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Molecular Analysis of Italian Patients with Congenital Glaucoma

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1. Introduction

Glaucoma is a heterogeneous group of optic neuropathies which can lead to optic nerve loss of optic nerve fibers and permanent loss of vision. It is the second most prevalent cause of bilateral blindness in the Western World and it affects several million people worldwide. (Ray et al., 2003). There are different types of glaucoma: adult-onset open-angle glaucoma, the commonest form of the disease, representing almost half of all cases; as juvenile-onset open-angle glaucoma, followed by juvenile-onset open-angle glaucoma (Weisschuh and Schiefer, 2003), and as congenital glaucoma (CG), a clinical and genetic entity distinct from the juvenile form.

CG may be associated with ocular malformation, such as aniridia (Lee et al., 2003) or congenital hydrocephalus and anterior segment dysgenesis (Mandal et al., 2002). It results from poorly understood developmental abnormalities of the ocular drainage structures and is clinically characterized by high intraocular pressure (IOP), epiphora, corneal oedema, photophobia, blepharospasm and ocular enlargement (Libby et al., 2003). The disease is bilateral in approximately 75% of cases. CG incidence varies substantially among countries: it is estimated to occur in 1/10,000 births in Europe, in 1/2500 in Saudi Arabia and 1/1250 in the gypsy population of Slovakia. (Online Mendelian Inheritance in Man, OMIM 231300).

An increase in IOP, untreated either with surgery or pharmacological therapy, results in ocular enlargement (buphthalmos) and rapidly progressive cupping of the optic nerve with severe and irreversible alteration of the visual field (Belmouden et al., 2002, Bruttini et al., 2003). Early recognition and therapy can significantly improve the child's visual function. Onset of an aggressive form of glaucoma occurs between birth and 3 years of age. The disease has higher prevalence in males (2:1 M/F).

Most cases of CG are sporadic and recessive inheritance of CG is common, with almost complete penetrance in populations with a high consanguinity rate. Reduced penetrance (40% in some populations) and various phenotypic forms suggests a polygenic inheritance pattern or multifactorial aetiologies.

Genetic linkage studies, started in 1995, identified two separate loci (GLC3A on 2p21 and GLC3B on 1p36) associated with the disease, thus confirming that CG is genetically heterogeneous (Ohtake et al., 2003) (Sarfarazi et al., 1995). A few years later Sarfarazi reported that the cytochrome P4501B1 gene (CYP1B1, OMIM 601771), located within the GLC3A locus, was mutated in individuals with CG (Akarsu et al., 1996).

CYP1B1 belongs to a multigene superfamily of monomeric mixed-function monooxygenases, responsible for the phase I metabolism of a wide range of structurally different substrates including steroids and retinoids (Stoilov et al., 1996, Hayes et al., 1997). A specific CYP1B1 metabolite is most probably required for normal eye development, and its deficiency (or toxic accumulation) may result in CG (Zhang et al., 2000).

The CYP1B1 gene maps to 2p22-p21, contains 3 exons and 2 introns, and is expressed in several normal human tissues (Stoilov et al., 2001). Northern hybridization analysis showed strong expression of CYP1B1 in the anterior uveal tract, which is involved in secretion of the aqueous humor and in the regulation of outflow facility, processes that could contribute to the elevated intraocular pressure characteristic of CG (Sutter et al., 1994).

An extensive allelic heterogeneity was illustrated by a comprehensive sequence analysis of the translated regions of the CYP1B1 gene in 22 CG families and in 100 randomly selected normal individuals. Sixteen mutations and 6 polymorphisms were identified. The positions affected by these changes were evaluated by building a 3-dimensional homology model of the conserved C-terminal half of CYP1B1. These mutations may interfere with the heme incorporation by affecting the hinge region and/or the conserved core structures (CCS) that determine the proper folding and heme-binding ability of P450 molecules. In contrast, all polymorphic sites were poorly conserved and located outside the CCS (Stoilov et al., 1998).

More than 50 CYP1B1 pathogenic mutations, including 34 missense/nonsense substitutions, 10 small deletions, 6 small insertions, a 27 bp duplication and a deletion involving part of intron 2 and exon 3, have been described in various ethnic groups (Human Gene Mutations Database). Two CYP1B1 mutations, Met1Thr and Trp57Stop, in a compound heterozygosis status, have been identified as the molecular basis of Peters' anomaly that consists of corneal opacity, defects in the posterior structures of the cornea, iridocorneal and/or keratolenticular adhesions (Stoilov et al., 1998).

Mutations in CYP1B1 were found also in patients with juvenile glaucoma (JOAG), an early-onset form of open angle glaucoma that can be caused by a mutation in the gene encoding myocilin (MYOC/TIGR).

Vincent et al. studied the role of the CYP1B1 and MYOC/TIGR and found that MYOC/TIGR mutations included cases of juvenile glaucoma (either with or without pigmentary glaucoma) and mixed-mechanism glaucoma, and CYP1B1 mutations involved cases of juvenile open-angle glaucoma as well as cases of congenital glaucoma. That paper emphasized the genetic heterogeneity of juvenile and congenital glaucoma and demonstrated that the spectrum of expression of MYOC/ TIGR and CYP1B1 mutations is greater than expected. It also appeared that CYP1B1 may act as a modifier of MYOC/ TIGR expression and that these 2 genes may interact through a common pathway (Vincent et al., 2001). Both CYP1B1 and the MYOC/TIGR genes are expressed in the iris, trabecular meshwork, and ciliary body of the eye (Vincent et al., 2002).

Different CYP1B1 mutations together with a common MYOC/TIGR mutation (Gln48His) have been reported in CG patients (Ming and Muenke, 2002, Chakrabarti et al., 2005), thus suggesting a role for the MYOC gene in congenital glaucoma via digenic interactions with CYP1B1.

In 22 Saudi Arabian families, 40 apparently unaffected individuals had mutations in CYP1B1 and haplotypes identical to their affected siblings. Analysis of these relatives suggested the presence of a dominant modifier locus that is not linked genetically to CYP1B1. Linkage and Southern analyses excluded 3 candidate modifier loci, the arylhydrocarbon receptor (AHR) on 7p15, the arylhydrocarbon receptor nuclear translocator

(ARNT) on 1q21, and the CYP2D6 gene on 22q13.1 (Kaur et al., 2005, Bejjani et al., 2000, Libby et al., 2003).

To determine the possible role of the genetic defects described in CG cases in Italy, molecular analysis was undertaken, and the CYP1B1 and MYOC genes were screened for mutations. We describe here the pathogenic mutations found, some of which are not published, and the haplotype for five intragenic SNPs (Single Nucleotide Polymorphisms) associated with the mutations identified.

2. Methods

2.1 Subjects

Peripheral blood samples were collected from 72 patients (10 subjects belonging to 5 families and 62 unrelated subjects) with a diagnosis of bilateral CG coming from different Italian regions. A clinical report with demographic data and clinical evaluation was obtained for every patient. Reports contain subjective symptoms (irritability, sensitivity to light, tearing of the eyes) and objective evaluation (corneal diameter enlargement, type of corneal oedema, rupture of Descemet membrane, increase of IOP). Age of disease onset and number of surgical procedures are described in Table I.

ID	Age at Diagnosis (mo)	N° of Surgery events	Genotype	SNP 2	SNP 3	SNP 4	SNP 5	SNP 6
206	1	6	[L52P] / [R390S]	C/G	G/T	G/C	T/C	A
110	1	4	[A106D] / [A106D]	C	G	G	T	A
161	<1	3	[1236-1237insC] / [1436-1448del13]	C	G	G	T	A
164	<1	3	[A106D] / [1775-1801dup27]	C	G	G	T	A
113	1	3	[1436-1448del13] / -	C	G	G	T	A
2	1	2	[W57X] / [1775-1801dup27]	C	G	G	T	A
67	1	2	[G61E] / [1436-1448del13]	C	G	G	T	A
71	1	2	[1236-1237insC] / [1775-1801dup27]	C	G	G	T	A
153	<1	2	[G61E] / [1436-1448del13]	C	G	G	T	A
12	2	4	[G61E] / [1236-1237insC]	C	G	G	T	A
25	3	8	[1775-1801dup27] / [1775-1801dup27]	C	G	G	T	A
42	3	7	[G61E] / - *	C	G	G/C	T/C	A/G
63	5	3	[R355X] / [E387K]	C/G	G/T	G/C	T/C	A
1	5	2	[W57X] / [1775-1801dup27]	C	G	G	T	A
16	6	2	[L26R] / [R368H]	C	G	G	T	A
203	8	3	[A443G] / -	C/G	G/T	G	T	A
31	<1	1	[G61E] / [P52L]	C	G	G	T	A
54	1	1	[M1T] / [R368H]	C	G	G	T	A
62	12	0	[R355X] / [E387K]	C/G	G/T	G/C	T/C	A
89	<1	0	[G61E] / [1236-1237insC]	C	G	G	T	A
122	17 y	0	[G61E] / [G61E]	C	G	G	T	A
179	<1	0	[A237E] / [1581-1582ins10]	C	G	C	T	A
91	4 y	0	[1236-1237insC] / -	C	G	G/C	T/C	A
114	29 y	0	[1436-1448del13] / -	C	G	G	T	A
132	3 y	0	[G61E] / -	C	G	G/C	T/C	A/G

Table I. Phenotype–Genotype Correlation in Italian CG patients with *CYP1B1* mutations. SNP 1–5 are: 1. 3947 C>G (R48G); 2. 4160 G>T (A119S); 3. 8131C>G (L432V); 4. 8184C>T (D449D); 5. 8195A>G (N435S).

2.2 Mutation screening

DNA was extracted from peripheral blood of patients and family members. The entire transcript region of the *CYP1B1* gene, organized in three exons of which only II and III are translated, was screened for mutations using four sets of primers:

set1 - cyp1b1Ex1F 5'-GCT TTG ACT CTG GAG TGG G-3'and cyp1b1Ex1R 5'-TCC ATC TGA AGA GGT CGC C-3' (424 bp); set2 - cyp1b1Ex2aF 5'-TGA GTG TCA CGC CTT CTC C-3' and cyp1b1Ex2aR 5'-CTC AGC ACG TGG CCC TC-3' (548 bp); set3 - cyp1b1Ex2bF 5'-ATG CGC AAC TTC TTC ACG-3' and cyp1b1Ex2bR 5'-AGA GGA GAA AAG ACC TGG C-3' (631 bp); set4 - cyp1b1Ex3F 5'-TGC TCA CTT GCT TTT CTC TC-3' and cyp1b1Ex3R 5'-ATT TTA CTC CTC ATC TCC GAA-3' (691 bp). Polymerase chain reaction (PCR) amplification was performed in a 50-µL volume consisting of 50 to 100 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.8 M of each primer and 1 U Taq polymerase (AmpliTaq Polimerase - Perkin Elmer) with 10% dimethyl sulfoxide (DMSO) only for set 2 and set 3. PCR amplification procedures were performed under the following conditions: for set 1 and set 4, 2 cycles consisting of 60 seconds at 94°C, 30 seconds at 52°C and 90 seconds at 72°C followed by 35 cycles each consisting of 30 seconds at 94°C, 30 seconds at 54°C and 90 seconds at 72°C, followed by a final extension of 7 minutes at 72°C; for set 2 and set 3, 12 cycles consisting of 60 seconds at 94°C, 120 seconds at 64°C with a decrement of 1° C every cycle and 180 seconds at 72°C followed by 25 cycles each consisting of 60 seconds at 94°C, 120 seconds at 52°C and 5 minutes at 72°C, followed by a final extension of 7 minutes at 72°C.

Patients with none or only one mutation in the CYP1B1 gene were screened for mutations in the transcript region of the MYOC/TIGR gene using three sets of primers:

Set 1 - myocEx1F 5'-GGC TGG CTC CCC AGT ATA TA - 3'and myoc1Ex1R 5'-CTG CTG AAC TCA GAG TCC CC- 3' (760 bp); set 2 - myocEx2F 5'-AAC ATA GTC AAT CCT TGG GCC- 3' and myocEx2R 5'- TAA AGA CCA TGT GGG CAC AA- 3' (230 bp); set 3 - myocEx3F 5'-TTA TGG ATT AAG TGG TGC TTC G- 3' and myocEx3R 5'- AGC ATC TCC TTC TGC CAT TG- 3' (870 bp). PCR amplification was performed in a 25-µL volume consisting of 50 to 100 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.8 M of each primer and 1 U Taq polymerase (AmpliTaq Polimerase - Perkin Elmer). PCR amplification procedures were performed as follows: 35 cycles consisting of 60 seconds at 94°C, 60 seconds at 55°C and 120 seconds at 72°C followed by a final extension of 7 minutes at 72°C.

PCR products were sequenced on an automated DNA sequencer (ABI-310; PE Applied Biosystems).

Every nucleotide change is indicated using the sequence of the cDNA published with the accession number NM_000104.2 for the CYP1B1 and NM_000261 for the MYOC/TIGR transcript. The position of mutations were described as in Recommendations for a Nomenclature System for Human Gene Mutations (Antonarakis et al., 1998).

2.3 Comparative sequencing alignment

The cytochrome P4501B1 and MYOC/TIGR sequences were obtained from GenBank provided by the National Center for Biotechnology Information, Bethesda, MD, USA (http://ww.ncbi.nlm.nih.gov/Genbank).

Computer-assisted sequence alignment was performed with the pattern-induced multisequence alignment program (PIMA; provided by Baylor College of Medicine Search Launcher and available at: http://searchlauncher.bcm.tmc.edu).

3. Results

3.1 *CYP1B1* gene sequence analysis

Twenty-five patients (34,7%) were carriers of mutations in the coding region of CYP1B1 gene: 17 were compound heterozygotes, 2 were homozygotes, 6 carried only one mutation in a single allele (Table II).

The analysis was also performed on relatives of mutated subjects in order to verify the allele segregation and to complete the family study.

Exon	Effect		Patients	N (%) of affected chromosomes (n=44)
2	T374C	M1T	54	1 (2.3%)
2	T449G	**L26R ^**	16	1 (2.3%)
2	C527T	**P52L*^**	31, 206	2 (4.5%)
2	G543A	W57X	1, 2	2 (4.5%)
2	G554A	G61E	12, 31, 67, 89, 122, 153, 42, 131	9 (20.5%)
2	C689A	**A106D^**	110, 164	3 (6.8%)
2	C1082A	**A237E^**	179	1 (2.3%)
2	1236-1237insC	Frameshit beginning from aa 288	12, 71, 89, 91, 161	5 (11.4%)
3	C1435T	R355X	62, 63	2 (4.5%)
3	1436-1448del13	Frameshit beginning from aa 355	67, 113, 114, 153, 161	5 (11.4%)
3	G1475A	R368H	16, 31	2 (4.5%)
3	G1531A	E387K	62, 63	2 (4.5%)
3	C1540A	R390S	206	1 (2.3%)
3	1581-1582ins10	Frameshit beginning from aa 404	179	1 (2.3%)
3	T1692G	**F440L*^**	31, 206	
3	C1700G	A443G	203	1 (2.3%)
3	1775-1801dup27	Frameshit beginning from aa 468	1, 2, 25, 71, 164	6 (13.6%)

* present on the same chromosome inherited from the unaffected father, in patients 31 and 206 who are also compound heterozygotes for other published pathological mutations (G61E and R390S respectively).
^ new variations

Table II. Frequencies of *CYP1B1* mutations in the Italian population

Out of the seventeen different CYP1B1 variations identified, twelve had been previously characterized as pathogenetic: six aminoacid substitutions (M1T, G61E, R368H, E387K, R390S and A443G), two stop codons (W57X, R355X), two insertions (1236-1237insC and

1581-1582ins TCATGCCACC), one deletion (1436-1448delGAGTGCAGGCAGA) and one duplication (1775-1801dupGGCGGTGCATTGGCGAAGAACTTTCTA). Five variations (L26R, P52L, A106D, A237E, F440L) are described here for the first time (Table II).

The new variants show the following characteristics: L26R, which affects the second nucleotide of codon 26, substitutes leucine, an aliphatic and hydrophobic residue with a polar and hydrophilic arginine. The variation L26R is present in patient 16 with the known mutation R368H. A237E presents a substitution of an alanine, weakly hydrophobic with small nonpolar side chains, with glutamic acid that carries a hydrophilic acidic group with strong negative charge and was found in patient 179 associated with the insertion 1581-1582ins10.

A106D was observed on three CG chromosomes (patient 110 is homozygous and patient 164 is compound heterozygote with the known mutation 1775-1801dup27). This change causes a missense substitution of alanine (weakly hydrophobic with small nonpolar side chains) with asparagine (with polar, uncharged side chains).

P52L changes proline, often present in the protein turning point and with small nonpolar side chains, to leucine (aliphatic and hydrophobic). F440L changes phenylalanine, a hydrophobic aminoacid usually orientated towards the interior of the folded protein, to leucine with the same characteristics as phenylalanine.

The variations P52L and F440L are present in cis in patients 31 and 206, who are also compound heterozygotes for other published pathological mutations (G61E and R390S respectively); these patients are from the same Italian region.

All new identified aminoacidic changes alter residues that are evolutionarily conserved in Eukariota and in the cytochrome P450 family (Figs. # 1 and 2).

The most frequent mutations in our CG patients are G61E, 1775-1801dup27, 1436-1448del13 and 1236-1237insC; they occur respectively in 20.5%, 13.6%, 11.4% and 11,4% of mutated chromosomes (Table II).

Two nucleotide variants were found in the untranslated exon 1: one of these (cDNA 198 C/T) was characterized as a single nucleotide polymorphism (NCBI SNPs databank rs 9341244) while the second one (C>A at 226 of cDNA) was identified for the first time in patient 91.

In these 72 Italian CG subjects we also identified 6 Single Nucleotide Polymorphisms previously described and 2 new synonymous changes (G/A 359E and C/T 363V) whose frequencies are 1/144 and 3/144 respectively.

3.2 CYP1B1 SNP haplotypes

We analyzed the following known CYP1B1 polymorphisms (Akarsu et al., 2002, Sutter et al., 2005, Fan et al., 2004) in our patients: g.3947C>G, g.4160G>T, g.8131C>G, g.8184C>T, g.8195A>G. (Table II). The most frequent haplotype in individuals with CYP1B1 mutations is 5'-C-G-G-T-A-3', as already described in other populations (Belmouden et al.,2002, Kaur et al., 2005, Bejjani et al., 1998, Stoilov et al., 2001). This haplotype is present in 41 of the 44 mutated alleles (93%), and in only 20 of the remaining 100 wild type alleles (20%); the difference is statistically significant (Q2= 22.23, p < 0.0001, CI95% 0.555-0.905).

Considering the seventeen mutations we found, fifteen are linked to the 5'-C-G-G-T-A-3' haplotype and two (E387K and R390S) are associated with two other different haplotypes.

3.3 MYOC/TIGR gene analysis

The report in published papers of patients with mutations in either CYP1B1 and MYOC genes prompted us to look for MYOC/TIGR variations in the six patients having only one mutation in CYP1B1.

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  1    --MGTSLSPNDPWPLNPLSIQQTTLLI L LSVLATVHVGQRLLRQ      42
  1    --MATSLGPDAPLQPSALSAQQTTLLI L LSVLAAVHAGQWLLRQ      42
  1    --MATSLSADSPQQLSSLSTQQTTLLI L FSVLAAVHLGQWLLRQ      42
  1    --MALERLGEALRGTPPL---QSSLLI L LCLLAAVHLGKLLLQR      39
  1    -MLAALIYTILAILLSVLATSYICIIY G VKRRVLQPVKTKNSTE      43


 43    R--RRQ-LRSAE P GPFAWPLIGNAAAVGQAAHLSF---------      74
 43    R--RRQ-PGSAE P GPFAWPLIGNAAAMGPAPHLSF---------      74
 43    W--QRK-PWSSE P GPFPWPLIGNAAAVGQASHLYF---------      74
 40    RRWRRQGQRLAE P GPFPWPLIGNAAQLGSAPHLSFEKLGSAPHQ      83
 44    INHNAYQKYTQA P GPRPWPIIGNLHLLDRYRDSPFAG-------      80


 75    --ARLARRYGDVFQIRLGSCPIVVLNGERAIHQ A LVQQGSAFAD     116
 75    --ARLARRYGDVFQIRLGSCRVVVLNGERAIRQ A LVQQGAAFAD     116
 75    --ARLARRYGDVFQIRLGSCPVVVLNGESAIHQ A LVQQGSIFAD     116
 84    SLARLASTYGAVFQLRSGRWPVVVVNGESAIRQ A LVRQGAAFAG     127
 81    -FTALAQQYGDIYSLTFGHTRCLVVNNLELIRE V LNQNGKVMSG     123


204    VMSAVCFGCRYSHDD---------PEFRELLSHNEEFGRTVG A G    238
204    VMSAVCFGCRYSHDD---------AEFRELLSHNEEFGRTVG A G    238
204    VMSAVCFGCRYNHDD---------AEFLELLSHNEEFGRTVG A G    238
       ------------------------------------------      
212    MFSQYMCSLRFDYDD---------VDFQQIVQYFDEIFWEIN Q G    246


409    ---VLGYHIPKDTVVFVNQWSVNHDPVKWPN-PENF DPARFLDK    448
409    ---VLGYHIPKDTVVFVNQWSVNHDPVKWPN-PEDF DPVRFLDK    448
409    ---VLGYYIPKNTVVFVNQWSVNHDPAKWPN-PEDF DPARFLDK    448
285    ---IMGYLIPKDTVIFVNQWSVNHDPAKWSN-PEDF DPTRFLDE    324
404    ---ISGYGVTKGTIVFINNYVLNTSEKFWVN-PKEF NPLRFLEP    443
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Fig. 1. Aminoacidic Sequence Alignment of CYP1B1 and homolous genes in Eukariota. Lane 1:CYP1B1 H.sapiens , lane 2 LOC483038 C.familiaris, lane 3: Cyp1b1 M.musculus, lane 4: LOC421466 G.gallus, lane 5: spo D.melanogaster.

```
              10        20        30        40        50        60        70        80
         ....*....|....*....|....*....|....*....|....*....|....*....|....*....|....*....|
12  PPGPSPLPVLGNLLQMDRKGLLRS-FLRLREKYGDVFTVYLGSRPVVVLCGTDAIREALVDQAEAFSGRGKIAVVDP---  87
51  PPGPFAWPLIGNAAAVGQAA--HLsFARLARRYGDVFQIRLGSCPIVVLNGERAIHQALVQQGSAFADR---PAFASfrv 125
33  PRSLPALPLVGSLLQLAGHPQLHLrLWRLQGRYGSLYGLWMGSHYVVVVNSYQHAREVLLKKGKAFAGRPRTVTTDL1-- 110
30  PPGPTPLPIFGNILQVGVKNISKS-MCMLAKEYGPVFTMYLGMKPTVVLYGYEVLKEALIDRGEEFSDKMHSSMLSK--- 105
30  PPGPTPLPVVGNLLQLETKDINKS-LSMLAKEYGSIFTLYFGMKPAVVLYGYEGVIEALIDRGEEFSGRGIFPVFDR--- 105
30  PPGPTPLPIIGNFLQIDVKNISQS-LTKFSKTYGPVFTLYLGSQPTVILHGYEAIKEALIDNGEKFSGRGSYPMNEN--- 105
20  PPGPTPFPILGNILQIGIQDISKS-FTKLSEVYGPVFTVYLGMKPTVVIHGYDAVKEALVDLGEEFSGRIVFPLTAK---  95
34  PPGPTPLPIIGNLLQLNLKDIPAS-LSKLAKEYGPVYTLYFGTSPTVVLHGYDVVKEALLQQGDEFLGRGPLPIIED--- 109
24  PPGPTPLPVLGNLLQVDFEDPRPS-FNQLRRRFGNVFSLQQVWTPVVVLNGLAAVREALVYRSQDTADRPPPAVYERlgy 102
37  PPGPVPWPVLGNLLQIDFQNMPAG-FQKLRCRFGDLFSLQLAFESVVVLNGLPALREALVKYSEDTADRPPLHFNDQsgf 115
```

```
              170       180       190       200       210       220       230       240
         ....*....|....*....|....*....|....*....|....*....|....*....|....*....|....*....|
158 NIICSIVFGKRFDYKDPVFLRLLDLFFQSFSLISSFSSQVFELFSgFLKHFPGTH---RQIYRNL-QEINTFIGQSVEKH 233
203 NVMSAVCFGCRYsHDDPEFRELLSHNEEFGRTVGAGS--LVDVMP-WLQYFPNPVrtvFREFEQLnRNFSNFILDKFLRH 279
182 NVVCSLCFNSSYRRGDPEFEAMLEYSQGIVDTVAKES--LVDIFP-WLQIFPNRD---LALLKRClKVRDQLLQQKFTEH 255
176 NVISSVIFQHRFDYSDEKFQKFIENFHTKIEILASPWAQLCSAYPvLY-YLPGIH---NKFLKDV-TEQKKFILMEINRH 250
176 NVICSVIFQNRFDYDDEKFKTLIKYFHENFELLGTPWIQLYNIFPiLH-YLPGSH---RQLFKNI-DGQIKFILEKVQEH 250
176 NVICSITFQNHFDYKDKEMLTFMEKVNENLKIMSSPWMQVCNSFPsLIDYFPGTH---HKIAKNI-NYMKSYLLKKIEEH 251
166 NVICSVIFQNRFDYTDQDFLSLMGKLNENFKILNSPWVQMCNNFPiLIDYLPGSH---NKILRNN-IYIRNYVLEKIKEH 241
180 NVICSILFNDRFQYNDKTFLNLMDLLNKNFQQVNSWCQMYNLWPtIIKYLPGKH---IEFAKRI-DDVKNFILEKVKEH 255
174 NVIASLTFGCRFEYNDPRIIKLLDLTEDGLKEEFNLVRKVVEAVP-VLLSIPGLA---ARVFPAQ-RAFMALIDGLIAEQ 248
187 NVIASLLFACRFEYNDPRFIRLLDLLKDTLEEESGFLPMLLNVFP-MLLHIPGLL---GKVFSGK-KAFVAMLDELLTEH 261
```

```
              410       420       430       440       450       460       470       480
         ....*....|....*....|....*....|....*....|....*....|....*....|....*....|....*....|
382 YFETPNTFNPGHFLDANG--ALKRNE--GFMPFSLGKRICLGEGIARTELFLFFTTILQNFSIA--SPVPPEDIDLTPre 455
433 KWPNPENFDPARFLDKDG--LINKDLtsRVMIFSVGKRRCIGEELSKMQLFLFISILAHQCDFRanPNEPAKMNFS-Y-- 507
408 EWDKPEEFNPGRFLDEQGqhIHSPSP--SYLPFGAGIRVCLGEVLAKMELFLFLAWVLQRFTLEc-PQDQPLPSLEGKf- 483
399 EFPNPEKFDPGHFLDEKG--NFKKSD--YFMAFSAGRRACIGEGLARMEMFLILTSILQHFTLK--PLVNPEDIDTTPvq 472
399 EFPNPEKFDPGHFLDESG--NFKKSD--YFMPFSAGKRACVGEGLARMELFLLLTTILQHFTLK--PLVDPKDIDPTPve 472
400 EFPNPEMFDPGHFLDENG--NFKKSD--YFLPFSAGKRACVGEGLARMQLFLFLTTILQNFNLK--SLVHPKDIDTMPvl 473
390 EFPNPDRFDPGHFLDASG--KFRKSD--YFMPFSTGKRVCVGEVLARMELFLFLTAILQNFTPK--PLVDPKDIDTTPlv 463
404 EFANPEKFDPGHFLDKNG--CFKKTD--YFVPFSLGKRACVGESLARMELFLFFTTLLQKFSLK--TLVEPKDLDIKPit 477
398 VWQKPFRFHPEHFLDAQG--RFVKQE--AFIPFSAGRRACLGEPLARMELFLFFTSLLQHFSFSv-PAGQPRPSEHGv-- 470
411 VWEKPLRFHPEHFLDAQG--NFVKHE--AFMPFSAGRRACLGEPLARMELFLFFTCLLQRFSFSv-PTGQPRPSDYGi-- 483
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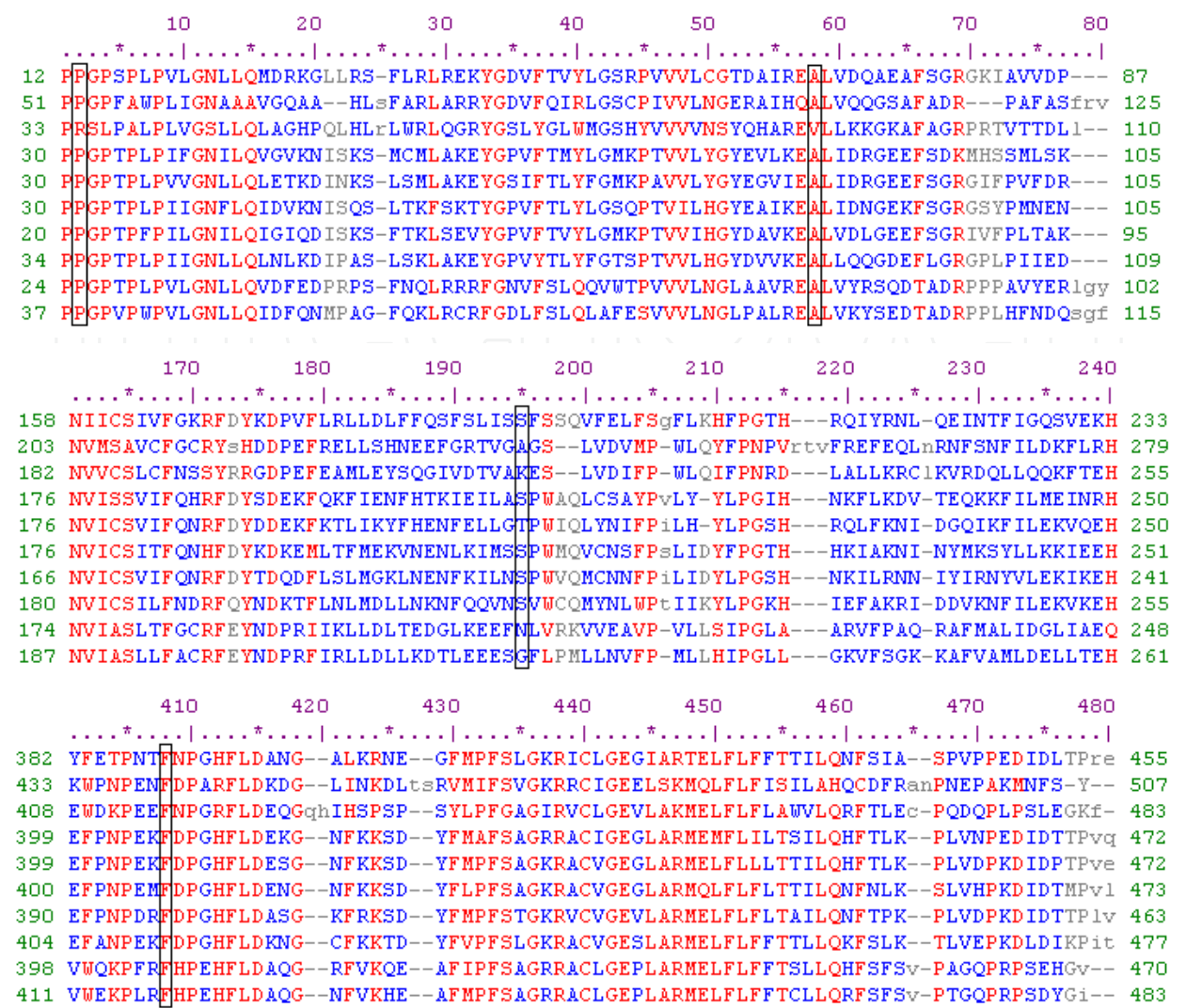
Fig. 2. Aminoacidic Sequence Alignment of CYP1B1 (second lane) and other P450 family members (CYP2b4, CYP17A1, CYP2C22, CYP2C3, CYP2C7, CYP2C1, CYP2C23, CYP2D14, CYP2D4) .

The analysis of the complete MYOC gene sequence identified, in three patients, two transitions (G>A at 249 and C>T at 1362 cDNA positions) causing the two aminoacidic variations R76K and A447V. Lysine in position 76 is similar to Arginine; Valine in position 447, an aminoacid with a large aliphatic hydrophobic side chain, usually located inside the protein, substitutes Alanine that has only small steric limits and can be located in the hydrophilic areas either outside or in the hydrophobic areas inside the protein.

In patient 42 the A447V is present with the G61E common CYP1B1 mutation; two mutations are distinctly inherited from the two parents. Patient 132, with a single maternally transmitted CYP1B1 G61E mutation, inherited R76K from the father while patient 203 received both the R76K and the CYP1B1 A443G from the unaffected father, who is homozygotes for R76K.

To confirm the polymorphic role of R76K, already suggested in literature (Colomb et al., 2003, Edward et al., 2004), we analyzed 100 chromosomes in a random Italian population, obtaining a frequency of 0,125 and 0,875 for the A and G alleles respectively.

4. Discussion

Molecular analysis of the CYP1B1 gene performed on 72 Italian patients with found eighteen different variations in 25 (34.7%) individuals. Four of these variations (L26R, P52L, A106D, A237E) are reported here for the first time and produced a non synonymous aminoacidic change. Their pathological role in CG development could be an explanation for the significant changes in the protein caused by the aminoacidic substitutions in Eukariota and cytochrome P450 family conserved regions. The new changes are in a compound heterozygous state with already described pathological mutations and are distributed in exon # 2 and 3.

The conservative aminoacid change F440L always found in cis with the P52L in 4 subjects, both in patients (with another CYP1B1 mutation) and in healthy carriers, could be classified as a rare polymorphism linked to the pathological P52L.

One of the two nucleotide variants we identified in the untranslated exon 1 had not been previously reported. In patient 91 a C to A at position 226cDNA was found combined with the pathological mutation 1236-1237insC. The presence in cis with the CG mutation also in the unaffected mother suggests that this ex1UTR variation does not play a pathological role in CG development.

We identified two new synonymous changes (G >A at the Glutamic Acid in position 359 and C>T at the Valine in position 363) in 2 different patients. These variations do not introduce aminoacidic changes and were not found in 100 analyzed chromosomes, so we can consider them as rare polymorphisms.

Clinical evaluation of patients cannot show a particular phenotype-genotype correlation even if mutations that produce truncated proteins seem more frequent in patients with a disease onset before the first year of life and more then two surgical procedures.

As also described in Indian and Saudi Arabian populations, we identified the M1T and W57X CYP1B1 mutations that have been previously associated only with Peters' anomaly, in three of our CG patients. These results support the concept that distinct CYP1B1 mutations are not responsible for the two different diseases and that a specific phenotype could result from interactions between CYP1B1 and other modifier genes.

The previously described CYP1B1 intragenic polymorphisms (R48G, A119S, L432V, D449D and N453S) were analyzed and the most frequent haplotype (93%) among affected individuals with CYP1B1 mutations appears to be 5'-C-G-G-T-A-3', which has been associated with fifteen different mutations. This particular haplotype was also observed in 94.7% of the Saudi Arabian and in the majority of the Brazilian CG chromosomes. Interestingly, in our 47 CG patients without mutations in CYP1B1, the frequency of the 5'-C-G-G-T-A-3' haplotype is the same as the general Italian population (23% vs 20%). The association of this specific uncommon haplotype with the majority of CYP1B1 mutations in geographically and ethnically distinct populations could confirm the hypothesis that this DNA sequence predisposes the gene to mutational events as already suggested.

The presence in other populations of patients with mutations in both CYP1B1 and MYOC genes prompted us to analyze the six patients that had only one mutation in CYP1B1 for the presence of MYOC/TIGR variations. The R76K was found in compound heterozygosis in patient 132 and 203 with the G61E and A443G CYP1B1 mutations respectively. The presence of MYOC R76K aminoacidic change in both alleles of patient 203's unaffected father, who also had a CYP1B1 mutation, defines the non pathological role of this variation, confirmed as a polymorphism in an Italian population frequency of 0.125 and 0.875 for the A and G alleles respectively.

In patient 42 we found, in double heterozygous status with the CYP1B1 G61E, the new MYOC variation A447V. This substitution affects a conserved aminoacidic position and introduces a residue with peculiar characteristics that can alter the protein functions. The two mutations are distinctly inherited from the unaffected heterozygous parents and the A447V was not found in 100 control chromosomes. This is the first report of CYP1B1 and MYOC mutations in Italian patients with congenital glaucoma.

The molecular characterisation of both CYP1B1 and MYOC/TIGR genes in affected subjects allowed us to better clarify the genetic basis of the CG disease. Our results confirm the major role of CYP1B1 in congenital glaucoma and also support an autosomic recessive role of MYOC/TIGR gene in a digenic inheritance model. The presence of only one CYP1B1 mutation in 5 patients confirms that other genes could interact with the function of the CYP1B1 codified protein in CG onset with different digenic inheritances. We did not look for and we did not find any genotype and phenotype correlations in our patients (Hollander et al., 2006). A study of Iranian patients did not find any myocilin mutations as cause of congenital glaucoma (Elahi et al., 2010).

5. Acknowledgements

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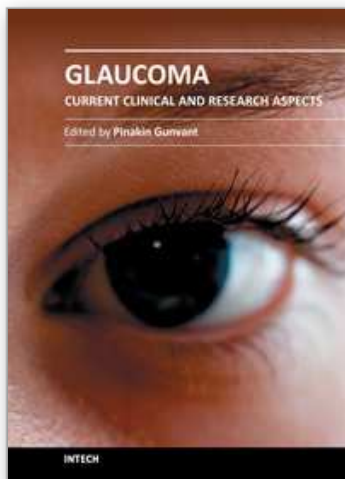
Glaucoma - Current Clinical and Research Aspects

Edited by Dr. Pinakin Gunvant

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