EXTENDED REPORT

Investigation of crystallin genes in familial cataract, and report of two disease associated mutations

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Aims: Mutations of seven crystallin genes have been shown to cause familial cataract. The authors aimed to identify disease causing crystallin mutations in paediatric cataract families from south eastern Australia.

Methods: 38 families with autosomal dominant or recessive paediatric cataract were examined. Three large families were studied by linkage analysis. Candidate genes at regions providing significant LOD scores were sequenced. Single stranded conformational polymorphism (SSCP) analysis was used to screen five crystallin genes in the probands, followed by direct sequencing of observed electrophoretic shifts. Mutations predicted to affect the coding sequence were subsequently investigated in the entire pedigree.

Results: A LOD score of 3.72 was obtained at the γ-crystallin locus in one pedigree. Sequencing revealed a P23T mutation of CRYGD, found to segregate with disease. A splice site mutation at the first base of intron 3 of the CRYBA1/A3 gene segregating with disease was identified by SSCP in another large family. Five polymorphisms were also detected.

Conclusions: Although mutations in the five crystallin genes comprehensively screened in this study account for 38% of paediatric cataract mutations in the literature, only two causative mutations were detected in 38 pedigrees, suggesting that crystallin mutations are a relatively rare cause of the cataract phenotype in this population.

Congenital or paediatric cataract is a clinically and genetically heterogeneous disorder. The phenotype varies considerably between and within families.1 Familial paediatric cataract is most commonly inherited in an autosomal dominant manner, although recessive and X linked forms also exist.2 Mutations in 14 genes have been described in association with the phenotype of isolated paediatric/congenital cataract. These include members of the crystallin and connexin families,3 membrane proteins MIP4 and LIM2,5, a cytoskeletal protein BFSP2,6 and transcription factors PDX37 and HSF4.8

Mutations in seven crystallin genes have been shown to cause monogenic paediatric cataract. These include αA-crystallin (CRYAA),9 βA1/A3-crystallin (CRYBA1/A3),10 βB2-crystallin (CRYBB2),11 γ-crystallin (CRYGC),12 and γD-crystallin (CRYGD).13 βB-crystallin (CRYBB1) mutations14–16 and βB1-crystallin (CRYBB1) mutations17–19 were reported in familial cataract after the commencement of this study.

While it is apparent that familial cataract is genetically heterogeneous, only 17 families with crystalline mutations have been described. It is unknown whether mutations of these genes are a significant cause of familial cataract. In order to detect causative mutations in our population of 38 familial paediatric cataract pedigrees from south eastern Australia, large pedigrees were assessed by linkage analysis, while the first five reported crystallin genes were screened in smaller pedigrees by single stranded conformational polymorphism (SSCP) analysis.

MATERIALS AND METHODS

Paediatric cataract families and genetic material

Approval was obtained from the human research ethics committees of the Royal Children’s Hospital, Melbourne, the Royal Victorian Eye and Ear Hospital, Melbourne, and the University of Tasmania, Hobart, and informed consent was obtained from all participants or their guardians. Ascertainment and recruitment of pedigrees has been previously described.22 All available family members were examined by one or more ophthalmologists (MGW, DAM, JEE, IMR-E, or JEC). Buccal mucosa swabs were collected and DNA extracted using the PureGene DNA Isolation Kit (Gentra Systems).

Premier extension preamplification

Premier extension preamplification (PEP)23 was used to provide sufficient DNA from buccal mucosa swabs for subsequent polymerase chain reaction (PCR) based amplification. Each 50 μl reaction contained 50–100 ng template DNA, 1000 pmol random PolyN 15-mer primer (Operon Technologies), 200 μM dNTPs (Promega), 2 mM Mg2+, and 5U Taq Polymerase (Qiagen). The reactions were amplified over 50 cycles of 92°C for 1 minute, 37°C for 2 minutes, and 55°C for 4 minutes with a final extension of 72°C for 10 minutes, and then diluted to 25–50 ng/μl for PCR.

Linkage analysis

Individuals from three large families were genotyped using fluorescently labelled primers to amplify microsatellite markers (table 1) from each of the five crystallin loci and analysed using an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Primer sequences and PCR conditions are available from the Genome Database (www.gdb.org).

Allele frequencies were calculated from founders in the pedigrees. Two point linkage analysis was carried out with MLINK, part of the FASTLINK package.24 Disease gene frequency was set to 0.0001. Penetration was set at 0.0 in wild type homozygotes and 0.95 in heterozygotes and mutant homozygotes. LOD scores were calculated over a range of recombination distances between 0 and 0.5 for each marker.

Single stranded conformational polymorphism (SSCP) analysis

The proband from each pedigree, including those investigated by linkage, was screened for mutations in CRYAA, CRYBA1/A3, CRYBB2, CRYGC, and CRYGD by SSCP. CRYAB and CRYBB1 had not been associated with cataract at the time of this
study and were not screened in this way. Both forward and reverse PCR primers (sequences available on request) were end labelled with γ32P-ATP by T4 polynucleotide kinase (New England Biolabs). Each exon was amplified by PCR in 10 μl reaction volumes containing 0.7 μM of each unlabelled primer and 0.11 μM of each labelled primer, 200 μM dNTPs (Promega), 0.5 U Taq polymerase (Qiagen), and 50 ng of DNA. PCR products were mixed with SSCP Stop Solution and denatured at 95 °C for 2 minutes, and snap cooled on ice. PCR products were mixed with SSCP Stop Solution and denatured at 95 °C for 2 minutes, and snap cooled on ice. DNA. PCR products were mixed with SSCP Stop Solution and denatured at 95 °C for 2 minutes, and snap cooled on ice.

DNA sequence analysis
PCR products were cycle sequenced using Big Dye Terminator Ready Reaction Mix (Applied Biosystems) and an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Denaturing high performance liquid chromatography (dHPLC)
Exon 2 of CRYGD was amplified by PCR and injected onto a Varian Helix dHPLC column at 64 °C. The acetonitrile concentration was varied from 45% to 68% over 7 minutes. Chromatograms were compared to those generated by samples of known sequence.

Restriction digest
Exon 3 of CRYBA1/A3 was amplified by PCR in pedigree crah08; 10 μl of PCR product were digested with 1 U of NlaIII (New England Biolabs) at 37°C for 1 hour. Products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

RESULTS
Thirty eight pedigrees were investigated including 32 with autosomal dominant inheritance, two autosomal recessive pedigrees, and four small families of uncertain inheritance.

Linkage analysis of large pedigrees
Four large pedigrees were selected for linkage analysis on the basis of simulations using SLINK24 25 (data not shown). A crystallin mutation was detected in pedigree crah08 before the linkage study, hence this family was not included in linkage analysis.

<table>
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<tr>
<th>Pedigree</th>
<th>Gene</th>
<th>Marker</th>
<th>0.0</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
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<th>α</th>
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*Two crystallin genes are represented by one microsatellite marker at these loci.
†CRYAB and CRYBB1 were not screened by SSCP as they were not reported in the literature at the time of the investigation.

SSCP analysis
Analysis of the proband from each pedigree revealed one causative mutation and four non-segregating polymorphisms (table 2). All mutations identified through SSCP of PEP DNA were subsequently confirmed by direct sequencing of genomic DNA, indicating that the use of PEP DNA did not introduce spurious SSCP positive PCR artefacts.

The G→A transition detected at the first base of the 5' donor splice site of intron 3 in the CRYBA1/A3 gene segregated with disease in pedigree crah08, as demonstrated by RFLP analysis (fig 2A). The clinical features of the phakic patients in this Australian family of European origin showed a consistent spectrum of: (1) Y-sutural opacities, (2) mild opacification throughout the region of the fetal nucleus, and (3) peripheral cortical dot opacities (fig 2B). The severity of the disease ranged from requiring surgery in childhood to unawareness of affected status before this study. Individual III:3 of pedigree crah08 is heterozygous for the mutation. Mild opacities consistent with his age (65 years) characterised as nuclear sclerosis with cortical opacities were noted following genotyping revealing his carrier status and could represent a mild form of the inherited phenotype. As this mutation and a second mutation at the same base have been previously shown to segregate with congenital cataract and not found in 150 control chromosomes of various ethnicity12 13 population controls from south eastern Australia were not screened. The mutation was not detected in any of the Australian probands.
DISCUSSION

Mutations of crystallin genes represent 45% of the reported familial cataract mutations to date. We have screened all genes by linkage, and the first five crystallin genes reported to cause cataract by SSCP, and detected two mutations that segregate with the cataract phenotype—CRYGD P23T and a CRYBA1/A3 splice site mutation—as well as five other non-disease causing polymorphisms.

This collection of pedigrees were ascertained through a comprehensive evaluation of almost all familial cataract in south eastern Australia, a source population of around five million, and participation rates were high. Only those families with inherited cataract where none of the family members required specialist medical care and sporadic cases later shown to be familial would have been overlooked.

Both the mutations detected in this study have been previously reported in other large congenital cataract pedigrees. The P23T mutation of CRYGD has been associated with differing phenotypes including lamellar cataract in an Indian pedigree and Cerulean cataract in a Moroccan family. Three other mutations of this gene have also been associated with cataract. As the P23T mutation has been found to segregate with congenital cataract in three independent studies and has not been detected in 400 control chromosomes, it is unlikely to represent a rare polymorphism. It is unclear how the P23T mutation affects the function of the CRYGD protein. The mutation is located in the second β sheet of the first Greek key motif of the protein, a region crucial to the correct folding of the protein. This residue is a proline or serine in all species represented in Genbank, but never a threonine. The backbone of the native sheet of the first Greek key motif of the protein

Table 2

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<tr>
<th>Gene</th>
<th>Exon/intron</th>
<th>Position</th>
<th>Base change</th>
<th>Expected effect on protein</th>
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*Intron position numbers refer to the number of nucleotides past the 3' end of the exon.
†This variant is likely to be in the presumed pseudogene CRYBB2-2, rather than CRYBB2.
ACKNOWLEDGEMENTS

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REFERENCES