Carrier of R14W in Carbonic Anhydrase IV Presents Bothnia Dystrophy Phenotype Caused by Two Allelic Mutations in RLBP1

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PURPOSE. Bothnia dystrophy (BD) is an autosomal recessive retinitis pigmentosa (arRP) associated with the c.700C>T mutation in the RLBP1 gene. Testing of patients with BD has revealed the c.700C>T mutation on one or both alleles. The purpose of this study was to elucidate the underlying genetic mechanisms along with a clinical evaluation of the heterozygous patients with BD.

METHODS. Patients with BD heterozygous for the RLBP1 c.700C>T were tested for 848 mutations by arrayed primer-extension technology. Further mutation detection was performed by PCR-restriction fragment length polymorphism (RFLP), sequencing, denaturing (d)HLPC and allelic discrimination. The ophthalmic examinations were performed in all c.700C>T heterozygotes.

RESULTS. The clinical findings in 10 BD heterozygotes were similar to those in the homozygotes. The presence of a second mutation, c.677T>A, corresponding to p.M226K was detected in all 10 cases. Segregation analysis showed that the mutations were allelic, and the patients were compound heterozygotes [c.677T>A]+[c.700C>T]. One of those patients was also a carrier of the c.40C>T corresponding to the p.R14W change in carbonic anhydrase IV (CAIV) associated with autosomal dominant RP, RP17. His mother, a carrier of the identical change was declared healthy after ophthalmic examination. This sequence variant was found in 6 of 143 tested blood donors.

CONCLUSIONS. The high frequency of arRP in northern Sweden is due to two mutations in the RLBP1 gene: c.677T>A and c.700C>T. BD is caused by the loss of CRALBP function due to changed physical features and impaired activity of retinoid binding. The CAIV p.R14W sequence variant found in one of the patients with a BD phenotype is a benign polymorphism in a population of northern Sweden. (Invest Ophthalmol Vis Sci. 2008;49:3172–3177) DOI:10.1167/iovs.07-1664

Retinitis pigmentosa (RP) is a clinically and genetically heterogeneous group of retinal dystrophies. RP loci have been mapped to numerous chromosomal localizations. At least 26 disease causative genes have been identified, and 14 loci have been mapped.1,2 A rather large group of patients with a variant of RP, Bothnia dystrophy (BD) (MIM 180090; Mendelian Inheritance in Man, National Center for Biotechnology Information, Bethesda, MD) has been identified in northern Sweden.3,4 The phenotype is characterized by night blindness in early childhood, retinitis punctata albescens (RPA) in young adulthood, and progressive macular and peripheral retinal degeneration.4 The disease is associated with a c.700C>T mutation in the RLBP1 gene resulting in an amino acid substitution p.R234W in the cellular retinaldehyde-binding protein (CRALBP) found in a homozygous state in affected individuals (amino acid numbering is based on the primary translation product where the translation initiator Met is +1, although it is not present in the mature protein) (http://www.hgvs.org/mutnomen/recsprot.htm).5 So far, a few cases of retinal degeneration associated with mutations in the RLBP1 gene have been reported. The p.R151Q missense mutation was found in the homozygous state in a consanguineous family of Indian origin and in one of a consanguineous kindred from Saudi Arabia diagnosed with RPA.6,7 Three additional mutations in the RLBP1 gene have been reported in the patients of European ancestry, who also demonstrated a phenotype distinguishable from classic RP.8 Two splice junction mutations were found in Newfoundland families with a severe rod–cone dystrophy (NFRCD).9,10 Most of the reported cases carried RLBP1 mutations in homozygous state although compound heterozygotes have also been described.9-12 One of compound heterozygotes, a Japanese patient with diagnosed retinitis punctata albescens (RPA) carried the c.700C>T mutation on one allele.11 However, sequence changes involving single nucleotides are not the only type of mutation that affects the RLBP1 gene. In a patient with RPA, a large homozygous deletion was recently described.13 In an inventory work on retinal dystrophies in northern Sweden, we found a relatively large group of patients with an autosomal recessive form of retinitis pigmentosa (arRP) of Bothnia type associated with the c.700C>T mutation. In addition to individuals homozygous for the c.700C>T mutation, patients heterozygous for the same mutation were identified. This observation led us to initiate an extended genetic analysis to clarify the underlying genetic mechanisms, along with a clinical evaluation of the c.700C>T heterozygous patients.

METHODS

Patients

Patients with a history of night blindness and a clinical diagnosis of arRP who resided in the four counties of northern Sweden with a population of 880,000 were included in the study. Of almost 200

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persons, DNA was available from 121. The study adhered to the tenets of the Declaration of Helsinki, and consent was obtained from all individuals.

**Molecular Genetic Analysis**

**DNA Extraction.** Extraction of genomic DNA was performed as described by Balciuniene et al.14

**PCR, Sequencing, and c.700C>T Testing.** PCR on genomic DNA was done as described elsewhere with primers previously reported.5 A standard temperature profile for PCR comprised initial denaturing at 95°C for 4 minutes, followed by 30 cycles consisting of denaturing at 95°C for 45 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. The final extension was at 72°C for 5 minutes. Gene-specific primers (20 μM) were used to amplify 100 ng of genomic DNA in a 20 μL-amplification mixture containing 10× PCR reaction buffer, 2 mM dNTP mix, and 0.3 U Taq-polymerase (Sigma-Aldrich, St. Louis, MO).

To detect RLBP1 c.700C>T mutation PCR fragments amplified with primers 5′-7R-3′ were separated on 3% agarose gel (NuSieve; FMC BioProducts, Rockland, ME) after digestion with MspI, taking an advantage of the fact that the c.700C>T mutation abolishes a recognition site for the MspI endonuclease.

The sequencing reactions were performed (Big Dye Terminator, ver. 3.1 Cycle Sequencing Kit; Applied Biosystems, Inc. [ABI], Foster City, CA) in a final reaction of 10 μL. The products of the sequencing reactions were run on a DNA analyzer (3730 xl; ABI).

**Arrayed Primer Extension (APEX) Analysis.** DNA from two patients with BD carrying the RLBP1 c.700C>T mutation were subjected to microarray genotyping designed and manufactured according to the APEX technology.15 (http://www.apexport.com). Genetic testing was performed using the aRP array for both patients, and in addition patient 223:3 was analyzed with the autosomal dominant retinitis pigmentosa (adRP) array, taking into account that the only one mutation was detected in the patients with BD. The aRP array included testing, performed according to the manufacturer’s instructions, for 501 known mutations in 16 genes (CERKL, CNGB1, CNGB3, MERTK, PDE6A, PDE6B, PNR, RH12, RGR, RLBP1, SAG, TULP1, CRB, RPE65, USH2A, and USH3A). adRP panel comprised 347 mutations in 13 genes (CA4, FSCN2, IMPDH1, NLR, PRP3, PRPF31, PRPF8, RDS, RH0, ROM1, RPL1, RP9, CRX).

**Allelic Discrimination Assay for RLBP1 c.677T>A Detection.** Two allele-specific MGB probes (TaqMan Assay-by-Design; ABI) were used. The primer and probe sequences are the following: forward primer 5′-CGGACGTCACTGACTGGAAGATG, reverse primer 5′-CCACGCTGGAGGGCT; reporter, 5′-VIC-CTGGAGCATGTCCA; and quencher, ACCTGGAGCGTTCCT-3′. The assay was performed as described by the manufacturer, with 2× PCR master mix (Universal; ABI), 200 nM FAM-labeled probe, 150 nM VIC-labeled probe, 900 nM of forward and reverse probe, 20 ng of genomic DNA in a 10-μL final volume. The PCR program included one cycle at 50°C for 2 minutes to activate uracil-N-glycosylase, one cycle at 95°C for 10 minutes; and 40 cycles of 92°C for 15 seconds for denaturing and 60°C for 60 seconds for annealing/extension. Color quantification and genotype generation was performed using an automated DNA analyzer (model 7000; ABI).

**Denaturing (d)HPLC Analysis.** The presence of the CAIV c.40C>T sequence variant was detected by temperature-modulated heteroduplex analysis (TMHA)16–18 (Wave Nucleic Acid Fragment Analysis System; Transgenicom, Omaha, NB) performed on PCR products containing exon 1 of the CAIV gene. The PCR primers were the following: forward 5′-cGGCTCCCGCTATTAAACCCCG and reverse 5′-ccggagactcagctgc-3′. The manufacturer’s software (Wavemaker; Transgenicom) was used for the prediction of melting characteristics. Chromatography was performed with buffers A, aqueous solution of 0.1 M triethylammonium acetate and solvent B, 0.1 M triethylammonium acetate in 25% acetonitrile. Chromatograms were recorded at 260 nm. The chromatogram of the wild-type ampiclon served as a reference for mutation calling. Samples with additional peaks or with a different peak appearance were scored as positive. These samples were analyzed by PCR-fragment restriction length polymorphism (RFLP) or direct sequencing.

**Mutagenesis and Production of the rCRALBP Dual Mutant p.[M226K;R234W].** Mutant rCRALBP cDNA carrying both the c.677T>A and c.700C>T substitutions were created using site-directed mutagenesis method (QuickChange; Stratagene, La Jolla, CA). Mutant human CRALBP cDNA carrying c.700C>T in the petT19b vector19 was used as a template to replace a Met with a Lys (residue 226). The following complimentary oligonucleotides were used: sense 5′-GAAGATGTTGGAAAAGTGTCCTGCTCTCCGAGATTG -3′ and antisense 5′-GAATCCGGGACTGTCTGCTCCACCATCCTG-3′.

Mutant for the c.677T>A clones were identified by restriction analysis with NsiI. The presence of both mutations was also verified by sequencing in both directions with the gene- and vector-specific primers.

**Clinical Examination**

Ten patients heterozygous for the c.700C>T mutation belonging to eight families were clinically evaluated. Eight patients had a routine clinical ophthalmic examination. In two cases, data were obtained from medical records. Standard full-field, single flash, and flicker ERGs were recorded (UTAS-E 2000; LKC Technologies Inc., Gaithersburg, MD) using Burian-Allen bipolar electrodes according to standard clinical procedures and the recommendations of the ISCEV (International Society of Clinical Electrophysiology of Vision)20 in selected cases. In addition the parents of case 223:3 (223:1 and 223:2, 62 and 61 years old) underwent ophthalmic examination and the full-field ERG was performed in 223:2.

**Results**

**Molecular Findings**

One hundred twenty-one individuals affected with arRP were tested for the presence of the c.700C>T mutation. PCR-RFLP analysis showed that 67 of them were homozygous and 10 were heterozygous for the c.700C>T mutation.

**arRP Genetic Testing**

Simultaneous evaluation of 501 mutations known as a cause of arRP was performed by arrayed APEX technology. Testing of two patients with BD (027:4 and 225:3, Fig. 1A) with the arRP panel revealed, besides the RLBP1 c.700C>T (p.R234W) mutation known to us, a second mutation, c.677T>A, resulting in p.M226K. This mutation abolishes an NsiI restriction site, and therefore all 10 persons heterozygous for the c.700C>T were tested by PCR-RFLP and demonstrated the presence of the c.677T>A mutation. DNA was available in six cases. Segregation analysis in all tested families showed that 700C>T and c.677T>A were allelic and that the patients were compound heterozygotes, c.[677T>A]+[700C>T] (Fig. 1). None of patients with BD homozygous for the c.700C>T mutation tested by allelic discrimination carried the c.677T>A mutation (data not shown). Allele frequency was determined by the same test in a matched control population; one carrier of the c.677T>A mutation was found among 233 tested individuals. The screening of the RP population from northern Sweden also revealed two homozygotes for the RLBP1 c.677T>A mutation.

Wild-type and mutant dual-mutant rCRALBP carrying both p.M226K and p.R234W were expressed in bacteria and the recombinant proteins purified. SDS-polyacrylamide gel electrophoresis of the crude soluble bacterial lysates and resuspended pellet fractions showed that the dual mutant was less soluble than wild-type rCRALBP (Fig. 2).

adRP Genetic Testing

Testing of a patient with BD (223:3) with the adRP panel, which included 347 known mutations in 13 genes, resulted in detection of only one sequence change: c.40C>T in exon 1 of RLBP1 which leads to amino acid substitution p.R14W. The p.R14W mutation was reported as a cause of adRP, RP17.21,22

The identification of this sequence variant prompted us to test all compound RLBP1 heterozygotes for the presence of c.40C>T. The c.40C>T incorporates an MscI restriction site; therefore, all patients of interest were tested by digestion of PCR products amplified with designed primers (the Methods section; data not shown). Nine carriers of RLBP1 c.[677T>A]+[700C>T] mutations were negative for CAIV c.40C>T, whereas its presence was confirmed only in 223:3. The mother to 223:3 (223:2) was also a CAIV c.40C>T carrier. Testing of 143 healthy blood donors by TMHA followed by sequencing revealed six carriers of CAIV c.40C>T (Fig. 3).

Clinical Findings

Ten patients, compound heterozygous for the RLBP1 c.[677T>A]+[700C>T] mutations presented a nonsyndromic type of retinal degeneration. Clinical data are summarized in Table 1. In the two youngest patients (case 242:7, age 7, and 243:3, age 27), visual acuity (VA) was preserved with no signs of advanced maculopathy. The 31-year-old man (case 223:3) had low VA. At examination of his fundus subtle macular mottling and RPA changes were present as well as degenerative pigmented changes in the peripheral retina (Fig. 4). With increasing age, all examined patients presented low VA with maculopathy and progressive retinal degeneration. The two elder siblings, 52 and 64 years old (20:1 and 20:2; Table 1), presented maculopathy with generalized peripheral atrophy of the retina. The oldest patient, aged 84, had only light perception with advanced retinal degeneration, narrow vessels, and a pale optic disc (211:1, Table 1).

All patients reported night blindness as an early symptom of the disease. The eight youngest subjects underwent full-field standard ERGs. In all cases examined, the scotopic rod responses were nonrecordable and the mixed rod-cone responses were severely reduced. The photopic responses, the cone b-wave, and the 30-Hz flicker of the full-field ERGs were subnormal in the younger patients, with reduced amplitudes and prolonged implicit time. Both parents of patient 223:3 were examined. The father, a carrier of the RLBP1 c.677T>A mutation (223:1, Figs. 1A, 1C) presented normal ophthalmic examination and the mother, a carrier of the RLBP1 c.700C>T and the CAIV c.40C>T mutations (223:2, Figs. 1A, 1B) demonstrated normal ERG responses of both the rods and cones.

Figure 2. SDS-PAGE of the rCRALBP dual mutant p[R234W;M226K] and the wild type. Crude bacterial lysates (~15 μg total protein) and purified recombinant proteins (1-2 μg each) from cells expressing wild-type rCRALBP and the dual mutant were analyzed by SDS-PAGE on a 12% gel and stained with Coomassie blue. (A) Wild-type rCRALBP purified under native conditions. (B) Wild-type rCRALBP purified under denaturing conditions. (C) Dual p.[R234W;M226K] mutant rCRALBP purified under native conditions. (D) Dual p.[R234W;M226K] mutant rCRALBP purified under denaturing conditions. U, noninduced resuspended pellet; I, induced resuspended pellet; E1-E5, purified protein elution fractions 1 to 5; MW, molecular weight markers.

Figure 1. Allelic c.677T>A and c.700C>T mutations in families to probands diagnosed with arRP of Bothnia dystrophy. Detection of both mutations was done by PCR-RFLP. (A) Pedigree charts of five families. Filled symbols: affected individuals; empty symbols: unaffected individuals. (B) PCR with primers 7F and 7R followed by digestion with the MspI enzyme. c.700C>T mutation abolishing an MspI restriction site results in a fragment of 215 bp. (C) PCR with primers 6F and 6R followed by restriction digestion with the NspI enzyme. c.677T>A mutation abolishing an NspI restriction site results in a fragment of 310 bp.
C.40C mutation. To date, at least 40 causative genes and loci have been identified, and an identical underlying genetic defect. Sixty-seven control cases, from which four carriers of the c.40C>T mutation are presented in chromatogram (A) and scatterplot (B). The overlay of most of the chromatograms represents the profile of the wild-type homoduplex. Four chromatograms demonstrate different peak appearance, indicating the presence of the CAIV c.40C>T mutation as confirmed by digestion or direct sequencing.

DISCUSSION

Retinitis pigmentosa is a group of inherited retinal dystrophies with loss of photoreceptors characterized by night blindness and progressive loss of peripheral vision often resulting in severely decreased visual function. The prevalence of nonsyndromic RP is approximately 1:4000 worldwide. However, in Västerbotten County in the northern part of Sweden, it is 1:2500. This can be explained by the founder effect, the isolation of population in small villages, and the low migration rate. To date, at least 40 causative genes and loci have been identified in nonsyndromic RP, but one can expect that only a limited number of mutated genes causes retinal degenerations in such populations as the northern Swedish. We identified a large group of patients with similar clinical appearance and an identical underlying genetic defect. Sixty-seven patients have a biallelic mutation in the RLBP1 gene, c.700C>T (p.R234W). In 10 patients with a phenotype similar to BD molecular testing revealed the c.700C>T mutation on only one allele. Several disease models such as uniparental disomy, digenic inheritance, and compound heterozygosity were taken into consideration. A genome-wide scan of families to RLBP1 c.700C>T heterozygous patients with BD did not show a statistically significant link to any chromosomal region due to low statistical power based on the small number of affected individuals in these families. However, haplotypes reconstructed by using microsatellite markers and SNPs in close proximity and in the RLBP1 gene were identical in all patients, which strongly indicates impairment of the RLBP1 gene and also excludes uniparental disomy and a large genomic deletion (data not shown).

In two c.700C>T heterozygous patients with BD, we found a second mutation, c.677T>A by using APEX technology. The presence of the c.677T>A corresponding to p.M226K was detected in 10 c.700C>T heterozygous patients with BD. Segregation analysis in five families showed that these mutations were allelic. Based on the allele frequency of c.700C>T (3/200) and c.677T>A (1/233) in a control population, we expect that c.700C>T was the first mutation to appear in northern Sweden. The 10 compound heterozygotes comprise a smaller group compared with the group of patients with BD reported earlier; however, their clinical evaluation and comparison with c.700C>T homozygotes revealed similarity in phenotypic findings such as night blindness, a decline of VA, the fundus appearance, and the results of electrophysiological examinations. Nevertheless, more thorough clinical examinations are needed for evaluation of the BD severity and the disease progression in both compound heterozygotes and the c.677T>A homozygotes.

CRALBP protein encoded by RLBP1 is mainly expressed in the RPE and Müller cells of the retina, where it functions as a carrier of 11-cis-retinol and 11-cis-retinal and thereby plays an important role in the visual cycle. During the visual cycle all-trans-retinal is converted to 11-cis-retinal in the retinal pigment epithelium (RPE) and is then transported back to the photoreceptor cells. CRALBP stimulates the enzymatic isomerization of all-trans- to 11-cis-retinal and, in vitro, facilitates the oxidation of 11-cis-retinol to 11-cis-retinal, retards 11-cis-retinol esterification, and mediates the hydrolysis of endogenous RPE 11-cis-retinyl ester.

Structure–function studies of recombinant p.M226K and p.R234W demonstrated differences in their ability to bind 11-cis-retinaldehyde (pM226K and R233W in PM226K rCRALBP was less soluble than wild-type protein and completely abolished binding to 11-cis retinaldehyde as did

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VA, visual acuity; CF, counting fingers; HM, hand movements; P, light perception; RPA, retinitis punctata albscens; +, findings present; –, findings not present; SM, subtle mottling of the macula; AM, macular atrophy of the macula.
rCRALBP carrying the R151Q mutation found in an atypical form of arRP in Indian families (R150Q). However, functional study on the recombinant p.R234W protein showed that the mutant protein was soluble and did not abolish interaction with the ligand. On the contrary, the p.R234W mutation led to increased binding of 11-cis and 9-cis retinal revealing at least a twofold higher affinity of the p.R234W mutant for retinoids compared with the wild-type protein. The presence of both mutations in patients with BD results in impaired function of CRALBP in the visual cycle. To access the role of both mutations, we expressed dual mutant in bacteria being aware that CAIV is not expressed in the affected individuals and such protein is not expressed in the affected individuals and showed that it was less soluble than wild-type protein which prevented the experiments on retinoid binding.

The initial data indicating the presence of the p.R234W alone in patients with BD led us to test for known mutations in the genes associated with adRP. Therefore, one of the p.R234W heterozygotes was tested for 347 mutations in 13 adRP genes and was found to be a carrier of the p.R14W mutation in CAIV, a mutation associated with adRP in South African families of European ancestry. Since CAIV is not expressed in the retina but is found in the choriocapillaris, the disease RP17 was proposed to be caused by apoptotic damage of nutrition cells, due to accumulation of unfolded proteins in the endoplasmic reticulum. Decreased level of physical interaction of the mutant R14W protein and impaired pH homeostasis was described as an alternative mechanism of RP17 evolution. To avoid confusion, we screened all 10 c.[677T>A]+[700C>T] patients with BD and 143 healthy control individuals for R14W. It appeared that this sequence variant was present in 4% of the population from northern Sweden. None of the RLBPI compound heterozygotes had CAIV of R14W heterozygous patient 223:3.

The phenotype of the RLBPI compound heterozygote (223:3) also carrying CAIV p.R14W could not be distinguished from the other patients with BD. In addition, we could not detect any signs of retinal degenerative changes in his 61-year-old mother carrying the same sequence variant. Whether any modifier genes switch off the mutant R14W protein or any factors resist its function remains to be investigated, but based on the results of the present study, it does not seem to be a pathogenic change, at least in population of northern Sweden.

In summary, the high frequency of arRP observed in northern Sweden is due to the presence of two mutations in the RLBPI gene: c.677T>A and c.700C>T. The patients are either homozygotes or compound heterozygotes. All 79 patients originating from Västerbotten County, with a population of 257,000 inhabitants, presented the BD-like phenotype. We conclude that Bothnia dystrophy is caused by the loss of CRALBP function due to changed physical features and impaired activity of retinoid binding. p.R14W in CAIV, reported as a cause of RP17, is not pathogenic of Bothnia dystrophy.

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References


