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Homozygosity mapping in autosomal recessive retinitis pigmentosa families detects novel mutations

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Purpose: Autosomal recessive retinitis pigmentosa (arRP) is a genetically heterogeneous disease resulting in progressive loss of photoreceptors that leads to blindness. To date, 36 genes are known to cause arRP, rendering the molecular diagnosis a challenge. The aim of this study was to use homozygosity mapping to identify the causative mutation in a series of inbred families with arRP.
Methods: arRP patients underwent standard ophthalmic examination, Goldman perimetry, fundus examination, retinal OCT, autofluorescence measurement, and full-field electroretinogram. Fifteen consanguineous families with arRP excluded for *USH2A* and *EYS* were genotyped on 250 K SNP arrays. Homozygous regions were listed, and known genes within these regions were PCR sequenced. Familial segregation and mutation analyzes were performed.
Results: We found ten mutations, seven of which were novel mutations in eight known genes, including *RP1, IMPG2, NR2E3, PDE6A, PDE6B, RLBP1, CNGB1,* and *C2ORF71,* in ten out of 15 families. The patients carrying *RP1*, *C2ORF71,* and *IMPG2* mutations presented with severe RP, while those with *PDE6A*, *PDE6B,* and *CNGB1* mutations were less severely affected. The five families without mutations in known genes could be a source of identification of novel genes.
Conclusions: Homozygosity mapping combined with systematic screening of known genes results in a positive molecular diagnosis in 66.7% of families.

Photoreceptor degeneration is the leading cause of inherited blindness [1]. This is partly explained by the extreme genetic heterogeneity of these conditions as more than 200 genes are currently registered in the RetNet database, reflecting the vast repertoire of genes necessary for photoreceptor or other retinal cell function. The most frequent clinical entity, nonsyndromic retinitis pigmentosa (RP; OMIM 268000), is also the most genetically heterogeneous with more than 50 disease-causing genes currently associated with this condition. Among these, 36 are known to be responsible for autosomal recessive (ar) inheritance, accounting for 50–60% of all arRP cases [2]. Two major genes are responsible for ar inheritance, *USH2A* [3,4] and *EYS* [5-8].

Homozygosity mapping in inbred multiplex families or isolated cases with presumed ar inheritance has proven successful for finding novel genes [9-19] and identifying mutations in previously described genes [20-35]. Homozygosity mapping saves time as it readily highlights regions containing already known disease-causing genes or new genes/loci. This strategy has also been successful in a variable proportion of cases from outbred families who carry a homozygous mutation due to a high level of inbreeding encountered in some populations [21,22,36]. In this study, we applied this strategy to a series of 15 families with consanguineous parents and found that two-thirds of the families carried a mutation in a known arRP gene.

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METHODS

Patients and clinical investigations: Consanguineous arRP families were selected from 423 families with arRP. Informed written consent and peripheral blood samples were obtained for genetic analysis from all family members according to approved protocols of the Montpellier University Hospital, in agreement with the Declaration of Helsinki.

Patients underwent standard ophthalmologic examination (refractometry, visual acuity, slit-lamp examination, applanation tonometry, funduscopy). Kinetic visual fields were determined with a Goldman perimeter with targets V_{4e}, III_{4e}, and I_{4e}. OCT measurement of the macula was performed using an OCT-3 system (Stratus model 3000; Carl Zeiss Meditec, Dublin, CA) with software version 3.0. Autofluorescence measurements were obtained with the HRA2 Heidelberg retinal confocal angiograph (Heidelberg

Engineering, Dossenheim, Germany), and fundus pictures were taken. Full-field ERG was recorded using a Ganzfeld apparatus (Metrovision, Pérenchies, France) with a bipolar contact lens electrode on maximally dilated pupils according to the ISCEV protocol [37].

Single nucleotide polymorphism genotyping and Sanger sequencing of candidate genes: Genomic DNA was isolated from leucocytes using a proteinase K digestion, followed by an ethanol precipitation [38]. DNA samples were quantified by a spectrophotometer, aliquoted and stored at +4 °C and -20 °C until use. From 31 consanguineous families (26 multiplex, five sporadic), 15 families that were not homozygous for *EYS* and *USH2A* microsatellite markers were selected and genotyped with 262,270 single nucleotide polymorphisms (SNPs; GeneChip Mapping 250 K Nsp Array; Affymetrix, Santa Clara, CA) at DNAVision, Charleroi, Belgium. Results were analyzed using the common homozygosity regions test of the transmitted allele search engine (TASE) [39]. TASE was designed to screen for common homozygous genotypes in all affected individuals that are heterozygous or wild type in unaffected individuals. Candidate chromosomal regions of homozygosity larger than 2 Mb were compared to the position of known genes and loci for retinal inherited diseases according to the RetNet database. All exons and exon–intron boundaries of the candidate genes were then sequenced. Each PCR was performed in a 25-µl reaction mix containing 50 ng of genomic DNA, 2 mM $MgCl_2$, 200 µM deoxyribo nucleotide triphosphate (dNTPs), 0.2 µM of each primer (designed with Primer 3 software), and 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA) in a buffer composed of 100 mM Tris-HCl, pH 8.3 and 500 mM KCl. Following the first denaturation at 95 °C for 9 min, amplification was carried out for 35 cycles at 95 °C for 30 s, at the melting temperature (Tm) of the primers (56 °C-60 °C) for 30 sec and at 72 °C for 1 min, ending with a final extension step at 72 °C for 10 min. PCR products were purified with ExoStar 1-step clean up (GE Healthcare, Little Chalfont, UK) and sequenced using the BigDye Terminator cycle sequencing ready reaction kit V3.1 on an Applied Biosystems 3130xL genetic analyzer, following the manufacturer's instructions. Sequencing results were analyzed with Sequencing Analysis v5.2 software (Applied Biosystems).

Mutation validation: Mutations identified were validated by reading the existing literature, performing familial segregation whenever possible, consulting the Human Gene Mutation Database (HGMD), and interrogating databases with mutation frequencies (1000 genomes, Exome Variant Server [EVS], dbSNP). Missense variations were systematically analyzed using Polyphen-2 and SIFT softwares.

RESULTS

Genotyping and mutation detection: From the 15 consanguineous families, eight families were simplex and seven were multiplex (Figure 1). An average of two SNP arrays per family was performed (range one to three arrays). The quality control (QC) call rate for all samples was always above 90%.

For each family, homozygous regions were classified as a function of SNP coverage (number of SNPs/Mb; Table 1). There were on average 7.7 homozygous regions (range 1–18 regions) with a mean size of 15.3 Mb (range 2.2–53 Mb) per family. The homozygous regions were compared with the position of the genes listed in the RetNet database, and the corresponding genes were systematically sequenced, with priority given to known nonsyndromic arRP genes. A total of 32 genes were sequenced for the 15 families. For eight of these families, only one gene was sequenced and this revealed a causative mutation.

We found that ten out of 15 families had a causative homozygous mutation in one of the genes screened (Table 2). In eight of the ten families, segregation analysis could be performed and the results obtained were in accordance with the autosomal recessive inheritance of the disease (Figure 1). Among the ten mutations, three were previously described (one mutation was only described in the dbSNP database). Seven mutations were either nonsense, frameshift, or large deletions and were presumed to lead to loss of protein function.

Three mutations were amino acid changes. The c.364C>T (p.Arg122Cys) in *NR2E3* is not found in the HGMD database nor in the EVS. It is located two amino acids downstream of the DNA-binding domain of the protein and is considered to be probably damaging with a score of 1.000 by Polyphen-2. The c.2284C>T (p.Arg762Cys) in exon 23 of *CNGB1* affects arginine 762 located in the extracellular domain, which is found in two isoforms of the protein but is missing in the third isoform (named GARP2, for glutamic acid-rich protein-2). This amino acid is conserved in all species in the Polyphen-2 software. It is not described in the EVS, 1000 genomes, and HGMD databases. It is predicted to be probably damaging by Polyphen-2 and damaging by SIFT. The c.1568T>G (p.Met523Arg) in exon 12 of *PDE6B* is located between the cGMP binding domain and the catalytic domain where many mutations known to cause RP are found. This mutation is not described in the EVS, 1000 genomes, and HGMD databases and is predicted to be possibly damaging by Polyphen-2 and damaging by SIFT.

In total, we found that ten out of 15 families, i.e., 66.7% of the sequenced families, had a mutation in a known gene.

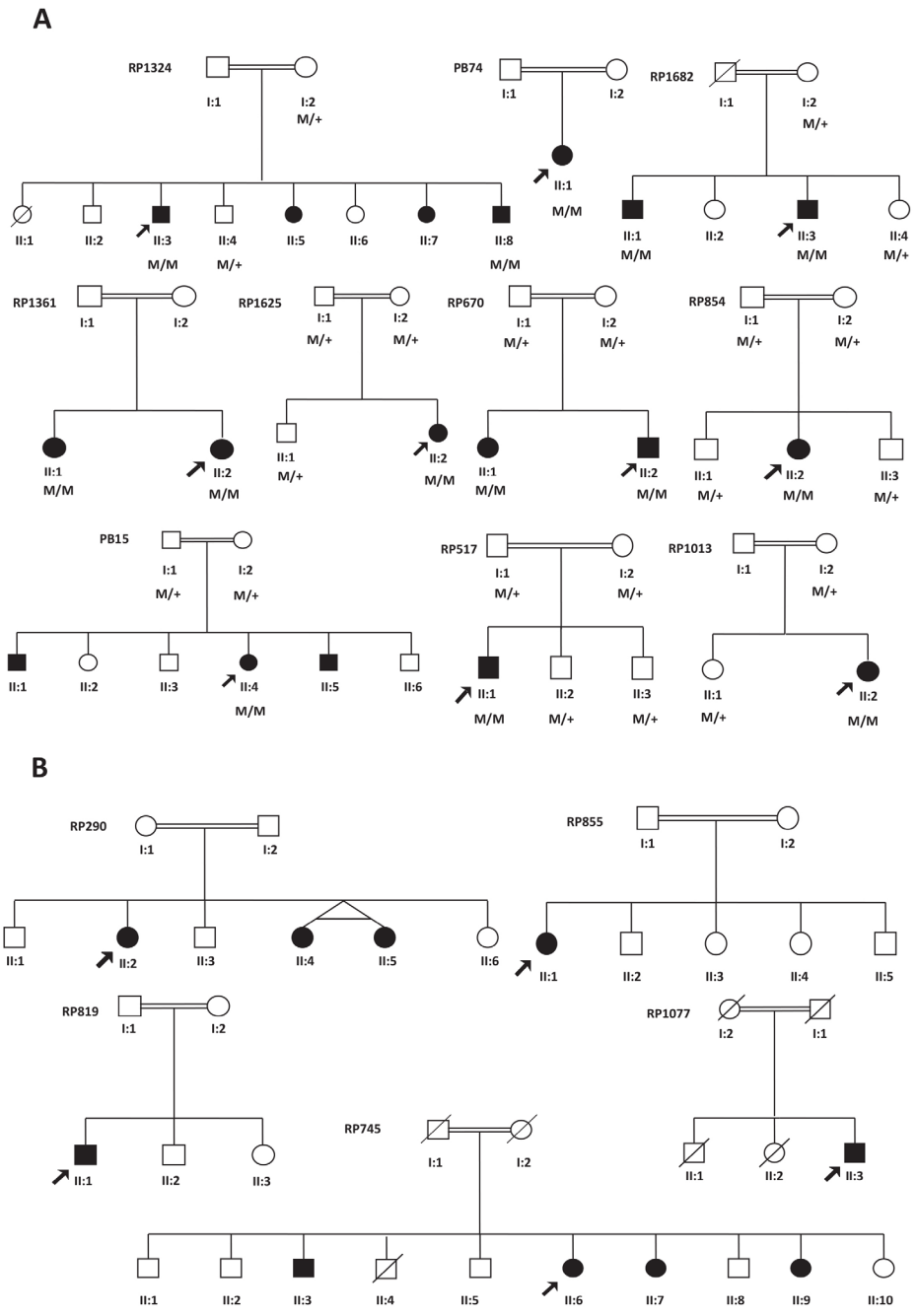

Figure 1. Pedigrees of 15 consanguineous families with autosomal recessive retinitis pigmentosa. **A:** Pedigrees of families with mutations in a known RP gene, and co-segregation in available family members. Filled symbols, affected; unfilled, unaffected persons. Squares, males; circles, females; arrows, index patients; M, mutated allele; +, wild-type allele. **B:** Pedigrees of unsolved families.

For the remaining five families (33.3%), all RP genes in homozygous regions were sequenced but no mutation was detected, suggesting that their proband could carry a mutation in a novel gene. We cannot, however, exclude that mutations in noncoding genomic sequences have been missed. These negative families will ultimately undergo exome sequencing.

Clinical findings in families with identified mutations: Clinical description was available for 12 patients from the ten families in which a mutation was found (Appendix 1). The clinical features were variable depending on the gene involved, although all patients had night blindness (not known

Table 1. Homozygous regions and candidate genes found in the studied families.

Family #	Chromosome	Length*	SNP/Mb	Start	Stop	RANK	*Genes*
PB15	15	8	97	94,755,230	103,000,000	1	
	3	20	69	81,385,867	101,821,068	2	***IMPG2***, *BBS3*
	11	8	48	48,019,397	56,670,113	3	
	8	6	18	42,604,791	49,328,524	4	
PB74	7	2.8	192	14,305,994	17,109,926	1	
	9	2.6	147	23,912,009	26,502,533	2	
	11	25.2	110	8,268,949	33,487,251	3	*TEAD1, USH1C*
	6	2.4	104	75,641,951	78,011,282	4	
	14	24.4	99	33,161,305	57,535,645	5	*OTX2*
	13	20	92	41,472,025	61,458,472	6	*RB1*
	2	11.3	77	229,188,012	240,448,167	7	*SAG, KCNJ13*
	3	30.2	73	86,088,197	116,313,537	8	*IMPG2, BBS3*
	1	2.9	47	49,428,303	52,348,388	9	
	4	3.6	45	0	3,586,240	10	***PDE6B***
	11	5.6	42	51,538,030	57,168,706	11	
	19	2.2	25	17,232,499	19,446,936	12	
	10	5.6	24	37,264,807	42,897,610	13	
RP290	4	22	111	164,572,108	1,886,612,779	1	
	13	24	110	19,611,203	43,979,500	2	
	4	17	109	14,126,510	31,208,706	3	*PROM1*
	14	18	95	72,775,668	90,693,572	4	*BBS8*
	11	37	78	27,547,379	64,200,379	5	*BEST1, ROM1*
	9	22	71	115,228,455	137,266,704	6	
	11	16	51	64,202,427	80,653,055	7	*BBS1, CABP4, EVR4, MYO7A*
RP517	18	10	147	64,702,698	75,485,034	1	
	10	15	104	102,878,609	124,720,093	2	
	15	6	99	88,605,547	94,989,511	3	***RLBP1***
	4	27	93	142,603,130	170,091,790	4	*LRAT*
	6	5	93	134,536,698	139,731,589	5	*AHI1, PEX7*
	10	42	92	13,549,280	55,658,110	6	*RBP3, PCDH15*
	10	16	90	73,315,427	90,014,680	7	*PCDH21, RGR*
	2	19	88	215,924,486	243,717,669	8	*SAG, KCNJ13*
	2	7	85	161,288,182	168,767,322	9	

Family #	Chromosome	Length*	SNP/Mb	Start	Stop	RANK	*Genes*
	7	10	77	139,122,579	149,147,283	10	
	16	22	68	54,368,954	76,163,081	11	*CNGB1, CDH3*
RP670	15	20	67	70,670,532	91,626,291	1	*RLBP1,* ***NR2E3***
	12	5	35	33,095,301	38,783,937	2	
	11	7	28	47,884,847	55,182,977	3	
RP745	18	15	131	59,022,364	74,333,187	1	
	7	13	96	36,733,691	49,999,517	2	
	5	27	81	38,657,850	65,932,343	3	
	7	32	74	70,138,345	102,504,683	4	*PEX1*
	7	20	61	50,006,472	70,113,409	5	
	11	8	34	47,873,883	55,620,859	6	
	17	17	33	14,152,804	30,823,007	7	*HRG4/UNC119*
RP819	16	7	145	6,137,184	12,877,511	1	
	11	9	106	76,325,821	85,436,868	2	*MYO7A*
	3	17	93	100,042,849	116,695,553	3	*IMPG2*
	10	11	89	99,038,933	110,389,800	4	*ARL3*
	5	20	82	72,943,817	92,857,172	5	
RP854	8	7	125	16,489,491	23,287,191	1	
	12	6	113	102,972,070	108,720,576	2	
	1	9	97	167,407,517	176,453,828	3	
	8	24	98	102,458,515	126,789,312	4	
	8	13	96	53,880,054	67,044,043	5	*RP1, TTPA*
	2	7	81	183,870,146	191,325,255	6	
	2	8	74	26,097,607	34,429,224	7	*ZNF513, C2ORF71*
	5	16	67	38,414,330	54,391,555		
RP855	11	12	119	85,869,911	97,383,737	1	
	15	5	117	33,415,288	38,491,401	2	
	10	19	114	105,391,725	123,911,345	3	
	13	6	106	84,581,120	90,263,015	4	
	6	8	104	117,641,016	125,607,559	5	
	5	23	103	101,320,305	123,846,658	6	
	7	15	103	155,645,303	170,683,241	7	
	18	9	101	38,011,865	47,209,971	8	
	5	5	101	26,045,009	31,423,339	9	

Family #	Chromosome	Length*	SNP/Mb	Start	Stop	RANK	*Genes*
	14	14	96	34,194,336	48,552,897	10	
	4	25	93	111,252,455	136,022,295	11	*BBS12*
	11	10	89	110,685,226	120,548,318	12	*MFRP*
	2	16	88	148,875,566	164,388,639	13	
	10	29	87	34,317,035	63,401,422	14	*RBP3, PCDH15*
	20	9	87	53,622,497	63,000,000	15	*PRPF6*
	6	20	84	23,895,019	44,154,325	16	*TULP1, PRPH2*
	21	5	73	42,506,107	48,000,000	17	
	11	48	72	35,991,050	83,847,610	18	*BEST1, ROM1, BBS1, CABP4, MYO7A*
RP1013	7	15	112	19,386,915	34,648,783	1	*KLHL7,PAP1*
	5	15	110	106,422,649	121,734,393	2	
	16	15	104	1,889,821	16,730,604	3	*ABCC6*
	8	7	103	25,445,326	32,079,101	4	
	7	9	92	120,144,923	128,748,534	5	*TSPAN12, IMPDH1, OPN1SW*
	2	36	89	188,652,307	224,429,597	6	
	8	11	86	58,758,850	69,756,364	7	*TTPA*
	3	53	85	68,243,465	121,132,268	8	*BBS3, IMPG2*
	8	30	85	73,152,837	102,974,357	9	*PEX2, CNGB3, C8ORF37*
	5	48	84	26,788,362	74,365,757	10	
	7	10	81	137,476,571	147,969,602	11	
	11	48	70	32,076,832	80,155,054	12	*BEST1, ROM1, BBS1, LRP5*
	2	11	68	23,712,008	34,302,743	13	*ZNF513,* ***C2ORF71***
RP1077	1	25	106	217,257,837	241,982,430	1	
	21	21	98	9,764,385	31,185,292	2	
	4	5	20	48,094,534	53,233,482	3	
	5	6	16	44,334,983	50,066,049	4	
	20	5	10	25,506,582	30,680,225	5	
	16	15	4	31,905,355	46,831,180	6	
	2	7	3	88,965,501	96,239,773	7	
	9	33	2	38,703,364	71,244,025	8	
RP1324	16	7	100	54,776,161	62,457,005	1	***CNGB1****, BBS2*
RP1361	5	16	112	145,313,228	162,831,477	1	***PDE6A***
	20	11	112	49,172,235	60,352,153	2	
	16	16	107	65,223,172	81,678,049	3	*OPA8, CDH3*

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Family #	Chromosome	Length*	SNP/Mb	Start	Stop	RANK	*Genes*
	8	25	77	37,328,843	63,110,766	4	*RP1, ADAM9*
	20	22	66	22,782,904	45,359,408	5	
RP1625	8	18	142	5,057,818	23,559,224	1	*RP1L1*
	1	13	120	58,394,231	72,327,802	2	*RPE65*
	4	5	98	31,636,008	36,732,290	3	
	7	7	91	123,580,016	131,407,605	4	*OPN1SW, IMPDH1*
	12	13	90	117,118,028	130,497,472	5	
	2	7	79	233,929,283	241,117,231	6	*SAG*
	8	32	79	40,071,825	72,824,945	7	***RP1**, TTPA*
	12	5	30	34,142,799	39,744,369	8	
	3	5	27	88,365,050	93,558,926	9	
	7	7	17	56,665,370	63,796,171	10	
RP1682	15	13	89	81,632,077	95,169,873	1	***RLBP1***
	5	5	74	172,682,382	177,906,494	2	

For each family, homozygous regions were classified according to the size of the region in Mb, and the coverage (number of SNP per Mb) to give a rank for prioritizing the molecular screening. Each region is then defined by its position on the corresponding chromosome (Start and Stop sections). The causative gene was bolded for families with positive molecular diagnosis. *: In Megabase pair.

Table 2. Mutations found in this study

Family #	Gene	cDNA change	Protein change	Prediction	Allele frequency	Previously described
PB15	*IMPG2*	c.636delA	p.Glu213ArgfsX17	deletion of the last 1012 AA	0	novel
PB74	*PDE6B*	c.1568T>G	p.Met523Arg	possibly damaging	0	novel
RP517	*RLBP1*	DelExons7–9	p.Ile176_Phe317del	deletion of the last 142 AA	nd*	[62]
RP670	*NR2E3*	c.364C>T	p.Arg122Cys	probably damaging	0	novel
RP854	*RP1*	c.3418delGG	p.Gly1140LysfsX4	deletion of the last 1013 AA	0	novel
RP1013	*C2ORF71*	c.403G>T	p.E135X	deletion of the last 1154 AA	0	novel
RP1324	*CNGB1*	c.2284C>T	p.Arg762Cys	probably damaging	0	novel
RP1361	*PDE6A*	c.769C>T	p.Arg257X	deletion of the last 603 AA	0.000093**	[40]
RP1625	*RP1*	c.1186C>T	p.Arg396X	deletion of the last 1760 AA	0.000076**	rs201493928
RP1682	*RLBP1*	c.488insA	p.Ile163AsnfsX1	deletion of the last 154 AA	0	novel

For each family with a positive molecular result, we indicate the name of the causative gene, the cDNA change, the protein change and its prediction, the allele frequency of the mutation, and when possible the reference of the mutation found. *nd: not determined ** based on Exome Variant Server database

for II:4 from PB15), retinal vessel attenuation and retinal atrophy in fundus, and strongly decreased ERG responses.

The 19- and 17-year-old II:1 and II:2 sisters from RP1361 had the same clinical presentation, revealing severe RP, which was consistent with the homozygous *PDE6A* null mutation that they carried as the produced protein is expected to be unstable and degraded by nonsense-mediated decay [40]. They showed few pigment deposits in the fundus (Figure 2A, B) and few atrophic spots in peripheral retina (Figure 2C). The retinal arterioles, however, were already narrow and the macular area showed a typical ring of autofluorescence best seen in patient II:2 (Figure 2D). The sisters also had an important bilateral macular edema (Figure 2E, F) with decreased visual acuity between 0.5 and 0.7. They had no scotopic ERG responses but both still had minimal photopic responses. In comparison, 46-year-old patient II:1 from PB74, who had a missense mutation in *PDE6B*, retained relatively good visual acuity (0.8 in both eyes). The 44- and 57-year-old II:8 and II:3 brothers from RP1324 also had severe RP due to a missense homozygous mutation in *CNGB1*. The younger brother had ocular trauma on the left eye in infancy with no light perception, while the contralateral eye had decreased visual acuity at 0.3 (in part due to a cataract as shown by the blurred fundus image [Figure 2G]), bone spicule pigment deposits in the retinal periphery, and narrowing of retinal vessels without atrophy of the optic disc. The IS/OS line was still present in the foveal area. He had tunnel vision at 20–30°, and ERG responses were absent. The elder brother had advanced RP with bare light perception in both eyes, atrophy of both peripheral retina and macula, and large pigment deposits distributed throughout the retina (Figure 2H).

The 29-year-old III:2 patient from RP1013 carried a null mutation in *C2ORF71*, was myopic, and had severe RP; visual acuity was decreased at 0.3 OD and 0.4 OS. Fundus examination showed bilateral, round, foveal atrophy with narrowed retinal vessels and atrophic optic discs (Figure 2I). Retinal autofluorescence testing revealed small atrophic spots grouped in the foveal area (Figure 2J). The visual field was tubular at 30°, and ERG photopic responses were still recordable although very low.

Both patients with a homozygous *RP1* mutation also had severe RP, were myopic, and showed bilateral macular involvement. The younger 10-year-old patient II:2 from RP1625 had decreased visual acuity at 0.6 on both eyes. The fundus showed an abnormal foveal reflex, a dark perifoveal area, narrowed retinal arterioles, atrophy of the peripheral retina, and a few small pigment deposits (Figure 2K). Retinal autofluorescence testing revealed many atrophic spots in the peripheral retina and a slightly increased autofluorescence around the fovea (Figure 2L). The outer nuclear layer and the IS/OS were absent except in the foveola where they remained only partly preserved (Figure 2M, N). The patient had tunnel visual field (10–20°), and ERG responses were absent. The older 37-year-old patient III:2 from RP854 had hand motion in both eyes. Fundus examination showed a bilateral round atrophy of the macula, narrowed retinal vessels, atrophic optic discs, and many bone spicule pigment deposits in the retinal periphery (Figure 2O, P). The visual field was undetectable, and ERG responses were absent. The 13-year-old patient from PB15 with a null mutation in *IMPG2* also had severe RP with myopia, macular involvement, decreased visual acuity at 0.2 in both eyes, and no ERG response.

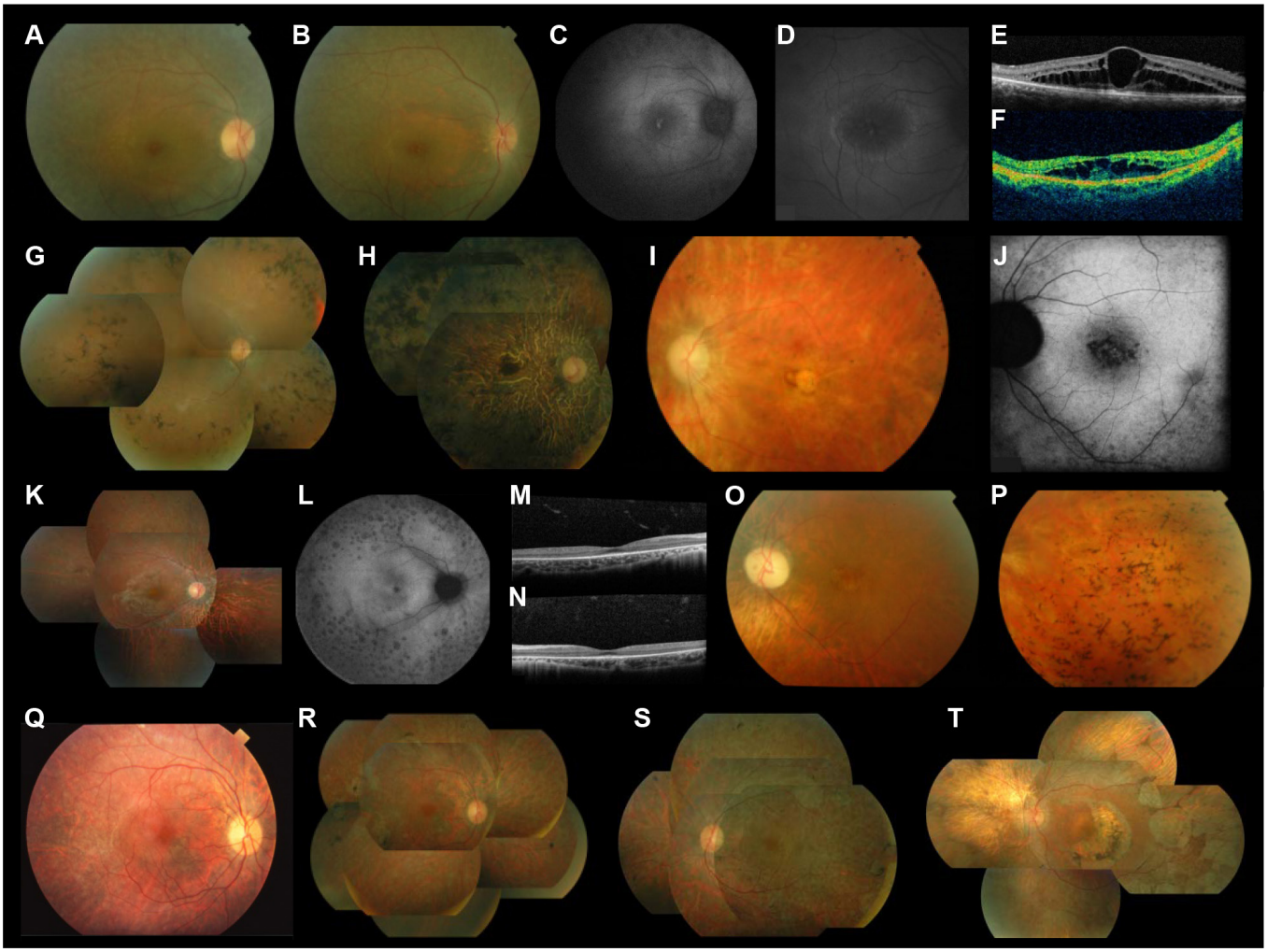

Figure 2. Clinical features of patients with mutations in autosomal recessive retinitis pigmentosa. **A-F**: Family RP1361 with PDE6A mutation; fundus photographs of right eyes of subjects II:1 (**A**) and II:2 (**B**), retinal autofluorescence in right eyes of subjects II:1 (**C**) and II:2 (**D**), OCT scan of the macula of the right eye of subjects II:1 (**E**) and II:2 (**F**). **G-H**: Family RP1324 with CNGB1 mutation; fundus photographs of right eyes of subjects II:8 (**G**) and II:3 (**H**). **I-J**: Family RP1013 with C2ORF71 mutation; fundus photograph (I) and retinal autofluorescence (J) of the left eye of subject II:2. **K-P**: Families RP1625 (K-N) and RP854 (**O**, **P**) with RP1 mutations; fundus photograph (**K**) and retinal autofluorescence (L) of the right eye of subject II:2 of family RP1625, OCT scan in the macula of the right (M) and left (N) eyes of subject II:2 of family RP1625, fundus photographs of the left eye of subject II:2 of family RP854 showing the macula (**O**) and the temporal periphery (P). **Q-T**: Families RP517 (**Q-S**) and RP1682 (T) with RLBP1 mutations; fundus photographs of the right eye at 32 years (Q) and 40 years (R) and of the left eye at 40 years (S) of subject II:1 of family RP517, and of the left eye (T) of subject II:3 of family RP1682.

Both patients with homozygous *RLBP1* mutations had less severe RP than the ten other patients and both had early onset night blindness. The 32-year-old patient from RP517 family had decreased visual acuity in accordance with foveal thinning, but the retinal vessels were moderately narrowed and the optic discs were not atrophic (Figure 2Q). The fundus had a whitish aspect and the retinal periphery showed rare clumps of pigment deposits. When this patient was examined at the age of 40, larger atrophic spots were visible (Figure 2R, S). The 58-year-old II:3 from RP1682 had a similar presentation although with more advanced disease. Visual acuity was still at 0.4 OD and 0.3 OS. The fundus showed large scallop-shaped spots of atrophy covering the mid-periphery of the retina (Figure 2T). There were semicircular atrophic spots around the fovea in both eyes that were secondary to previous laser treatment of macular edema.

DISCUSSION

With the advent of clinical trials for inherited retinal dystrophies, the causative gene needs to be identified. Molecular identification permits the diagnosis of the RP subtype,

improved patient follow up, and prediction of disease course. Gene identification is also necessary for gene therapy and to organize patient series for clinical trials. However, molecular diagnosis in arRP, the most genetically heterogeneous form of inherited retinal disease, currently requires screening 36 genes, a process which has never been completed by any research group by Sanger sequencing because it is time and money consuming.

As a preliminary approach to exome sequencing, we used SNP genotyping for homozygosity mapping of consanguineous families and found that two-thirds of the 15 families carried a homozygous causative mutation in a known gene. At the time of our study, it was more economical to perform homozygosity mapping with SNP 250 K arrays to select for families negative for known genes. Presently, the cost of targeted sequencing of RP genes or exome sequencing using next generation sequencing (NGS) is equivalent to that of mapping with SNP arrays, therefore making this approach more affordable.

Given that an average of 13% of arRP cases (range 5% to 18%) may have a mutation in *EYS* [5-8] and that 16% (range 12% to 20%) may have a mutation in *USH2A* [3,4], then 76% of patients with arRP are estimated to have a mutation in a known gene, meaning that about a quarter of the arRP patients would have mutations in yet undiscovered genes. In this series, we did not find any homozygous or heterozygous mutations in candidate genes within homozygous regions for the five negative families. These regions represent good candidates to find novel genes by whole exome sequencing. Future analyzes will then be shortened by directly exploring the homozygous regions. Our results showed a high percentage (70%) of novel mutations, indicating that there is considerable allelic heterogeneity in arRP. Similar results were found in a recent study in which 63% of novel mutations were found by targeted sequencing using NGS in a Chinese patient cohort with arRP [41].

The analysis of patient phenotypes showed some variations in disease severity. We found that the three patients with mutations in a connecting cilium gene, i.e., *RP1* and *C2ORF71*, had severe RP with early macular degeneration, while the patients with mutations in a phototransduction gene, i.e., *PDE6A*, *PD6B*, and *CNGB1*, had less severe RP [40,42-45]. Indeed, patients with arRP due to *RP1* mutations were frequently reported with legal blindness by their twenties and thirties. Previous case reports described onset in childhood, flat ERG by 18 years, macular involvement before 20 years, or even total blindness before 20 [46-53]. Macular involvement is found earlier in RP due to cilia-associated genes, such as Bardet–Biedl syndrome genes and the recently described *ARL2BP* gene [54]. Conversely, *PDE6A* and *PDE6B* phenotypes show great variation in the severity of disease and frequent macular edema [55]. Yet, it remains difficult to preselect genes for screening based only on macular involvement and severity of the disease.

It is of note that the two patients with a mutation in *RLBP1* were not diagnosed as having retinitis punctata albescens (RPA). Apart from typical RPA, *RLBP1* mutations have been reported in two subclinical forms of RPA, Bothnia retinal dystrophy [56] and Newfoundland rod–cone dystrophy [57], as well as in rare cases of arRP [58]. In our patients, the specific, small, white, dot-like deposits usually observed on the fundus were not present [59]. It is possible that the dots were present at early stages and had progressively vanished in the course of the disease, thus preventing the correct diagnosis, as previously reported [60]. It is also important to mention that the two patients with this RPA form of arRP had the least severe phenotype among the 12 patients examined. It is known that signs of retinal degeneration (retinal vessel attenuation, optic disc pallor) progress more slowly in RPA than in typical arRP [61].

APPENDIX 1. SUMMARY OF CLINICAL FEATURES.

To access the data, click or select the words "Appendix 1." *: apparent age at onset; Ch: childhood; fl: flickers; NB: night blindness; nd: not determined; ND: not done; nl: normal; PP: photophobia; PV: peripheral vision impairment; OD: ocular dextra; OS: ocular sinistra; HM: hand motion; LP: light perception.

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