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Mutation analysis of *GABRR1* and *GABRR2* in autosomal recessive retinitis pigmentosa (*RP25*)

EDITOR—Retinitis pigmentosa (RP, MIM 268000) is the most frequent form of retinal dystrophy world wide. The clinical findings are night blindness and narrowing of the visual field. Examination of the fundus of the eye in RP patients usually shows bone spicula pigmentation of the retina, waxy pallor of the optic disc, attenuation of the retinal blood vessels, and no results detectable by electroretinogram.[1]

RP shows notable allelic and non-allelic heterogeneity[2] (RET-GEN-NET http://www.sph.uth.tm.edu/Retnet/home.htm). By using classical linkage strategies and the direct and indirect candidate gene approach, the number of RP loci identified has grown increasingly since 1989 and to date more than 30 autosomal RP loci have been identified, including syndromic and non-syndromic forms of the disease. Autosomal recessive RP (ARRP) is the commonest form of RP and to date at least 13 independent ARRP loci have been identified.[3–15]

Our group proposed the hypothesis that the alteration of functions related to neurotransmission in the external plexiform layer of the retina could be related to RP.[14] In order to test this model, we used homozygosity mapping to analyse different genes involved in retinal neurotransmission. Using this indirect candidate gene approach, we identified the locus *RP25* in an important subgroup of ARRP patients from our cohort. In fact, around 14% of the ARRP families from southern Spain showed linkage to *RP25*.[14] *RP25* is an ARRP locus located on the long arm of chromosome 6 between markers D6S257 and D6S1644 (MIM 602772). This chromosomal region contains the *GABRR1* and *GABRR2* genes, both being expressed in the retina. These genes encode the rho1 and rho2 subunits of the C type receptor for γ-aminobutyric acid (GABAc receptor).[16 17] The GABAc receptor is expressed in the horizontal and bipolar cells of the retina.[18 19] For this reason, we considered both genes to be attractive candidates for mutation analysis.

In order to identify the intron-exon boundaries of *GABRR1*, we selected the gene that encodes the β1 subunit of the GABAa receptor whose complete genomic structure is known (M59212). Comparing the cDNA of the *GABRR1* gene (M62323) and the cDNA of the *GABRB1* gene (X14767),[20] we obtained regions of high homology, approximately 59%, in the fragments corresponding to exons 6, 7, 8, and 9. However, the homology observed in the fragments corresponding to exons 1, 2, 3, 4, and 5 was less than 42%.

Afterwards, we localised by homology the different putative exons of the cDNA of the *GABRR1* gene, which permitted the design of exonic primers to amplify exon-exon fragments containing all the introns. All the primers had the universal M13 primers attached 5' (see table 1 for more details). The large PCR products were purified and then sequenced by the biochemical method of Sanger using dideoxynucleotides as terminators (fmol®DNA Sequencing System Promega, Madison, WI). Electrophoresis was carried out in the automatic sequencer Alf-Express (Amersham-Pharmacia Biotech) at 1500 V and 50°C using Long Ranger Singel™ (FMC) matrix.

The sequences obtained were analysed with the Alf-Manager™ program and were aligned afterwards with the cDNA sequence of the GABAc receptor (M62323) using the command Bestfit for GCG or the Multalin programs (Multiple Alignment with Hierarchical Clustering) or both.[21]

Using this approach, we identified the four fragments corresponding to the last four introns of the *GABRR1* gene (table 1).The information regarding introns 1, 2, 3, 4, and 5 of the *GABRR1* gene has been published elsewhere.[22]

In order to perform mutation screening of *GABRR1* and *GABRR2*, the index patients of the ARRP families that showed linkage to *RP25*, RP5.II.1, RP73.II.1, RP167.II.8, and RP214.II.5, were selected.[14] The DNA samples were PCR amplified using intronic primer pairs (tables 2 and 3). The products obtained were analysed by direct sequencing and fluorescent single strand conformational polymorphism analysis (SSCP) in the Alf-Express automatic sequencer (Amersham-Pharmacia Biotech) at 15 W. The migration patterns of each of the fragments were analysed using the Fragment Manager™ program. The DNA fragments corresponding to exons 4 and 8 of the *GABRR1*

Table 1 PCR conditions of individual introns of the GABRR1 gene

Intron	*Forward primer (5'→3')*	*Reverse primer (5'→3')*	*Size*	*Temp (°C)*	*Cycles*
Intron 6	M13F*-atggacttcagccgatttc	M13R*-agtcattgcccttttttcc	~5 kb	59	42
Intron 7	M13F-cttaaagacagatgaacgg	M13R-tggagcaagaagaagaag	~4 kb	60	40
Intron 8	M13F-aacttatttccccgctac	M13R-gaggaacacgaacacaaa	1.4 kb	57	35
Intron 9	M13F-tacctctgggtcagcttt	M13R-gggatagtgaaaacatgg	1.6 kb	58	23

*M13F: cgccagggttttcccagtcacgac
M13R: tttcacacaggaaacagctatgac

Table 2 PCR amplification of individual exons of the GABRR1 gene

Exon	*Forward primer (5'→3')*	*Reverse primer (5'→3')*	*bp*	*Temp (°C)*
1	M13F*-gaacagaccaataatgtctt	M13R*-cctaaatccttctatccc	291	50
2a	M13F-cttggttgatctgagtacc	M13R-ctttctgatccttggtg	272	49
2b	M13F-caccccatcacacctatc	M13R-gtcccttggctaatttcctgc	327	58
3	M13F-caagtaaaaacagtgaatgc	M13R-ctttgtgaatccccctgc	202	53
4	M13F-cagtgggtttgtgtgtgtc	M13R-gtgaaaccaatgcttttc	373	50
5	M13F-ctacatattggaaggaagc	M13R-gaattatcaggagctgtgtg	275	49
6	M13F-ctgatgctggcccctgtc	M13R-gctgaagcctgccctgac	256	62
7	M13F-aggagccatgatgtgtact	M13R-tgcagatgcttggaatatgc	282	60
8	M13F-ggacaaatgagcagagac	M13R-ttccagagctagatcagg	384	59
9a	M13F-gagagatgatgctgagct	M13R-gtatttatcaatggcgtggg	324	57
9b	M13F-gctatgtgagcatgagaatc	M13R-gggatagtgaaaacatgg	284	55

Table 3 PCR amplification of individual exons of the GABRR2 gene

Exon	*Forward primer (5'→3')*	*Reverse primer (5'→3')*	*bp*	*Temp (°C)*
1	cagccttagccctaacagc	gtggcacagtgggatggc	318	48
2	ctcactcaatgcattgaag	cttcctcatgcatggtgc	268	52
3	gatggaaggtgccttaac	gtgtagtgggcctggtggtgc	172	52
4	aaaccacttaatgcca	ctttctggtatgtgtggtc	337	48
5	ccaataattcaccgcacaag	catgagactgagcactgcc	219	56
6	gttacttcaccctgcatc	cagccttaaccccaagg	279	52
7	gtttgctttcacctctc	gagttcttaactgatgag	267	48
8	agggcagttctagaccgc	catgctgctgggtgaaaaa	299	52
9	cttaatgatgttctttgtgc	cggacttgttgaccac	426	48

gene were digested with the restriction endonucleases *Msp*I (Roche Diagnostic) and *Eag*I (Amersham-Pharmacia Biotech) respectively before SSCP analysis.

The sequences obtained were aligned with the previously published cDNA sequence (M62323), the sequence we obtained after the analysis of the intron-exon boundaries, and the sequence provided by Hackam *et al.*[22] A total of 12 variants were found, 10 in *GABRR1* (table 4), four of which are described in this work, and two in *GABRR2* (table 5). However, none of them appear to be disease causative since they were found in the controls.

All the polymorphisms detected (tables 4 and 5) were confirmed by restriction analysis following the manufacturer's instructions. The 5'UTR-*Rsa*I and IVS2+45C→G polymorphisms were genotyped by PCR digestion using a *Rsa*I (Roche Diagnostic) site and *Mae*III (Amersham-Pharmacia Biotech) site, respectively, introduced into the PCR primer next to the nucleotide change (table 4).

The 12 polymorphisms identified in *GABRR1* and *GABRR2* were genotyped in all families previously linked to *RP25*. In the analysis of M20V of *GABRR1* and V84V of *GABRR2* in the consanguineous family RP5, patients RP5.II.1 and RP5.II.3 were observed to have homozygous M20V and V84V changes, while a third patient, RP5.II.2, was heterozygous for these variants. The analysis of the other changes, namely IVS2+45C→G, IVS6-33C→T, and A389A of *GABRR1* and V84V of *GABRR2*, in the consanguineous family RP167, showed that patient RP167.II.8 was homozygous for the normal alleles, while patient RP167.II.3 was homozygous for the mutated ones (fig 1). These results exclude the *GABRR1* and *GABRR2* genes as the cause of RP in both consanguineous families (RP5 and RP167). Since the *RP25* locus was identified by homozygosity mapping, these data argue against the involvement of these genes in *RP25*.

The *RP25* locus is the third gene involved in RP and the seventh one related to retinal degeneration localised on chromosome 6. According to the data from the human transcription map,[24] the initial *RP25* critical region colocalises with two loci involved in retinal degeneration, an autosomal dominant Stargardt-like locus (*STGD3*)[25] and an autosomal dominant cone-rod dystrophy locus (*CORD7*),[26] sharing a region of 4.8 cM. These disorders are different, but it cannot be excluded that the same gene could be responsible for *STGD3*, *CORD7*, and *RP25*. This allelic heterogeneity has already been reported for the *peripherin/RDS* gene, the *ABCR* gene, and the *CRX* gene.[27–30] Recently, a kindred with autosomal dominant cone-rod dystrophy with features of Stargardt-like disease where genetic analysis has shown linkage to *CORD7* and *STGD3* on chromosome 6q14 has been identified.[31] On the other hand, linkage analysis in one family of Pakistani origin has refined the *RP25* critical region from 16.1 cM[14] to 2.4 cM between D6S1053 and D6S430.[32] However, according to the physical and genetic maps available, the data provided by Khaliq *et al*[32] would not be consistent with the overlap of *RP25* and *CORD7*/*STGD3*.

On the whole, the data reported argue against the involvement of the *GABRR1* and *GABRR2* genes in *RP25*. However, the exclusion of both genes does not rule out other genes involved in neurotransmission within the critical region. In order to address the search for additional candidate genes for *RP25*, our current efforts include

Table 4 Sequence polymorphisms identified in the GABRR1 gene

Exon	*Nucleotide change*	*Amino acid substitution*	*Restriction site changed*	*Size of alleles (bp)*		*No*	*Allele 1 frequency*	*PIC†*
				(1)*	*(2*)*			
1	5'UTR-*Rsa*I‡	None	*Rsa*I**	243	213, 30	56	0.03	0.056
1	(nt) c104A→G§	M20V	*Apa*LI	188, 103	291	NA	NA	NA
1	(nt) c108A→G‡	H21R	*Hha*I	191, 100	291	55	0.16	0.253
2a	IVS1-14T→A§	None	*Mse*I	198, 74	272	NA	NA	NA
2a	IVS1-5A→G§	None	*Nla*III	156, 83, 33	156, 116	NA	NA	NA
2a	IVS2+42T→C‡	None	*Mbo*II	144, 128	128, 102, 42	57	0.45	0.372
2a	IVS2+45C→G‡	None	*Mae*III††	159, 33	192	43	0.38	0.360
4	(nt) c466T→C§	D140D	*Eag*I	210, 164	374	NA	NA	NA
6	IVS6-33C→T§	None	*Dra*III	323	274, 49	NA	NA	NA
9	(nt) c1213A→G¶	A389A	*Bsr*DI	487	397, 90	83	0.69	0.336

*Allele 1 is always defined as the polymorphic allele, allele 2 as the wild type allele.
†PIC: polymorphism information content.
‡Polymorphisms described in this work.
§Polymorphisms previously described by Hackam *et al.*[22]
¶Polymorphism previously described by Marcos *et al.*[23]
The primers used to genotype the 5'UTR-*Rsa*I polymorphism were CT1F (gaccaataatgtcttaagagagaaaaagt**a) and CT1R (cttttcctaaatccttctatccctaaatgt).
††The primers used to genotype the IVS2+45C→G polymorphism were CGF(cttggtttgatctgagtacctagactttct) and CGR (gccctgctgaaaatcactacagttgagg**g**t).
No: controls tested.
NA: not available.

Table 5 Sequence polymorphisms identified in the GABRR2 gene

Exon	*Nucleotide change*	*Amino acid substitution*	*Restriction site changed*	*Size of alleles (bp)*		*No*	*Allele 1 frequency*	*PIC†*
				(1)*	*(2*)*			
3	(nt) c250A→G‡	V84V	*Rsa*I	172	101, 71	NA	NA	NA
9	(nt) c1289C→T‡	T430M	None	NA	NA	NA	NA	NA

*Allele 1 is always defined as the polymorphic allele, allele 2 as the wild type allele.
†PIC: polymorphism information content.
‡Polymorphisms previously described by Hackam *et al.*[22]
No: controls tested.
NA: not available.

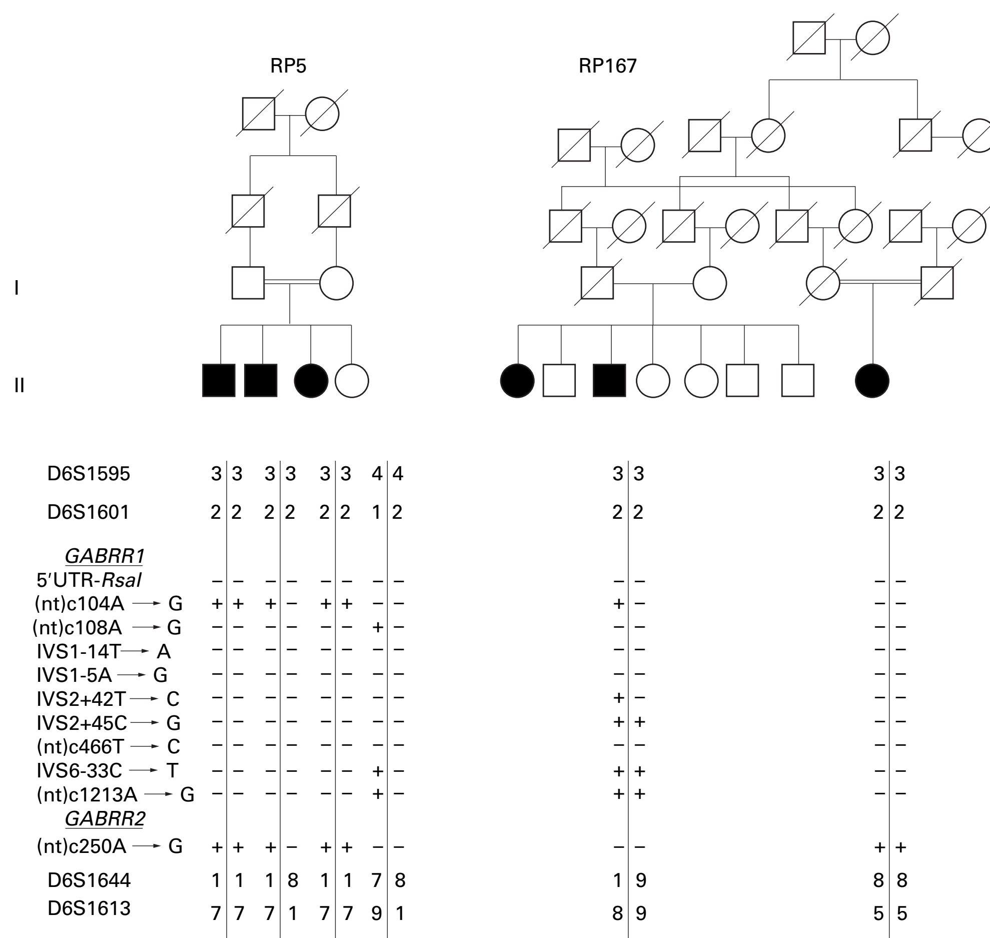

Figure 1 Segregation of the polymorphisms of the GABRR1 and GABRR2 genes in two families linked to RP25, RP5 and RP167.

building a physical map across the current critical region to localise the STSs, ESTs, and polymorphic markers to the critical region.

Data access: RET-GEN-NET, htp://www.sph.uth.tm.edu/Retnet/home.htm. Online *Mendelian Inheritance in Man* (OMIN), http://www.ncbi.nlm.nih/htbin-post/OMIN Généthon, http://www.genethon.fr. GeneMap '99, http://www.ncbi.nlm.nih.gov/genemap/ We would like to express our gratitude to all those affected by RP for their cooperation, essential for the achievement of this study. We are very grateful to Santiago Rodríguez de Córdoba, who provided invaluable comments on this article. This study was supported by Fondo de Investigaciones Sanitarias (grant 99/0010-02), the Fundación ONCE, Consejería de Salud/Comunidad Autónoma de Andalucía (grant 98/144), and the Asociación Andaluza de Retinosis Pigmentaria. IM is the recipient of a fellowship from the Instituto de Salud Carlos III (grant 99/4250, Ministerio de Sanidad y Consumo, Spain).

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