ANNALS OF human genetics

doi: 10.1111/j.1469-1809.2008.00448.x

Linkage Validation of *RP25* Using the 10K GeneChip Array and Further Refinement of the Locus by New Linked Families

I. Barragán[1,2,#], M. M. Abd El-Aziz[3,4,#], S. Borrego[1,2], M. F. El-Ashry[3,5], C. O'Driscoll[3], S. S. Bhattacharya[3] and G. Antiñolo[1,2,*]

[1] *Unidad Clínica de Genética y Reproducción, Hospitales Universitarios Virgen del Rocío, Seville, Spain*
[2] *Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Seville, Spain*
[3] *Department of Molecular Genetics, Institute of Ophthalmology, London EC1V 9EL, UK*
[4] *Department of Clinical Pathology, Tanta University Hospital, Tanta, Egypt*
[5] *Department of Ophthalmology, Tanta University Hospital, Tanta, Egypt*

Summary

Retinitis pigmentosa (RP) is a clinically and genetically heterogeneous group of retinal dystrophies, characterised by rod photoreceptor cell degeneration with autosomal recessive RP (arRP) as the commonest form worldwide. To date, a total of 26 loci have been reported for arRP, each having a prevalence of 1–5%, except for the *RP25* locus which was identified as the genetic cause of 14% of arRP cases in Spain. In order to validate the original linkage of *RP25*, we undertook a total genome scan using the 10K GeneChip mapping array on three of the previously linked families. The data obtained supported the initial findings of linkage. Additionally, linkage analysis in 18 newly ascertained arRP families was performed using microsatellite markers spanning the chromosome 6p12.1-q15 interval. Five out of the 18 families showed suggestive evidence of linkage to *RP25*, hence supporting the high prevalence of this locus in the Spanish population. Furthermore, the finding of a crossover in one of these families is likely to have refined the disease interval from the original 16 cM to only a 2.67 cM region between *D6S257* and *D6S1557*.

Keywords: Retinitis pigmentosa, *RP25* locus, retinal disease gene, chromosome 6

Introduction

Retinitis pigmentosa (RP) is a clinically and genetically heterogeneous group of retinal dystrophies, affecting approximately 1 in 4000 individuals worldwide (Inglehearn 1998). Patients typically present with night blindness and constriction of peripheral visual fields due to degeneration of rod photoreceptor cells. Clinical manifestations include waxy pallor of the optic disc, attenuation of retinal blood vessels with later atrophy of the retinal pigment epithelium. Electroretinographic (ERG) changes are present with abnormalities of both rod and cone ERGs, with the scotopic alteration the first to be reported. However, in advanced RP both rod and cone ERG responses are undetectable (Jiménez-Sierra et al. 1989).

Notable allelic and non-allelic heterogeneity are characteristic of RP and the disease can present with different modes of inheritance, such as X-linked (xl), autosomal dominant (ad), autosomal recessive (ar) and digenic. The autosomal recessive form of RP is the commonest worldwide, accounting for approximately 39% of cases in Spain (Ayuso et al. 1995). To date, 26 loci have been reported as responsible for arRP, of which 21 genes have been identified (http://www.sph.uth.tm.edu/Retnet/). However, all together the reported loci are responsible for only ~10–15% of the recessive RP cases (Tuson et al. 2004). This is in comparison to the *RP25* locus which was identified as the genetic cause of 10–20% of arRP cases in Spain (Ruiz et al. 1998; Woods et al. 2006).

*Corresponding author: Guillermo Antiñolo, MD, PhD, Unidad Clínica de Genética y Reproducción, Hospitales Universitarios Virgen del Rocío, Avenida Manuel Siurot s/n, 41013 Seville, Spain. Tel: +34 95 50127 72; Fax: +34 95 501 34 73; E-mail: guillermo.antinolo.sspa@juntadeandalucia.es
#Contributed equally.

RP25 was originally identified by homozygosity mapping through targeting functional candidate genes. This resulted in mapping of 4 Spanish families with arRP to a 16.1 cM interval between microsatellite markers *D6S257* and *D6S1644* (Ruiz et al. 1998). Subsequently, evidence of linkage to the same region has been reported in three additional Spanish families with arRP, as well as in other ethnic groups (Khaliq et al. 1999; Barragan et al. 2005a; Abd El-Aziz et al. 2007). The isolation of the *RP25* gene would therefore represent a major achievement in RP research. Interestingly, several loci responsible for various types of retinal dystrophies have been reported to co-localise with *RP25*, among which is included Leber congenital amaurosis (LCA5), a severe form of retinal degeneration for which the gene has recently been identified (Dharmaraj et al. 2000; den Hollander et al. 2007).

Extensive bioinformatics analysis and mutation screening of candidate genes within the *RP25* interval led to the exclusion of 17 genes, *GABRR1*, *GABRR2*, *ELOVL4*, *GlcAT-S*, *TFAP2B*, *RAB23, RIM1, ELOVL5*, *GLULD1, SMAP1*, *EEF1A1*, *IMPG1*, *KHDRBS2*, *PTP4A1*, *KIAA1411*, *OGFRL1* and *FAM46A,* as disease causing (Marcos et al. 2000, 2002, 2003; Li et al. 2001; Barragan et al. 2005a; Barragan et al. 2005b; Abd El-Aziz et al. 2005, 2006; Barragan et al. 2007). This together with our recent work involving large scale bioinformatics analysis of the *RP25* interval and mutation screening of additional 43 genes ruled out ~55% of the genes from the interval as responsible for RP (submitted for publication).

The considerable technological advances in genotyping and statistical analysis of genetic markers within pedigrees are the basis of the development of genomewide linkage studies for the elucidation of causative genes (Zhang et al. 2005; Zhao et al. 2005). The initial genomewide linkage analyses employed panels of about 400 microsatellites at 10-cM average intermarker distance (Stambolian et al. 2004). However, recent progress in Single Nucleotide Polymorphism (SNP) discovery and genotyping has provided the opportunity to use this type of marker for linkage analyses as well (Sellick et al. 2004). Besides, various studies highlighted the advantageous use of SNPs compared to microsatellite markers for linkage purposes (Evans & Cardon 2004; Middleton et al. 2004; John et al. 2004).

Herein we report the validation of linkage to the *RP25* locus by genomewide linkage analysis using the 10K SNP GeneChip array in 3 previously mapped families. Additionally, linkage analysis in 18 newly ascertained arRP families was performed using microsatellite markers spanning the chromosome 6p12.1-q15 interval.

Materials and Methods

Subjects and DNA

This study involved 21 Spanish arRP families comprising 34 affected and 68 unaffected individuals, all derived from Ophthalmology Services to our Genetic Service (UCGR) (Fig. 1). The studied families included three of the originally *RP25* linked families, RP5, RP214 and RP299, and the remaining eighteen were newly ascertained.

A group of matching control individuals was also recruited. Informed consent was obtained from all participants and the study conformed to the tenets of the Declaration of Helsinki (June 1964). All subjects underwent a peripheral blood extraction for genomic DNA extraction from leukocytes using the standard protocols (Dracopoli et al. 1994).

Genotyping Using GeneChip® Human Mapping 10K Array

The three previously studied families, RP5, RP214 and RP299, were subjected to a total genome scan using the GeneChip Human Mapping 10K array in order to validate the original linkage data (Affymetrix, High Wycombe, UK). DNA samples from six affected (**RP5** II-1, II-2, II-3; **RP214** II-5; **RP299** II-2, II-3) and 5 unaffected (**RP5** I-1, I-2, II-4; **RP214** I-2; **RP299** I-1) members from the 3 families (Fig. 1) were genotyped using the 10K Array Xba 142 2.0, (Affymetrix, High Wycombe, UK), employing the methodology described elsewhere (Sellick et al. 2004; Abd El-Aziz et al. 2007).

The selection of family members to be genotyped with the 10K array was based on the previous linkage data (Ruiz et al. 1998; Barragan et al. 2005a). For example, in the case of family RP214, two individuals (the available parent I-2 and affected offspring II-5) were sufficient to validate the original linkage of *RP25* based on homozygosity mapping. Similarly, for family RP299 (non-consanguineous), by using one parent (I-1) and two affected offspring (II-2 and II-3) linkage to *RP25* was established based on identifying the largest region of shared haplotype between the affected members.

Analysis of the 10K Array Data

The SNP data were analysed using ExcludeAR3, AR1liteCHIP2 macros and the ALOHOMORA program for RP5, RP214 and RP299 families, respectively (Woods et al. 2004; Ruschendorf & Nurnberg, 2005).

Autozygosity mapping was suggested as the method of choice for identifying recessive loci using the 10K array (Lander and Botstein, 1987). Hence, ExcludeAR3 and AR1 liteCHIP2 macros were utilised to analyse the three affected members and the affected member in both RP5 (consanguineous) and RP214 families (non-consanguineous), respectively. Even though

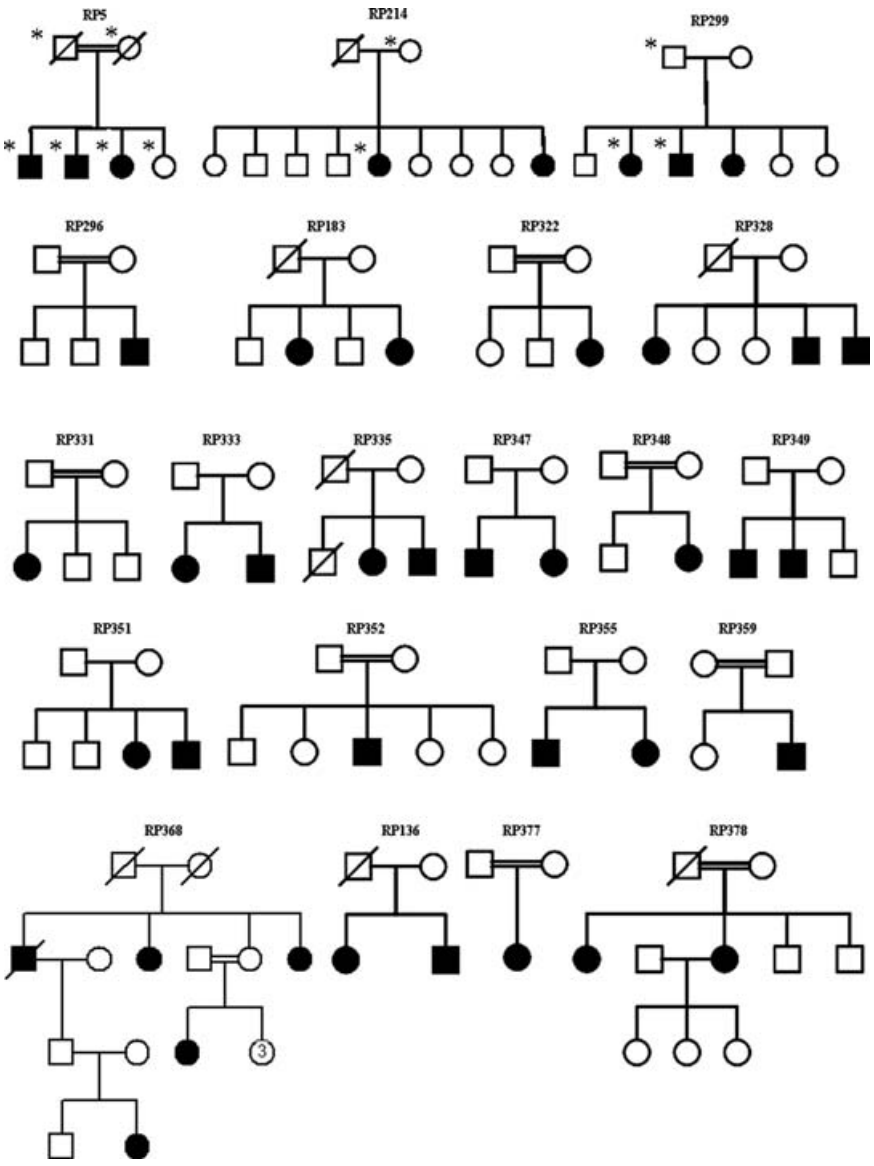

Figure 1 Shows family pedigrees of individuals who were included in the study. Open and closed symbols denote unaffected and affected individuals respectively. Deceased family members are denoted by diagonal slashes and asterisks indicate individuals that were analysed using the GeneChip 10K array.

family RP214 was not described as consanguineous, the original linkage data revealed a region of homozygosity compatible with linkage that was shared between its 2 affected members. Therefore we have used the ExcludeAR1 program to analyse the 10K array data from this family. Upon identifying the disease gene for RP25, we expect the mutation in this family to be homozygous.

For family RP299 (non-consanguineous) the ExcludeAR software was not suitable for analysing the linkage data since it identifies only homozygous intervals. Therefore, the ALOHOMORA program was used to identify genetic intervals of shared haplotype between the two affected members of this family. We have also utilised the ALOHOMORA software to analyse family RP5 in order to confirm the data obtained from the ExcludeAR3 macro. However, family RP214 could not be analysed by using this method since only one affected member was available and consequently the pedigree was uninformative for comparison of haplotypes among affected members as well as for LOD score calculations.

Within ALOHOMORA, Mendelian errors and the correct relationships within families were checked for by using the PedCheck and GRR programs, respectively (O'Connell and Weeks, 1998; Abecasis et al. 2001a). Identification and deletion of non-Mendelian errors and unlikely genotypes were performed. Uninformative SNPs were removed from the data by Merlin (Abecasis

et al. 2001b). For parametric linkage analysis, the data was then converted for Genehunter where the analysis was done in subsets of markers in a non-overlapping moving window. The haplotyping data were then used from the Genehunter file.

The analysis of the 10K array data using any of the computer programs mentioned earlier is based on identifying regions of shared haplotype between the affected members in the case of the RP5 and RP299 families or observing significant regions of homozygosity as for the RP214 family. The breakpoints were identified based on the observation of a crossover between the affected members in the RP5 and RP299 families or the loss of homozygosity in the case of the RP214 family. This was then followed by combining the data from the three families in order to identify significant regions of shared haplotype.

Genotyping and Microsatellite Marker Analysis of the *RP25* Locus

A total of 93 members from the newly ascertained arRP families (Fig. 1) were subjected to genotyping analysis using microsatellite markers covering the *RP25* region. For this purpose 19 microsatellite markers were utilised, of which 11 were selected from the ABI PRISM® Linkage Mapping Sets V2.5 (Applied Biosystems, Madrid, Spain) and the remaining 8 were synthesised commercially according to the information obtained from the Ensembl database (www.ensembl.org).

Multiplex PCR amplification was employed for genotyping the microsatellite markers using genomic DNA templates and the Qiagen Multiplex PCR Kit (Qiagen, Barcelona, Spain). PCR products were injected in an automated ABI-3730 sequencer, data collection and allele identification were performed using GeneMapper software (Applied Biosystems). Multipoint parametric linkage analysis was then carried out with GENEHUNTER Complete Linkage Analysis version 2.1_r3beta software (http://linkage.rockefeller.edu/soft/gh/). The phenotype was analysed as an autosomal recessive trait with full penetrance and with a disease-gene allele frequency of 0.0001.

Results

Genomewide Linkage Analysis of the Originally Linked *RP25* Families

The data obtained from ExcludeAR3 and AR1liteCHIP2 macros for families RP5 and RP214 revealed the largest region of homozygosity spanning the *RP25* interval across the human genome (Table 1). This is supportive of the

Table 1 Regions of homozygosity obtained using macros in RP5 family (ExcludeAR3) and RP214 family (ExcludeAR1). Regions are marked as 'significant' by the program if the random chance of the observed number of consecutive SNPs in the stated genetic distance being homozygous (for AR1) combined with the chance of them being concordant in the affected siblings (for AR3) is less than one in 1000 as described in Woods et al. 2004.

RP5 Homozygous Region by Size	cM	Chrom	SNP's	Significant?	Start cM	Finish cM
largest	**50.3**	**6**	**188**	**significant**	**73**	**124**
second	17.3	11	6	not significant	91	108
third	16.6	9	7	not significant	169	185
fourth	15.0	5	14	significant	187	202
fifth	9.9	20	9	not significant	3	13
sixth	9.7	10	9	not significant	112	122
seventh	7.9	5	4	not significant	239	247
eighth	7.8	2	12	significant	101	109
ninth	7.4	4	5	not significant	65	72
tenth	7.3	19	6	not significant	102	110
RP214 Homozygous Region by Size	cM	Chrom	SNP's	Significant?	Start cM	Finish cM
largest	**28.2**	**6**	**107**	**significant**	**80**	**112**
second	24.2	2	30	not significant	301	326
third	17.8	1	44	not significant	193	211
fourth	14.6	10	13	not significant	107	121
fifth	12.9	6	11	not significant	70	83
sixth	12.7	16	11	not significant	134	146
seventh	12.0	17	10	not significant	73	85
eighth	11.1	9	4	not significant	174	185
ninth	10.8	7	9	not significant	108	119
tenth	10.5	22	7	not significant	22	32

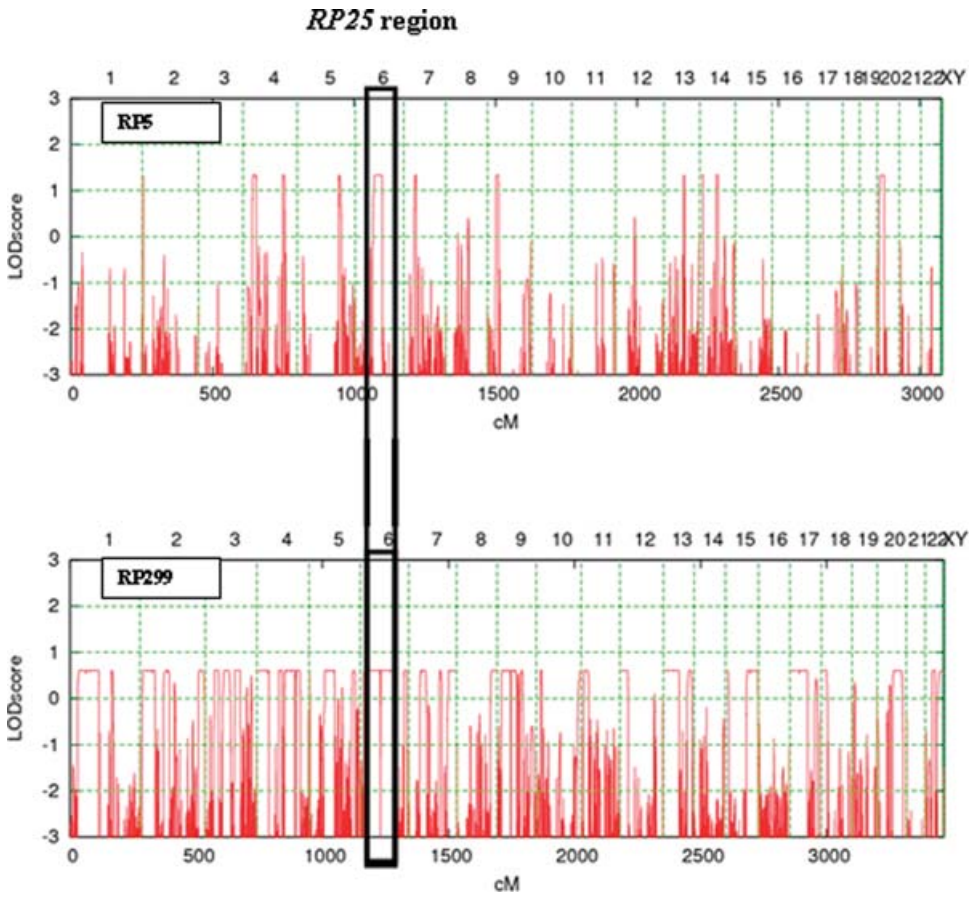

Figure 2 Schematic representation of the total genome analysis for families RP5 and RP299 generated by the ALOHOMORA software. The genetic interval (cM)/each chromosome (1–23) on the x axis is plotted against the LOD score on the y axis. The red peaks on each chromosome represent the LOD score for each of the single nucleotide polymorphisms (SNPs) used. The higher and the greater the width of the red peak the more likely it is to represent the true locus for linkage. The area enclosed by bold lines shows the RP25 locus as the largest region across the whole genome common to both families in which haplotypes within each family are identical in affected members.

original linkage data in these families (Ruiz et al. 1998). Table 1 is generated by ExcludeAR software which lists the homozygous intervals across the human genome in a descending order based on the number of SNPs and the genetic size of each interval. The *RP25* locus was considered as the disease interval in both RP5 and RP214 families since 188 and 107 SNPs were observed to be homozygous in both families, respectively.

Additionally, the data obtained from the ALOHOMORA software for both RP299 and RP5 families, highlighted the *RP25* locus as the region of shared haplotype between the affected members of both families across the human genome (Fig. 2).

Therefore, the GeneChip mapping data generated from genotyping of the three previously linked families confirmed the initial findings of linkage to the *RP25* region.

However, this did not lead to further refinement of the *RP25* genetic interval and a region of 16 cM interval has been confirmed as the disease interval in RP25 families.

Targeted *RP25* Linkage Analysis in arRP Families

Genetic linkage to *RP25* was identified in five, RP349, RP351, RP355, RP377 and RP328, out of the 18 newly ascertained arRP families (Fig. 3). In these five families, all the studied microsatellite markers in the vicinity of *RP25* generated positive LOD scores. We pooled the linkage data from all families except for RP377 and a maximum LOD score of 3.63 for microsatellite marker D6S1573 at $\theta = 0$ was generated (Table 2, Fig. 4). The reason behind

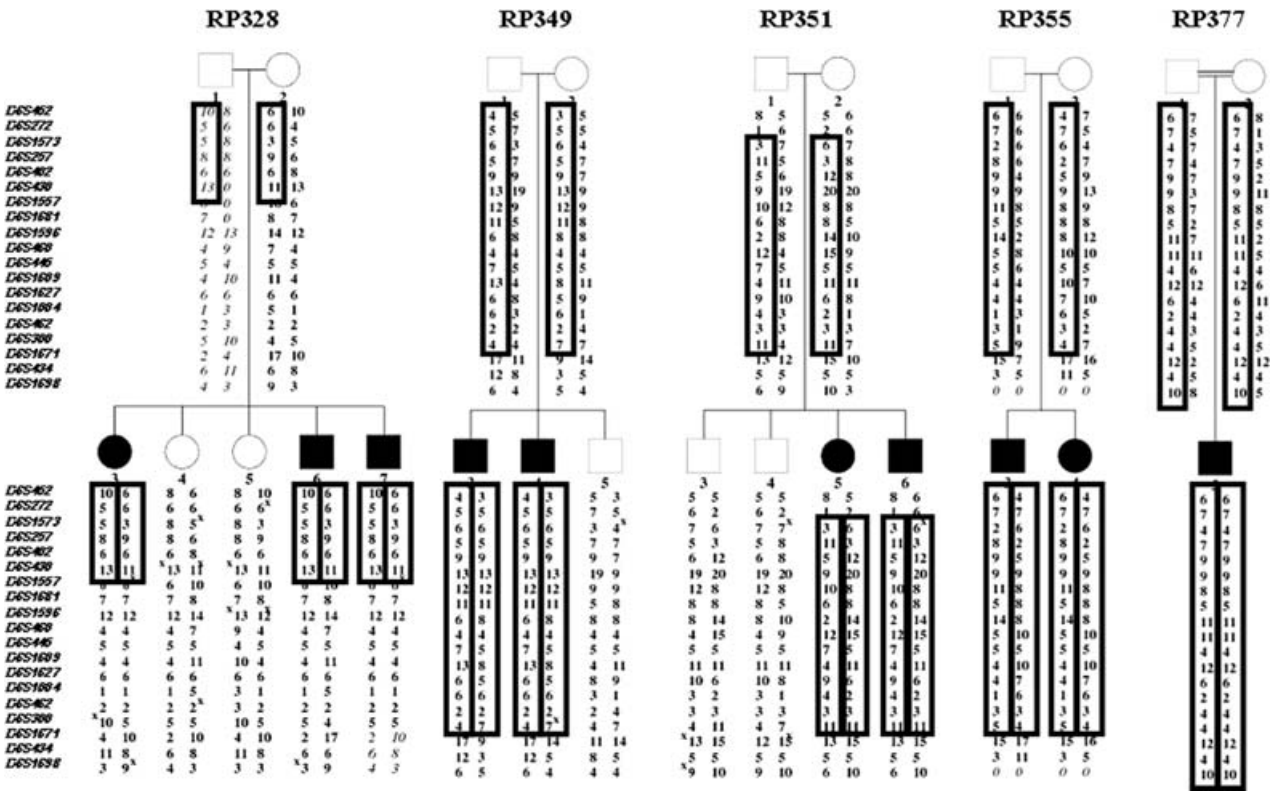

Figure 3 Haplotypes of families RP328, RP349, RP351, RP355 and RP377 using microsatellite markers on 6p12.1-q12. Recombination events between markers *D6S272* and *D6S1557* are shown in families RP351 and RP328, respectively.

Table 2 LOD score values of the individual and combined linkage data for families RP328, RP349, RP351 and RP355. Combined LOD scores have been calculated at a theta value of 0.

		LOD Score/FAMILY			
Marker	Combined LOD Score/p-value	RP328	RP349	RP351	RP355
D6S452	$-\infty$/0.013	1.25	0.73	$-\infty$	0.56
D6S272	$-\infty$/0.007	1.37	0.71	$-\infty$	0.60
D6S1573	**3.63/0.001**	1.45	0.73	0.85	0.60
D6S257	**3.47/ 0.001**	1.29	0.73	0.85	0.60
D6S402	**3.17/ 0.001**	1.00	0.72	0.85	0.60
D6S430	**2.82/ 0.001**	0.65	0.73	0.85	0.59
D6S1557	$-\infty$/0.013	$-\infty$	0.73	0.85	0.60
D6S1681	$-\infty$/0.013	$-\infty$	0.73	0.85	0.60
D6S1596	$-\infty$/0.013	$-\infty$	0.73	0.85	0.60
D6S460	$-\infty$/0.013	$-\infty$	0.72	0.85	0.59
D6S445	0.47/ 0.013	-1.7	0.73	0.85	0.59
D6S1609	$-\infty$/0.013	$-\infty$	0.73	0.85	0.60
D6S1627	0.08/ 0.013	-2.1	0.73	0.85	0.60
D6S1004	$-\infty$/0.013	$-\infty$	0.73	0.85	0.60
D6S462	0.47/0.013	-1.7	0.72	0.85	0.60
D6S300	$-\infty$/0.071	$-\infty$	0.50	0.85	0.60
D6S1671	$-\infty$/0.495	-1.9	$-\infty$	$-\infty$	$-\infty$
D6S434	$-\infty$/0.495	-1.6	$-\infty$	0.09	$-\infty$
D6S1698	$-\infty$/0.246	-0.87	$-\infty$	0.85	-0.29

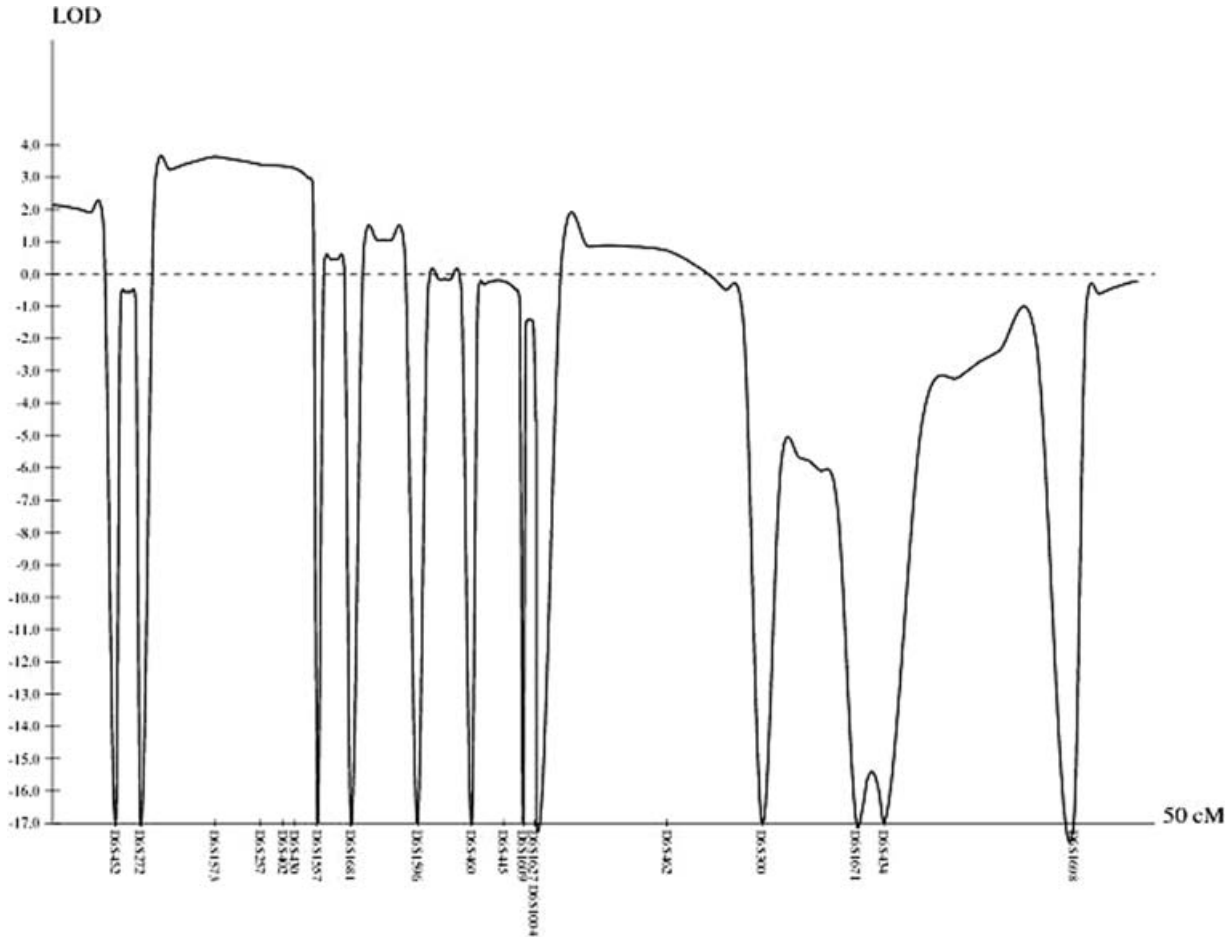

Figure 4 Graphic depiction of the LOD score variation with genetic distance calculated using the combined linkage data of families RP328, RP349, RP351, RP355 and RP377.

excluding RP377 is that Genehunter does not allow LOD score calculation if there are 2 founders and a non-founder in the pedigree.

Interestingly, in one of the linked families (RP328) a novel crossover was observed with microsatellite marker *D6S1557* (Fig. 3). This may potentially lead to the refinement of the *RP25* genetic interval from the original 16 cM to 2.67 cM between microsatellite markers *D6S257* and *D6S1557*. We believe that the observed crossover in this family and the consequent genetic refinement of the *RP25* interval to 2.67 cM are compatible with our recent identification of ~100-Kb deletion within the refined interval in one of the *RP25* linked families (submitted for publication).

It is interesting to note that there was no common haplotype between the studied families within the *RP25* interval. Ultimately, this can rule out the possibility that the mutation in the causative gene will be due to a common founder effect in all families.

Discussion

In this paper we present validation of linkage to the *RP25* locus in 3 original families as reported by us previously (Ruiz et al. 1998). Furthermore, we show a suggestive evidence of linkage to the same locus in 5 new Spanish arRP families of which one is likely to have refined the *RP25* interval from 16.1 cM to a 2.67 cM between *D6S257* and *D6S1557*.

Previously, positional cloning via family based genomewide linkage analyses has been considered as a powerful approach for the elucidation of both Mendelian and common diseases (Sellick et al. 2004; Farrall & Morris 2005). Likewise, recent advances in SNP identification and genotyping technology have extended the use of dense SNP maps for linkage purposes in place of microsatellite panels (Evans & Cardon 2004).

Recently, targeted linkage analysis and genomewide screening approaches have been combined as useful tools

for mapping rare and genetically heterogeneous disorders such as RP (Abd El-Aziz et al. 2007; Pomares et al. 2007).

In this study, we undertook a genomewide linkage analysis in three of the originally linked families using the 10K GeneChip array in order to prove *RP25* as the true locus for linkage and to eliminate the possibility of other loci existing across the human genome. The data generated from this analysis revealed the *RP25* locus as the most likely region of linkage and hence supported the original linkage data (Ruiz et al. 1998) and raises an interesting possibility that a disease gene should be present within the *RP25* interval.

Secondly, we have identified 5 out of 18 newly ascertained Spanish arRP families as linked to the *RP25* locus using *RP25* targeted linkage analysis. This has further confirmed the high prevalence of *RP25* which, would increase from 13.7% (Ruiz et al. 1998) to 27.7% among the Spanish population. In addition, a novel crossover in one of the newly linked families, RP328, has further refined the *RP25* interval from 16.1 cM to 2.67 cM. In parallel, we have identified a deletion within the 2.67 cM interval in one of the previously described *RP25*-linked families by using comparative genomic hybridization (CGH) analysis (submitted for publication) therefore highlighting the significance of the current finding.

In summary, we have confirmed the original linkage to the *RP25* locus, and identified five new Spanish families likely to be linked to the same locus. Additionally, a refined interval of 2.67 cM between *D6S257* and *D6S1557* has been established as a probable disease interval for *RP25*. This will help in prioritizing the genes within the refined interval for mutation screening which will eventually lead to cloning of the *RP25* gene. It is interesting to note that the previous and the current genetic data could not identify any regions of common haplotype between affected individuals from different families from Spain across the *RP25* interval. Different mutations within one gene are therefore expected. On the other hand, more than one gene responsible for the RP phenotype in different families may exist. The identification of the gene and/or the genes responsible for the RP25 phenotype would consequently complement our knowledge on the pathophysiology of the retinal degenerations and give rise to possibilities for the development of new therapies.

Acknowledgments

We would like to thank the families who participated in the study. This study is funded by Fondo de Investigación Sanitaria, Spain (PI050857), the British Retinitis Pigmentosa Society (Grant ref.GR556), NIHR Biomedical Research Centre for Ophthalmology (BMRC) and Special Trustees of Moorfields Eye Hospital.

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Received: 14 November 2007
Accepted: 2 April 2008