

DISEASE-ASSOCIATED VARIANTS OF THE ROD-DERIVED CONE VIABILITY FACTOR (RdCVF) IN LEBER CONGENITAL AMAUROSIS

Rod-derived cone viability variants in LCA

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1. INTRODUCTION

Leber congenital amaurosis (LCA) is the most early and severe form of all inherited retinal dystrophies, responsible for congenital blindness. The genetic heterogeneity of LCA has been accepted for a long time but it turned out to be largely higher than all odds. So far, 11 genes have been mapped on human chromosomes and eight identified. *i*) the retinal specific guanylate cyclase gene (GUCY2D, retGC1; 17p13.1; LCA1; MIM 600179), *ii*) the gene encoding the 65-kD protein specific to the retinal pigment epithelium (RPE65; 1p31; LCA2; MIM180069), *iii*) the cone-rod homeobox-containing gene (CRX; 19q13.3; LCA7; MIM 60225), *iv*) the gene encoding the arylhydrocarbon receptor interacting protein-like 1 (AIPL1; 17p13.1; LCA; MIM 604392), *v*) the gene encoding the retinitis pigmentosa GTPase regulator-interacting protein 1 (RPGRIP1; 14q11; LCA6; MIM 605446), *vi*) the human homologue of the *drosophila melanogaster* crumbs gene (CRB1; 1q31; LCA8; MIM 604210), *vii*) the gene encoding the tubby-like protein 1 (TULP1; 6q21.3; LCA10; MIM 602280), *viii*) the retinol dehydrogenase 12 (RDH12; 14q24; LCA11; MIM 608830), *ix*) LCA3 (14q24; MIM 604232), *x*) LCA5 (6q11-16; MIM 604537) and *xi*) LCA9 (1p36; MIM608553).

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Interestingly, all LCA genes hitherto identified are involved in strikingly different physiologic pathways resulting in a wide physiopathologic variety.

So far, 50% of all cases have been related to a known disease-causing gene. If in 60% of these cases, the affection develops like a congenital severe cone-rod dystrophy, in about 40% of them, the disease appear to develop as a severe yet progressive rod-cone dystrophy and may represent the extremity of a spectrum of severity of retinitis pigmentosa (RP), (Hanein et al., 2004).

On the other hand, it has been shown that factors secreted from rods are an essential requirement for cone viability (Mohand-Saïd et al., 1998). One such trophic factor has just been identified by expression cloning and named rod-derived cone viability factor (RdCVF). RdCVF is a novel protein specifically expressed by photoreceptors. By immunodepletion with specific antibodies it has been demonstrated that RdCVF is required for cone rescue in mouse retinal cultured explants. The injection of recombinant RdCVF is able to prevent 40% of cones from degeneration in the *rd1* mouse over a period of two weeks. These results provide a biochemical basis for the previously described paracrine interaction between rod and cone photoreceptors that appears to play a key role in maintaining cone cell viability.

The aim of the present study was to look for mutations in LCA patients in RdCVF, by screening the thioredoxin-like 6 gene which encode this novel protein (TXNL6; Leveillard et al., 2004).

2. MATERIAL AND METHODS

2.1. Patients Panel

A total of 200 unrelated patients were either seen at the Ophthalmology-Genetics Center of Necker-Enfants Malades Hospital or sent to the laboratory by referent ophthalmologists or geneticists from France or other countries worldwide.

Our inclusion criteria were: *i*) severe impairment of visual function detected at birth or during the first months of life with pendular nystagmus, roving eye movements, eye poking, inability to follow light or objects and normal fundus, *ii*) extinguished ERG, *iii*) exclusion of ophthalmologic or systemic diseases sharing features with LCA. Detailed clinical data were required for each patient *ie i*) age and mode of onset, *ii*) light behaviour since birth, *iii*) natural history of the visual impairment since the first months of life including the subjective impressions of parents, *iv*) refraction data, *v*) ophthalmologic findings (anterior chamber and fundus), *vi*) visual acuity (if measurable) and *vii*) electrophysiology recordings. The course of the disease was determined by interviewing the patients or their parents and a pedigree was established.

Genomic DNA was extracted from whole blood or immortalized lymphoblast cell lines of patients using standard methods. When a mutation was identified we examined the parents and other family members when available in both sporadic and familial cases.

2.2. Controls Panel

Genomic DNA obtained from 125 unrelated healthy individuals and a cohort of 55 patients affected with Stargardt (STGD) disease were used as a control panel for molecular studies.

Table 2.1. Sequences of forward and reverse primers used for the mutational screening of the TXNL6 gene.

Exon number	Forward sequence (5'-3')	Reverse sequence (5'-3')
1	GAGAGGAGCCAGTCAGCAGA	TGGATGCTTCACTTTCAGCG
2	TCAGCATCAGGGATGTGGAT	TGGAGGTTCATCAACAAACC

2.3. Mutational Screening of TXNL6

Mutational screening of TXNL6 gene was performed on genomic DNA from the patients using primers designed to flank the splice junctions of each coding exon (Table 2.1). After standard PCR amplification (conditions available on request), products were screened for mutations using denaturing high-pressure liquid chromatography (DHPLC). heteroduplex formation was induced by heat denaturation of PCR products at 94°C for 10min, followed by gradual reannealing from 94°C to 25°C over 30min. DHPLC analysis was performed with the WAVE DNA fragment analysis system [Transgenomic, Cheshire, UK]. PCR products were eluted at a flow rate of 0.9ml/min with a linear acetonitrile gradient. the values of the buffer gradients (buffer A, 0.1 M triethylammoniumacetate; buffer B, 0.1 M triethylammoniumacetate/25% acetonitrile), start and end points of the gradient, and melting temperature predictions were determined by the WAVEMAKER software (Transgenomic, Cheshire, UK). Optimal run temperatures were empirically determined.

PCR fragments displaying DHPLC abnormal profiles were further sequenced using the Big Dye Terminator Cycle Sequencing Kit v3 (ABI Prism, Applied Biosystems, Foster City, USA on a 3100 automated sequencer).

2.4. Mutation Nomenclature

We have chosen to number the A of the start codon (ATG) of the TXNL6 cDNA sequence (Genbank accession number BC014127) as nucleotide 1.

2.5. Statistical Test

Comparison of the genotype and allele frequencies in patients and controls were performed by the Fisher’s exact test (two sided).

3. RESULTS

3.1. Mutational Screening of the TXNL6 Gene

Sequence analysis of the 2 TXNL6 exons allowed to identify eleven variant alleles (9/11 different) in 7/200 unrelated LCA patients excluding all eight known LCA genes and in 2/56 other patients for whom the genetic screening of LCA genes is still ongoing. Single or compound heterozygosity for non-conservative amino acid substitutions was identified in seven patients (3 consanguineous) and two patients (1 consanguineous), respectively (Table 2.2). For both compound heterozygous patients, the variants were inherited from healthy parents and co-segregated with the disease.

Table 2.2. Sequence changes in patients affected with LCA.

Family	Exon	Base Change	ALLELE 1		Base Change	ALLELE 2		Control Panel
			Predicted	Exon		Predicted Change	Change	
*91 F	Intron2	c.327-9G>A	“?”		“?”	“?”		0/180
94 S	2	c.485 A>G	p.Asn162Ser	2	c.533 C>T	p.The178Ile		0/180
*103 F	2	c.533 C>T	p.The178Ile		“?”	“?”		0/180
105 S	2	c.334 G>T	p.Gly112Trp		“?”	“?”		0/180
*110 S	2	c.485 A>G	p.Asn162Ser		“?”	“?”		0/180
211 S	1	c.189 G>A	p.Glu64Lys		“?”	“?”		0/180
247 F	1	c.282 G>C	p.Met94Ile		“?”	“?”		0/180
*284 S	1	c.46 G>A	p.Asp18Asn	2	c.533 C>T	p.The178Ile		0/180
285 S	1	c.275 A>G	p.Lys92Arg		“?”	“?”		0/180

* Indicate consanguinity of the parents of LCA patients. S: sporadic case, F: familial case. A of the start codon (ATG) of the cDNA sequences of TXNL6 (Genbank accession numbers BC014127) as nucleotide 1.

Table 2.3. Sequence changes in 125 control individuals and 55 STGD patients.

Exon	Base change	Predicted change	Frequency
2	c.461 A>G	p.Glu154Val	33%
1	c.83 G>C	p.Arg28Pro	30%
1	c.93 G>C	p.Glu31Asp	2%
Intron 1	c.326+7A>C	“?”	2%
1	c.108 G>A	p.Leu91Leu	1%
2	c.457 G>A	p.Gln153Lys	1%

A of the start codon (ATG) of the TXNL6 cDNA sequence (Genbank accession number BC014127) as nucleotide 1.

All 11 variant alleles resulted from non-conservative amino acid substitutions. None of them was found either in 125 unaffected control individuals or in 55 patients affected with typical Stargardt disease (Table 2.3).

3.2. TXNL6 Variants in LCA

The proportions of TXNL6 variant alleles in LCA patients *versus* controls and STGD patients were compared by the Fisher exact test. A statistically significant difference in TXNL6 genotype frequencies was evidenced between LCA patients and control individuals: $P_{LCA} = 9/200$ vs $P_{controls} = 0/125$, $p = 0.024$. This difference was even more significant when STGD patients were added to the control populations: $P_{LCA} = 10/200$ vs $P_{controls+STGD\ patients} = 0/180$, $p = 0.006$. TXNL6 allele frequencies were also compared: $P_{LCA} = 9/400$ vs $P_{controls} = 0/250$, $p = 0.009$ and $P_{LCA} = 10/400$ vs $P_{controls+STGD\ patients} = 0/360$, $p = 0.0008$.

4. DISCUSSION

We report here the identification of compound or single heterozygosity for TXNL6 variant alleles in 2 and 7 unrelated LCA patients, respectively.

None of the 9 different variants identified in LCA patients was found in a control population of 125 healthy individuals and 55 patients affected with Stargardt disease supporting the involvement of these alterations in LCA.

The identification of compound heterozygous variants in a LCA patient born to non-consanguineous parents was consistent with a recessive inheritance. However, four patients born to consanguineous were either compound heterozygous (1/4) or single heterozygous (3/4) for TXNL6 substitutions. This observation ruled out a simple recessive transmission and supported the view that TXNL6 variants may act as modifiers of the phenotype or may be disease-causing mutations in a multiallelic mode of inheritance.

The absence of variants in control individuals and STGD patients makes the hypothesis of a modifier role of TXNL6 in LCA less likely than a possible multiallelic inheritance. From this point of view, one has to mention that a triallelism have been demonstrated in Bardet-Biedl syndrome by the identification of several patients harbouring two mutations in the BBS2 gene and one mutation in the BBS6 gene and some asymptomatic individuals carrying two BBS2 gene mutations (Katsanis et al., 2002). These data could be related to the microarray-based mutation analysis of all LCA genes (>260 mutations) in large cohorts of LCA patients showing that *i*) more than two (expected) variants in a substantial fraction of patients and that *ii*) the third allele segregated with a more severe disease phenotype in several families (Allikmets et al., 2004). Along the same lines, it is worth noting that all nine patients harbouring TXNL6 variants are affected with a severe form of congenital cone-rod dystrophy according to the description recently by Hanein et al. (2004).

In conclusion, our data suggest that TXNL6 variants may be associated to 7.5% of LCA patients in our series. Further experiments are now necessary to confirm this hypothesis by showing, for instance, that the TXNL6 variants identified in LCA patients are responsible for a significant reduction of the capacity of the RdCVF protein to maintain cone cell viability.

6. ACKNOWLEDGEMENTS

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7. ELECTRONIC DATA BASES

Online Mendelian inheritance in Man: <http://www4.ncbi.nlm.nih.gov/OMIM/>

8. REFERENCES

- Hanein, S., Perrault, I., Gerber, S., Tanguy, G., Barbet, F., Ducroq, D., Calvas, P., Dollfus, H., Hamel, C., Loppönen, T., Munier, F., Santos, L., Shalev, S., Zafeiriou, D., Dufier, J.L., Munnich, A., Rozet, J.M., Kaplan, J., 2004, Leber congenital amaurosis: comprehensive survey of the genetic heterogeneity, refinement of the clinical definition, and genotype-phenotype correlations as a strategy for molecular diagnosis. *Hum Mutat* **23**(4):306-17.
- Kaplan, J., Bonneau, D., Frezal, J., Munnich, A., Dufier, J.L., 1990, Clinical and genetic heterogeneity in retinitis pigmentosa. *Hum Genet* **86**:635-42.

- Katsanis, N., Eichers, E.R., Ansley, S.J., Lewis, R.A., Kayserili, H., Hoskins, B.E., Scambler, P.J., Beales, P.L., Lupski, J.R., 2002. BBS4 is a minor contributor to Bardet-Biedl syndrome and may also participate in triallelic inheritance. *Am J Hum Genet* **71**(1):22-9.
- Leveillard, T., Mohand-Said, S., Lorentz, O., Hicks, D., Fintz, A.C., Clerin, E., Simonutti, M., Forster, V., Cavusoglu, N., Chalmel, F., Dolle, P., Poch, O., Lambrou, G., Sahel, J.A., 2004, Identification and characterization of rod-derived cone viability factor. *Nat Genet* **36**(7):755-9.
- Mohand-Said, S., Deudon-Combe, A., Hicks, D., Simonutti, M., Forster, V., Fintz, A.C., Leveillard, T., Dreyfus, H., Sahel, J.A., 1998, Normal retina releases a diffusible factor stimulating cone survival in the retinal degeneration mouse. *Proc Natl Acad Sci U S A* **7**;95(14):8357-62.
- Allikmets, R.L., Zernant, J., Perrault, I., den Hollander, A., Dharmaraj, S., F.P., Cremers, M., Kaplan, J., Koenekoop, R.K., Maumenee, I., 2004, Multiallelic inheritance and/or modifier alleles in leber congenital amaurosis: analysis with the lca disease chip, ARVO Meeting (2444/B79).

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