Autosomal recessive congenital cataract in consanguineous Pakistani families is associated with mutations in GALK1

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Purpose: To identify the pathogenic mutations responsible for autosomal recessive congenital cataracts in consanguineous Pakistani families.

Methods: All affected individuals underwent detailed ophthalmologic and medical examination. Blood samples were collected and genomic DNA was extracted. A genome-wide scan was performed with polymorphic microsatellite markers on genomic DNA from affected and unaffected family members and logarithm of odds (LOD) scores were calculated. All coding exons of galactokinase (GALK1) were sequenced to identify pathogenic lesions.

Results: Clinical records and ophthalmological examinations suggested that affected individuals have nuclear cataracts. Linkage analysis localized the critical interval to chromosome 17q with a maximum LOD score of 5.54 at θ=0, with D17S785 in family PKCC030. Sequencing of GALK1, a gene present in the critical interval, identified a single base pair deletion: c.410delG, which results in a frame shift leading to a premature termination of GALK1: p.G137fsX27. Additionally, we identified a missense mutation: c.416T>C, in family PKCC055 that results in substitution of a leucine residue at position 139 with a proline residue: p.L139P, and is predicted to be deleterious to the native GALK1 structure.

Conclusions: Here, we report pathogenic mutations in GALK1 that are responsible for autosomal recessive congenital cataracts in consanguineous Pakistani families.

Congenital cataracts are one of the major causes of vision loss in children worldwide [1,2]. It can occur in an isolated fashion or as one component of a syndrome affecting multiple tissues [3]. Congenital cataracts vary markedly in severity and morphology, affecting the nuclear, cortical, polar, or subcapsular parts of the lens, or in severe cases the entire lens [4]. To-date, fourteen loci have been associated with autosomal recessive cataracts with seven of these also causing autosomal dominant cataracts [5-18]. Of these loci, mutations in nine genes, eph-receptor type-A2 (EPHA2), connexin50 (GJA8), glucosaminyl (N-acetyl) transferase 2 (GCNT2), heat-shock transcription factor 4 (HSF4), lens intrinsic membrane protein (LIM2), beaded filament structural protein 1 (BFSP1), alphaA-crystallin (CRYαA), betaB1-crystallin (CRYβB1), and betaB3-crystallin (CRYβB3) have been found [5,7,9,12,14-18].

Galactokinase (GALK1) is involved in the first step of metabolism of galactose, the conversion of galactose to galactose-1-phosphate at the expense of ATP. In the absence of GALK1 the accumulating galactose is converted to galactitol by aldose reductase. Human GALK1 has been mapped to chromosome 17q25.1; it contains 8 exons and encodes for a 392 amino acid protein harboring two ATP binding sites [19]. Stambolian and colleagues first identified mutations in GALK1 in families with cataracts [19]. Here, we report two consanguineous Pakistani families with congenital cataracts. The critical interval was localized to chromosome 17q with significant two-point logarithm of odds (LOD) scores for both families and haplotype analyses supported the linkage results. Sequencing of GALK1 identified a single base pair deletion that results in premature termination and a missense mutation that leads to a non-conservative substitution in these two families, respectively. These variations were not present in 96 ethnically matched control samples.

METHODS

Clinical ascertainment: A total of 100 consanguineous Pakistani families with nonsyndromic cataracts were recruited to participate in a collaborative study between the National...
Centre of Excellence in Molecular Biology, Lahore, Pakistan, and the National Eye Institute, Bethesda, MD, to identify novel loci associated with congenital cataracts. Institutional Review Board (IRB) approval was obtained from the National Centre of Excellence in Molecular Biology and the National Eye Institute. The participating subjects gave informed consent consistent with the tenets of the Declaration of Helsinki. A detailed medical history was obtained by interviewing family members. Ophthalmic examinations were conducted with slit-lamp microscopy. Approximately 10 ml of blood samples were drawn from affected and unaffected members of the family and stored in 50 ml Sterilin® falcon tubes (BD Biosciences, San Jose, CA) containing 400 µl of 0.5 M EDTA. Blood samples were kept at −20 °C for long-term storage.

DNA extraction: DNA was extracted by a nonorganic method as described previously [5,10]. Briefly, aliquots of 10 ml blood samples were mixed with 35 ml of TE buffer (10 mM Tris-HCl, 2 mM EDTA, pH 8.0) and the TE-blood mixture was centrifuged at 3,000 rpm (1,800× g) for 20 min. The supernatant was discarded and the pellet was re-suspended in 6.25 ml of protein digestion cocktail (50 µl [10 mg/ml] of proteinase K, 6 ml TNE buffer [10 mM Tris HCl, 2 mM EDTA, 400 mM

| Exon | Forward | Reverse | Product size (bp) | Annealing temperature (°C) |
|------|---------|---------|------------------|-----------------------------|
| 1    | GAACCGGCTAGGCTCCTGAGG | CTTCTCCCTTCCAACGTGGG | 496 | 55 |
| 2    | AGCTGGCCTCTCAAGGATCTTC | GGGGACTCTTTTCCATCTTG | 488 | 55 |
| 3    | GTTTGGGTTCCTGTCAAAATTT | GTGGTCTTCAATGCACTCCAG | 384 | 57 |
| 4    | CAGTGTCCTCCAGCTTCCTAC | ACCTGGGCTGGAGTTACAAATG | 387 | 55 |
| 5    | CCCTGGCAGTGCAGACTTC | AACGAGCCCTTCTGAGATTG | 449 | 55 |
| 6    | CCACCTCTACCCGCTCCAG | CCATAACCCAGACGCACAGC | 413 | 57 |
| 7    | GAGGCGACACACGGCCTTGT | CGCTGGTCCTGAGAGGTTAG | 422 | 55 |
| 8    | CTGCACGGGTGACACTGTGGT | TTGCAGACCCCCGATAGGAAG | 307 | 55 |
NaCl] and 200 μl of 10% sodium dodecyl sulfate) was added to the re-suspended pellets and incubated overnight in a shaker (250 rpm) at 37 °C. The digested proteins were precipitated by adding 1 ml of 5 M NaCl, followed by vigorous shaking and chilling on ice for 15 min. The precipitated proteins were pelleted by centrifugation at 3,000 rpm (1,800× g) for 20 min and removed. The supernatant was mixed with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and the aqueous layer containing the genomic DNA was carefully collected. The DNA was precipitated with isopropanol and pelleted by centrifugation at 4,000 rpm (2,400× g) for 15 min. The DNA pellets were washed with 70% ethanol and dissolved in TE buffer. The DNA concentration was determined with a SmartSpec plus Bio-Rad Spectrophotometer (Bio-Rad, Hercules, CA).

Genotype analysis: A genome-wide scan was performed with 382 highly polymorphic fluorescent markers from the ABI PRISM Linkage Mapping Set MD-10 (Applied Biosystems, Foster City, CA) having an average spacing of 10 cM. Multiplex polymerase chain reaction (PCR) was completed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). Briefly, each reaction was performed in a 5 μl mixture containing 40 ng genomic DNA, various combinations of 10 mM dye-labeled primer pairs, 0.5 ml 10× GeneAmp PCR Buffer (Applied Biosystems), 1 mM dNTP mix, 2.5 mM MgCl₂, and 0.2 U Taq DNA polymerase (Applied Biosystems). Initial denaturation was performed for 5 min at 96 °C, followed by 10 cycles of 15 s at 94 °C, 15 s at 55 °C, and 30 s at 72 °C and then 20 cycles of 15 s at 89 °C, 15 s at 55 °C, and 30 s at 72 °C. The final extension was performed for 10 min at 72 °C. PCR products from each DNA sample were pooled and mixed with a loading cocktail containing HD-400 size standards (Applied Biosystems). The resulting PCR products were separated in an ABI 3100 DNA Analyzer (Applied Biosystems) and genotypes were assigned with GeneMapper software (Applied Biosystems).

Linkage analysis: Two-point linkage analyses were performed using the FASTLINK version of MLINK from the LINKAGE Program Package (provided in the public domain by the Human Genome Mapping Project Resources Centre, Cambridge, UK) [20,21]. Maximum LOD scores were calculated with ILINK from the LINKAGE Program Package. Autosomal recessive cataract was analyzed as a fully penetrant trait with an affected allele frequency of 0.001. The marker order and distances between the markers were obtained from the Marshfield database and the National Center for Biotechnology Information (NCBI) chromosome 17 sequence maps. For the initial genome scan, equal allele frequencies were assumed, while for fine mapping allele frequencies were estimated from 96 unrelated and unaffected individuals from the Punjab province of Pakistan.

Mutation screening: Primer pairs for individual exons were designed using the primer3 program. The sequences and annealing temperatures are given in Table 1. Amplifications were performed in a 25 μl reaction containing 50 ng of genomic DNA, 400 nM of each primer, 250 μM of dNTPs, 2.5 mM MgCl₂ and 0.2 U Taq DNA polymerase in the standard PCR buffer provided by the manufacturer (Applied Biosystems). PCR amplification consisted of a denaturation step at 96 °C for 5 min followed by 40 cycles, each consisting

Figure 2. Slit lamp photographs of the affected individual 14 of family PKCC030 show nuclear cataracts that developed in early infancy.
of 96 °C for 30 s followed by 57 °C (or primer set-specific
annealing temperature; see Table 1) for 30 s and 72 °C for 1
min. PCR products were analyzed on a 2% agarose gel and
purified by ethanol precipitation. The PCR primers for each
exon were used for bidirectional sequencing using BigDye
Terminator Ready reaction mix (Applied Biosystems),
according to the manufacturer’s instructions. Sequencing
products were precipitated and resuspended in 10 μl
formamide (Applied Biosystems) and denatured at 95 °C for
5 min. Sequencing was performed in an ABI PRISM 3100
Automated Sequencer (Applied Biosystems) and sequencing
results were assembled with ABI PRISM sequencing analysis
software version 3.7 and analyzed with SeqScape software
(Applied Biosystems).

**Prediction analysis:** Evolutionary conservation of the
mutated amino acids in other GALK1 orthologs was
examined using the UCSC genome browser. The degree of
evolutionary conservation of positions at which missense
mutations exist and the possible impact of an amino acid
substitution on the structure of GALK1 was examined with
SIFT and PolyPhen tools available online.

**RESULTS**

A large consanguineous family, PKCC030 (Figure 1)
consisting of six affected and nine unaffected individuals was
recruited from the Punjab province of Pakistan. A detailed
medical history was obtained from all family members. Medical records of previously conducted ophthalmic
examinations with slit lamp biomicroscopy were suggestive
of nuclear cataract in affected individuals of PKCC030
(Figure 2). According to the medical records available to us
all the affected individuals developed cataract in the first year
of their life except individual 14 who developed cataract in
the 2nd year of her life. The medical examination concluded
that affected individuals did not present any extra-ocular
anomalies, although according to family elders, higher intake
of dairy products usually results in vomiting and diarrhea in
all affecteds.

Initially, linkage to known autosomal recessive cataract
loci was excluded by haplotype analysis using closely
flanking markers (data not shown). Next, we completed a
genome-wide scan with 382 fluorescently-labeled short
tandem repeat (STR) markers. A maximum two-point LOD
score of 5.54 at θ=0 was obtained with D17S785 during the
genome-wide scan (Table 2). Additional STR markers from
the Marshfield database were designed to analyze the critical
interval, which further provided evidence of linkage to
chromosome 17q with LOD scores of 5.49 and 2.76 at θ=0
with markers D17S1839 and D17S801, respectively (Table
2).

Visual inspection of the haplotypes supported the results
of linkage analysis (Figure 1). This places the critical interval

### Table 2. Two-point LOD scores of chromosome 17q markers.

| Marker     | cM   | Mb   | 0   | 0.01 | 0.05 | 0.1  | 0.2  | 0.3  | 0.4  | Zmax | θmax |
|------------|------|------|-----|------|------|------|------|------|------|------|------|
| D17S944*   | 82.56| 61.43| -∞  | −4.07| −1.55| −0.68| −0.14| −0.03| −0.02| 0.40 | 0.85 |
| D17S949*   | 93.27| 68.46| −2.50| −0.38| 0.19 | 0.32 | 0.29 | 0.15 | 0.04 | 0.33 | 0.13 |
| D17S1839   | 102.46| 73.80| 5.49 | 5.37 | 5.16 | 4.65 | 3.57 | 2.37 | 1.13 | 5.49 | 0.00 |
| D17S785*   | 103.53| 74.43| 5.54 | 5.43 | 5.21 | 4.70 | 3.62 | 2.43 | 1.19 | 5.62 | 0.00 |
| D17S801    | 103.53| 74.50| 2.76 | 2.69 | 2.47 | 2.19 | 1.58 | 0.95 | 0.38 | 2.76 | 0.00 |
| D17S784    | 116.86| 77.80| -∞  | −0.88| 0.24 | 0.49 | 0.45 | 0.25 | 0.08 | 0.52 | 0.13 |

The asterisk indicates that the STR markers were included in the genome-wide scan.

Figure 3. Forward and reverse sequence chromatograms illustrating a single base pair deletion in GALK1. A: Individual 11 homozygous for wild type allele. B: Individual 13 heterozygous for mutant and wild type allele. C: Individual 14 homozygous for the single base pair deletion in exon 3; c.410delG. This deletion leads to a frame shift in the open reading frame of GALK1, which results in a premature termination of the protein: p.G137fsX27.
Figure 4. Pedigree drawing of family PKCC055 and alignment of L139 in GALK1 orthologs. A: Illustration of the chromosome 17q haplotypes and segregation of c.416T>C variation with the disease phenotype. B: Conservation of L139 in other GALK1 orthologs is illustrated with primates colored green; placental mammals blue; and vertebrates are purple. The arrow points to amino acid L139.
in a 34.3 cM (16.37 Mb) interval, flanked by markers D17S944, proximally and marker D17S784, distally. Lack of homozygosity in alleles of affected individuals 8, 12, 14, 17, 18, and 19 at D17S949, further suggests that the pathogenic mutations resides in a 23.59 cM (9.34 Mb) interval flanked by D17S949 proximally and D17S784 distally.

The critical interval harbors GALK1, a gene previously associated with cataractogenesis [19]. We sequenced all coding exons, exon-intron boundaries and the 100 bases of the 5’- and 3’-regions of coding exons, exon-intron boundaries and the 100 bases of the orthologs as shown in Figure 4B. SIFT predictions were matched control samples. This is the first report associating GALK1 with autosomal recessive congenital cataracts in families of Pakistani origin.

Glucose is a highly consumed monosaccharide, which is converted to glucose-6-phosphate in a four step process known as the glycolytic pathway. Any disruption of these processes can potentially result in galactosemia. GALK1 is involved in the first step of metabolism of galactose, the conversion of galactose to galactose-1-phosphate at the expense of ATP. In the absence of GALK1, the accumulating galactose is converted to galactitol by aldose reductase. Accumulation and subsequent osmotic swelling of galactitol results in cataracts [22], probably due to osmotic swelling. All affected individuals reported in this study developed cataracts in their infancy whereas the unaffected heterozygous carriers of the pathogenic mutations in GALK1 did not present any signs or symptoms of caractogenesis, even in their forties.

Identification of the pathogenic mutations in GALK1 and the phenotype of cataracts associated with these mutations will increase our understanding of lens biology at a molecular level, which will lead to better treatments and therapeutics.

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DISCUSSION
Here, we report pathogenic mutations in GALK1 that are responsible for autosomal recessive congenital cataracts in two Pakistani families. Both families mapped to chromosome 17q with significant LOD scores and these results were supported by visual inspection of the haplotype analyses, localizing the critical interval to a region harboring GALK1. Sequencing of GALK1 indentified a homozygous single base pair deletion in the first family, which results in premature termination of GALK1 and a missense mutation in a second family, which leads to a non-conservative substitution. These variations were not present in 96 ethnically matched control samples.
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