Molecular Vision **1999; 5:10** <http://www.molvis.org/molvis/v5/p10>
Received 22 March 1999 | Accepted 2 July 1999 | Published 2 July 1999

Four polymorphic variations in the PEDF gene identified during the mutation screening of patients with Leber congenital amaurosis

Robert Koenekoop,[1]Ana Luisa Pina,[1]Malgali Loyer,[1] Joe Davidson,[1]Johane Robitaille, [2]Irene Maumenee[2], Joyce Tombran-Tink[3]

[1]McGill Ocular Genetics Laboratory, Montreal Children's Hospital Research Institute, Montreal, Canada; [2]Johns Hopkins Center for Hereditary Eye Diseases, Baltimore, MD; [3]Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, National Institutes of Health, Bethesda, MD

Purpose: Leber congenital amaurosis (LCA) has been mapped to chromosome 17p13.1. From the candidate genes mapped to this region, thus far, only Retinal Guanylate Cyclase (RetGC), has been found to have pathogenic LCA mutations, in families from North African origin. However, early reports, demonstrated eight LCA families linked to 17p13.1, but only four of them showed mutations in RetGC. Mapped in proximity to this locus is the candidate gene Pigment Epithelium Derived Factor (PEDF), a factor implicated in photoreceptor differentiation and neuronal survival. Our purpose in this study was to identify mutations and polymorphisms in the PEDF gene in LCA patients of diverse ethnic origin.
Methods: Automated genotyping with four 17p13.1 markers flanking the PEDF gene was performed to assess homozygosity and PCR-SSCP combined with direct sequencing was used to detect mutations in the PEDF gene in 17 LCA patients.
Results: Homozygosity of markers D17S796 and D17S804 was found and four new intragenic basepair alterations were discovered: a Met72Thr polymorphism in exon 3 (T331C), a Thr130Thr polymorphism in exon 4 (T506C), a G to A transition in intron 5 (nine base pairs upstream from splice acceptor site), and a Tyr321Tyr polymorphism in exon 7 (C1079T) were detected.
Conclusions: We report the discovery of four new polymorphic alterations in the PEDF gene in LCA patients and exclude by RFLP analysis the PEDF gene as a common cause of Leber congenital amaurosis. These single nucleotide polymorphisms will aid in future linkage analysis of complex multifactorial diseases involving retinal and RPE dysfunctions.

Leber congenital amaurosis (LCA) is the earliest form of retinitis pigmentosa (RP) and is characterized by autosomal recessive inheritance, visual impairment at birth, nystagmus, and an abolished electroretinogram (ERG). Finding the cause of LCA commands considerable interest because it is a common cause of retinal blindness in children [1-4], and its gene identification will allow study of the molecular developmental biology of the human eye. Genetic heterogeneity of LCA has been suspected since Waardenburg's report [5] of normal children born to two affected parents, and rare reports of autosomal dominant inheritance [6]. Patients with LCA might maintain visual function, despite progressive retinal changes supporting the hypothesis that some forms of LCA are an aplasia and not a degeneration with impaired development of rod and cone photoreceptors [7,8]. Other pathological findings ranging from diffuse retinal atrophy [6], to ganglion cell abnormalities [9] absent rods [10], or cones [11], immature [12], or normal rods and cones [13], also support the aplasia hypothesis.

Recently, the genetic heterogeneity of this disease has been confirmed, when mutations in three genes were found to be associated with the LCA phenotype: the guanylate cyclase gene [14], RPE65 [15] and CRX [16]. Also a recent linkage study reported a new LCA locus on chromosome 14q24, but the gene remains to be identified [17]. Of these genes, guanylate cyclase is located in 17p13.1, the first locus for LCA described [18,19]. Until now, only LCA patients of Maghrebian origin from North Africa have been linked to 17p13.1 [14]. However, in the same report, it was demonstrated that from eight families linked to 17p13.1, only four show mutations in RetGC. Another candidate gene, Pigment Epithelium-Derived Factor (PEDF), also localizes to 17p13.1 [20]. PEDF is a serine protease inhibitor (serpin) expressed in fetal retinal pigment epithelial cells. In vitro studies [21] show, that in PEDF-treated retinoblastoma cells (which represent primitive photoreceptor cells), neurite like projections develop, and neuron specific enolase including neurofilament NF-200 are expressed, unlike untreated cells. Based on linkage data and a compelling physiological profile, PEDF is still, an obvious and intriguing candidate gene for photoreceptor degenerations, particularly LCA.

Correspondence to: Dr. Robert Koenekoop, Ophthalmology Department, Montreal Children's Hospital, 2300 Tupper, Montreal, Quebec H3H 1P3, Canada; Phone: (514) 934-4400 ext. 2891; FAX: (514) 934-4443; email: rkoeoph@mch.mcgill.ca

METHODS

Patients with LCA from around the world (5 multiplex, 12 simplex) entered the study. Criteria of inclusion were: (1) severe visual impairment at birth or shortly after, (2) nystagmus, (3) a diminished ERG, and (4) other systemic diseases were also allowed. Exclusions were Zellwegers disease, abetalipoproteinemia, infantile phytanic acid storage disease, and neuronal ceroid lipofuscinosis. Most patients have been

previously reported [7,22]. PCR-SSCP was performed to detect mutations, and automated genotyping was done with microsatellite markers to detect homozygosity. For all PCR-SSCP, our 25 µl amplification mixture contained 100 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (W/V) gelatin, 12.5 µM of each dNTP (dCTP, dGTP, dTTP), 6.25 µM dATP, 0.6 U Taq polymerase (GIBCO-BRL, Burlington ON), 10 ng/ µl of each intronic primer (described in Table 1) and α-^{35}S-dATP (12.5 mCi/ml, NEN, Boston MA) as a tracer. Denaturation at 94 °C for 30 s, was followed by annealing at primer specific temperatures (see Table 1) for 30 s, and elongation at 72 °C for 90 s in a DNA thermocycler (Perkin-Elmer, Oakville ON) for a total of 32 cycles. PCR products were run on 6% polyacrylamide gels with 5% or 10% glycerol. All abnormal SSCP variants were further analyzed by direct sequencing using dideoxy termination kits and α-^{35}S-dATP (Sequenase PCR product sequencing kit, US Biochemicals, Cleveland OH). Every change in the DNA was then confirmed by a restriction diagnostic digest. Nonradioactive PCRs were performed with the same conditions as before, including 0.2 mM dNTP. For restriction digestion of exon 7, 5 Units of MaeIII (Boehringer Mannheim, Indianapolis IN) were used and the digest products were run on a 10% polyacrylamide gel. Automated genotyping using fluorescein labelled microsatellite markers selected from the Whitehead genome data base flanking the PEDF gene were run on the 373 DNA sequencer (Applied Biosystems, Foster City CA). Allele analysis was performed using the GENESCAN and GENOTYPER (Applied Biosystems) programs. Normal controls consisted of 50 children (equal sex ratio) from the province of Quebec (100 chromosomes). Allele frequencies are noted per chromosome.

Table 1. Intronic primer sequences are shown with location, PCR fragment length, and the annealing temperature (T-ann)

Primer sequences	Fragment length	T-ann
EXON 1		
5'-ATCACAGAATCCAAATTGAGTGCAG	277 bp	65 °C
5'-TCTTTTCTGCTCCCTGGAGTGCCCC		
EXON 2		
5'-GAAGGGGTAGCGGGGCAGTGCAGTG	237 bp	68 °C
5'-GTTCCCTGTCCTGTTTCCCGTGCTG		
EXON 3		
5'-GACAGTCCCTGTGCATCTCTGCGAG	336 bp	68 °C
5'-TCAGCCACGTTTACGCAGAGGCCAC		
EXON 4		
5'-TGAGTATAGTGTCTGTGTTCTGGGA	310 bp	66 °C
5'-AAGACCCCCCCAGCCTGCAGCATGG		
EXON 5		
5'-GCTCTCAAAGACGGGATGCTTGTCG	354 bp	65 °C
5'-AGGGCTACAGAGTAAGACTCCATCG		
EXON 6		
5'-CAGCATGGCGCCACTGTCTTTCTGG	273 bp	65 °C
5'-TACCCTGTTTTGCTTCTCTATCCTC		
EXON 7		
5'-GGAAGGCAGCTCCTGGCTGTGTCTG	362 bp	68 °C
5'-CACAGTGGAAGGCCCAAAGACCCTG		
EXON 8		
5'-ATCCCAGCTTGCTTGCAAAGGGATC	435 bp	66 °C
5'-TGAAACCTTACAGGGGCAGCCTTCG		

RESULTS

We established homozygosity for 17p13 alleles in one Greek LCA proband for markers D17S796 and D17S804 but not D17S938 or D17S786 (data not shown). We also found four new intragenic base pair alterations, namely a Met72Thr polymorphism in exon 3 (T331C), a Thr130Thr polymorphism in exon 4 (T506C), a G to A transition in intron 5 (nine base pair upstream from splice acceptor site), and a Tyr321Tyr polymorphism in exon 7 (C1079T). Alterations in restriction sites are listed in Table 2. Normal controls were checked for the presence of each base pair change by restriction digestion. The

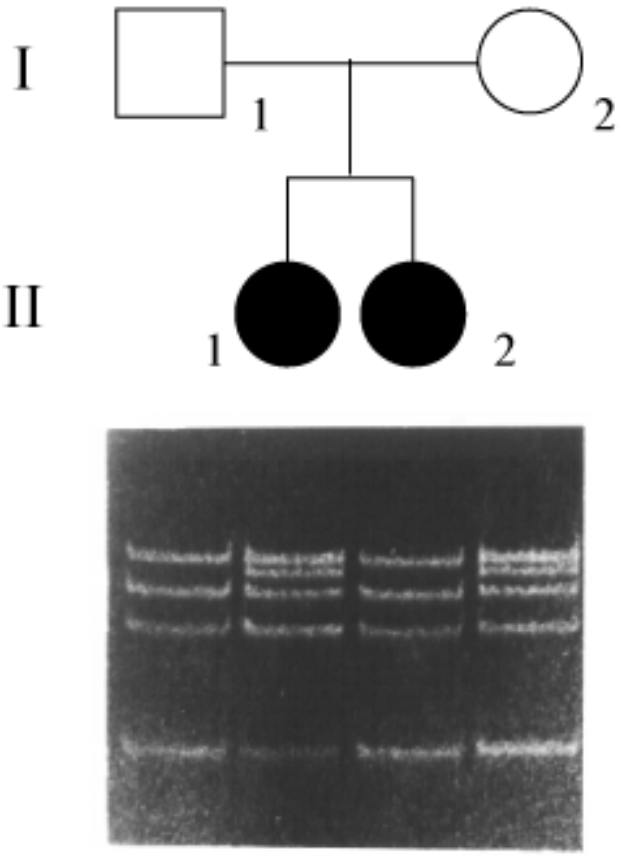

Figure 1. Example of a restriction diagnostic digest for the Tyr321Tyr polymorphism. The more frequent human allele has MaeIII sites that generate bands of 136, 115 and 96 bp (a 14 bp fragment cannot be seen) as shown in the first and third lanes from left to right. A homozygote for the Tyr321Tyr polymorphism (with one MaeIII site destroyed) would have bands of 136, 129 and 96 bp. A heterozygote has bands at 136, 129, 115, and 96 bp (as shown in the second and fourth lanes on the gel image). An example from a Greek LCA pedigree shows that one affected sib is heterozygous for the polymorphism (second lane), the other has two copies of the common allele (third lane). Lanes in the image correspond to each of the family members of the pedigree (lane one, the father; lane two, affected sib number 1; lane three, affected sib number 2, and lane four, the mother). The low molecular weight band in each lane is compatible with primer dimer formation.

frequency of each polymorphism is shown in Table 2. Pedigree analysis with the new RFLPs excluded PEDF as the causative gene in three of five families. One family is shown in Figure 1. The families excluded using the same polymorphism (data not shown) were from the middle East, another was an Amish family from Northern Germany, and one family was from Greece. Linkage exclusion of the 17p13.1 LCA1 locus in the large Amish family has also been reported by others [23].

DISCUSSION

Early reports, on the molecular basis of LCA, demonstrated its mapping to 17p13.1 and its genetic heterogeneity [18,19]. Shortly after, Perrault et al. [14], described LCA1, caused by mutations in the RetGC gene. They analyzed eight families linked to 17p13 and demonstrated that only four of them have mutations in the RetGC gene. Thus, the possibility exists that more than one gene in the same region can cause LCA. In this report we explored the possibility that PEDF, a gene that may promote photoreceptor differentiation and neuronal survival, can cause LCA in families of diverse ethnic origin.

Our results here excluded the PEDF gene as a common cause for Leber congenital amaurosis [24]. However, PEDF remains an important candidate gene for inherited retinal diseases that map to chromosome 17p13 [24] such as, autosomal dominant retinitis pigmentosa [25-28], autosomal dominant cone-rod dystrophy [29,30], and central areolar choroidal dystrophy [31]. For this group of diseases, our four newly described single nucleotide polymorphisms will aid in future linkage analyses. Evidence that PEDF may have an effect on the differentiation of rods and cones is demonstrated by experiments that show differentiation of retinoblastoma cells into neuronal-like cells [21]. This suggests that PEDF may be a factor involved in a programmed series of events that includes both proliferation and commitment to a final differentiated neuronal phenotype [20]. Further exploration towards the elucidation of the role of PEDF in normal and pathological retinal development remains to be done.

ACKNOWLEDGEMENTS

This study was supported by the E. Mildred Kanigsberg Estate Fund and an FRSQ award (970260-103) to RK and a Retinitis Pigmentosa Foundation fellowship to ALP.

Table 2. Four new polymorphisms with their respective exons, created or destroyed restriction enzyme sites and relative frequencies.

Exon	Polymorphism	Enzyme	Frequency in LCA Patients	Frequency in Normals
3	Met72Thr T331C	BssSI Created	9/34 (26%)	14/48 (29%)
4	Thr130Thr T506C	BstEII Created	9/34 (26%)	5/26 (19%)
5	G+9 A Intronic	BstUI Destroyed	4/34 (12%)	6/48 (12.5%)
7	Tyr321Tyr C1079T	MaeIII Destroyed	8/34 (23.5%)	13/24 (27%)

REFERENCES

1. Leber T. Uber retinitis pigmentosa und angeborene Amaurose. Archiv Für Ophthalmologie 1869; 15:1-25.
2. Franceschetti A. L'importance diagnostique de l'ERG dans les degenerescences tapeto-retiniennes avec retrecissement du champ visuel et hemeralopie. Confin Neurol 1954; 14:184-6.
3. Schappert-Kimmijster J, Henkes HE, Van den Bosch J. Amaurosis congenita (Leber). Arch Ophthalmol 1956; 61:211.
4. Heckenlively J.R., Foxman S.G. Congenital and early onset forms of retinitis pigmentosa. In: Heckenlively JR, editor. Retinitis pigmentosa. Philadelphia: J. B. Lippencott; 1988. p. 107-124.
5. Waardenburg P, Schappert-Kimmijster J. On various recessive biotypes of Leber's congenital amaurosis. Acta Ophthalmol (Copenh) 1963; 41:317-20.
6. Sorsby A, Williams CE. Retinal aplasia as a clinical entity. Br Med J 1960; 1:293.
7. Heher KL, Traboulsi EI, Maumenee IH. The natural history of Leber's congenital amaurosis. Age-related findings in 35 patients. Ophthalmology 1992; 99:241-5.
8. Fulton AB, Hansen RM, Mayer DL. Vision in Leber congenital amaurosis. Arch Ophthalmol 1996; 114:698-703.
9. Noble KG, Carr RE. Leber's congenital amaurosis. A retrospective study of 33 cases and a histopathological study of one case. Arch Ophthalmol 1978; 96:818-21.
10. Kroll AJ, Kuwabara T. Electron microscopy of a retinal abiotrophy. Arch Ophthalmol 1964; 71:683-90.
11. Gillespie FD. Congenital amaurosis of Leber. Am J Ophthalmol 1966; 61:874.
12. Mizuno K, Takei Y, Sears ML, Peterson WS, Carr RE, Jampol LM. Leber's congenital amaurosis. Am J Ophthalmol 1977; 83:32-42.
13. Horsten GP. Development of the retina of man and animals. Arch Ophthalmol 1960; 63:232-42.
14. Perrault I, Rozet JM, Calvas P, Gerber S, Camuzat A, Dollfus H, Chatelin S, Souied E, Ghazi I, Leowski C, Bonnemaison M, Le Paslier D, Frezal J, Dufier JL, Pittler S, Munnich A, Kaplan J. Retinal-specific guanylate cyclase gene mutations in Leber's congenital amaurosis. Nat Genet 1996; 14:461-4.
15. Marlhens F, Bareil C, Griffoin JM, Zrenner E, Amalric P, Eliaou C, Liu SY, Harris E, Redmond TM, Arnaud B, Claustres M, Hamel CP. Mutations in the RPE65 cause Leber's congenital amaurosis. Nat Genet 1997; 17:139-41.
16. Freund CL, Wang QL, Chen S, Muskat BL, Wiles CD, Sheffield VC, Jacobson SG, McInnes RR, Zack DJ, Stone EM. De novo mutations in the CRX homeobox gene associated with Leber congenital amaurosis. Nat Genet 1998; 18:311-2.
17. Stockton DW, Lewis RA, Abboud EB, Al-Rajhi A, Jabak M, Anderson KL, Lupski JR. A novel locus for Leber congenital amurosis on chromosome 14q24. Hum Genet 1998 103:328-33.
18. Camuzat A, Dollfus H, Rozet JM, Gerber S, Bonneau D, Bonnemaison M, Briard ML, Dufier JL, Ghazi I, Leowski C, et al. A gene for Leber's congenital amaurosis maps to chromosome 17p. Hum Mol Genet 1995; 4:1447-52.
19. Camuzat A, Rozet JM, Dollfus H, Gerber S, Perrault I, Weissenbach J, Munnich A, Kaplan J. Evidence of genetic heterogeneity of Leber's congenital amaurosis (LCA) and mapping of LCA1 to chromosome 17p13. Hum Genet 1996; 97:798-801.
20. Tombran-Tink J, Pawar H, Swaroop A, Rodriguez I, Chader GJ. Localization of the gene for pigment epithelium-derived factor (PEDF) to chromosome 17p13.1 and expression in cultured human retinoblastoma cells. Genomics 1994; 19:266-72.

21. Steele FR, Chader GJ, Johnson LV, Tombran-Tink J. Pigment epithelium-derived factor: neurotrophic activity and identification as a member of the serine protease inhibitor gene family. Proc Natl Acad Sci U S A 1993; 90:1526-30.
22. Schroeder R, Mets MB, Maumenee IH. Leber's congenital amaurosis. Retrospective review of 43 cases and a new fundus finding in two cases. Arch Ophthalmol 1987; 105:356-9.
23. Robitaille JM, Palincsar LK, Zhu D, Maumenee-Hussels IE. Linkage analysis in an Amish family with Leber's congenital amaurosis. Invest Ophthalmol Vis Sci 1996; 37:S998.
24. Davidson JK, Tink J, Loyer M, Traboulsi E, Maumenee I, Koenekoop RK. Pigment epithelium derived factor as a candidate gene for Leber's congenital amaurosis. Invest Ophthalmol Vis Sci 1996; 37:S991.
25. Joshi AR, Mullen L, Small KW. The retina: genetic studies of several retinopathies located on the short arm of chromosome 17. Curr Opin Neurol 1997; 10:31-5.
26. Greenberg J, Goliath R, Beighton P, Ramesar R. A new locus for autosomal dominant retinitis pigmentosa on the short arm of cromosome 17. Hum Mol Genet 1994; 3:915-8.
27. Goliath R, Tombran-Tink J, Rodriquez IR, Chader G, Ramesar R, Greenberg J. The gene for PEDF, a retinal growth factor is a prime candidate for retinitis pigmentosa and is tightly linked to the RP13 locus on chromosome 17p13.3. Mol Vis 1996; 2:5 . <http:www.molvis.org/molvis/v2p5>
28. Kojis TL, Heinzmann C, Flodman P, Ngo JT, Sparkes RS, Spence MA, Bateman JB, Heckenlively JR. Map refinement of locus RP13 to human chromosome 17p13.3 in a second family with autosomal dominant retinitis pigmentosa. Am J Hum Genet 1996; 58:347-55.
29. Small KW, Syrquin M, Mullen L, Gehrs K. Mapping of autosomal dominant cone degeneration to chromosome 17p. Am J Ophthalmol 1996; 121:13-8.
30. Kelsell RE, Evans K, Gregory CY, Moore AT, Bird AC, Hunt DM. Localisation of a gene for dominant cone-rod dystrophy (CORD6) to chromosome 17p. Hum Mol Genet 1997; 6:597-600.
31. Lotery AJ, Ennis KT, Silvestri G, Nicholl S, McGibbon D, Collins AD, Hughes AE. Localisation of a gene for central areolar choroidal dystrophy to chromosome 17p. Hum Mol Genet 1996; 5:705-8.