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RESEARCH ARTICLE

Autosomal Recessive Retinitis Pigmentosa is Associated with Missense Mutation in *CRB1* in a Consanguineous Pakistani Family

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ABSTRACT

Retinitis pigmentosa (RP) causes severe visual impairment early in life. The purpose of this study was to identify families with autosomal recessive RP (arRP), their exclusion analysis and identification of novel loci/genes/mutations in affected individuals. Twenty five consanguineous Pakistani families suffering from non syndromic Retinitis pigmentosa (RP) were ascertained from different areas of Pakistan to participate in this study. 85 affected and 210 normal individuals of selected families and from the healthy population took part in this research work. The clinical records of affected family members were retrospectively analyzed and ophthalmological examinations were performed in selected families. Genomic DNA was extracted from peripheral blood samples of phenotypically healthy and affected individuals. Exclusion analysis was done initially for the screening of the causative gene using highly polymorphic microsatellite markers. Genetic investigations using polymorphic microsatellite markers revealed homozygosity with *CRB1* gene on chromosome 1 in affected individuals of family RP1. All other families and 210 healthy or control samples showed exclusion with the selected loci/genes. Haplotypes were constructed to appraise the results statistically. For further confirmation of linkage, sequence analysis of the linked gene (*CRB1*) was done. Sequence analysis revealed a novel missense mutation (c.1459T>C) in exon 6 of the *CRB1* gene segregating with disease phenotype. This mutation resulted in a substitution of proline for serine at amino acid 487 (p.Ser487Pro). These findings suggest a founder effect of the p.Ser487Pro mutation in the Pakistani population and extend the spectrum of known *CRB1* mutations. It was therefore concluded that the missense mutation in *CRB1*, leading to premature termination of the protein, is responsible for RP phenotype in the affected individuals of RP1.

INTRODUCTION

Retinitis pigmentosa is an inherited monogenic ocular disorder. It is a major form of incurable blindness affecting one out of 4000 people worldwide. Non-syndromic or simple RP is inherited either as autosomal recessive, autosomal dominant or X-linked pattern. Autosomal dominant RP (adRP) constitutes 20 to 25% of all cases; arRP comprises 15 to 20%; X-linked recessive RP ranges from 10 to 15% and the remaining 40 to 55% of cases in which family record is missing are termed as simplex RP (Boughman et al., 1980; Boughman and Caldwell, 1982; Inglehearn, 1998 and Stefano et al., 2011). RP is characterized by night blindness known as nyctalopia, peripheral vision loss leading to tunnel vision, progressive degeneration of photoreceptors starting from mid-periphery approaching to fovea and macula resulting in visual impairment of variable severity that can in rare cases result in complete blindness **(**Berson, 1993; Hartong et al., 2006). Statistically the number of men affected from this disease may be more than women because X-linked recessive RP is expressed in males only. Non syndromic RP is clinically and genetically very heterogeneous disorder involving many genes and loci. Different diseases result due to different mutations in the same gene and the same transmutation can result in different phenotypes. Due to this complexity in RP genetics the exact phenotype-genotype correlations are not yet possible (Hims et al., 2003). The first causative

gene of RP, *Rhodopsin* (*RHO*) was identified in 1990 (Dryja, *et. al*., 1990). To date, 40 genes have been identified to cause adRP, arRP and simplex RP. The most common among these identified genes are *ABCA4* and *RP1* responsible for 30 to 60% of autosomal recessive RP. These genes are mostly expressed in photoreceptors or retinal pigment epithelium (RPE) so directly affect the photo-transduction cascade (Stefano et al., 2011). Over 60 different mutations have been identified in the *RPE65* gene responsible for about 2% of autosomal recessive RP and 16% of LCA patients (Samardzija et al., 2008).

Mutations in crumbs homolog 1 (*CRB1*; MIM: 604210) are also known to cause severe retinal dystrophies, ranging from Leber congenital amaurosis (LCA) to RP (Bujakowska et al., 2011; den Hollander *et al*, 2004; Riveiro- Alvarez *et al*, 2008). The *CRB1*is a protein which is encoded by the *CRB1*gene in humans (den Hollander *et al*, 1999). As this gene has homology with the *Drosophila* Crumbs protein that is required for adhesion and polarity in embryonic epithelia so it was predicted that the role of *CRB1* in vertebrate photoreceptors may be in cell adhesion and photoreceptor morphogenesis. *CRB1* has 12 exons and is located on chromosome 1q31-q32 and may give rise to four known isoforms, the longest of which consists of 1406 amino acids. The CRB1 protein contains 19 epidermal growth factor-like domains, three laminin A globular-like domains, a transmembrane domain and a 37 amino acid cytoplasmic tail with a C-terminal ERLI motif (den Hollander *et al.*, 1999; Tepass *et al.*, 1990). Retinal disorders as a result of mutations in *CRB1* gene can be accompanied by additional specific features i.e. there may be preservation of the para-arteriolar retinal pigment epithelium (PPRPE) and coats-like vasculopathy (Simonelli et al, 2007; Jacobson et al, 2003; Siemiatkowska et al, 2011).

MATERIALS AND METHODS

Approval for this study was obtained from Institutional Ethical Committee, National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan. Twenty five consanguineous Pakistani families suffering from nonsyndromic RP were ascertained from different areas of Pakistan to participate in a collaborative study between NIBGE and Allied Hospital, Faisalabad Pakistan. The relevant clinical information regarding the time and age of onset of vision loss, symptoms, severity of vision loss, loss of vision in siblings, vision status of parents, and extended family members were investigated. To have inference at genetic level pedigrees were drawn using Cyrillic software 2.1.3 (Cherwell Scientific Publishing Ltd, Oxford). All the affected individuals were clinically evaluated by taking fundus photographs (fundus camera Topcon TRC 50DX, Japan).

After having informed consent the venous blood samples were collected from available healthy and affected members of the family and were stored in potassium EDTA containing vacutainers at -20°C. DNA was extracted from blood samples by using a standard (Phenol: Chloroform) protocol (Sambrook *et al*., 1989). A total of 295 DNA samples (85 affected and 210 normal) from twenty five families were taken for this study. The quality of the genomic DNA was tested by running the samples on 1% agarose gel, and the quantification of purified DNA was done using NanoDrop™ 1000 spectrophotometer at a wavelength of 260nm.

The search for a disease gene begins with linkage analysis. Owing to Retinitis pigmentosa's remarkable genetic heterogeneity (31 genes identified for arRP), the screening of all the reported RP loci/gene by linkage or exclusion analysis would have been laborious.

Therefore, for selection of genes and their markers literature was reviewed, genes already reported in literature causing the same disorder in patients from the same geographical region were selected on priority. The linkage study was performed by mapping homozygosity around these commonly reported loci using highly polymorphic STR markers selected from UCSC Human Genome Browser (http://www.genomebrowser.org) having an average heterozygosity of >70%. Marker's physical position, locus annealing temperature and other attributes were selected using Human Genome Browser. Two to three microsatellite markers were used for each gene or locus (Table 1). PCR was performed using Programmable Thermal Cycler PTC-06 (ICCC). The PCR products obtained by amplifying STRs were resolved on 8% nondenaturing polyacrylamide gel. Haplotype analysis was done for the confirmation of initial linkage.

Mutation screening

Primers were designed using the PRIMER3 program (http://frodo.wi.mit.edu/) for mutation screening of all coding exons of *CRB1* gene and exon-intron boundaries. PCR Amplifications were performed in 25 mL reaction containing 50 ng of genomic DNA, 400 nM, of each primer, 250 µM dNTPs, 2.5 mM $MgCl_2$ and 3 unit Taq DNA polymerase in the standard PCR buffer provided by the manufacturer (Applied Biosystems). PCR amplification consisted of a denaturation step at 96°C for 5 min, followed by 40 cycles, each consisting of 96°C for 45s followed by 57°C (or primer set specific annealing temperature) for 45 s and at 72°C for 1 min.

The genomic regions of all coding exons of *CRB1* gene as well as its corresponding exon-intron transitions were sequenced using Big Dye Terminator Ready

Table 1: STR Markers used for linkage analysis

Marker	Locus	Genes	Primers (5' to 3')	Annealing Temp. ^{0}C
D1S2816-F	1q31-q32	CRB1	TTCCCCAAATGTATTACTGC	55.5^{0}C
D1S2816-R			AAAGGAGTACCCAATCCCAG	
D1S2840-F	1q31-q32	CRB1	GACAAGTCATCTTACACCTCAGTTC	57^{0}C
D1S2840-R			CCAACATAATTTCTGGGCTG	
D1S1183-F	1q31-q32	CRB1	TCTTCATTTTTTTCTCTCCTC	56^{0}C
D1S1183-R			ACAAACTCTGAAGCTGAAGA	
D1S448-F	1p31.2	RPE65	TGCAGAGATAGACTTTCGCT	57°C
D1S448-R			AGTTAGTCATCCTTACCCAGC	
D1S1162-F	1p31.2	RPE65	CCACACTATCATTTACCAGA	57°C
D1S1162-R			GGTTTCCTATGTTCCAAGC	
D1S2813-F	1p22.1	ABCA4	CTTTTGACTCACTGGAAGACAT	55.5^{0}C
D1S2813-R			CCCCACCGTATCTGGTAT	
D1S2849-F	1p22.1	ABCA4	AGCTGAGATCGTGCCA	56^{0}C
D1S2849-R			TCCCTAACCCTCCAGAACT	
D2S1896-F	2q14.1	MERTK	GAGTTGCAATTATAAGCCATTG	55^{0}C
D2S1896-R			GCACAAGAGTGTCCCTGA	
D2S160-F	2q14.1	MERTK	TGTACCTAAGCCCACCCTTTAGAGC	60^{0}C
D2S160-R			TGGCCTCCAGAAACCTCCAA	
D4S1536-F	4p12-cen	CNGA1	GGCTATGGCAAGGGATACTA	57^{0}C
D4S1536-R			AGTTTGAACAACAGTGTGGG	
D4S405-F	4p12-cen	CNGA1	ATCAGGAGATGTTGCCTTGC	60^{0}C
D4S405-R			CAGGGCTATGATTGGATGTC	
D4S1592-F	4p12-cen	CNGA1	GATTGTACCACTGCCCTCC	59°C
D4S1592-R			CCACCATACCTGGCCTTG	
D8S509-F	8q11	RP1	GCTGGGCTTAATGCCT	56^{0}C
D8S509-R			TGTGAGGTCCCAATGGT	
D8S532-F	8q11	RP1	GCTCAAAGCCTCCAATGAC	57^{0}C
D8S532-R			GACTTCGTGATCCACCTGC	
D8S260-F	8q11	RP1	AGGCTTGCCAGATAAGGTTG	57^{0}C
D8S260-R			GCTGAAGGCTGTTCTATGGA	

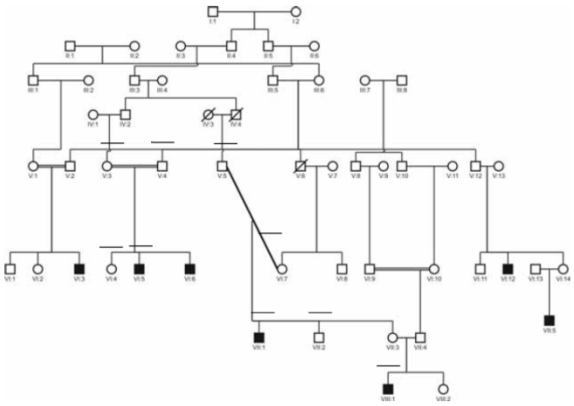

Fig. 1: Pedigree drawing of family RP1. Squares represent males, circles represent females, filled symbols are affected individuals, double line shows consanguinity and diagonal line on circle or square shows deceased family members. The Roman numbers indicate the generation number of the individuals within a pedigree while the Arabic numbers indicate their positions within a generation. A bar indicates the subjects from whom blood was collected for molecular analysis and they were also physically examined.

reaction mix according to manufacturer instructions (Applied Biosystems Foster City, CA). Sequencing was performed on an ABI PRISM 3100Automated sequencer (Applied Biosystems) Putatively pathogenic differences between the wild type sequence (human reference genome according to UCSC Genome Browser: hg19, GRCh37) and the patient's sequence mentioned in this report were validated by conventional Sanger sequencing. Chromatograms were analyzed by using computer program Sequencher v.4.1.2 (Gene Codes Corporation). Chromatograms from the sequence of normal and affected individuals were compared with the corresponding control gene sequence to identify abnormal and deviant nucleotide base-pair (Figure 3).

RESULTS AND DISCUSSION

The prerequisite for this strategy was availability of large consanguineous families with multiple affected individuals. Such families are selected and considered best for linkage analysis to find out the gene that is responsible for some trait (disease) in an individual. A number of studies have focused that consanguinity elevates the mortality levels as there is a greater chance of two related individuals sharing a common disease

variant and both of them passing it on to the child and this inbreeding results in congenital defects and recessive genetic disorders (Tamim et al., 2003; Schulpen et al., 2006).

Such large families are common in Pakistani population where consanguinity is very high (Jabber et al., 1998; Hussain et al., 2001). Keeping in view these facts and as part of our ongoing effort to characterize the set of genes that cause inherited retinal degenerations in Pakistani populations, we recruited 25 index highly consangeneous cases with clear nonsyndromic arRP. Homozygosity mapping was the tool of choice because it is a powerful approach to confine and identify the genes underlying autosomal recessive genetic disorders (Lander and Botstein, 1987). It was also postulated that a recessive gene could be mapped using the offspring of consanguineous unions in"Homozygosity Mapping" that provides a rapid mean of mapping autosomal recessive genes in consanguineous families by identifying chromosomal regions that show homozygous identity-by-descend (IBD) segments in pooled samples (Lander and Botstein, 1987; Miano et al., 2000).

As the search for the diseased gene begins with the linkage analysis of the candidate gene so linkage analysis was done with candidate genes (table 1). Twenty five families with 85 diseased and 210 normal individuals of these families and from healthy populations were studied in this research work. Initially all families (except RP1) showed exclusion with commonly reported genes/locus. As it was postulated that all the diseased individuals would be homozygous for the marker that was adjacent to the gene responsible for the disorder while unaffected or carrier individuals would be heterozygous (Smith, 1953) so heterozygosity for the different combinations of the parental alleles in both affected and normal individuals confirmed exclusion in all the families except RP1 to six selected known genes on different chromosomes associated with arRP. The family RP1 showed linkage to *CRB1* with the marker D1S2840 and D1S2816 (which are closely linked to the human *CRB1* gene on chromosome 1q31.2). The haplotype analysis showed that three affected members were homozygous for these markers while the seven unaffected members were found to be heterozygous carriers. This family showed exclusion with other five genes (*MERTK, RPE65*, *ABCA4*, *RP1 and CNGA1*). These results helped to conclude the linkage of this family to *CRB1* gene on chromosome 1q31-q32. To confirm the linkage sequence analysis was done. The sequence analysis of family RP1 revealed a novel missense mutation (c.1459T>C) in *CRB1* gene analogous to exon 6. At protein level this mutation resulted in a substitution of proline for serine at amino acid 487 (p.Ser487Pro). The sequence chromatograms showed that affected individual were homozygous for c.1459T>C mutation while unaffected but carrier individual were heterozygous for this mutation (Figure 3).

The familyRP1 was recruited from the KPK province of Pakistan having three affected individuals. After having medical history, three affected and one unaffected individual underwent detailed ophthalmic examination. According to patients history all affected individuals started experiencing visual problems during the first decade of their life and the vision was reduced only to perception of light in individual VI-5 and VII-1 and tunnel vision in individual VIII-1 at the time of examination (table 2).

The more severe phenotype in 2 affected individuals (VI-5 and V11-1) of RP1 comprising of extensive pigmentation in peripheral and mid-peripheral region of retina coupled with macular atrophy might be due to the relatively older age of examination. Where as in 3rd affected individual few disperse pigmentary spicules at peripheral retina along with vascular attenuation might be due to earlier age of examination (Figure 1).

The presence of a missense mutation in *CRB1*, a gene previously associated with arRP, segregation of the mutation with the disease phenotype in the family, along with absence of the variation in matched controls strongly suggest that this mutation is responsible for RP phenotype in family RP1. Mutations in *CRB1* were previously reported as the cause RP in families of different ethnic origins (Beryozkin et al., 2013; Bujakowska et al, 2011; den Hollander et al., 1999 and Siemiatkowska et al, 2011). In majority of cases of RP due to *CRB1* gene, mutations are segregated in an autosomal recessive fashion (den Hollander et al., 2008; Hanein et al, 2004; Hartong et al, 2006). In the current study, it was also shown that mutation in *CRB1* was the cause of RP segregating in an autosomal recessive fashion among the consangenious Pakistani families.

Mutations in *CRB1* result in retinal thickening and lack of distinct layering in the fully developed adult retina (Jacobson et al, 2003). As *CRB1* has a central role in localization of phototransduction proteins to the apical membrane of the photoreceptors so mutated or nonfunctional *CRB1* may slow down the photo-transduction cascade and that is why there is progressive dystrophy of the photoreceptors and RPE in patients of RP (Rashbass et al, 2000). In our study all the affected individuals started experiencing visual problems in the first decade of their life similarly the *CRB1* mutations have been reported to be a relatively common cause of autosomal recessive early-onset retinal degeneration in the Israeli and Palestinian populations (Beryozkin et al, 2013).

Missense mutations in CRB1 gene have been shown to have great effect on the retinal functions which results in RP and Leber congenital amaurosis (LCA). It is predicted that missense mutations affecting cysteine

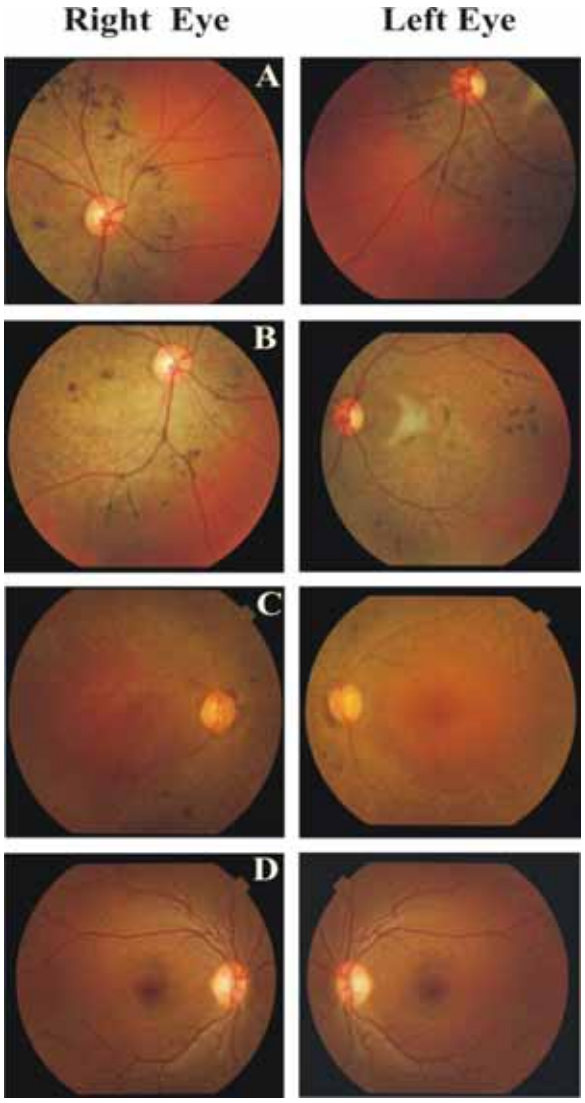

Fig. 2: Fundus photographs of affected members of family RP1 demonstrating features associated with RP in (A) Affected individual VI-5; (B) affected individual VII-1; (C) affected individual VIII-1; showing vascular attenuation, pallor optic disc and peripheral and mid peripheral pigmentation (D) unaffected/normal individual.

residues will affect the secondary structure of the protein due to disruption of disulfide bridges (den Hollander et al, 2004). Laminin AG-like domains have been identified in different proteins. Some of the residues of these domains are extremely conserved, and have important role in protein-protein interaction modules. Protein interactions are greatly affected as a result of missense mutations in these conserved residues (Beckmann et al, 1998). It was also predicted that the normal and proper retinal function were impaired due to missense mutations in the laminin AG-

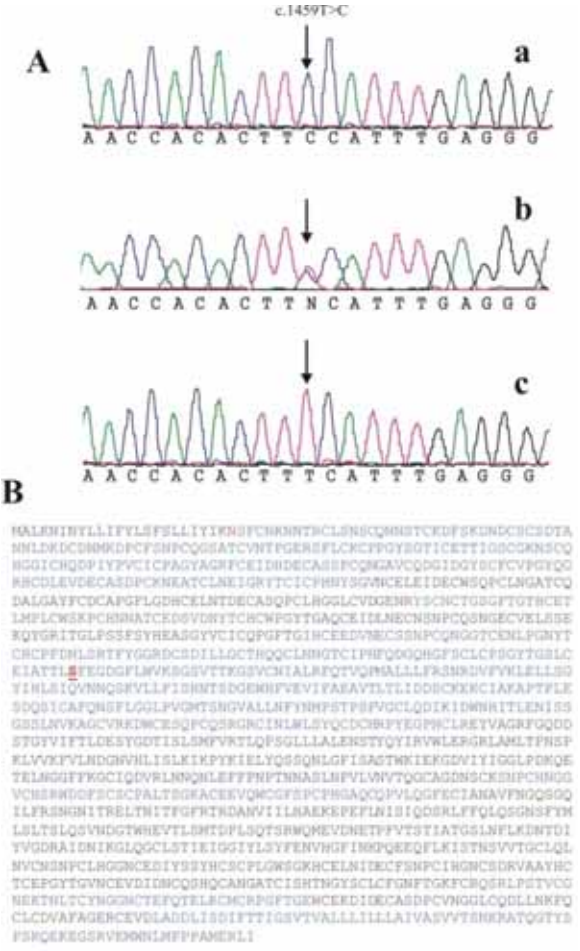

Fig 3A: Sequence Chromatogram of *CRB1* in family RP1. a: affected individual homozygous for c.1459T>C b: unaffected/carrier individual heterozygous; c. control sample. **3B: *CRB1* Protein Sequence with mutation (p. Ser487Pro).** The affected amino acid (S) is underlined at amino acid 487.

like and EGF-like domains which ultimately affected protein-protein interactions, calcium binding and protein folding (Tepass et al, 1990; Davis et al, 2007; Gosens et al, 2008). A missense mutation in *CRB1* has also been identified as a molecular defect responsible for RP phenotype in a consanguineous Pakistani family RP1. The sequence analyses of all the coding exons of *CRB1*gene in the linked family RP1 revealed a novel missense mutation (c.1459T>C) in exon 6, segregating with disease phenotype. This mutation resulted in a substitution of proline for serine at amino acid 487 (p.Ser487Pro). On the basis of our results it was strongly suggested that the missense mutation in *CRB1*, leading to premature termination of the protein, is responsible for RP phenotype in the affected individuals of this consanguineous Pakistani family.

Table 2: Clinical characteristics of affected individuals of RP1 diagnosed with autosomal recessive Retinitis pigmentosa (arRP)

Patient ID	Age at the time of examination (years)	Age of onset of disease(years)	Visual acuity	First Symptom	Disease progression	Fundus findings
V1-5	48	10	PL	Night blindness	Progressive	Pallor optic disc, Vascular attenuation, prominent bony spicules along vascular arcades.
V1I-1	45	12	PL	Night blindness	Progressive	Pallor optic disc, Vascular attenuation, prominent pigment depositon.
V11I-1	18	9	Tunnel vision	Night blindness	Progressive	Few disperse pigmentary spicules, pallor optic disc and vascular attenuation.

*PL perception of light

Conclusion

In conclusion, a missense mutation in *CRB1* has been identified as a molecular defect responsible for RP in a consanguineous Pakistani family. To the best of our knowledge this is the first report of missense mutation at nucleotide position 1459 (c.1459T>C). At protein level this mutation resulted in a substitution of proline for serine at amino acid 487 (p.Ser487Pro).

It is conceivable that many loci/genes/mutations are yet to be identified for this phenotype. Identification of these genes/loci would be helpful in future to understand RP at molecular level. This study will provide further assistance in defining the functions of *CRB1* gene products, as well as will help to ascertain the cause of the disease. The development of carrier screening and genetic counseling as a result of this research would lead to crucial impacts on the society as a whole.

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