

Recurrent mutations in a single exon encoding the evolutionarily conserved olfactomedin-homology domain of TIGR in familial open-angle glaucoma

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Primary open-angle glaucoma (POAG) is a highly prevalent cause of irreversible blindness which associates cupping of the optic disc and alteration of the visual field, elevation of intraocular pressure being a major risk factor. Provided diagnosis is made at an early stage, treatments are available to prevent visual impairment. A locus, GLC1A, has been mapped on chromosome 1q23–q25 in several families affected with juvenile-onset POAG (JOAG) and also in some families affected with juvenile and middle-age onset POAG. Recently, three mutations of the TIGR (Trabecular meshwork-Induced Glucocorticoid Response) gene were shown to be responsible for the disease in several American families and in unrelated POAG patients. We now describe five new mutations in eight French families. All mutations known to date appear to concentrate in the evolutionarily conserved C-terminal domain of TIGR which bears homology to frog olfactomedin, an extracellular matrix glycoprotein of the olfactory epithelium, to rat and human neuronal olfactomedin-related proteins and to F11C3.2, a protein from *Caenorhabditis elegans*. Moreover, this conserved domain of TIGR is encoded by a single exon to which mutation screening could be limited. Surprisingly, the TIGR message, which is abundantly transcribed in the trabecular meshwork and also in the ciliary body and the sclera, is not expressed in the optic nerve whose degeneration is, however, the primary lesion of POAG.

INTRODUCTION

Primary open-angle glaucoma (POAG) is one of the most prevalent causes of irreversible blindness in developed countries

(1). Its definition associates characteristic cupping of the optic disk and alteration of the visual field (2). Elevation of intraocular pressure is often present and is a major risk factor (2). The disease is painless and often diagnosed at a late stage, when visual field defects are severe. Identification of POAG patients at an early stage when treatment can still prevent irreversible glaucomatous optic nerve atrophy is therefore essential (3).

The increased frequency of POAG among relatives of POAG patients strongly suggests that genetic factors markedly influence POAG susceptibility (1). JOAG, the juvenile form of POAG (onset before the age of 30) was shown to be inherited in a simple autosomal dominant manner (4). A locus for JOAG, termed GLC1A, was mapped to chromosome 1q21–q31 (5) and confirmed by subsequent studies (6,7). Two other families with both juvenile-onset and adult-onset POAG were also linked to GLC1A, suggesting variable expressivity of the gene (8,9). The GLC1A region was progressively reduced to 3 cM and physically mapped to a maximum 3 Mb region (10). Recently, study of candidate genes within the susceptibility region led to identifying the TIGR (Trabecular meshwork Induced Glucocorticoid Response protein) gene as that responsible for the disease in several American families and in unrelated POAG patients (11).

Little is known yet about the TIGR gene. In the present report, we have characterized its genomic organization and its expression pattern as well as its mutations in French families affected with GLC1A-linked POAG.

RESULTS AND DISCUSSION

Genomic organization of the TIGR gene

Based on the physical map and the YAC contig previously described (10) (Fig. 1A), a BAC contig was established across the region containing the TIGR gene. The 54D3 BAC, positive for a STS derived from the TIGR sequence, was subcloned and sequenced. Comparison of genomic and cDNA TIGR sequences revealed a simple organization consisting of three exons of 681,

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126, and 1323 nucleotides (nt), respectively (Fig. 1B and C). Of note, Southern blot analysis of genomic DNA digested with 12 restriction endonucleases and hybridized with each of three exon-specific probes indicated that the TIGR gene was a single-copy gene (data not shown). All exon/intron boundaries conformed to the GT/AG consensus for intronic donor and acceptor splice signals (12). The size of the second intron was ~1.9 kb, whereas that of the first intron has not yet been determined. The transcription initiation site was determined by amplification of the 5' end and located 77 bp upstream of the ATG codon which is localized in the first exon. Analysis of the 3' untranslated region showed three potential polyadenylation sites, of which the most 3' is predominantly used, as judged from the cDNA sequence. This yielded a calculated size of 2130 nt for the TIGR mRNA, in good agreement with the ~2 kb size observed by northern blot analysis (see below). Of interest, two polymorphic (GT)_n repeats, NGA17 and NGA19, with heterozygosity of 0.48 and 0.63 respectively, were identified in the 5' and the 3' region of the gene. They provide useful tools for linkage disequilibrium studies.

Analysis of the promoter sequence revealed the presence of a TATA box between 20 and 30 bp upstream of the transcription start site. No CAAT box, which is often seen in tissue-specific genes, was identified. However, sequencing of 5 kb upstream (data partially shown, Fig. 1C) indicated the existence of a substantial number of regulatory elements. These notably included 16 steroid hormone response elements. Of these, 11 glucocorticoid response elements (13) and one progesterone response element (14) were clustered within 1 kb of sequence immediately upstream of the transcription start site. Three other potential binding sites for receptors of vitamin D and retinoic acid were present between 2.5 and 4.5 kb upstream of the transcription initiation site. In addition, there were two shear stress response elements (5'-GAGACC-3') which have been shown to be involved in endothelial cell gene regulation (15). Altogether, the

presence of these numerous regulatory elements might account for the sensitivity of TIGR expression to steroid hormone regulation and also to mechanical forces which may be exerted during intraocular pressure variation.

The TIGR cDNA sequence contains an open reading frame of 504 amino acids (Fig. 1C). Search for motifs in the Prosite database revealed two potential sites for N-glycosylation and glycosaminoglycan extension, eight leucine residues evenly spaced every seven residues defining a typical leucine zipper motif which is characteristic of many transcription factors, and a tripeptide C-terminal targeting signal for microbodies, a class of small, single-membraned organelles to which belong peroxisomes, glyoxysomes and glycosomes (16). Whether these motifs are functional is currently unknown. A BLAST search identified three other proteins with significant homology to the C-terminal half of the TIGR protein, which is exactly encoded by the third exon, as described in detail below. In contrast, no known protein was homologous to the N-terminal part of the TIGR protein, that encoded by exon I or exon II.

Mutations of the TIGR gene in eight French families

For mutation analysis, the genomic organization data were used to design seven primer sets covering the three TIGR exons and their boundaries. Genomic DNA samples from representative glaucomatous patients belonging to eight unrelated families, in which disease had been previously linked to GLC1A (Table 1) (9,17) were thus amplified and sequenced. In three of these families (nos 9, 22 and 23), median age at onset was ≤20 years and the disease course was severe, poorly controlled by topical drugs and requiring surgical treatment to lower intraocular pressure to normal levels. In other families, both cases of juvenile and middle age onset were observed, with a median age at onset varying between 30 and 35 years but a nearly complete penetrance above 50 years.

Table 1. Mutations of TIGR gene in French JOAG and POAG families

Mutation	Family	No. of patients	Median age at diagnosis (range, years)	No. of healthy carriers, median age (range)	No. of healthy controls ^a
1109 C→T Pro370Leu	9	4	10 (6–14)	0	5
	22	10	11 (7–27)	1 (2)	14
1430 T→G Ile477Ser	3	20	33 (11–51)	10 29 (6–68)	51
1440 C→A Asn480Lys	1	39	30 (10–65)	11 21 (10–48)	89
	14	6	35 (21–51)	0	3
	27	7	32 (14–75)	0	5
1495 A→T Ile499Phe	6	7	31 (20–40)	2 33 (20,46)	10
736 G→A Gly246Arg	23	7	20 (11–28)	1 (21)	6

^aHealthy subjects of the family that were genotyped and lacked a TIGR mutation.

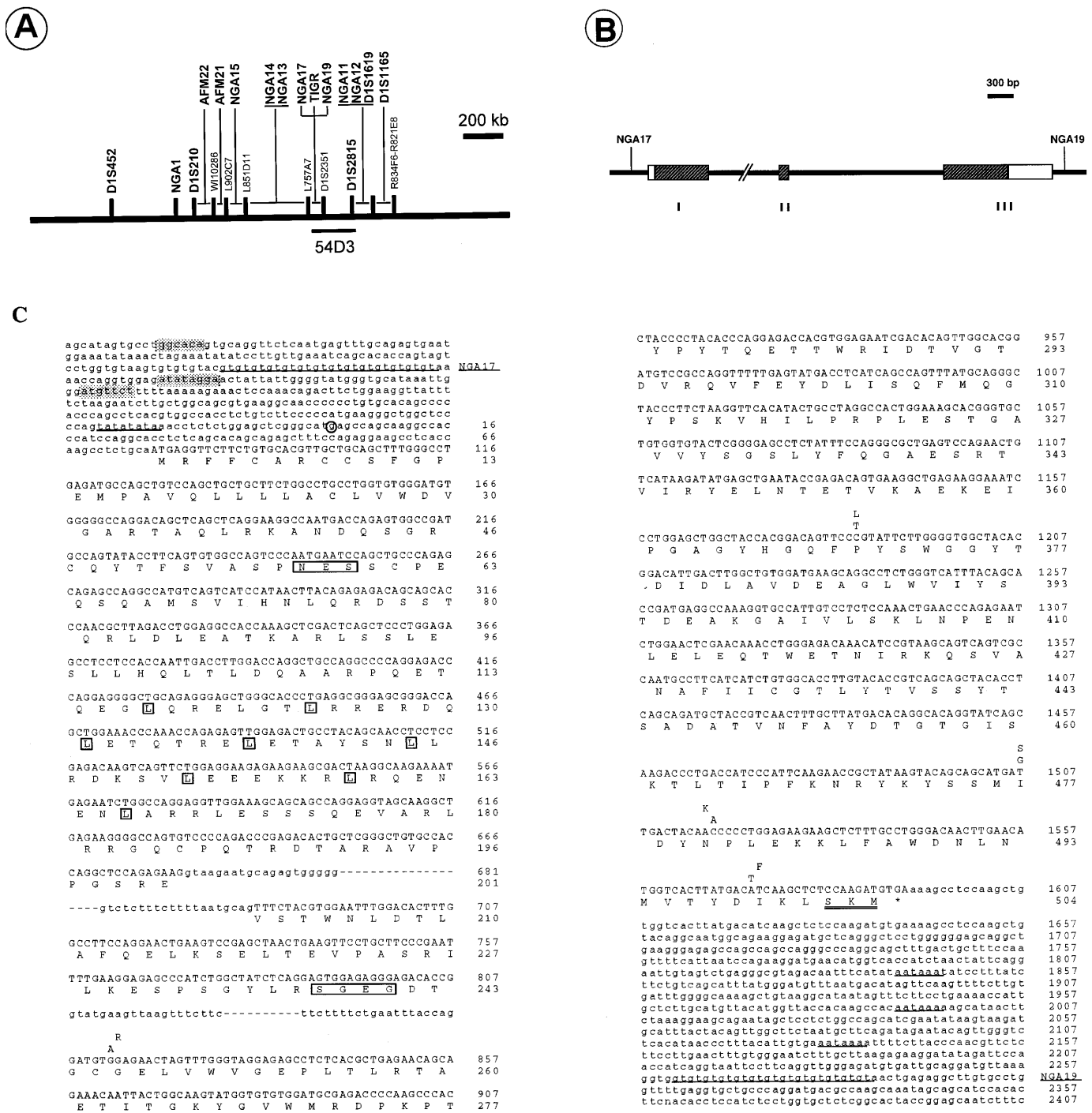


Figure 1. Map of the GLC1A region and exon-intron organization of the TIGR gene. (A) Physical map integrating data from our YAC contig (10) and from a BAC contig (our unpublished data). The markers at the top were positioned approximately in the indicated intervals by STS-content analysis of BACs. Grouped markers were not dissociated by recombination events or by STS content analysis. The position of the TIGR-containing BAC 54D3 is also shown. Polymorphic markers are printed in boldface. (B) Genomic organization of the TIGR gene. Closed and open boxes represent coding and untranslated exonic regions, respectively. The size of the first intron was not determined. (C) Nucleotide and deduced amino acid sequence of the human TIGR gene. The transcribed sequence is numbered from the transcription start site (circled) as determined by amplification of the 5' end. The coding sequence is in capital letters. The (GT)_n repeats, in the 5' and 3' region (NGA17 and NGA19, respectively), the TATA box and the potential polyadenylation sites are underlined. Three putative steroid hormone response elements are shaded. The amino acid sequence (one letter code) is shown under the nucleotide coding sequence. It is numbered from the first in-frame AUG codon. Eight leucine residues evenly spaced every seven residues defining a typical leucine zipper motif, and two potential sites for N-glycosylation and glycosaminoglycan extension are boxed. A microbodies C-terminal targeting signal is doubly underlined. Mutations of the nucleotide sequence that were observed in French families and the resulting amino acid changes are indicated above the wild-type sequence. EMBL accession numbers for TIGR exon I, exon II and exon III are Z97171, Z97174, Z97177, respectively.

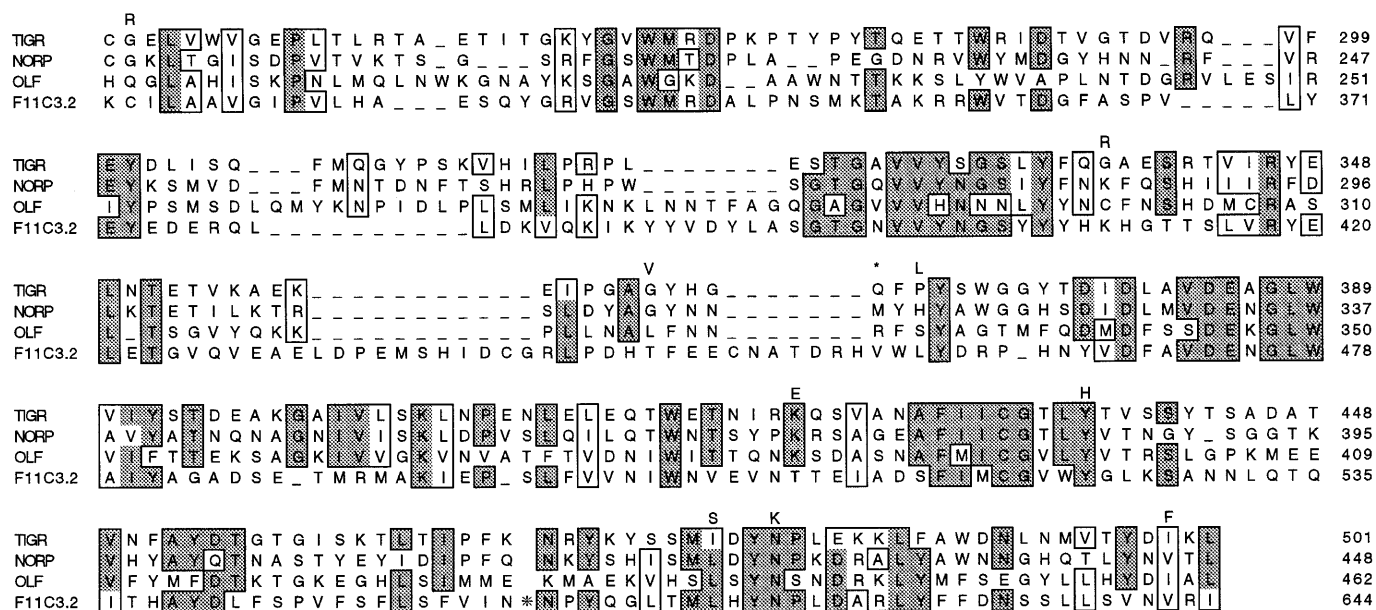


Figure 2. Homology with olfactomedin and evolutionary conservation of the exon III-encoded domain of TIGR. OLF: olfactomedin (bullfrog), NORP: neuronal olfactomedin-related protein (rat), F11C3.2 (*C. elegans*). The human olfactomedin-related brain protein was not used for alignment as it is highly conserved, differing from the rat protein at only five positions. The star in the F11C3.2 sequence indicates a 56 amino acid insertion. Identical residues and conservative changes in three or more of the sequences are shaded and boxed, respectively. Disease-associated mutations shown above the TIGR sequence included the five mutations mentioned in Table 1, the three mutations initially described, G364V, Q368* and Y437H (11) and two other substitutions, K423E, identified in two patients from a French Canadian family (8) and Q337R, characterized in a Scottish family (M.Sarfazai, personal communication).

Five new mutations, different from the three initially described (11), were identified (Fig. 1C and Table 1). All these mutations resulted in non-conservative amino acid substitutions. These mutations were not detected among 150 unrelated controls by dot-blot hybridization of PCR-amplified genomic DNA with sequence-specific oligonucleotides. Furthermore, all but two patients carried the mutation associated with disease in their family. The two exceptions, two ocular hypertensive siblings in Family 3, were previously identified as phenocopies (9). Reciprocally, screening of 208 healthy subjects identified 25 persons who carried the mutation of their family (Table 1). Their age varied from 2 up to 68 years, reflecting the age-dependency of the penetrance of the disease gene. These healthy carriers are at high risk of developing glaucoma.

Analysis of the pattern of recurrence of two mutations

Two of the mutations, P370L and N480K, were seen in two and three families, respectively. To discriminate between a founder effect and a *de novo* recurrence, disease-associated haplotypes across the GLC1A region were determined by typing 15 microsatellite markers (shown in Fig. 1A). This revealed that mutation P370L was associated with two distinct haplotypes in Families 9 and 22 as alleles differed at all markers tested (not shown). Therefore, mutation P370L is likely to have occurred twice independently. In contrast, mutation N480K was associated with a haplotype common to Family 1 and Family 14 (including NGA14 and markers distal to it, $P < 10^{-5}$) and also Family 27 (including NGA15 and markers distal to it, $P < 10^{-6}$). This indicated that this mutation had arisen from a common ancestor, who probably lived in the Pas-de-Calais, in Northern France. Mutation N480K appears to have disseminated to a significant

extent. Family 1 itself was representative of a group of four families with ancestors in the Pas-de-Calais and currently grouping 60 patients who all carry the N480K mutation and share a common haplotype extending over 9 cM, from D1S445 to D1S218 (A.P.Br  zin *et al.*, manuscript in preparation). Furthermore, when 120 unrelated POAG patients, collected regardless of a family history of glaucoma, were screened by dot-blot hybridization for the mutations described here or previously (11), only mutation N480K was detected in one of them. This patient also lived in the Pas-de-Calais, bore the extended haplotype of Family 1, and, in fact, had at least four glaucomatous first-degree relatives. A similar founder effect has also been observed with the I499F mutation which was seen in another family aside from Family 6. Altogether, these findings indicate that both independent occurrence and founding effect can cause recurrence of mutations in POAG families.

Concentration of mutations in the evolutionarily conserved olfactomedin homology domain

Remarkably, the third exon of the TIGR gene appears to be the focus of all pathogenic mutations known to date. In addition to the three initially reported (11) and the five described just above, these mutations also include a Q337R change characterized in a Scottish family (M.Sarfazai, personal communication) and a K423E mutation which we identified in two patients from a French Canadian family (8). Of major interest, the peptidic sequence encoded by TIGR exon III displays significant homology to three other proteins, including frog olfactomedin (18), mammalian brain olfactomedin-related proteins (19) and the F11C3.2 protein from *Caenorhabditis elegans* (Fig. 2). Frog olfactomedin, the first member described of this new protein

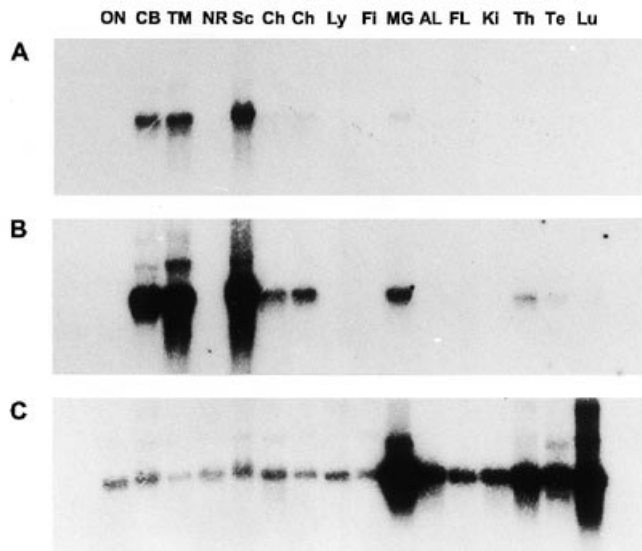


Figure 3. High expression of the TIGR message in the non-neural parts of the eye. Total RNA was prepared from optic nerve (ON), ciliary body (CB), trabecular meshwork (TM), neural retina (NR), sclera (Sc), choroid (Ch), lymphoid cells (Ly) and fibroblasts (Fi). PolyA⁺-enriched RNA was from mammary glands (MG), adult liver (AL), fetal liver (FL), kidney (Ki), thymus (Th), testis (Te), and lung (Lu). After northern blotting, samples were hybridized with a TIGR cDNA probe (A and B, 6 and 26 h exposure, respectively) and re-probed with a β -actin probe (C).

family, shares 35% identical residues with the exon III-encoded TIGR peptide. This homodimer-forming 57 kDa glycoprotein is specifically synthesized in very large quantities in the olfactory tissue by sustentacular cells and acinar cells of Bowman's glands and then exported into the deep mucous layer of the chemosensory surface of the olfactory epithelium. Less is known of the olfactomedin-related glycoprotein which was identified in rat neurons and shows 50% identical residues with TIGR. Finally, the F11C3.2 protein was predicted from computer analysis of nucleotide sequence from chromosome III of *C.elegans*. The significant conservation (~50% sequence similarity) of the olfactomedin-homology domain of TIGR across distant branches of the phylogenetic tree, including mammals, amphibians and nematodes, together with the concentration of the pathogenic mutations at its level, strongly suggests that it plays an important, yet unknown, physiological role.

Intriguingly, some of the mutations do not affect evolutionarily conserved residues. Still, at least two of these mutations (G246R and P370L) consistently determine severe JOAG. This indicates that there is not necessarily a correlation between the evolutionary conservation of a residue and its functional importance, perhaps because from an evolutionary perspective, these non-conserved residues reflect the specialization of the protein.

Expression of the TIGR gene in non-neural parts of the eye

To gain further insight into TIGR function, its expression pattern was studied by northern blot. Total RNA was extracted from the main ocular structures which are putatively involved in the pathogenesis of glaucoma, including the trabecular meshwork, the ciliary body, the sclera, the optic nerve and the neural retina. These RNA samples were migrated together with

polyA-enriched RNA preparations from extraocular tissues and hybridized with a TIGR cDNA probe (Fig. 3). As revealed after a 6 h exposure, one major and abundant 2 kb transcript was expressed in the trabecular meshwork, in the ciliary body and in the sclera. It was also found in the choroid, albeit at a much lower level, but not in the neural retina or in the optic nerve. Among extraocular tissues tested, only the mammary gland, the thymus and the testis expressed the TIGR message. Nevertheless, this extraocular expression was weak compared with ocular expression. These findings indicated that the TIGR gene was very predominantly expressed in the non-neural parts of the eye.

Recently however, the TIGR gene, designated as myocilin, was identified in a retina cDNA library (20). Northern blot analysis using polyA⁺-enriched mRNA from retina revealed a weak expression of TIGR. The protein product was localized in the ciliary rootlet and the basal body of the connecting cilium of the photoreceptor cells. This localization is intriguing as it does not account for the marked increase of intraocular pressure most often associated with GLC1A-linked glaucoma, and also because photoreceptor cells are not damaged in glaucomatous eyes (21).

CONCLUDING REMARKS

Our observation of new TIGR mutations in familial POAG confirms the initial report of Stone *et al.* (11) and further establishes this gene as the cause of GLC1A-linked POAG, including juvenile-onset and adult-onset POAG.

Despite the number of TIGR mutations now known, their diversity might be limited as judged by the recurrence of some of them in several families and by their concentration in the third exon of the TIGR gene. The evolutionary conservation of the peptide encoded by this exon suggests an important function for TIGR. Its high expression in the trabecular meshwork and in the ciliary body, which are the sites of aqueous humor resorption and production respectively, accounts for the major elevation of intraocular pressure often observed in GLC1A-linked POAG. However, its equally high expression in the sclera remains to be explained. Very interestingly, as the primary lesion of POAG is the degeneration of optic nerve fibers, POAG-causing mutations of TIGR, to our knowledge, provide the first example of a hereditary neurodegenerative disease of the central nervous system caused by mutation in a gene expressed in a non-neural cell type.

Our findings also have important clinical implications. Given the simple intron–exon organization of the TIGR gene and the concentration of the pathogenic mutations in the third exon, the search for mutations should be greatly simplified and this should facilitate the identification of at-risk individuals, an essential task as lesions of the optic nerve can be prevented by effective treatments.

MATERIALS AND METHODS

Patients and families

Patients and their families were recruited as described previously (9,17). Their clinical status was determined using previously described standard diagnostic criteria (9,17). Family 3 (9), Families 6, 14, 22 and 23 (families A, C, D, B in ref. 17) have already been presented. A detailed description of Families 1 and 27 will be given elsewhere.

Physical map and polymorphic markers

Based on the YAC contig previously described (10), a BAC contig covering the GLC1A region was constructed. The Research Genetics BAC library was screened by hybridization of high density membranes with existing and newly identified STS probes.

The polymorphic simple repeat markers, NGA11, NGA12, NGA13, NGA14 and NGA15 were generated as previously described (10). NGA17 and NGA19 were identified by sequencing the TIGR gene. These markers were all deposited in GDB. For allele scoring, amplification was carried out with fluorescently-labelled primers and PCR products were size-fractionated on a 6% polyacrylamide-urea gel using an ABI373A automated sequencer.

Characterization of the TIGR gene

The 54D3 BAC was positive for STS derived from the TIGR cDNA sequence. It was subcloned and sequenced. Exon boundaries were recognized by comparison of the genomic and the cDNA sequences. To map the transcription start site, sclera total RNA was reverse transcribed using the CapFinderTM PCR cDNA synthesis Kit (Clontech). The resulting first strand cDNA was submitted to two successive rounds of amplification using the CapSwitch II oligonucleotide as the forward primer for both PCR, and the two nested TIGR specific primers, 1286b (5'-AAGGTGCCACAGATGATGAAGG-3') and 630b (5'-CAGTTAGCTCGGACTTCAGTTCCTGG-3') as reverse primers. Resulting PCR products were subcloned and sequenced.

Mutation analysis

The following seven pairs of primers, coupled to M13 universal and reverse primers, were used to cover the TIGR coding sequence (upper and lower case letters refer to exon and intron located sequences, respectively):

Exon I

fwd: aatctgtctggcagcgtg,	rev: AGCTGGATTGTTGGGAC
fwd: TGCAATGAGGTTCTTCTG,	rev: TCCAACCTCTGTTTGGG
fwd: CAGTCATCCATAACTTAC,	rev: atatcacctgctgaactc

Exon II

fwd: catagtcaatccttgggc,	rev: gggaacagagagagagag
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Exon III

fwd: ggtaagtgtgtcttcg,	rev: AATACGGGAAGTGTCCGTGG
fwd: AGAAGGAAATCCCTGGAG,	rev: CATAAGTGACCATGTTCAAG
fwd: ATTGACTACAACCCCTG,	rev: gctgtgtgaacatgaac

Genomic DNA (100 ng) was amplified using standard conditions. PCR products were gel-purified and aliquots (300 ng) were sequenced using the Amersham dideoxy cycle sequencing protocol in the presence of fluorescently-labelled DYEnamic -21M13 forward and -29M13 reverse primers. Two patients and two control members in each family were sequenced bidirectionally to establish the presence of a mutation. For rapid mutation detection, PCR products were dot-blotted on a nylon membrane and hybridized with a 17mer oligonucleotide probe matching either the wild-type or the mutated sequence. Filters were washed to obtain a sequence-specific signal.

Specimen collection and RNA analysis

Enucleated human eyes from patients with eye diseases other than glaucoma were dissected immediately after sectioning of the optic nerve, whose anterior intraorbital portion was removed and stored. The eye was then opened along the ora serrata and the ciliary body was dissected. The vitreous was removed and the neurosensory retina was detached from the retinal pigment epithelium (RPE). The choroid and the RPE were then dissected. Lastly, the sclera was cleaned from remaining pigmented tissue. One sample of trabecular meshwork was also excised during an evisceration. In addition, trabeculectomy specimens were obtained during standard surgical procedures in patients with glaucoma (22). All fragments were immediately stored in liquid nitrogen.

Total RNA from ocular specimens, from blood mononuclear cells and from fibroblasts was extracted with the acid-phenol method (23). PolyA⁺-enriched RNA samples were purchased from Clontech. Northern blot analysis was carried out using standard procedures.

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