MUTATION IN BRIEF

Novel frameshift mutations in *CRX* associated with congenital retinal blindness

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Mutations in *CRX*, a photoreceptor-specific transcription factor, cause congenital retinal blindness (Leber congenital amaurosis), cone-rod dystrophy (CORD), and retinitis pigmentosa (RP), all of which feature severe visual impairment. Upon screening 55 patients with congenital retinal blindness, 75 patients with cone-rod dystrophy, 13 with cone dystrophy, and 36 with recessive or isolate RP for changes in the *CRX* sequence, we found two patients with congenital retinal blindness who carried heterozygously one of two novel frameshift mutations. The first mutation, Tyr191(1-bp del), was a *de novo* change and the second change, Pro263(1-bp del) was inherited from the proband's affected father. Both mutations are predicted to encode mutant versions of CRX with altered carboxy termini. We also found a previously reported missense mutation, Arg41Gln, heterozygously in a 47-year-old patient with a form of RP. The missense change Val242Met was found in an isolate case of CORD and no controls; however, its pathogenicity remains uncertain because only limited segregation analysis was possible. A nonpathogenic missense change, Ala158Thr, was found to be a variant present at relatively high frequency among African-Americans. © 2001 Wiley-Liss, Inc.

KEY WORDS: congenital retinal blindness; Leber congenital amaurosis; cone-rod dystrophy; retinitis pigmentosa; CRX; transcription factor; homeobox gene; mutation detection; inherited retinopathy

INTRODUCTION

CRX (cone-rod homeobox gene; Genbank: AF024711) is a 299-residue transcription factor expressed in the rod and cone photoreceptors of the retina and in the pineal gland of vertebrates (Chen et al., 1997; Furukawa et al., 1997). It belongs to the otd/Otx (orthodenticle homeobox) family of homeobox genes that are involved in the development of anterior head structures of both invertebrates and vertebrates. In addition to the homeobox domain (residues 39-98), CRX shares with other members of the OTX family a 10-residue sequence called the WSP domain (residues 161-170) and the OTX tail located at the carboxy terminus of the protein (residues 284-295)

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(Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997). In the retina, CRX is required to activate the expression of many photoreceptor-specific genes, including genes coding proteins involved in the phototransduction cascade. This activation is achieved synergistically with the transcription factor NRL and possibly with other transcription factors that bind to specific DNA sequences located upstream of the target genes (Chau et al., 2000; Chen et al., 1997; Liu et al., 2001; Mani et al., 1999; Mitton et al., 2000).

Mutations in the *CRX* gene have been previously associated with three hereditary photoreceptor diseases. One of the diseases is called Leber congenital amaurosis (LCA) or congenital retinal blindness (CRB; OMIM: 602225); it is characterized by blindness or severely reduced vision within the first months of life, nystagmus, and absent or barely recordable electroretinographic responses. The second disease, called cone-rod dystrophy (CORD; OMIM: 120970) results in impaired color vision and progressive reduction of central vision acuity with profound loss of cone ERG amplitudes and some reduction of rod ERG amplitudes in the early stages (Berson et al., 1968). In the third disease, retinitis pigmentosa (RP), rod photoreceptors degenerate more rapidly or a comparable rate than cone photoreceptors. The visual loss in CRB, CORD, and RP is due to maldevelopment, malfunction, and/or degeneration of both cone and rod photoreceptors.

MATERIALS AND METHODS

Ascertainment of patients

All index cases in this study were examined by one of the authors (E.L.B., G.A.F. or A.B.F.), who made the diagnosis of CRB, CORD, cone dystrophy, or RP through ophthalmologic examination including visual field tests and electroretinography. Patients known to have pathogenic mutations in the *RPE65* gene were excluded; of the 55 patients with CRB in this study, 34 had been previously screened for *RPE65* mutations. None of the patients with CORD or a related disease were previously screened for *RPE65* mutations. Modality of inheritance of the diseases was established by family history. Informed consent was obtained from all participants before they donated 2 to 30 ml of their intravenous blood for this research, in accordance with our respective institutional guidelines. DNA was prepared from peripheral blood leukocytes by standard procedures. Blood samples were also obtained from control individuals with no visual symptoms and no known blood relatives with hereditary retinal degeneration.

Mutation detection

CRX exons were PCR amplified from genomic DNA using the primers described by Sohocki et al. (1998), with the exception of the upstream primer for exon 2, the sequence of which was 5'-GGATGGAATTCTTGGCATCCCAC-3'. ^{33}P-radiolabeled PCR products were investigated for mutations by standard SSCP experiments, after restriction with enzymes *Rsa*I (exon 2), *Sty*I (exon3a), *Hha*I (exon 3b), or *Bsa*HI (exon 3c). All samples showing abnormal SSCP patterns were subsequently amplified a second time and sequenced with an ABI Prism 377 sequencer in both sense and antisense directions.

For analysis of parentage, we analyzed 9 microsatellite markers previously reported to be highly sensitive in detecting incorrect parentage using minor modifications of the methods described in that report (Alford et al., 1994; Hammond et al., 1994).

RESULTS

We evaluated 55 patients with CRB, 75 patients with CORD, 13 with cone dystrophy, and 36 with recessive or isolate RP. All patients and controls were unrelated. All variants found in our set of patients are listed in Table 1. Three patients were found to have likely pathogenic mutations that were not present in 88-103 normal controls: two patients with CRB had novel mutations and one patient with RP had the missense change Arg41Gln that has been previously reported (Sohocki et al., 1998; Swain et al., 1997). One patient with RP was found to have the previously reported mutation Val242Met of uncertain pathogenicity.

One novel mutation, Tyr191(1-bp del), was found heterozygously in an isolate case of CRB. This patient had severely reduced vision and wandering eye movements from birth. Mental and physical development at age 4 years were normal. Visual acuity was possible light perception. The patient was hyperopic (+ 6.00 sphere in both eyes). The fundi had pale disks, granularity in the maculas, no foveal reflexes, and attenuated retinal vessels. Analysis of leukocyte DNA from both parents and an unaffected sibling showed that none of these family members carried the mutation. Analysis of 9 highly informative microsatellite markers indicated that the

designated parents were highly likely to be the affected child's biological parents ($p > 0.997$). Possible mosaicism in the parents was evaluated by digesting the amplicon obtained from their leukocyte DNA and containing codon 191 with the restriction endonuclease *Nla*III which has a recognition sequence created by the Tyr191(1-bp del) mutation. We could not detect a partial digestion of the amplified fragment through the analysis of either double or single stranded fragments in non-denaturing polyacrylamide gels (data not shown). We concluded that the mutation was *de novo*. The alteration of the translational frame caused by Tyr191(1-bp del) leads to a truncation of the C-terminus of the CRX protein because of a new stop 2 codons downstream.

The second novel change, Pro263(1-bp del), was found heterozygously in a proband and his affected father, both of whom reported poor vision since birth. The proband's unaffected mother did not carry the mutation; the proband had no siblings. The proband (examined at age 24) and his father (examined at age 44) had vision of light perception or less. Both had keratoconus. The fundi had pale disks, attenuated retinal vessels, and retinal pigment deposits. The deletion of one base at codon 263 alters and extends the carboxy terminus of CRX, creating a mutant version of the protein with 369 residues rather than the normal 299. The carboxy-terminal 107 residues of this mutant would be identical to that produced by Leu237(1-bp del), a previously reported mutation also causing CRB (Silva et al., 2000).

The missense mutation Arg41Gln was present heterozygously in one patient with RP. The patient was the offspring of parents who were second cousins, but there was no family history of retinal degeneration. The patient reported night vision deficiency since childhood and additional visual difficulties beginning in his late 30's (e.g., trouble tracking the ball when playing tennis). At age 47, the patient had corrected visual acuity of 20/200 (right eye) and 20/40 (left eye); his refractive error was -0.75 spherical equivalent in both eyes. The patient had slight constriction of his peripheral visual fields and had a central scotoma 20° in diameter measured with the I4e test light in a Goldmann perimeter in both eyes. The right macula had an atrophic region involving the fovea; the left macula had a granular appearance. The arterioles were minimally attenuated and there were rare bone spicules. The rod-plus-cone ERG amplitude in response to 0.5-Hz flashes of light was 77 μV, and the cone ERG amplitude in response to 30-Hz flashes of light was 59 μV (both amplitudes are averages between the two eyes; normal amplitudes are ≥ 350 μV and ≥ 50 μV, respectively). The cone ERG implicit time was abnormally prolonged (40 msec in each eye; normal is ≤ 32 msec) indicating a progressive retinal degeneration.

Another change, Val242Met, first detected by Swain et al. (1997) in a CORD patient, was also found heterozygously in an isolate RP patient from our set. At age 43 years, this African-American patient had corrected visual acuity of 20/40 (right eye) and 20/400 (left eye) (average refractive error = -8.8 diopters spherical equivalent). The patient had slight constriction of his peripheral visual fields and no central scotomas with the V4e test light. The fundi showed attenuated arterioles. The rod-plus-cone ERG amplitude in response to 0.5-Hz flashes of light was 150 μV, and the cone ERG amplitude in response to 30-Hz flashes of light was 22 μV. The cone ERG implicit time was 38 msec. Segregation analysis was limited to the analysis of DNA from the proband's mother and one of the siblings. These relatives, both unaffected, did not carry the mutation. This change was not present among a group of 103 normal control individuals, including 15 unrelated controls who were African-American.

In addition to these pathogenic *CRX* mutations, we found a nonpathogenic change, Ala158Thr, in an African-American patient with CRB. This change had been previously reported as polymorphism (allele frequency = 0.02, based on 55 normal controls of unspecified race) (Swain et al., 1997). It was also reported in one Caucasian patient with CRB (Swain et al., 1997; Swaroop and Sieving, pers. comm.). We investigated the Ala158Thr change by analyzing 15 unrelated African-Americans and found 3 heterozygous carriers (allele frequency = 0.1). Altogether, these data suggest that Ala158Thr is a polymorphism that is relatively common in African-Americans.

DISCUSSION

Almost all *CRX* mutations so far identified have been found to be dominant, since they were detected as *de novo* mutations found in heterozygotes or in families with a history of dominantly inherited retinal disease. We report two novel mutations in patients with CRB that also appear to be dominant: Tyr191(1-bp del) and Pro263(1-bp del). Tyr191(1-bp del) was found to originate as a *de novo* event, whereas Pro263(1-bp del) was identified in a family with dominant CRB. Both mutations are single base-pair deletions within the last exon of the *CRX* gene. Since there is no intron located downstream of these frameshifts, it is likely that the alleles are translated to mutant CRX proteins with an abnormal carboxy terminus (Hentze and Kulozik, 1999). Like these two novel mutations, all previously reported pathogenic frameshifts in *CRX* that have been clearly associated with retinopathy are located within the last exon and would be expected to result in defective versions of the CRX protein [Rivolta et al.;

companion manuscript]. This pattern suggests that the frameshifts cause disease by a mechanism other than by haploinsufficiency.

We found the mutation Arg41Gln in a patient with RP with macular involvement as an early feature. Like previously reported patients with this mutation (Sohocki et al., 1998), our case had a reduction in the rod ERG amplitudes and markedly delayed cone implicit times. Abnormal rod and cone function and central scotomas were also present in patients with a different *CRX* mutation involving the same codon, Arg41Trp (Swain et al., 1997). In view of the roughly comparable rod and cone malfunction in patients with *CRX* mutations involving codon 41, the phenotype is probably better described as a form of RP rather than CORD.

The pathogenicity of the Val242Met allele remains uncertain. Swain et al. (1997), who encountered this change in an isolate patient with CORD, were not able to perform segregation analysis. They did not find this missense among 192 normal controls, but the possibility remained that Val242Met was a rare nonpathogenic variant fortuitously not present in their set of controls. The segregation analysis we performed on the family of our proband with this change was consistent with its pathogenicity, but the small family size prevented a definitive conclusion regarding this allele's pathogenicity. We also did not find this change among controls; however, even when one combines our data with that from Swain et al. (1997), there is no statistically significant association of Val242Met with retinal degeneration (2 affected patients with the mutation out of 259 analyzed versus 0 with the mutation out of 280 controls; $p = 0.23$ with Fisher's exact test).

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Table 1. Patients with sequence variants in *CRX*

Patient ID	***Diagnosis***	***Exon/intron***	***Amino acid change***	***Sequence change‡***	***Pathogenic****	***References†***
Many		IVS1		IVS1 +12 C to T	no	1
007-051	Isolate RP	2	Arg41Gln	CGG to CAG	yes	1,2
289-026	CORD	IVS2		IVS2 -18 T to A	no	
182-007	Cone dystrophy	IVS2		IVS2 -15 G to A	no	2
286-001	Rod monochromacy	3	Gly122Asp	GGC to GAC	no	3
048-079	CRB	3	Gly122Asp	GGC to GAC	no	3
048-076	CRB	3	Ala158Thr	GCC to ACC	no	1
048-084	CRB	3	Tyr191(1-bp del)	TAT to -AT	yes	
003-299	Recessive RP	3	Ser199Ser	TCC to TCT	no	2
289-017	Isolate RP	3	Val242Met	GTG to ATG	uncertain	1
048-005	CRB	3	Pro263(1-bp del)	CCC to CC-	yes	

‡ All sequence changes were found in heterozygotes except for the polymorphism IVS1 +12 C to T which was found in homozygotes and heterozygotes.
* Mutations were considered nonpathogenic if there was convincing evidence for such in the cited previous reports.
† Previous reports of the same sequence change: 1) Swain et al., 1997; 2) Sohocki et al, 1998; 3) Sohocki et al., 2001.