The \textit{GJA8} allele encoding CX50I247M is a rare polymorphism, not a cataract-causing mutation

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\textbf{Purpose:} The aim of this study was the genetic, cellular, and physiological characterization of a connexin50 (CX50) variant identified in a child with congenital cataracts.

\textbf{Methods:} Lens material from surgery was collected and used for cDNA production. Genomic DNA was prepared from blood obtained from the proband and her parents. PCR amplified DNA fragments were sequenced and characterized by restriction digestion. Connexin protein distribution was studied by immunofluorescence in transiently transfected HeLa cells. Formation of functional channels was assessed by two-microelectrode voltage-clamp in cRNA-injected \textit{Xenopus} oocytes.

\textbf{Results:} Ophthalmologic examination showed that the proband suffered from bilateral white, diffuse cataracts, but the parents were free of lens opacities. Direct sequencing of the PCR product produced from lens cDNA showed that the proband was heterozygous for a G>T transition at position 741 of the \textit{GJA8} gene, encoding the exchange of methionine for isoleucine at position 247 of CX50 (CX50I247M). The mutation was confirmed in the genomic DNA, but it was also present in the unaffected mother. When expressed in HeLa cells, both wild type CX50 and CX50I247M formed gap junction plaques. Both CX50 and CX50I247M induced gap junctional currents in pairs of \textit{Xenopus} oocytes.

\textbf{Conclusions:} Although the CX50I247M substitution has previously been suggested to cause cataracts, our genetic, cellular, and electrophysiological data suggest that this allele more likely represents a rare silent, polymorphic variant.

Congenital cataracts are responsible for approximately 10\% of childhood blindness worldwide. They are clinically and genetically heterogeneous. More than 30 loci have been linked to the cataract phenotype, and at least 17 cataract-associated genes have been characterized including those encoding crystallins, transcription factors, cytoskeletal proteins, and membrane proteins (reviewed in [1,2]). Among these, mutations in the \textit{GJA3} and \textit{GJA8} genes (that encode the lens gap junction proteins, CX46 and CX50) have been shown to underlie congenital cataracts, which most often exhibit dominant inheritance [3]. The CX46 and CX50 mutants that have been characterized do not support intercellular communication when expressed by themselves (and some of these mutants act as dominant negative inhibitors of wild type connexin function) supporting the hypothesis that decreased gap junctional intercellular communication contributes to cataract formation.

A nucleotide transition in \textit{GJA8} (T741G) that co-segregated with the cataract phenotype was previously identified in a Russian family [4]. The cellular and functional properties of this mutation have not been characterized. In the current study, we have examined the cellular and physiological consequences of the encoded amino acid substitution (CX50I247M) and report the identification of the same substitution in members of another family in which the transition does not co-segregate with the cataract phenotype.

\textbf{METHODS}

The study adopted the tenets of the Declaration of Helsinki. Family members gave informed consent after explanation of the study design and goals and their roles. The study was approved by the Institutional Ethical Committee of the University of Gießen, Germany.

Clinical details regarding the health histories of family members were recorded at the Center of Ophthalmology at the University of Gießen (Germany). A senior pediatric ophthalmologist (W.S.) performed surgery on the proband. Lens material from cataract surgery was frozen immediately on dry ice and kept at -80 °C for a few days only [5]. RNA samples from lenses were reverse transcribed to cDNA using the Ready-to-Go kit (GE Health Care, Freiburg, Germany). Using this cDNA as template, PCR was performed to amplify crystallin, alpha A (\textit{CRYAA}); crystallin, alpha B (\textit{CRYAB});
crystallin, beta A4 (CRYBA4); crystallin, beta B1 (CRYBB1); crystallin, beta B2 (CRYBB2); crystallins, gamma A-D (CRYGA-D); crystallin, gamma C (CRYGC); GJA8; ferritin, light polypeptide (FTL); lens intrinsic membrane protein 2, (LIM2); and major intrinsic protein of lens fiber (MIP/AQP0). The selection of these genes was based upon the frequency of mutations in these genes leading to congenital cataracts.

The parents were examined for the presence of lens opacities with a slit-lamp (Zeiss, Oberkochen, Germany). Blood samples (5-10 ml) were collected from the proband and her parents, and they were used to isolate genomic DNA [6]. The conditions for PCR reactions performed using genomic DNA as template have been described previously [7-9]. Primers were obtained from Utz Linzner (Helmholtz Center Munich, Institutes of Experimental Genetics and of Pathology, Munich, Germany) or from commercial sources (Invitrogen, Karlsruhe, Germany, MWG, Vaterstetten, Germany or Sigma Genosys, Steinheim, Germany). Sequencing was performed commercially (Sequiserve, Vaterstetten, Germany or GATC Biotech, Konstanz, Germany). The presence of the mutant allele in the PCR fragments was confirmed by LweI digestion.

The Cooperative Health Research in the Augsburg Region (KORA) Survey 2000 (S3) which studied a population based sample of 4,261 subjects aged 25–74 years during the years 1999–2001 [10] was used as a population-based control. 179 randomly chosen individuals without cataracts from this cohort were analyzed for the putative GJA8 mutation.

The proband, LB, suffered from bilateral, diffuse white lens opacities. She underwent cataract surgery shortly after birth. Both parents were healthy; slit lamp examination showed no evidence of lens opacities (Figure 1). Using a functional candidate approach, we checked several genes including GJA8 for sequence alterations. We identified a T→G exchange in GJA8 cDNA at position 741 (Figure 2A). This substitution changes the amino acid codon at position 247 from isoleucine to methionine (CX50I247M). It also creates a new SfaN/LweI restriction site in the mutated sequence (Figure 2B). Using LweI digestion of the PCR fragments obtained from genomic DNA, we observed the same transition in the unaffected mother (Figure 2C, arrows). Sequencing of genomic DNA from both parents confirmed that the mother was heterozygous like the daughter, and the father was wild type. None of the other genes analyzed (CRYAA, CRYAB, CRYBA4, CRYBB1, CRYBB2, CRYGA-D, CRYGS, FTL, LIM2, and AQP0) showed alterations.

We also used LweI digestion of genomic DNA to test for the presence of the CX50I247M allele in 179 controls obtained from a population-based study (KORA). Since no additional LweI restriction site was observed in these samples, the frequency of the CX50I247M allele must be less than 0.3%.

The capacity of CX50I247M to form gap junctions was assessed by immunofluorescence microscopy of HeLa cells transfected with wild type CX50 or CX50I247M. Similar to wild type CX50, CX50I247M localized at appositional...
membranes, where it formed gap junction plaques, and in the perinuclear region, probably the Golgi compartment (Figure 3A,B).

The ability of CX50I247M to form functional gap junctional channels was characterized by two-electrode voltage-clamp in Xenopus oocyte pairs. Pairs of oocytes injected with CX50I247M cRNA developed gap junctional conductances with mean values that were not significantly different from those determined in oocyte pairs injected with wild type CX50 cRNA (Figure 4). Pairs of control oocytes injected with no connexin cRNA showed no coupling.

**DISCUSSION**

In this study, we demonstrated a heterozygous mutation in GJA8 of a child with severe congenital cataracts (LB). The mutated sequence encodes CX50I247M, a CX50 variant in which the isoleucine at position 247 (within the cytoplasmic COOH-terminus) is replaced by methione. CX50I247M formed gap junction plaques and supported intercellular communication similarly to wild type CX50.

Very few of the identified cataract-associated connexin mutations lie in the COOH-terminus. Indeed, removal of the COOH-terminus (139-150 amino acids) of CX50 causes only modest effects on voltage-dependent gap junction channel gating [18,19]. Similar to the effects caused by truncation of ovine Cx50 [20], removal of the COOH-terminus of human CX50 results in a decrease in sensitivity to intracellular pH (pH$_i$) [18]. Truncation of mouse CX50 also appeared to cause a decrease in sensitivity to pH$_i$ as evidenced by the delay in the decrease in junctional conductance induced by 100% CO$_2$ perfusion and the slower recovery of gap junctional conductance following washout [19]. Truncated human and mouse CX50 both show decreased junctional conductance [18,19]. Thus, this region may be important for regulation of CX50 channel function, but it is dispensable for channel activity per se. Two of the mutations in lens connexin genes linked to hereditary cataracts that affect the COOH-terminus cause frame shifts [5,21]. In the Cx46 mutant, CX46fs380 (that contains a frame shift at codon 380), the new protein sequence caused by the frame shift contains
a retention/retrieval signal that leads to loss of function [22]
and localization of the mutant connexin in the cytoplasm [13].

The CX50 variant, CX50I247M, was previously reported
in three members of a three generation Russian family, and it
cosegregated with a zonular pulverulent cataract trait [4].
However, this mutation did not co-segregate with the disease
in our study; it was also present in the healthy mother of our
proband. (Indeed, the genetic alteration responsible for the
cataract in our patient has not been identified.)

The segregation of the CX50I247M allele with cataract
in the Russian family remains puzzling [4], because it seems
to be a rare allele with a frequency of less than 0.3%. A
plausible explanation for the contradicting findings between
the previous study and ours is the possibility of a close linkage
to another gene, which is really causative for these cataracts.
If this hypothesis were true, there should be another cataract-
related gene close to the CX50-encoding gene GJA8.
Referring to the database of Mendelian hereditary disorders
(OMIM), a few further cataract loci are reported on human
chromosome 1; however, two of them have already been
attributed to GJA8. Another one is the gene encoding
glycerophosphate O-acyltransferase (GNPAT) for which a
syndromic cataract would be expected rather than an isolated
one as we have reported. Therefore, one might speculate that
there is another yet unidentified cataract-associated gene in
this region. Examination of the ENSEMBL database for this
region reveals a considerable number of genes that have not
yet been annotated including some genes for non-coding
RNAs.

Our cellular and functional studies support the conclusion
that CX50I247M is an inconsequential variant. Taken
together, our results strongly suggest that CX50I247M
represents a rare polymorphic site rather than a causative
mutation.

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REFERENCES

1. Graw J. The genetic and molecular basis of congenital eye defects. Nat Rev Genet 2003; 4:876-88. [PMID: 14634635]
2. Hejtmancik JF. Congenital cataracts and their molecular genetics. Semin Cell Dev Biol 2008; 19:134-49. [PMID: 18035564]
3. Berthoud VM, Beyer EC. Oxidative stress, lens gap junctions, and cataracts. Antioxid Redox Signal 2009; 11:339-53. [PMID: 18831679]
4. Polyakov AV, Shagina IA, Khlebnikova OV, Evgrafov OV. Mutation in the connexin 50 gene (GJA8) in a Russian family with zonular pulverulent cataract. Clin Genet 2001; 60:476-8. [PMID: 11846744]
5. Schmidt W, Klopp N, Illig T, Graw J. A novel GJA8 mutation causing a recessive triangular cataract. Mol Vis 2008; 14:851-6. [PMID: 18483562]
6. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988; 16:1215. [PMID: 3344216]
7. Graw J, Klopp N, Illig T, Preising MN, Lorenz B. Congenital cataract and macular hypoplasia in humans associated with a de novo mutation in CRYAA and compound heterozygous mutations in P. Graefes Arch Clin Exp Ophthalmol 2006; 244:912-9. [PMID: 16453125]
8. Santhiya ST, Maniasstry SM, Rawlley D, Malathi R, Anishetty S, Gopinath PM, Vijayalakshmi P, Namperumalamsamy P, Adamski J, Graw J. Mutation analysis of congenital cataracts in Indian families: identification of SNPS and a new causative allele in CRYBB2 gene. Invest Ophthalmol Vis Sci 2004; 45:5959-607. [PMID: 15452067]
9. Santhiya ST, Shyam MM, Rawlley D, Vijayalakshmi P, Namperumalamsamy P, Gopinath PM, Loster J, Graw J. Novel mutations in the? -crystallin genes cause autosomal dominant congenital cataracts. J Med Genet 2002; 39:352-8. [PMID: 12011157]
10. Illig T, Bongardt F, Schopfer A, Holle R, Muller S, Rathmann W, Koenig W, Meisinger C, Wichmann HE, Kolb H. The endotoxin receptor TLR4 polymorphism is not associated with diabetes or components of the metabolic syndrome. Diabetes 2003; 52:2861-4. [PMID: 14578307]
11. Ebihara L, Berthoud VM, Beyer EC. Distinct behavior of connexin56 and connexin46 gap junctional channels can be predicted from the behavior of their hemi-gap-junctional channels. Biophys J 1995; 68:1796-803. [PMID: 7612821]
12. Arora A, Minogue PJ, Liu X, Reddy MA, Ainsworth JR, Bhattacharya SS, Webster AR, Hunt DM, Ebibara L, Moore AT, Beyer EC, Berthoud VM. A novel GJA8 mutation is associated with autosomal dominant lamellar pulverulent cataract: further evidence for gap junction dysfunction in human cataract. J Med Genet 2006; 43:e2. [PMID: 16397066]
13. Minogue PJ, Liu X, Ebibara L, Beyer EC, Berthoud VM. An aberrant sequence in a connexin46 mutant underlies congenital cataracts. J Biol Chem 2005; 280:40788-95. [PMID: 16204255]
14. Thomas BC, Minogue PJ, Valiumus V, Kanaporis G, Brink PR, Berthoud VM, Beyer EC. Cataracts are caused by alterations of a critical N-terminal positive charge in connexin50. Invest Ophthalmol Vis Sci 2008; 49:2549-56. [PMID: 18326694]
15. Berthoud VM, Minogue PJ, Guo J, Williamson EK, Xu X, Ebibara L, Beyer EC. Loss of function and impaired degradation of a cataract-associated mutant connexin50. Eur J Cell Biol 2003; 82:209-21. [PMID: 12800976]
16. Ebibara L. Expression of gap junctional proteins in Xenopus oocyte pairs. Methods Enzymol 1992; 207:376-80. [PMID: 1382193]
17. Tong JJ, Liu X, Dong L, Ebibara L. Exchange of gating properties between rat Cx46 and chicken Cx45.6. Biophys J 2004; 87:2397-406. [PMID: 15454438]
18. Xu X, Berthoud VM, Beyer EC, Ebibara L. Functional role of the carboxyl terminal domain of human connexin 50 in gap junctional channels. J Membr Biol 2002; 186:101-12. [PMID: 11944087]
19. DeRosa AM, Mui R, Srinivas M, White TW. Functional characterization of a naturally occurring Cx50 truncation. Invest Ophthalmol Vis Sci 2006; 47:4474-81. [PMID: 17003442]
20. Lin JS, Eckert R, Kistler J, Donaldson P. Spatial differences in gap junction gating in the lens are a consequence of connexin cleavage. Eur J Cell Biol 1998; 76:246-50. [PMID: 9765054]
21. Mackay D, Ionides A, Kibar Z, Rouleau G, Berry V, Moore A, Shiel A, Bhattacharya S. Connexin46 mutations in autosomal dominant congenital cataract. Am J Hum Genet 1999; 64:1357-64. [PMID: 10205266]
22. Pal JD, Liu X, Mackay D, Shiel A, Berthoud VM, Beyer EC, Ebibara L. Connexin46 mutations linked to congenital cataract show loss of gap junction channel function. Am J Physiol Cell Physiol 2000; 279:C596-602. [PMID: 10942709]