Mutation Screening of 299 Spanish Families with Retinal Dystrophies by Leber Congenital Amaurosis Genotyping Microarray

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Purpose. Leber Congenital Amaurosis (LCA) is one of the most severe inherited retinal dystrophies with the earliest age of onset. This study was a mutational analysis of eight genes (*AIPL1*, *CRB1*, *CRX*, *GUCY2D*, *RPE65*, *RPGRIP1*, *MERTK*, and *LRAT*) in 299 unrelated Spanish families, containing 42 patients with initial diagnosis of LCA: 107 with early-onset autosomal recessive retinitis pigmentosa (ARRP; onset <10 years of age) and 150 with non–early-onset ARRP (onset, >10 years of age).

Methods. Samples were studied by using a genotyping microarray (Asper Biotech, Ltd., Tartu, Estonia) followed by a family study in cases with potential digenism/triallelism.

Results. The frequencies of alleles carrying disease-causing mutations found in the authors'cohort using the chip were 23.8% (20/84) for LCA with 13 families carrying mutations, 6.1% (13/214) for early-onset ARRP with 12 families carrying mutations, and 4.3% (13/300) for non–early-onset ARRP with 12 families carrying mutations. *CRB1* was the most frequently found mutated gene in affected Spanish families. Five families with anticipated digenism or triallelism were further studied in depth. Digenism could be discarded in all these cases; however, triallelism could not be ruled out.

Conclusions. *CRB1* is the main gene responsible for LCA in the Spanish population. Sequence changes p.Asp1114Gly (*RPGRIP1*), p.Pro701Ser (*GUCY2D*), and p.Tyr134Phe (*AIPL1*) were found at similar frequencies in patients and control subjects. The authors therefore suggest that these changes be considered as polymorphism or modifier alleles, rather than as disease-causing mutations. The LCA microarray is a quick and reasonably low-cost first step in the molecular diagnosis of LCA. The diagnosis should be completed by conventional laboratory analysis as a second step. This stepwise proceeding permits detection of novel disease-causing mutations and identification of cases involving potential digenism/triallelism. Previous accurate ophthalmic diagnosis was found to be indispensable. (*Invest Ophthalmol Vis Sci.* 2007;48:5653–5661) DOI:10.1167/iovs.07-0007

Leber congenital amaurosis (LCA, MIM 204000; Mendelian Inheritance in Man; National Center for Biotechnology Information, Bethesda, MD) is the earliest and most severe form of all inherited retinal dystrophies and is responsible for congenital blindness.[1–6] The diagnosis is usually based on onset at birth or during the first months of life of total blindness or heavily impaired vision, congenital nystagmus, and nonrecordable electroretinogram (ERG). Sluggish or absent pupillary reaction and eye poking (Franceschetti sign) may be accompanying signs. LCA is generally inherited in an autosomal recessive manner, although autosomal dominance has been described in some families.[7–9] Nonsyndromic LCA has been associated so far with mutations in 10 genes: *AIPL1* (17p13.1),[10,11], *CEP290* (12q21.3),[12–14] *CRB1* (1q31-q32.2),[15–17] *CRX* (19q13.3),[18–20] *GUCY2D* (17p13.3),[2] *IMPDH1* (7q31.3-q32),[21–23] *RPE65* (1p31),[24] *RPGRIP1* (14q11),[25–27] *RDH12* (14q23.3-q24.1),[28,29] and *TULP1* (6q21.3).[30,31]

The LCA genes identified are involved in different physiologic pathways in the retina.[10,32–42] The genetic and phenotypic heterogeneity in LCA renders the establishment of molecular diagnoses complicated.[43] Distinguishing LCA from early-onset autosomal recessive retinitis pigmentosa (ARRP) is further complicated, because these disorders represent a continuum of phenotypes often caused by mutations in the same gene(s).

We have screened a cohort of 299 families with diagnoses of LCA, early-onset ARRP, or non–early-onset ARRP. In these individuals we investigated six genes associated with LCA (*AIPL1*, *CRB1*, *CRX*, *GUCY2D*, *RPE65*, and *RPGRIP1*) and two associated with early-onset RP (*LRAT*[44,45] and *MERTK*[46]), by using the an LCA genotyping microarray (Asper Biotech, Ltd., Tartu, Estonia) that employs arrayed primer extension (APEX) technology to assess the frequencies of affected genes and respective mutations involved in the selected forms of severe RD among Spanish patients.

The complete genetic and phenotypic evaluation of patients carrying mutations of one of these genes is a mandatory prerequisite for identifying those who may benefit from upcoming novel therapeutic strategies.

Materials and Methods

Patients

A total of 299 unrelated Spanish families with retinal dystrophy were studied. Informed consent was obtained from all persons involved in the study or from their legal guardians, in accordance with the tenets of the Declaration of Helsinki (Edinburgh, 2000). Of these patients, three different cohorts were investigated individually, depending on their respective clinical ophthalmic diagnosis: 42 patients with LCA

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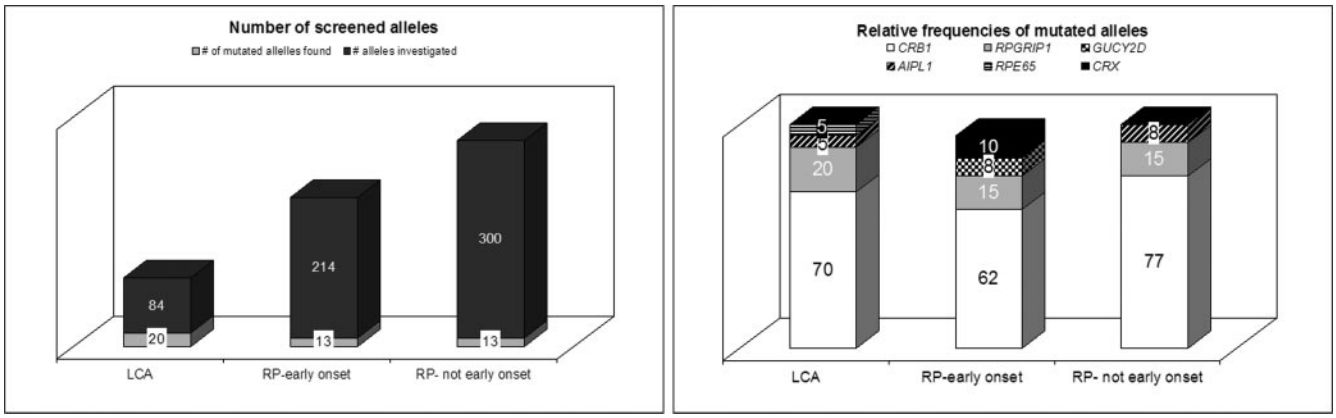


FIGURE 1. *Left*: 84, 214, and 300 alleles were screened in patients with diagnoses of LCA, early-onset RP, and non–early-onset RP, respectively. Within these groups 20 (23.8%), 13 (6.1%), and 13 (4.3%) alleles with disease-causing sequence changes were detected, respectively. *Right*: percentages of alleles with detected sequence changes found in the different clinical groups. *CRB1* is the gene with the most frequent mutations in all three patient groups, followed by *RPGRIP1*. Changes in *RPE65* were found only in the LCA group and changes in *CRX* only in the early-onset RP group.

(characterized by severe visual loss, nystagmus, nonrecordable or severely reduced ERG, and sluggish pupillary responses with onset before the age of one year[47]), 107 patients with early-onset ARRP (onset before the age of 10 years); and 150 patients with non–early-onset ARRP (onset after the age of 10 years). One affected individual of each family was analyzed with the LCA microarray, respectively.

Whenever sequence changes in two different genes were found (which was considered potential digenism or triallelism), we extended the study to the rest of the family.

Genomic DNA obtained from 192 unrelated healthy individuals was used as a control panel for molecular studies of the most frequent mutations found in this study and also for CRB1 new mutations.

Genotyping

We used a genotyping microarray with the APEX technology designed for LCA and early-onset ARRP. The microarray contained 344 disease-associated sequence variants and six polymorphisms in eight LCA or

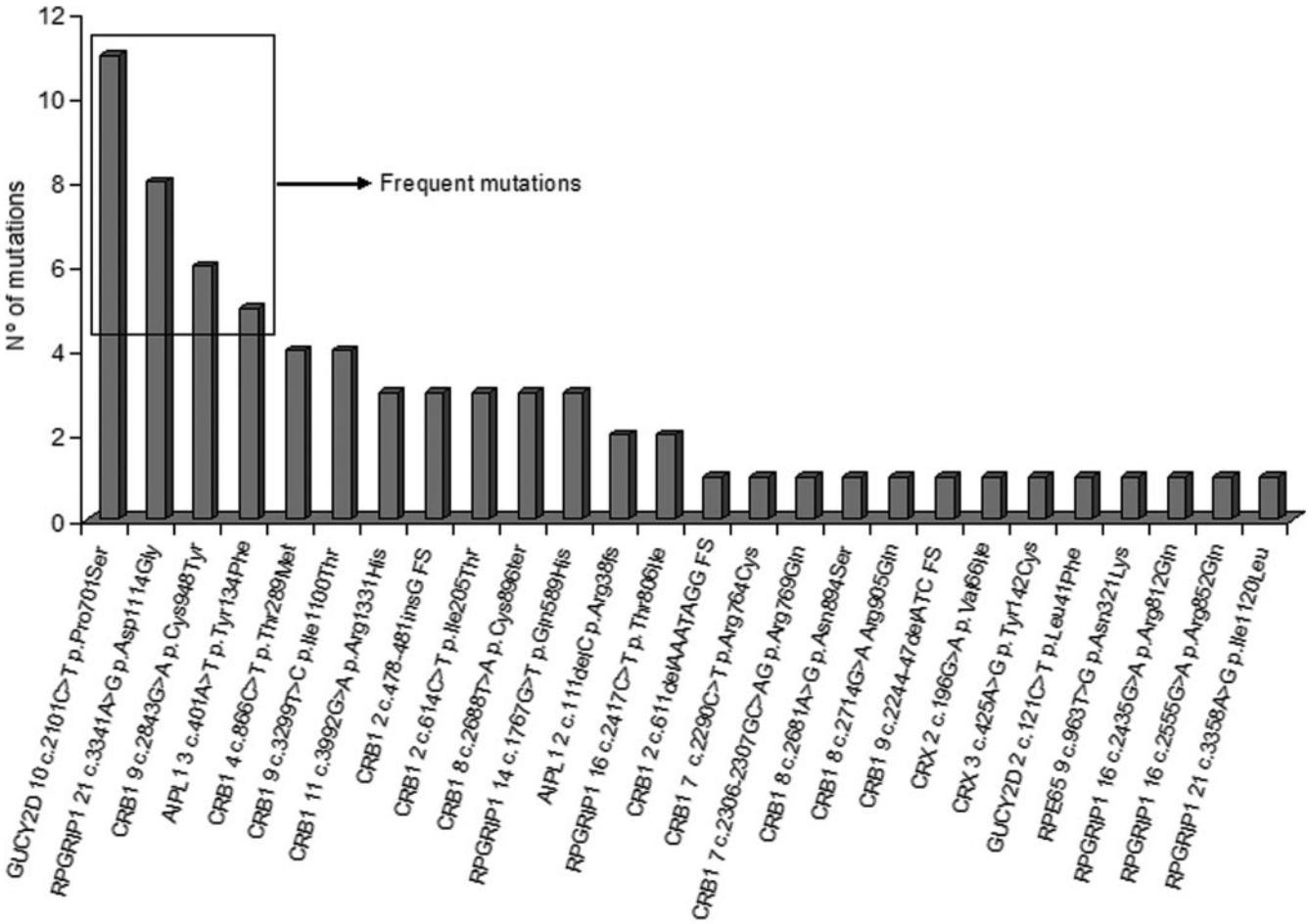
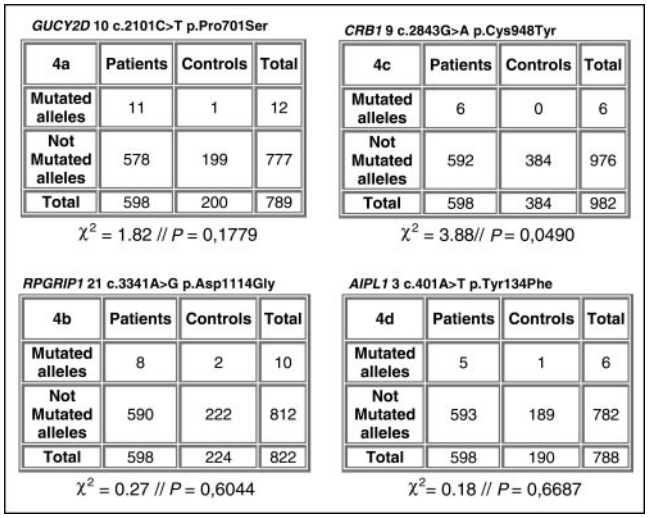


FIGURE 2. Frequent mutations found with the LCA microarray.

TABLE 1. χ^2 Analysis in the Four Most Frequently Found Mutations



GUCY2D 10 c.2101C>T p.Pro701Ser

4a	Patients	Controls	Total
Mutated alleles	11	1	12
Not Mutated alleles	578	199	777
Total	598	200	789

$\chi^2 = 1.82$ // $P = 0{,}1779$

CRB1 9 c.2843G>A p.Cys948Tyr

4c	Patients	Controls	Total
Mutated alleles	6	0	6
Not Mutated alleles	592	384	976
Total	598	384	982

$\chi^2 = 3.88$// $P = 0{,}0490$

RPGRIP1 21 c.3341A>G p.Asp1114Gly

4b	Patients	Controls	Total
Mutated alleles	8	2	10
Not Mutated alleles	590	222	812
Total	598	224	822

$\chi^2 = 0.27$ // $P = 0{,}6044$

AIPL1 3 c.401A>T p.Tyr134Phe

4d	Patients	Controls	Total
Mutated alleles	5	1	6
Not Mutated alleles	593	189	782
Total	598	190	788

$\chi^2 = 0.18$ // $P = 0{,}6687$

early-onset RP genes (*AIPL1*, *CRB1*, *CRX*, *GUCY2D*, *LRAT*, *MERTK*, *RPGRIP1*, and *RPE65*) (http://www.asperophthalmics.com/LeberCongenitalAmaurosisDNAtest.htm; Asper Biotech, Ltd.). The complete description of this methodology can be found at www.asperbio.com and has been published in Zernant et al.[48] By design, the chip includes all variants from the coding region and adjacent intronic sequences of the respective LCA and early-onset RP genes that were known at the time of the design of the chip.

The most recent array contains 10 genes and 423 known mutations; however, at the time we began this study, only the chip containing 344 mutations in 8 genes was available.

Complementary studies were performed with dHPLC, direct sequencing, and haplotype analyses of *CRB1* (*D1S408*, *D1S2816*, *D1S2757*, and *D1S1660*).

A χ^2 test was use for calculations of the most frequent mutations found, to test for differences in cohorts of affected patients and unaffected, healthy individuals.

RESULTS

We performed a mutational screening of allele frequencies detected with the chip in the three groups of patients. Figure 1 shows the number of alleles screened in each patient group as well as the percentage of mutated alleles found in different genes in the three patient groups. A total of 84, 214, and 300 alleles were screened in patients with LCA, early-onset RP, and non-early-onset RP, respectively. Within these groups 24 (29%), 21 (10%), and 25 (8%) alleles with sequence changes were detected. *CRB1* was gene with the most frequently found mutations in all three patient groups. There were considerable differences among the groups in the frequency of mutations in *RPGRIP1* and *GUCY2D*. Changes in *RPE65* were found only in the LCA group and changes in *CRX* only in the early-onset RP group.

Analyzing the frequencies of the 344 sequence changes that can be detected with the LCA microarray, we found p.Pro701Ser (*GUCY2D*), p.Asp1114Gly (*RPGRIP1*), p.Cys948Tyr (*CRB1*), and p.Tyr134Phe (*AIPL1*) to be the four most common sequence changes in our cohort of affected patients (Fig. 2). When testing for these variants in a control population of healthy volunteers, they turned out to have frequencies higher than what would have been expected for alleles associated with recessive diseases that are highly penetrant but show a low overall incidence. Only the p.Cys948Tyr (*CRB1*) mutation was not found in the control population (Table 1).

Considering these unexpectedly high frequencies, we performed a χ^2 test to test for differences among affected patients and healthy control subjects to assess whether these changes are to be considered mutations or rather polymorphisms. For the changes p.Asp1114Gly (*RPGRIP1*), p.Pro701Ser (*GUCY2D*), and p.Tyr134Phe (*AIPL1*), the allele frequencies among affected patients and control subjects were not significantly different ($P \gg 0.05$; Table 1).

In contrast, p.Cys948Tyr mutation has significantly different frequencies in these populations, based on a 5% confidence level ($P = 0{,}0490$).

We identified seven families in which a digenic or triallelic trait that could be considered sequence changes affecting two distinct genes were observed (Tables 2, 3, 4; families LCA-0012, LCA-0032, LCA-0038, LCA-0042, RP-0137, RP-0310, and RP-0643). We were able to perform detailed genetic analyses of additional family members in five of the seven families (three families with LCA and two families with early-onset ARRP). In these families, we performed segregation analysis to assess the probability of digenic or triallelic inheritance. The respective pedigrees and allele status are shown in Figure 3.

TABLE 2. LCA Results

Family Number	Mutation 1	Mutation 2
LCA-0035 // RP-1008	*AIPL1* 2 c.111delC p.Arg38fs	
LCA-0010 // RP-0717	*CRB1* 2 c.478–481 insG FS	*CRB1* 2 c.478–481 insG FS
LCA-0019 // RP-0902	*CRB1* 2 c.611delAAATAGG FS	
LCA-0012 // RP-0489	*CRB1* 2 c.614C>T p.Ile205Thr	***GUCY2D*** 10 c.2101C>T p.Pro701Ser*
LCA-0042 // RP-1050	*CRB1* 2 c.614C>T p.Ile205Thr	***GUCY2D*** 10 c.2101C>T p.Pro701Ser*
LCA-0004 // RP-0899	*CRB1* 8 c.2688T>A p.Cys896ter	*CRB1* 9 c.2843G>A p.Cys948Tyr*
LCA-0038 // RP-0578	*CRB1* 8 c.2688T>A p.Cys896ter	*RPGRIP1* c.14 1767G>T p.Gln589His
LCA-0027 // RP-0907	*CRB1* 9 c.2843G>A p.Cys948Tyr*	
LCA-0011 // RP-0641	*CRB1* 9 c. 2843G>A p.Cys948Tyr*	*CRB1* 9 c.2244–47delATC FS
LCA-0028 // RP-0909	*CRB1* 9 c.2843G>A p.Cys948Tyr*	*CRB1* 9 c.3299T>C p.Ile1100Thr
LCA-0032 // RP-1009	*CRB1* 9 c.2843G>A p.Cys948Tyr*	*RPE65* 9 c.963T>G p.Asn321Lys
LCA-0037 // RP-0961	*RPGRIP1* 14 c. 1767G>T p.Gln589His	
LCA-0039 // RP-1006	*RPGRIP1* 16 c.2417C>T p.Thr806Ile	
LCA-0029 // RP-0869	*RPGRIP1* 16 c.2435G>A p.Arg812Gln	
LCA-0002 // RP-0746	***RPGRIP1*** 21 c.3341A>G p.Asp1114Gly*	
LCA-0034 // RP-0936	***RPGRIP1*** 21 c.3341A>G p.Asp1114Gly*	

Light gray: disease-causing role not proven unequivocally; dark gray: families with suspected digenism or triallelism.
* Frequent mutations.

TABLE 3. Early-Onset ARRP Results

Family Number	Mutation 1	Mutation 2
RP-0029	*AIPL1* 3 c.401A>T p.Tyr134Phe*	
RP-0082	*AIPL1* 3 c.401A>T p.Tyr134Phe*	
RP-0882	*AIPL1* 3 c.401A>T p.Tyr134Phe*	
RP-0617	*CRB1* 11 c.3992G>A p.Arg1331His	
RP-0091	*CRB1* 2 c.478–481 insG FS	
RP-0310	*CRB1* 2 c.614C>T p.Ile205Thr	*RPGRIP1* 21 c.3341A>G p.Asp1114Gly*
RP-0457	*CRB1* 8 c.2681A>G p.Asn894Ser	
RP-0137	*CRB1* 8 c.2714G>A Arg905Gln	*RPGRIP1* 21 c.3341A>G p.Asp1114Gly*
RP-0280	*CRB1* 9 c.2843G>A p.Cys948Tyr*	
RP-0025	*CRB1* 9 c.3299T>C p.Ile1100Thr	*CRB1* 9 c.3299T>C p.Ile1100Thr
RP-0862	*CRX* 2 c.196G>A p.Val66Ile	
RP-0632	*CRX* 3 c.425A>G p.Tyr142Cys	
RP-0591	*GUCY2D* 10 c.2101C>T p.Pro701Ser*	
RP-0933	*GUCY2D* 10 c.2101C>T p.Pro701Ser*	
RP-0050	*GUCY2D* 2 c.121C>T p.Leu41Phe	
RP-0841	*RPGRIP1* 16 c.2417C>T p.Thr806Ile	
RP-0061	*RPGRIP1* 21 c.3341A>G p.Asp1114Gly*	
RP-0726	*RPGRIP1* 21 c.3358A>G p.Ile1120Leu	

Light gray: disease-causing role not proven unequivocally; dark gray: families with suspected digenism or triallelism.

* Frequent mutations.

In family LCA-0012 we found the nonaffected father to carry the same two mutations as both affected children (*CRB1*: p.Ile205Thr and *GUCY2D*: p.Pro701Ser).

With the use of the chip, we found one mutation affecting *CRB1* (p.Cys896ter) and another affecting *RPE65* (p.Asn321Lys) in family LCA-0032. Detailed examination in our laboratory by automated sequencing revealed a second mutation in *CRB1.*

When we screened patient V:1 in family LCA-0038 by using the chip, we initially found two mutations affecting different genes: p.Cys896ter in *CRB1* and p.Gln589His in *RPGRIP1*. Automatic sequencing of the four affected patients in the family in our laboratory revealed two more mutations in *CRB1*: c.1690G>T p.Asp564Thr and c.3002A>T p.Ile1001Asn. Affected individuals harbored compound heterozygous mutations affecting *CRB1*, except for individual III:5 in whom no second mutation in *CRB1* could be found. Three of the four affected family members also carried the p.Gln589His mutation in *RPGRIP1.*

In family RP-0137, we found the two mutations Arg905Gln in *CRB1* and p.Asp1114Gly in *RPGRIP1.* One affected individual harbored both mutations, whereas the other affected individual did not carry any of these mutations.

In family RP-0310, we found the unaffected mother carrying the same two variants (p.Ile205Thr in *CRB1* and p.Asp1114Gly in *RPGRIP1*) as the affected son.

TABLE 4. Non-Early-Onset ARRP Results

Family Number	Mutation 1	Mutation 2
RP-0351	*AIPL1* 2 c.111delC p.Arg38fs	
RP-0366	*AIPL1* 3 c.401A>T p.Tyr134Phe*	
RP-0423	*AIPL1* 3 c.401A>T p.Tyr134Phe*	
RP-0040	*CRB1* 11 c.3992G>A p.Arg1331His	
RP-0376	*CRB1* 11 c.3992G>A p.Arg1331His	
RP-0041	*CRB1* 4 c.866C>T p.Thr289Met	
RP-0540	*CRB1* 4 c.866C>T p.Thr289Met	*CRB1* 4 c.866C>T p.Thr289Met
RP-0643	*CRB1* 4 c.866C>T p.Thr289Met	*GUCY2D* 10 c.2101C>T p.Pro701Ser*
RP-0487	*CRB1* 7 c.2290C>T p.Arg764Cys	
RP-0620	*CRB1* 7 c.2306–2307GC>AG p.Arg769Gln	
RP-0243	*CRB1* 8 c.2688T>A p.Cys896ter	
RP-0569	*CRB1* 9 c.3299T>C pIle1100Thr	
RP-0094	*GUCY2D* 10 c.2101C>T p.Pro701Ser*	
RP-0498	*GUCY2D* 10 c.2101C>T p.Pro701Ser*	
RP-0653	*GUCY2D* 10 c.2101C>T p.Pro701Ser*	
RP-0900	*GUCY2D* 10 c.2101C>T p.Pro701Ser*	
RP-0120	*GUCY2D* 10 c.2101C>T p.Pro701Ser*	*GUCY2D* 10 c.2101C>T p.Pro701Ser*
RP-0881	*RPGRIP1* 14 c.1767G>T p.Gln589His	
RP-0633	*RPGRIP1* 16 c.2555G>A p.Arg852Gln	
RP-0621	*RPGRIP1* 21 c.3341A>G p.Asp1114Gly*	
RP-0647	*RPGRIP1* 21 c.3341A>G p.Asp1114Gly*	
RP-0807	*RPGRIP1* 21 c.3341A>G p.Asp1114Gly*	

Light gray: disease-causing role not proven unequivocally; dark gray: family with suspected digenism or triallelism.

* Frequent mutations.

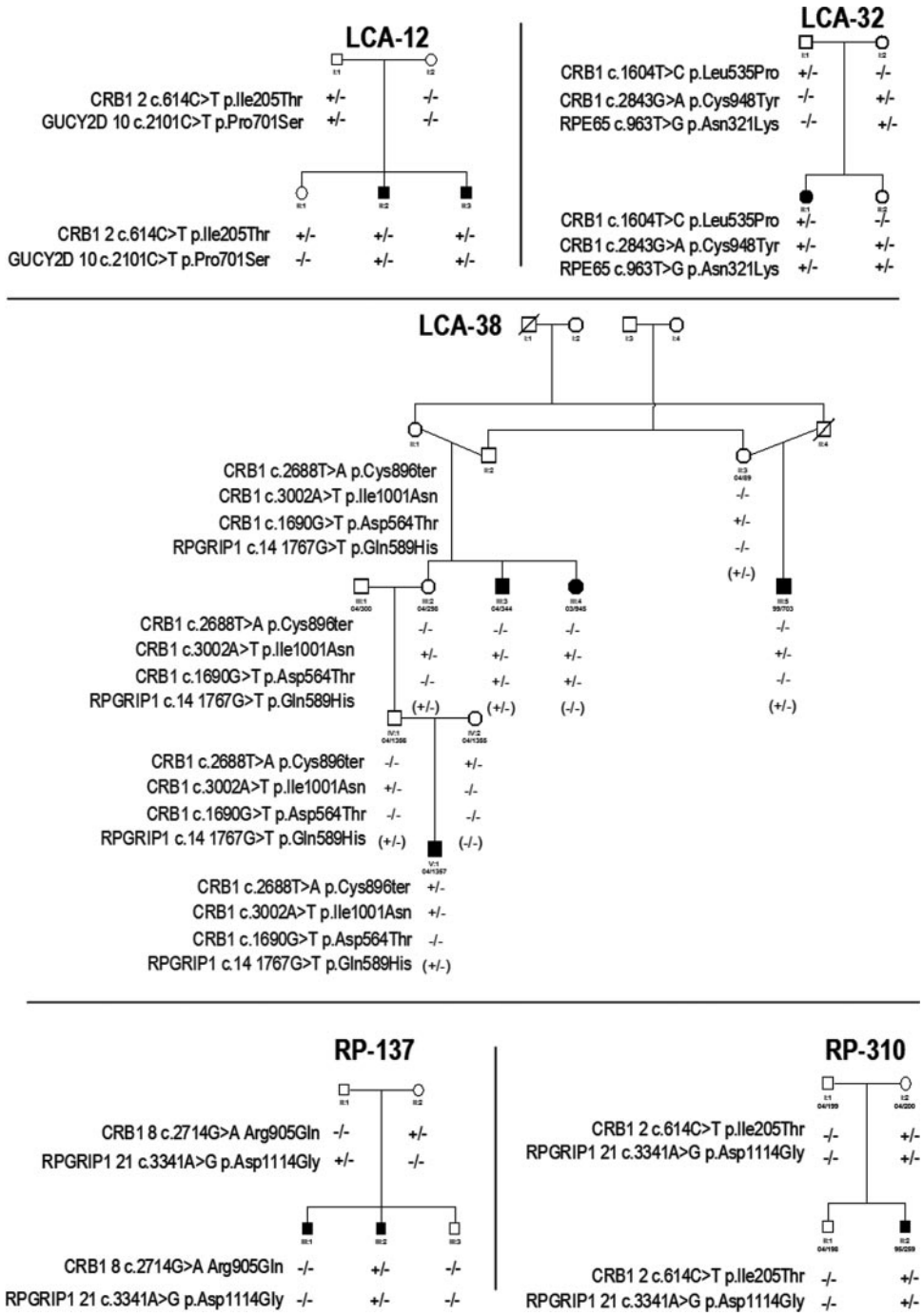


FIGURE 3. Pedigree of the five families with suspected digenism-triallelism.

DISCUSSION

The LCA microarray detected 23.8% (20/84) of mutated alleles for LCA with 13 families carrying mutations, 6.1% (13/214) for early-onset (ARRP) with 12 families carrying mutations, and 4.3% (13/300) for non–early-onset ARRP with 12 families carrying mutations.

Digenic and Triallelic Inheritance

Variants in two different genes were observed in some families and were further investigated in five of them (families LCA-0012, LCA-0032, LCA-0038, RP-0137 and RP-0310; Fig. 3). In these families, the possibility of triallelic or digenic inheritance was further investigated.

In family LCA-0012, the disease-causing role of the mutations in *CRB1* and *GUCY2D* remains unclear after segregation analysis. It can be hypothesized that another not yet known mutation in one of these genes may lead to the phenotype. Digenic inheritance seems highly unlikely, as individual I:1 carried both mutations, but was not affected. Whether the second mutated gene has a modifier effect remains to be elucidated by further studies.

In family LCA-0032, the phenotype segregates with compound heterozygous mutations in *CRB1*. However, we also found a mutation in *RPE65* in affected and nonaffected members. From this pedigree, we cannot exclude a possible triallelic inheritance of that phenotype.

In family LCA-0038, digenic inheritance seemed rather unlikely, as affected and nonaffected family members harbored

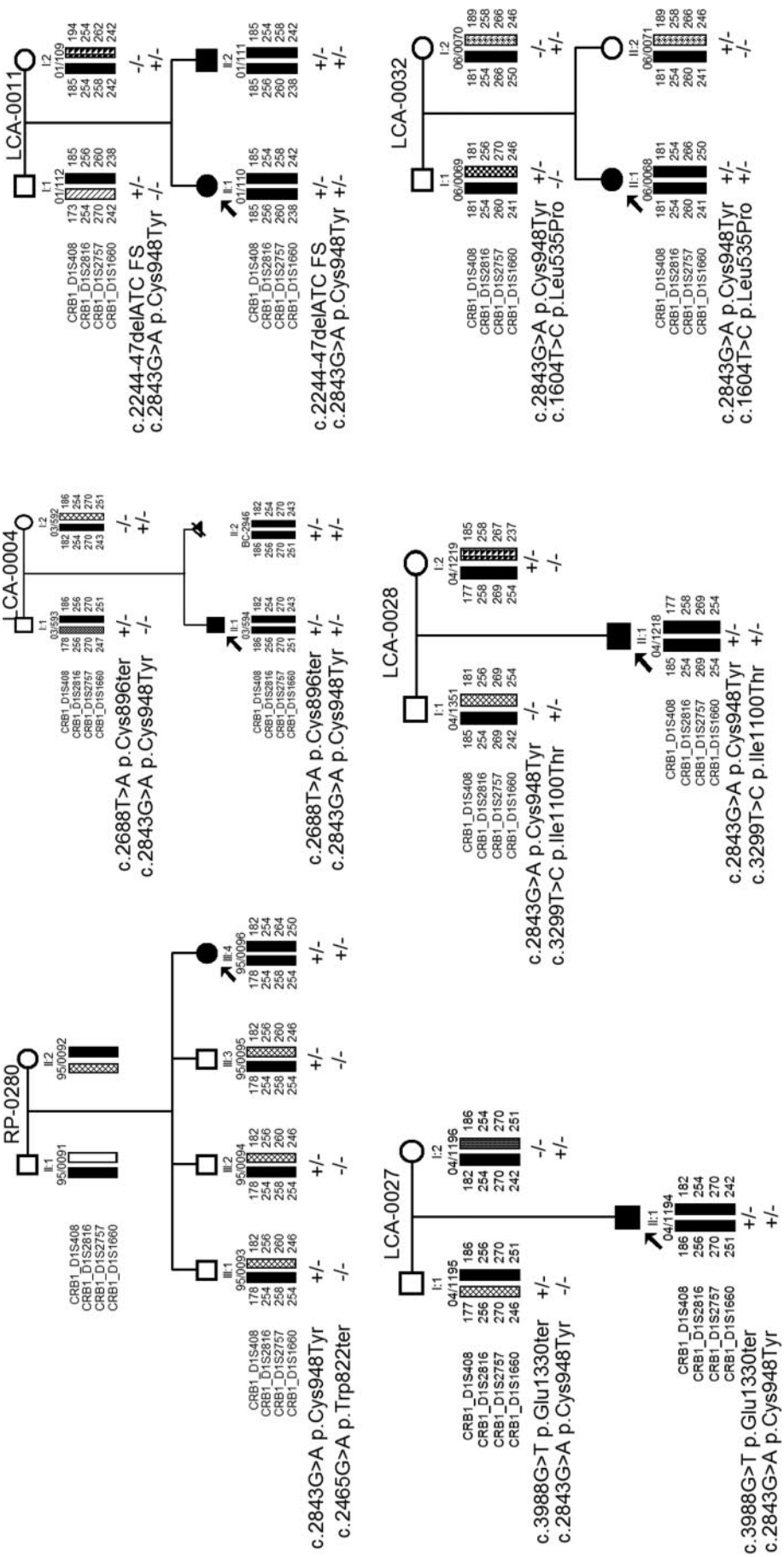


FIGURE 4. Segregation analysis of families RP-0280, LCA-0004, LCA-0011, LCA-0027, LCA-0028, and LCA-0032.

heterozygous mutations in *PRGRIP1* and *CRB1*, respectively. Triallelism is unlikely as well, as affected individual III:4 did not carry the third affected allele (*PRGRIP1*). However, a modifier effect cannot be excluded. Further comprehensive genotype–phenotype studies could resolve this question. Of interest, in affected individual III:5 we found only one heterozygous mutation in *CRB1* and *RPGRIP1*, respectively, although there are four different mutations (three in *CRB1* and one in *RPGRIP1*) found in this family. Therefore, a fifth yet unknown mutation must be considered in that family.

In family RP-0137 there is no obvious correlation between mutated alleles and the disease from segregation analysis. This finding stresses that mutations identified by the microarray are not necessarily related to the disease and that haplotype analysis is indispensable.

In family RP-0310, the disease-causing role of the mutations in *CRB1* and *RPGRIP1* remains unclear after segregation analysis, as affected and nonaffected individuals carried these mutations. Although digenic inheritance is rather unlikely, it can be hypothesized that another parentally inherited yet unknown mutation in one of these genes may lead to the phenotype. Whether the second mutated gene has a modifier effect remains unclear.

Polymorphisms or Mutations

Using the chip to perform mutation analysis yielded results that must be validated to consider their pathogenic role in an individual person. Some of the sequence changes found on the chip were detected at relatively high frequencies in our patients collectively (Fig. 2). It might be wondered whether some of these sequence changes can be regarded as polymorphisms rather than disease-causing mutations. To address this question, we studied the frequencies of these four sequence changes (p.Pro701Ser (*GUCY2D*), p.Asp1114Gly (*RPGRIP1*), p.Cys948Tyr (*CRB1*), and p.Tyr134Phe (*AIPL1*) in a Spanish normal population to test for differences between affected and nonaffected populations (Table 4).

In fact, it turned out that only for the p.Cys948Tyr (*CRB1*) sequence change is there a statistically significant difference between the two populations. We also found that p.Cys948Tyr (*CRB1*) cosegregated with the disease in some of our families (RP-0641, RP-0907, RP-0909 (Tables 2, 3) and RP-0280 (Fig. 4). Therefore, we think it is reasonable to consider this sequence change as a disease-causing mutation, in concordance with previously reported findings.[6,15,49,50]

In contrast, for the other three sequence changes, p.Pro701Ser (*GUCY2D*), p.Asp1114Gly (*RPGRIP1*), and p.Tyr134Phe (*AIPL1*), we did not find significantly different frequencies in the affected and control groups. Therefore, it is necessary to discuss the pathogenic role of these variants in more detail.

For the p.Pro701Ser (*GUCY2D*) change Zernant et al.[48] have shown complete segregation of this variant with the disease in three extended pedigrees, lack of nonsymptomatic homozygotes, and compound heterozygosity in one affected individual. We identified one affected individual in our study harboring the p.Pro701Ser change in the homozygous state (RP-0120). Given the high frequency among normal individuals, we think even these hints of a pathogenic role of this sequence change does not rectify its classification as a mutation.

For the p.Asp1114Gly (RPGRIP1) change, Lu et al.[50] recently determined that the altered protein that carries the substitution in the RID domain of *RPGRIP1* abolishes the interaction with *RPGR*. Despite evidence of the altered function of that mutant, we found no proof of a pathogenic role based on segregation analysis in our population. Differences in frequency between patient and control groups did not reach significance. Therefore, we cannot provide additional clues supporting a pathogenic role of that variant.

Regarding p.Tyr134Phe (*AIPL1*) Gerber et al.[26] have reported about the pathogenic role. In our study, we could not identify affected individuals carrying that change in the homozygous state. Therefore, we cannot provide additional proof of a possible pathogenic role of this variant.

Given the high frequency among normal individuals and considering the data mentioned herein, we think that a disease-causing role of p.Asp1114Gly (*RPGRIP1*), p.Pro701Ser (*GUCY2D*) and p.Tyr134Phe (*AIPL1*) has not been shown. Therefore, we think it is reasonable to exclude these changes from our further analyses of pathogenic mutations in our cohort.

Similarly, there is a need to discuss the role of the p.Ile205Thr variant in *CRB1*. This change was initially described as a disease-causing mutation in a Spanish ARRP family,[51] but recently has been reported in two articles[49,52] to be

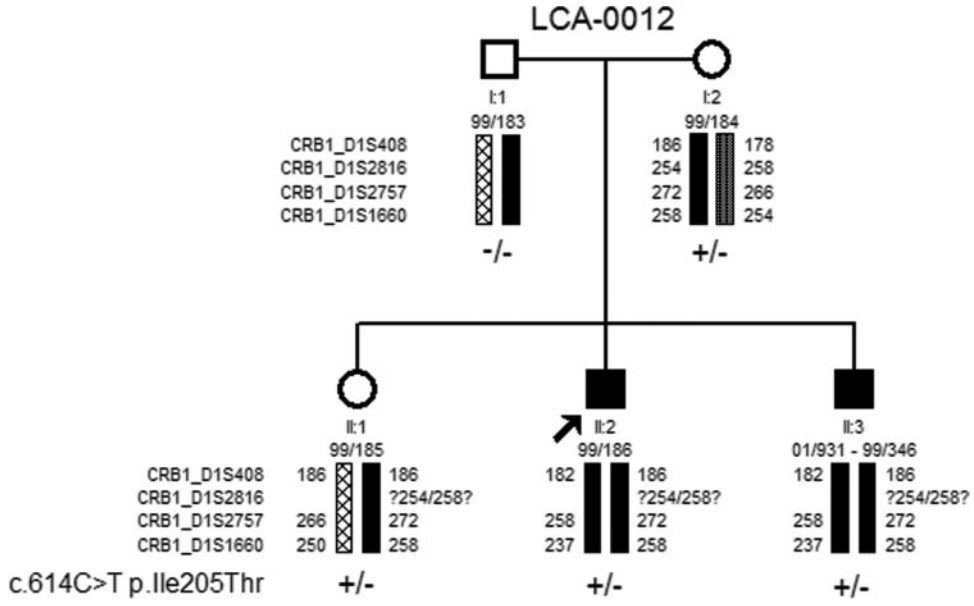


FIGURE 5. Segregation analysis of family LCA-0012.

TABLE 5. Respective Frequencies of Mutant Alleles in the LCA Cases Compared with Those from Other Countries

	Nijmegen (The Netherlands)*		Montreal (Canada)*		Baltimore (USA)*		Spain	
Alleles	**82**	**%**	**118**	**%**	**210**	**%**	**84**	**%**
AIPL1	10	12.20	6	5.08	6	2.86	1	1.19
CRB1	0	0.00	2	1.69	14	6.67	14	16.67
CRX	0	0.00	1	0.85	2	0.95	0	0.00
GUCY2D	4	4.88	11	9.32	17	8.10	0	0.00
RPE65	0	0.00	1	0.85	5	2.38	1	1.19
RPGRIP1	3	3.66	3	2.54	6	2.86	4	4.76
Total	17	20.73	24	20.34	50	23.81	20	23.81

* Zernant et al.[48]

a polymorphic sequence change. We found this change in a heterozygous state in three families, LCA-0012, LCA-0042, and RP-0310. In LCA-0012 (Fig. 5) and RP-0310, haplotypes for *CRB1* cosegregated with the disease, in LCA-0042 there was only one affected son; therefore, segregation analysis is not informative. We have not observed this change together with other *CRB1* pathogenic alleles in the same family, nor can microsatellites exclude it. We think it is reasonable to consider that change to be a mutation in our further analyses. It is up to future studies in different populations and to functional investigations to clarify the role of this change in retinal disease.

Frequencies of Disease-Causing Mutations

Taking these considerations into account, the frequencies of alleles carrying proven disease-causing mutations were 23.8%, 6.1%, and 4.3% (Fig. 1). *CRB1* was the most frequent mutated gene in the affected Spanish families (Fig. 1). There was a gradient in mutation frequencies with respect to onset and severity of retinal disease. Considering this fact, it is crucial to establish accurate clinical diagnosis including a thorough history of initial symptoms, to classify the samples correctly.

The most frequent mutated gene in this study was *CRB1*, with 32 of 46 mutated alleles in all three groups. *CRB1* represented 69% of all mutations. However this proportion is different from data presented by Zernant et al.[48] (Table 5). Initially, *CRB1* mutations were detected in 10% to 13% patients with LCA,[6,15,17] and the study of Yzer et al.[50] showed that *CRB1* was the most frequent mutated gene (9/19 patients) in a cohort of predominantly Belgian and Dutch patients. In contrast, den Hollander et al.[52] studied a cohort of 44 LCA patients (24 from Quebec and 20 from other countries worldwide) and identified only one mutated allele for *CRB1*.

We found only one *RPE65* mutation in our population (Fig. 1), in contrast with the frequency reported in other populations.[51] Consistent with our findings Marcos et al.[53] found no mutations of *RPE65* in 72 Spanish families.

CONCLUSIONS

In conclusion, *CRB1* was the most frequent mutated gene in LCA in this Spanish population.

We think the LCA microarray should be only the first step in the molecular diagnosis when the clinical diagnosis is appropriate for LCA or early-onset RP. This method allows for a fast and economic screening of the main genes implicated in the disease, as well as for the identification of respective mutations. The combination of microarray and conventional laboratory analysis is an optimal approach to verify the pathogenic role of the identified sequence changes and to detect new disease alleles.

However, the relative low percentage of affected alleles found in this study with the microarray suggests that more LCA-associated genes and mutations are still to be identified.

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