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Exon screening of the genes encoding the β- and γ-subunits of cone transducin in patients with inherited retinal disease

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Purpose: To screen the exons of the genes encoding the β3-subunit (*GNB3*) and γc-subunit (*GNGT2*) of cone transducin for mutations in a large number of unrelated patients with various forms of inherited retinal disease including cone dystrophy, cone-rod dystrophy and macular dystrophy.

Methods: Exons of the two genes were screened for mutations by denaturing gradient gel electrophoresis (DGGE) and/or single strand conformation polymorphism electrophoresis (SSCP); any variants were sequenced directly.

Results : Although many sequence variants were found in both genes, none could be associated with disease. Additionally, the gene structure and sequence of the coding exons of *GNB3* were determined and compared with those of the dog homolog. Both human and canine *GNB3* have nine coding exons and their two predicted amino acid sequences have 97% identity.

Conclusions: The results indicate that *GNB3* and *GNGT2* are unlikely sites of mutations responsible for inherited retinal degenerations that predominantly effect cone-mediated function (cone and cone-rod dystrophies) or have a predilection for disease in the macula (macular dystrophies).

A rare autosomal recessive canine disorder selectively affecting cones (*cd*) was originally identified in the Alaskan malamute dog [1]. Immunocytochemical studies comparing the proteins of pre-degenerate *cd* and normal dog retinas showed initially, the absence of cone-specific β3-transducin subunit. At the same time, there were no differences between pre-degenerate and normal retinas in α-transducin, phosducin, the α-, β- and γ-subunits of cGMP-phosphodiesterase, opsin, S-antigen, IRBP and the middle and short wavelength sensitive cone markers cos-1, and cos-2 [2]. More recently, the cone specific γc-transducin was also shown to be absent from the pre-degenerate *cd* retina [3]. Although, both β3- and γc-transducin are missing from the pre-degenerate *cd* retina, the mRNAs encoding each of the proteins are expressed and show no sequence differences from normal [3,4] suggesting that the primary defect is in another gene.

Cone and rod transducins are both heterotrimeric G proteins consisting of single α-, β- and γ-subunits. Although each of the subunits in cones is similar to its counterpart in rods they are nonetheless distinct. Functionally, however, the two transducins have the same role in the visual response of their corresponding photoreceptors. In both cases the transducin protein is separated into an α-subunit and a β/γ-subunit complex after interaction with light activated opsin. Theα-subunit goes on to activate cGMP-phosphodiesterase causing the breakdown of cGMP and the closing of cGMP-gated cation channels on the plasma membrane. This leads to an interruption of the dark current of the photoreceptor and results in vision. In recovery, the β/γ-subunit complex binds back to the α-subunit, shutting down the cGMP-phosphodiesterase and its concomitant breakdown of cGMP. When cGMP is replenished by guanylate cyclase the dark current is resumed and the visual signal to the brain ends.

The absence and apparent involvement of β3- and γc-transducin in a canine cone disease (even though the primary lesion is in another gene) and their central role in visual transduction makes the human genes encoding these proteins (*GNB3* and *GNGT2*) prime candidates for study in human inherited retinal disease. This is supported by the fact that several other visual transduction proteins have been associated with inherited retinal disease, including opsin [5], the α-subunit of rod transducin [6], the α- and β-subunits of rod cGMP-phosphodiesterase [7-10], arrestin [11], and others [12]. Since single base changes are the most common form of mutation, we adopted the strategy of exon screening of the *GNB3* and *GNGT2* genes.

METHODS

Exon/intron boundaries of the β3- and γc-transducin genes—Using the published sequences of human transducin-β3 cDNA [13] and sequences of the canine *GNB3* gene determined in our laboratory [4], we designed exon to exon primers for polymerase chain reaction (PCR) amplification. For the 5' and 3' UTRs (untranslated regions) we designed primers using sequences from canine exons 1 and 10, respectively. Direct sequencing of PCR products was performed with the Fmol DNA sequencing system (Promega, Madison,WI) with [γ-^{32}P]

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dATP. For exon 1, and introns 1, 8, and 9, it was necessary to first subclone PCR amplified fragments into a plasmid. Plasmid inserts were then sequenced using M13 primers and the Sequenase Kit (USB, Cleveland, OH) with [α-^{35}S] dATP. We compared our results with results taken from a recent 222,930-bp sequencing project spanning chromosome 12p13 and including the *GNB3* gene [14]. Our results confirmed the exon/intron sequence boundaries described. The sequences of the *GNGT2* gene exons and their intron boundaries have recently been published [15].

Sequence comparison of the dog and human *GNB3* coding regions shows that both contain 1020 bp predicting 340 amino acids with 97% identity. Both homologs have 10 exons and 9 introns with exons 2-10 containing the coding region. Except for introns 2 and 8 that are larger in the human, the exons and introns of the dog and human genes are similar in size.

Subjects— Included in this study were 186 unrelated patients with retinal degenerative disease and 51 unrelated control subjects. Controls self reported normal vision; their ages and ethnicities are not known. Table 1 lists the numbers of patients studied by modes of inheritance and clinical diagnoses. For the *GNB3* gene, 164 were screened by single strand conformation polymorphism electrophoresis (SSCP) with two different concentrations of glycerol; for the *GNGT2* gene, 148 patients (126 of which were part of the *GNB3* screening) were screened by SSCP with only one concentration of glycerol and by denaturing gradient gel electrophoresis (DGGE). Exons in which potential mutant sequence variants were present were screened in 30 and 51 controls for *GNB3* and *GNGT2*, respectively. A number of patient and control DNAs were lost after the *GNGT2* screening, and some new patient DNAs were acquired. All participants were fully informed of the nature of the investigations, and the research was performed in accordance to institutional guidelines and the Declaration of Helsinki.

Patients were evaluated clinically and with visual function tests including electroretinography (ERG) and perimetry. The retinal degenerative diseases were grouped into three categories: cone-rod dystrophy (CRD), cone dystrophy (CD) and macular dystrophy (MD). CRD patients had relatively early reduction in visual acuity, later impairment of peripheral vision, and subnormal cone and rod ERGs. CD patients also had progressive visual acuity loss and subnormal cone ERGs but rod ERGs that were normal or near normal. MD patients, although varying in clinical presentation, all had central retinal abnormalities and normal ERGs. In this category were patients with atrophic or hemorrhagic macular degeneration of uncertain type as well as patients with a diagnosis of Stargardt disease/fundus flavimaculatus, defined as an early-onset progressive maculopathy with atrophy and fleck-like lesions [16]. Patients with age-related macular degeneration were not included nor were any patients suspected of having toxic retinopathy.

Exon screening by SSCP and/or DGGE— DNA was extracted from blood samples by standard methods. For SSCP, exons of the *GNB3* and *GNGT2* genes were amplified directly from genomic DNAs in the presence of [α-^{32}P]dCTP with primer pairs that flanked each exon (Table 2). The PCR buffer was at pH 9.0 and contained 1.5 mM $MgCl_2$. The PCR protocol was as follows: 94 ϒX for 2 min followed by 30 cycles of (94 ϒX, 1 min, 60 ϒX, 30 s, 72 ϒX, 45 s) followed by 5 min at 72 ϒX. The amplicons were denatured with NaOH and heat before being electrophoresed in 6% non-denaturing acrylamide gels buffered with 1x TBE (90 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.3). The *GNB3* gene was screened by SSCP alone, but each *GNB3* exon was run in two separate gels; one with 10% glycerol, the other with no glycerol. The *GNGT2* gene was screened by DGGE and SSCP: each *GNGT2* exon was run only in one SSCP gel with 5% glycerol. For DGGE each of the three exons of *GNGT2* was amplified by the same

Table 1. Diagnoses of patients screened for mutations in *GNB3* and *GNGT2*

GNB3 - 164 patients		GNGT2 - 148 patients	
Autosomal dominant	35	Autosomal dominant	35
CRD	24	CRD	22
CD	6	CD	9
MD (S/FF - 0)	5	MD (S/FF - 1)	4
Autosomal recessive/multiplex	37	Autosomal recessive/multiplex	38
CRD	29	CRD	29
CD	2	CD	1
MD (S/FF - 3)	6	MD (S/FF - 5)	8
Simplex	92	Simplex	75
CRD	58	CRD	46
CD	18	CD	18
MD (S/FF - 11)	16	MD (S/FF - 9)	11

The number of patients in the MD category with the diagnosis of Stargardt disease/fundus flavimaculatus (S/FF) are shown in parentheses. Table abbreviations: Cone-rod dystrophy (CRD), Cone dystrophy (CD), Macular dystrophy (MD).

primer pairs as for SSCP, but with the exception that one of the pairs had a "GC clamp" associated with it. The 36-bp "GC clamp" was attached to the primer with the highest GC content; its sequence (Table 2) was kindly provided by Drs. John Nakamoto and Neil vanDop (Department of Endocrinology, UCLA). Each GC-clamped amplicon was then run in two 14% acrylamide denaturing gradient gels (10% to 50% denaturing gradient and 40% to 80% denaturing gradient) in a 60 ϒX bath as previously described [17].

DGGE is a costly, labor-intensive procedure. Consequently, after the screening of the small (3 exons) *GNGT2* gene (by DGGE and SSCP), and before screening the larger (9 coding exons) *GNB3* gene, we compared the DGGE/SSCP screening method with the one involving only SSCP (described above for *GNB3*). Summing this study of *GNGT2* and a previous study we found a total of 12 variants in 25 exons of two genes. In our studies of 77 exons of five genes screened by the SSCP/two gel method we found 35 variants. All genes were screened in a large number of patients. Since the number of variants was about one for every two exons with either method, we screened the *GNB3* gene by the easier SSCP/two gel method.

Table 2. Primer pairs used to amplify the exons of the genes encoding the β3- and γc-subunits of cone transducin

Transducin-β3	
exon 2	CCTCAACACCGACCCCATGTTC
	CCCCCTGGGCCACGCTCCTCAT
exon 3	GCCCTGAAGGCCCATGCACCT
	CGCTATTGCGGGTCGGGGAGCT
exon 4	CGTAACCTGCTCACCCTGATA
	CTCCCCTTCCCGCCTTCCTCA
exon 5	CCGACGCGGCGGACGTT
	GGCGCCCTTAGGAAGGTAGCA
exon 6	GGCTCCGGTCCCATCTCTGCT
	CACTGGAAGCCCAGGGTTCCC
exon 7	ACCCTGGGCTTCCAGTGGGCT
	CCCACTCCCAAGCCCCAGCAC
exon 8	GTGCTGGGGCTTGGGAGTGGG
	TGTGTTGTGGGGAGTGTCGGG
exon 9	CCAGGTCTGATCCCTGACCCA
	CAGCTGGAAGGACCCTCCCCA
exon 10	ACAGGCTGACTCCTTTCCCTC
	GAGATGGGGTCGGGCAGGGGG
Transducin-γc	
exon 1	GGTTTCCAGGCCATCTGCTTTATC
	*AGATCCCAGCCCAGTGCAACCTCA
exon 2	AGAGCTGATGAGATTTATGATCCT
	*CCTGCTCTTCCCTTCCTGTTCCTG
exon 3	CCAGAGACAGTAACAAGGACCTAA
	*GCTTGGCTGAAGAGGGACAGA

*For DGGE these primer pairs had the following 5' GC clamp: GCCGCCCGCCCGCCCGCCGCGCCGCCCGCCGCCCGC

Direct Sequencing of variant exons— The same primers used to amplify exons for SSCP were used to amplify variant exons for sequencing. The amplicons were purified and sequenced directly by the standard dideoxy method using [γ-^{32}P]dATP and the Fmol sequencing kit (Promega).

RESULTS

Exon screening - GNB3 and GNGT2— A total of four sequence variants were found in the *GNB3* gene, and four in the *GNGT2* gene. In *GNB3* they were: (1) an insert of a single G in a cluster of G's starting after the ninth base pair downstream of exon 4 (in intron 4) which did not produce any change in the 14-bp splice site; (2) a GtoA transition 38 bp downstream from exon 4 (and 57 bp upstream from exon 5) which did not create a new or alter an existing branchpoint; (3) a GtoA transition predicting Gly272Ser in exon 9; and (4) a CtoT transition (C825T) predicting Ser275Ser in exon 9. In *GNGT2* they were: (1) a GtoT transversion 31 bp upstream of the transcription start site which did not appear to be in any known regulatory sites; (2) an AtoG transition predicting Gln17Arg in exon 2; (3) a GtoT transversion predicting Leu11Phe in exon 2; and (4) a C deletion at the seventeenth base pair after the stop codon in exon 3. For each of these variants Table 3 shows the number of patients, their diagnoses, the number of controls and whether or not an additional variant is present in the same patient or control.

One AR-MD patient carried the transversion upstream of the transcription start site and the Gln17Arg missense in *GNGT2* and the GtoA transition in intron 4 of *GNB3*. However, these variants did not segregate with disease in the patient's family. Another patient (AR-CRD) carried the transversion upstream of the transcription start site and the C deletion downstream of the stop codon in *GNGT2* and the *GNB3* intron 4 insert. DNAs from this patient's family were not available for testing, but none of his variants appear to predict any significant change to the protein encoded by this gene. The Ser275Ser variant of *GNB3* was present in an approximately equal frequency in patients (53/164 = 32%) and controls (10/30 = 33%). The Gly272Ser of the same gene was also present in approximately equal frequency in patients (12/164 = 7%) and controls (2/30 = 7%). The two variants together were present in five patients and one control. All of the variants found in *GNGT2* were present only in simplex or AR patients. Gln17Arg was present in 9/148 patients (one patient that carried other variants is referred to above) and 4/51 controls. These are approximately equal frequencies of 6% and 8%, respectively. The Leu11Phe was found in a simplex patient and a control. Neither parent of this proband had disease nor was any second variant found in her *GNB3* or *GNGT2* exons.

The GtoT transversion upstream of the transcription start site was present in 8/148 patients (5%) and 1/51 controls (2%). No second variant was found in six of the eight patients; the other two patients are discussed above. Likewise, two of the three patients carrying the C deletion downstream of the stop codon revealed no additional sequence variants, and the other patient is discussed above.

DISCUSSION

The map locations of the *GNB3* gene at human chromosome 12p13 [18] and the *GNGT2* gene at human chromosome 17q21 [15] do not correspond to any currently known loci of inherited retinal disease. However, because the cone specific β3- and γc-subunits of transducin are absent in the pre-degenerate *cd* dog (even though the genes encoding these proteins appear not to be the primary site of the genetic lesion), and because they play significant roles in phototransduction, it was logical to perform mutation analysis of the *GNB3* and *GNGT2* genes in patients with retinal degenerations since there are loci for these diseases that have not yet been mapped.

We were not able to assign disease-causing potential to any of the four variants found in non-coding regions of the two genes we studied. The two intron 4 variants in *GNB3* neither altered existing nor produced new splice sites or branch points. One AR patient carried the GtoA transition in *GNB3* along with two *GNGT2* variants, but these did not segregate with disease in the patient's family. Another patient carried the G insert in *GNB3* along with the upstream (of the transcription start site) transversion and the downstream (of the stop codon) deletion in *GNGT2*, but DNAs from his family were not available for testing. Nevertheless, because the DNA sequences around the two *GNGT2* variants just mentioned did not correspond to any known regulatory sequences we could not assign any deleterious effect to them. Likewise, we could not find disease-causing potential in any of the four variants found in the coding regions of the two genes. The Leu11Phe in *GNGT2* was present in only one simplex patient. Neither of the parents of this patient had disease nor was a second variant found. Additionally, this variant was present in a control subject. The Gln17Arg appears to be a polymorphism being present in equal frequencies in patients (6%) and controls (8%). It was present only in simplex and AR patients, and did not segregate with disease in the family of the one patient that carried other variants. Its presence in four controls (two of the controls were over 50 years) rules out AD disease. Both the Ser275Ser and Gly272Ser were present in relatively equal frequencies in patients and controls suggesting that they too are polymorphisms. Furthermore, neither are in the functional N-terminal or C-terminal binding domains of the transducin-β3 protein.

Table 3. Sequence variants present in the GNB3 and GNGT2 genes

Variant(s)	Patients	Other Variants
GtoA in intron 4 of GNB3	AR-CRD, simplex MD	none found
Gly272Ser/GNB3	Mixed Diagnoses 7/164 patients 1/30 controls	none found
Ser275Ser/GNB3	Mixed Diagnoses 48/164 patients 9/30 controls	none found
Ser275Ser/GNB3	Mixed Diagnoses 5/164 patients 1/30 controls	Gly272Ser
GtoT upstream of transcription start site GNGT2	Mixed Diagnoses 5 AR and 1 Simplex 1/51 controls	none found
GtoT upstream of transcription start site GNGT2	AR-MD	GtoA in intron 4/GNB3 Gln17Arg/GNGT2
GtoT upstream of GNB3 transcription start site GNGT2	AR-CRD	G insert in intron 4/ C deletion downstream of stop/GNGT2
C deletion downstream of stop/GNGT2	AR-CRD, simplex CRD 0/51 controls	none found
Leu11Phe/GNGT2	simplex- CRD 1/51 controls	none found
Gln17Arg/GNGT2	Mixed Diagnoses 2 AR and 6 Simplex 4/51 controls	none found

Therefore, in our screening for exonic mutations in *GNB3* and *GNGT2* in 164 and 148 patients, respectively, with a number of different forms of retinal disease, we found several sequence variants, but none that could be associated with disease. In another study, exons of the gene encoding the α-subunit of cone transducin (GNAT2) were screened in patients with Stargardt disease. As in our case, no sequence variants could be associated with disease [19].

It should be noted that neither *GNB3* nor *GNGT2* (nor any gene for which negative exon screening results have been obtained) can be definitively ruled out as the site of mutations responsible for disease. This is because: (1) the SSCP or DGGE techniques may not detect all sequence variants although this would be a very small percentage; (2) mutations may be present in 5', 3' or intronic sequences not screened; and (3) mutations in this gene may be rare and/or present only in specific diseases such that not enough patients of a particular disease type were screened to detect them. Nevertheless, based on our data, it is unlikely that either the *GNB3* or *GNGT2* genes are common sites of mutations responsible for the inherited retinal diseases of cone dystrophy, cone-rod dystrophy or the (non-age related) macular dystrophies that we tested.

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