onto 2% agarose and visualized by Goldenview (SBS Genetech, Shanghai, China) staining.

Results

Clinical Features

A Chinese family with MZ twins presenting discordant phenotypes was recruited to help identify genetic factors that might explain the occurrence of a congenital cataract for one of the twins, but not the other (Figure S1). A detailed medical history was obtained by interviewing all family members. The cataract in the older affected twin was present at birth and developed during childhood. Slit lamp examination of the affected lens revealed a bilateral nuclear cataract phenotype. No other ocular or systemic abnormalities were found upon physical examination in either the affected individual or other family members. The younger twin sister had a normal phenotype.

No Mutation Observed on Known Candidate Genes

No differences in the sequences of 11 candidate known genes (CRYGC, CRYGD, CRYAA, CRYAB, CRYBA1, CRYBB1, CRYBB2, MIP, HSF4, GJA3, and GJA8) were found between the patient and her twin sister. These findings rule out the possibility that the cataract was caused by mutation in these 11 cataract-associated genes (the part of sequencing results listed in Figure S2).

No Differential Variant Found by Exome Sequencing

To identify additional putative genetic differences that might explain the selective occurrence of a cataract in one twin, we performed exome sequencing; an average of 4.88 Gb of sequence data was generated per individual. After mapping to the human reference genome (NCBI 37.1, hg 19), we achieved targeted exome sequences with an average sequencing depth of 70-fold and coverage of 97.91%. After annotation of variants, we focused only on non-synonymous variations (NS), splice donor-site or acceptor-site mutations (SS), and insertions/deletions (InDel) that were more likely to be pathogenic. Given discordant phenotypes in twins, we selected NS/SS/InDel that was different between the twin pair. As a result, 442 NSs and 65 Indels variants were detected. As a validation of the specificity of the method, the 11 candidate genes found by previous mutation screening to be conserved in the twins were not among the 442+65 variants. Further variations that were present in four public genetic variant databases db SNP 135, 1,000 genome, Hapmap, YH and our inhouse database were excluded. As a result, 65 NSs plus 7 Indels were detected. And only 10 NSs in genes were presented to express in eyes according to Ensemble and GeneCard (Online Web Resources) and prioritized for validation (Table S2).

The 10 genes are PLEKHO2, FRYL, RBP3, P2RX2, GSR, TRAM1, VEGFA, NARS2, CADPS, and TEKT4. Primers were designed for the 10 candidate genes, and exons were amplified by PCR and confirmed by gene sequencing. However, Sanger sequencing could not confirm differential mutations, which were discovered by exome sequencing analysis. These results suggest that the exome assay had a

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certain amount of false positivity and need verification by Sanger sequencing for identifying mutations. Nevertheless, the Sanger sequencing of 10 additional candidate genes further limits the possible set of genetic mutations that might be associated with the cataract formation in one twin.

No Epigenetic Differences Discovered in the Twins

Variable methylation provides an additional mechanism of regulating gene expression at the epigenetic level. Hypermethylated sequences on promoter fragments of each of the six candidate genes could be detected in DNA samples from both the patient and her twin sister after MSRE treatment. This indicates that there is no difference in the promoter methylation between the twins for these six genes.

Discussion

Mutation screening analysis for the twin pair ruled out disease-causing mutations of 11 candidate genes highly correlated with cataracts. These results suggests that exon mutations affecting coding regions that arise as early post-twinning mutational events in these 11 genes are not a cause of discordance among these MZ twins for the congenital cataract. On the basis of these results, we hypothesized that possible mutations may exist in other genes.

To test for the presence of mutations associated with cataracts aside from the 11 candidate genes, we performed exome-sequencing analysis. As a result, 442 variants were detected. We further screened 10 of them that are presented to be expressed in the eye according to Ensemble and GeneCard, PLEKHO2, FRYL, RBP3, P2RX2, GSR, TRAM1, VEGFA, NARS2, CADPS, and TEKT4. However, differential genes discovered in the exome sequencing analysis could not be confirmed by Sanger sequencing in our research, which rules out mutations in 10 additional suspect genes.

The results show that the signals of exon sequencing value of the differential variants were low in general, so that the differences between case and control were not reliable, and the reliability of the results was not high. Because of the limited differences observed, we picked out the 10 genes with difference and predicted expression in eyes to verify further. No difference was found in the sequences of the case and the control. It proved that when the signal value of exome sequencing was too low, the reliability of the results was not high, and the detection results needed further verification. Exon sequencing results found no differences between the case and control, and it complemented the results of cataract-related candidate gene sequencing, illustrating that differences probably do not exist between the gene sequences of phenotypically different twins.

In addition to genomic differences, there is also growing evidence that epigenetic events occurring early in embryogenesis participate in the development of phenotypic discordance in MZ twin pairs (Kaminsky et al., 2009; Yamazawa et al., 2008). Epigenetic aberrations have been shown to be intimately associated with many eye diseases, including glaucoma (Wiggs, 2012), age-related macular degeneration (Hunter et al., 2012), and retinoblastoma (McCarthy, 2012). However, little research has been published related to the epigenetic effects on congenital cataract pathogenesis. DNA methylation profiling was performed in human lens epithelial cells for CRYAA, which encodes a structural protein in the lens, to evaluate the role of epigenetic regulation at this locus in the development of age-related cataract. CRYAA transcript and protein levels are both downregulated, and the CpG island in the CRYAA promoter is hypermethylated in human age-related cataract (Zhou et al., 2012).

We performed promoter methylation analysis of six genes, including TRAM1, CRYAA, HSF4, VEGFA, GJA3, and FTL. Hypermethylated sequences could be detected in DNA samples from both the patient and her twin sister after MSRE treatment. Therefore, epigenetic events of these six genes occurring early in embryogenesis could not explain the discordance in the developmental phenotype of the MZ twin pair.

Though we were unable to identify a genetic or epigenetic mutation to explain the discordant phenotype, we ruled out the aberrant function of genomic exome with suspected roles in cataract formation. And we inferred the genomic consistency between the pair of the twins, whereas it could still be possible that a mutation in an unidentified gene might be involved. However, further investigation will be necessary to explore this possibility. Mutation is an important source of genetic variation in the human genome. It can introduce deleterious nucleotide changes to genes or provide fuel for phenotypic evolution. Such stochastic events could also lead to possible disruption of an organ's function, particularly so if the mutation were present in a large proportion of the cells within a tissue. Such mutations could exist if they were introduced early in embryogenesis and their identification could identify important control points in disease etiology, such as has happened for congenital cataract. Rapid advances in molecular genetics have demonstrated the importance of somatic mutation in a great variety of human diseases (Erickson, 2010). For instance, a recent study has revealed the existence of early embryonic somatic mutations causing Dravet syndrome (Vadlamudi et al., 2010), as well as another novel finding of mosaic AKT1 mutation in a Proteus syndrome patient (Lindhurst et al., 2011). In addition, the identification of early somatic mutations would provide preliminary insights into somatic mutation rates, which have been previously estimated in in vitro cell models (Araten & Luzzatto, 2006; Araten et al., 2005; Glaab & Tindall, 1997; Lichtenauer-Kaligis et al., 1996; Umar et al., 1998), or disease-gene data (Hornsby et al., 2008; Iwama et al., 2009). MZ twins provide a natural experiment to address these questions since any differences between MZ co-twins would arise due to somatic changes. Previous studies identified large structural

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variants such as different copy number profiles (Bruder et al., 2008) and chromosomal aneuploidies (Razzaghian et al., 2010). Genetic differences in MZ twins have also been demonstrated in epigenetic markers (Fraga et al., 2005) and DNA changes (Vadlamudi et al., 2010). They provide fuel for phenotypic variation, which might lead to tissue disruption and disease in later life. It has been speculated that, for any gene, a small number of individuals in the population carry a somatic mutation in a large majority of cells that have occurred in early development (Frank, 2010). Further evidence is necessary to support this hypothesis.

Supplementary Material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/thg.2015.34

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