Molecular characterisation of congenital glaucoma in a consanguineous Canadian community: a step towards preventing glaucoma related blindness

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Abstract
Glaucoma is a leading cause of irreversible blindness in Canada. Congenital glaucoma usually manifests during the first years of life and is characterised by severe visual loss and autosomal recessive inheritance. Two disease loci, on chromosomes 1p36 and 2p21, have been associated with various forms of congenital glaucoma. A branch of a large six generation family from a consanguineous Amish community in south western Ontario was affected with congenital glaucoma and was studied by linkage and mutational analysis to identify the glaucoma related genetic defects. Linkage analysis using the MLINK component of the LINKAGE package (v 5.1) showed evidence of linkage to the 2p21 region (Zmax=3.34, \( \theta = 0 \)), D2S1348 and D2S1346. Mutational analysis of the primary candidate gene, \( \text{CYP1B1} \), was done by direct cycle sequencing, dideoxy fingerprinting analysis, and fragment analysis. Two different disease causing mutations in exon 3, 1410del13 and 1505G \( \rightarrow \) A, both segregated with the disease phenotype. The two different combinations of these alleles appeared to result in a variable expressivity of the phenotype. The compound heterozygote appeared to have a milder phenotype when compared to the homozygotes for the 13 bp deletion. The congenital glaucoma phenotype for this large inbred Amish family is the result of mutations in \( \text{CYP1B1} (2p21) \). The molecular information derived from this study will be used to help identify carriers of the \( \text{CYP1B1} \) mutation in this community and optimise the management of those at risk of developing glaucoma.


Keywords: congenital glaucoma; \( \text{CYP1B1} \); gene; genetic counselling

Glaucoma constitutes a leading cause of irreversible blindness in Canada. Congenital and infantile glaucoma are associated with anomalous development of the trabecular meshwork and anterior chamber angle, which leads to an increased resistance to aqueous outflow and raised intraocular pressure (IOP). Clinical characteristics of congenital glaucoma most often manifest during the first years of life and include tearing, blepharospasm, and photophobia. Corneal oedema, Haab striae (breaks in Descemet membrane), enlarged cornea, optic nerve cupping, and buphthalmos may also develop if the pressure is not controlled. When the diagnosis of congenital glaucoma is delayed, the resulting visual outcome is often poor. Even though treatments are available, earlier detection is needed in order to prevent or minimise the glaucoma related visual loss.

The primary cause of the disease is unknown. However, congenital glaucoma is heritable and the majority of cases are autosomal recessive. Cases of incomplete penetrance are well documented. The incidence of congenital glaucoma varies depending on geographical location: 1:2500 births in the Middle East, 1:10 000 births in western countries, and 1:1250 births in the Gypsy population of Slovakia.

Congenital glaucoma is genetically heterogeneous with two associated loci on chromosomes 1p36 and 2p21, respectively. Other forms of early onset glaucoma, mapped to chromosomes 6p25 and 4q25, are usually associated with other congenital anterior segment anomalies which only occasionally result in glaucoma at birth or in infancy and are not considered as “primary congenital glaucoma”.

A large, consanguineous Amish family affected with congenital glaucoma, from south western Ontario, was recruited and studied in an attempt to identify the disease causing genetic defect in that community. In addition, several members of this family were characterised with an atypical form of iris hypoplasia that was studied separately.

Materials and methods

CLINICAL ASSESSMENT
The project was approved by the Toronto Hospital Human Subjects Review Committee and The Hospital for Sick Children Research Ethics Board. After informed consent, all participants were questioned on their personal medical history and a family tree was drawn. All participants, except for the nuclear family of the proband, were examined in their home where no electricity was available. As a result, no ocular photographs were taken. The proband and his immediate family had a comprehensive eye examination at The Hospital for Sick Children. The affected status was defined by an intraocular pressure greater than 25 mm Hg in the first years of life and by the presence...
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and 5''ccagctcgattcttgacaa3'-forward).20

U56438) (5'catgattcacagaccactgg3'-reverse

Mutational analysis of

CYP1B1

described.71 9 Additional exon 3 primers were

the gene

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CYP1B1

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MUTATIONAL ANALYSIS OF

CYP1B1

13q14.13 17 18 The markers used for each loci

markers covering the three previously reported

was done by linkage analysis using additional

sia variant observed in some family members

Molecular characterisation of the iris hypoplasia

and cornea can only occur in the first few years

of life, people with buphthalmos in their teens

and adulthood are presumed to have a form of

congenital or infantile glaucoma. Eye charts of

older affected people were reviewed. Affected

subjects and their relatives were examined with

a portable slit lamp and direct ophthalmoscopy.

The charts from their respective eye physici-

ans were reviewed when available.

GENOTYPING

DNA was prepared from whole blood (10-20

ml) using a non-organic procedure.11 The

selection of short repeat tandem repeat polymor-

phisms (STRPs) was done using genetic map

information from publications and genome

databases, Génethon, CHLC, and Marshfield.

Primers were obtained from Research Genetics

Inc (screening set 6A) or from ACGT Inc

(Toronto, ON). A fluorescent dye label was

incorporated on the 5' end of one of the prim-

ers and the protocol used for genotyping was

previously described.16 The genotyping was

done blinded to the affected status.

LINKAGE ANALYSIS OF CONGENITAL GLAUCOMA

Four STRP markers that mapped to the region

2p21, around CYP1B1, were used and their
telomeric to centromeric order with inter-

marker distances (in cM) were as follows:

D2S1788 - (3) - D2S177 - D2S1348 -

D2S1346. Linkage analysis was performed

with the MLINK component of the LINK-

AGE package (v 5.1). Lod scores were

obtained with the assumption of an autosomal

recessive mode of inheritance, full penetran-
tance, a disease gene frequency of 0.0001, and equal
allele frequencies.

LINKAGE ANALYSIS OF IRIS HYPOPLASIA

Molecular characterisation of the iris hypopla-
sia variant observed in some family members

was done by linkage analysis using additional

markers covering the three previously reported

loci on chromosomes 4q25, 6p25, and

13q14.11 17 18 The markers used for each loci

including the intermarker distance (in cM) are as

follows: 4q25 (D4S3240 - D4S2623 - (3) -

D4S406 - (4.5) - D4S2392), 6p25 (D6S1600 -

(1.4) - D6S967 - D6S344 - (6.9) - D6S477),

and 13q14 (D13S1493 - (7.5) - D13S894 -

(1.65) - D13S1253 - (3.8) - D13S263 - (6.3) -

D13S788).

MUTATIONAL ANALYSIS OF CYP1B1

CYP1B1

consists of three exons, only two of

which code for the protein. PCR primers used

for the mutational analysis of exons 2 and 3 of

the gene CYP1B1 were previously

described.7 9 Additional exon 3 primers were

designed from the mRNA sequence (Acc No

U56438) (5’catgattcacagaccactgg3'-reverse

and 5’ccagctcgattcttgacaa3'-forward).20

SEQUENCING OF CYP1B1

Mutational analysis of CYP1B1 used direct

sequencing from genomic DNA. Methods of

PCR amplification were as previously described.23 Gene specific primers tailed with

M13 universal primer 5’taaaacgacggcgt3’ or

M13 reverse primer 5’cagctcgattctggacaa3’ were used. The amplification was purified using

QIAquick PCR Purification Kit™ (Qiagen,

Mississauga) according to the manufacturer's

protocol. The column purified amplion was

then sequenced on a MicroGene Blaster™
adornated DNA sequencing unit (Visible

Genetic Inc (VGI), Toronto) using Cy5.5

labelled M13 universal or M13 reverse primers

and the Thermo Sequenase™ Cycle Sequenc-
ing Core Kit (US 79610, VGI) as previously

published.23

FRAGMENT ANALYSIS OF CYP1B1

Family members were screened for the 13 bp
deletion by fragment analysis as follows. M13
tailed primers were used to amplify a 320 bp

fragment in the 5' region of exon 3. Then 1 µl

of unpurified amplion, 1.5 mmol/l MgCl2, 1 ×

PCR buffer II, 2 mmol/l of each dNTP, and

0.75 pmol of M13 universal primer (CY5.5

labelled) and the unlabelled reverse primer in

the initial amplification were used in a second

PCR reaction. The PCR reaction used the fol-

lowing conditions: 94°C for three minutes,

(94°C for 30 seconds, 51°C for 30 seconds,

70°C for five seconds) × 28 cycles and 70°C for

an eight minute extension. Formamide loading
dye (1.5 µl) was mixed with 1.5 µl of the final

amplified product, denatured for one minute,

and then electrophoresed on a 6% Surefior™

sequencing gel (VGI, Toronto). Two control

size markers were run with each sample.

DIDEOXY FINGERPRINTING ANALYSIS OF CYP1B1

Screening for the 1505G→A missense muta-
tion was done by dideoxythymidine finger-

printing analysis. A cycle sequencing reaction

was performed with only the T termination mix from Thermo Sequenase™ Cycle Se-

quencing Core Kit and the M13 reverse

primer. The conditions for the cycle sequenc-
ing reaction are the same as described above.

PROTEIN MODELLING

Homology modelling was performed using

Swiss-Model version 2.0. The reference struc-
ture used was P450BM-P which is the best

model for eukaryotic P450s.19 22 The predicted

structure for CYP1B1 was viewed in the

WEELAB VIEWER™ PRO (Molecular Simu-

lation Inc, San Diego).

Results

CLINICAL ANALYSIS

Six bilaterally affected people and 38 unaf-

fected people were recruited. The penetrance

appeared complete and the pedigree (fig 1) was

consistent with autosomal recessive inheri-

tance. However, the severity of the phenotype

appeared to be variable between family mem-

bers. Buphthalmos and increased intraocular

pressure in the first year of life characterised

the affected status of the sixth generation,

whereas the fourth generation appeared to have

an overall milder course. No specific anterior
segment malformation was noted. The clinical information is summarised in table 1 and detailed as follows.

VI.1 (4 years old) presented in the first week of life with corneal oedema and the typical high iris insertion seen in congenital glaucoma, but normal corneal diameters (11.25 mm). The optic nerve could not be seen at presentation. His glaucoma has been poorly controlled despite maximum medical and surgical treatments. At 4 years of age he required bilateral laser cyclophotoablation (destruction of the ciliary body) and still continues on multiple topical antiglaucoma medications. His course has been complicated by a severe cone-rod retinal dystrophy confirmed by electroretinography, developmental delay, and failure to thrive. Karyotype and extensive investigations to characterise the nature of his non-ophthalmic findings have been unrevealing. His current vision is at best hand motion and felt largely to be because of the retinal dystrophy. The right optic nerve cup is approximately 0.3 but is difficult to assess. Corneal diameters are now enlarged, 14.0 mm OD and 14.25 mm OS.

Table 1  Clinical characteristics of subjects with congenital glaucoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Age at diagnosis</th>
<th>Corneal diameter at diagnosis OD;OS</th>
<th>IOP at diagnosis OD;OS</th>
<th>Last vision OD;OS (age)</th>
<th>Last C/D ratio (age) OD;OS</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI.1</td>
<td>&lt;1 wk</td>
<td>11.25 OU (normal)</td>
<td>18; 21 (mm Hg)</td>
<td>LP; HM</td>
<td>0.3; N/A</td>
<td>Medical treatment, surgery × 3, laser surgery</td>
</tr>
<tr>
<td>VI.2</td>
<td>&lt;1 wk</td>
<td>12 mm; 12.75 (enlarged OS)</td>
<td>22; 49 (mm Hg)</td>
<td>Amblyopia OD</td>
<td>0.1 OU</td>
<td>Medical treatment, surgery × 3, laser surgery</td>
</tr>
<tr>
<td>IV.7</td>
<td>-14 y</td>
<td>Enlarged OD</td>
<td>Raised OU</td>
<td>Amblyopia OD</td>
<td>0.9; 0.1</td>
<td>Medical treatment OU, trauma OU in 1969</td>
</tr>
<tr>
<td>IV.13</td>
<td>-14 y</td>
<td>Enlarged OU</td>
<td>Not available</td>
<td>20/15 OD teenage</td>
<td>None</td>
<td>Enucleation at 2 y, traumatic cause?</td>
</tr>
<tr>
<td>IV.14</td>
<td>Birth</td>
<td>Enlarged OU</td>
<td>Raised OU</td>
<td>Enucleated OU</td>
<td>None</td>
<td>Enucleation at 2 y, traumatic cause?</td>
</tr>
<tr>
<td>IV.15</td>
<td>Birth</td>
<td>Buphthalmos OU</td>
<td>Buphthalmos OU</td>
<td>37 mm Hg OU</td>
<td>Not available</td>
<td>Enucleated OU</td>
</tr>
</tbody>
</table>

OD: right eye; OS: left eye; OU: both eyes.
C/D: cup disc ratio of the optic nerve.
IOP: intraocular pressure.
LP: light perception, HM: hand motion.
CSM: central, steady and maintained.
VI.2 (5 years old) also presented in the first weeks of life with cloudy, enlarged corneas and findings consistent with congenital glaucoma. His condition was also difficult to control due to severe white blood count and increased intraocular pressures. Despite maximum medical treatment, he failed to respond and surgery was eventually performed at the age of 6 months. His corneal diameters are now 14.25 mm OD and 14.00 mm OS. Optic nerve cuppings are 0.4 OU and pressures are well controlled on medical therapy.

IV.7 (56 years old) was diagnosed at age 14 years with right eye (OD) buphthalmos and raised pressures in both eyes (OU). His intraocular pressures were controlled medically until the age of 30, when the pressure rose to 36 mmHg and the vision OD decreased to 20/200. Surgery of the right eye was required to control the disease which is currently stabilised with the addition of medical therapy.

IV.13 (63 years old) had bilateral buphthalmos diagnosed in his late teenage years but retained a good central vision OD with medical treatment until 1997 when he was last seen. He suffered traumatic visual loss OS in 1969.

IV.14 (61 years old) was born with glaucoma and bilaterally enucleated at 2 years of age. It is unclear if the enucleation was related to glaucoma or to a severe trauma she suffered in early childhood.

IV.15 (58 years old) was diagnosed at the age of 14 years with bilateral buphthalmos, mild optic nerve pallor, and intraocular pressures of 37 mm Hg in both eyes. He was controlled medically until 1967 when surgery was required to control the pressure in both his eyes. He has remained stable for the last 30 years with sporadic use of his glaucoma drops.

Twenty people were also affected with a type of iris hypoplasia (VI.8) in fig 1 that did not segregate with the congenital glaucoma phenotype. The absence of iris colarette and a variable degree of thinned anterior leaflet of the iris from the pupillary border to the iris base with the iris base being the thinnest characterised this phenotype. The stromal strands had a tight appearance and the pigment epithelium of the iris could be seen through the strands to a variable degree. These people were otherwise normal; no transillumination defects were observed, nor were the classical features of Rieger syndrome. The iris hypoplasia appeared to have an autosomal dominant mode of inheritance.

LINKAGE ANALYSIS OF IRI S H YPOPLASIA

A subset of the branch of the family was genotyped and studied by linkage analysis (fig 1). This included five affected subjects and 14 unaffected subjects. Affected subject VI.2 was recruited after linkage analysis was completed.

The first candidate locus was the CYP1B1 locus on chromosome 2p21 because of its suggested role in congenital glaucoma. Two point linkage data for the four STRP markers are summarised in table 2. Evidence for linkage to the locus at 2p21 was obtained with markers D2S1348 and D2S1346 (Zmax=3.34, 0max=0).

A haplotype analysis showed that the affected haplotypes segregated perfectly with the congenital glaucoma phenotype in this family (fig 1). A recombination event observed in affected subject IV.14 places the candidate gene CYP1B1 centromeric to D2S177. This recombination event also clarifies the order of the two previously non-recombinant markers, D2S177 and D2S1346 (fig 1).

Table 2 Two point linkage data

<table>
<thead>
<tr>
<th>Marker</th>
<th>LOD score at Zmax</th>
<th>Zmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2S1778</td>
<td>3.34 3.0 2.64 1.91 1.16 0.44 0.000</td>
<td>3.34</td>
</tr>
<tr>
<td>D2S1346</td>
<td>3.34 3.0 2.64 1.91 1.16 0.44 0.000</td>
<td>3.34</td>
</tr>
<tr>
<td>D2S1348</td>
<td>3.34 3.0 2.64 1.91 1.16 0.44 0.000</td>
<td>3.34</td>
</tr>
</tbody>
</table>

IMD: intermarker distance.

No correlations were observed between the CYP1B1 genotypes and the iris hypoplasia phenotype confirming that the iris hypoplasia observed in this family is unrelated to the congenital glaucoma. There was no evidence of linkage of the iris hypoplasia phenotype to the previously documented loci (data not shown).

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supporting the genetic heterogeneity of this condition and suggesting the possibility of an additional iris hypoplasia locus. No person with iris hypoplasia had congenital glaucoma with the exception of IV.7 and IV.13.

Discussion

Linkage and haplotype analysis identified the disease causing locus on chromosome 2p21 with CYP1B1 being the disease causing gene for the glaucoma segregating in this Amish family. CYP1B1 encodes for a cytochrome P450 enzyme that belongs to the multigene superfamily of monomeric mixed function mono-oxygenases responsible for phase 1 metabolism of numerous structurally diverse substrates. It is postulated that the oxygenation of a CYP1B1 substrate, still unknown in the eye, would allow the proper functioning of signal transduction pathways involved in eye growth and differentiation.12 By directly sequencing CYP1B1 in this family, two mutations in exon 3 were identified. The 1410del13 mutation introduces a stop codon 203 bp downstream of the deletion. The truncation of the protein (amino acids 422-453) eliminates the carboxy-terminus end, which includes the essential haem domain. This may have a deleterious effect on the function of the protein (data not shown).22 This mutation is suggested to create a functional null allele.6 The other mutation altered amino acid 387 from glutamic acid to lysine. Glu387 is located in helix K, which is one of the highly conserved core structural elements. The core structural elements are suspected to be involved in proper folding of the protein and in active haem binding.22,23 Glu387 is also conserved across all documented species and different P450 enzymes and is one of three absolutely conserved residues identified in P450s.22 This conservation of the amino acid supports its importance and suggests that this mutation is likely to affect the proper function of the protein.

The phenotypes between the two affected generations show some differences in severity. Children who were homozygous for the deletion appeared to be more severely affected than those in generation IV who were compound heterozygotes, with perhaps the exception of IV.14. This is not surprising since an important part of this protein is eliminated by the 1410del13 mutation. This homozygous deletion was previously reported but no phenotype was described,12 whereas a case homozygous for the 1505G→A mutation was previously reported with a severe phenotype.8 One could speculate that any homozygous impairment of the haem binding domain could lead to a severe phenotype. This combination of the mutations described has not been previously reported and this is the first report of the respective genotype-phenotype correlation. As more mutations are identified, the definition of phenotype-genotype correlation may help to improve the management.

All 44 members of the Amish family were screened for both mutations and the carriers of the disease were identified along with couples whose future children may be at risk of developing glaucoma. Identifying two mutations suggests that there are two distinct founders for the glaucoma segregating in this family. The mutant allele containing the 13 bp deletion is found in both branches of this family increasing the risk of the development of congenital glaucoma in future children considering the high degree of “intracommunity” relationships (not all illustrated in the pedigree shown). The information has been returned to the community to improve genetic counselling and management. The opportunity has also been given to other members of the Amish community to assess their carrier status and to proceed with early screening of their children at risk. Although genetic counselling is less likely to alter mating patterns of this highly consanguineous population, early identification of at risk couples will hopefully improve the management and outcome of offspring and provide an opportunity to prevent or minimise glaucoma related visual loss.

Data access: Genethon: www.genethon.fr/, Marshfield: www.marshfield.org/genetics/, CHILC: www.chilc.org/, Molecular Simulation Inc: www.msi.com, SWISS-MODEL, version 2.0: www.expasy.ch/swissmod/SWISS-MODEL.html. GenBank: www.ncbi.nlm.nih.gov/Web/Search/index.html. The authors are grateful to Gail Billingsley MSc for her constructive discussions, to the families for their enthusiastic participation, to Dr J R Walker who kindly provided medical records, and to Dr V Siu, Dr D Williams-Lyn and Ms E Perruzza for their support in the organisation of this project. This work was funded by the Glaucoma Research Society of Canada, the Weston Foundation, PSI grant No 97-02, Fight For Sight, and the Canadian Genetic Disease Network.

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