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A Homozygous c.2536G>A Mutation in CRB1 Gene Manifesting Autosomal Recessive Retinitis Pigmentosa in a Large Consanguineous Kashmiri Family

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Authors' Contribution
ZL Designed the study and wrote manuscript. KB provided concept and design of the study and acquired, analysis and interpreted the data. AAA, GA,THT and AR collected blood samples and did clinical examination. HH acquired whole exome sequencing data. MNK and AMK supervised the study and revised the manuscript.

Key words
Retinitis pigmentosa, LCA, Consanguineous marriage, CRB1 gene, Kashmiri.

ABSTRACT

Retinitis pigmentosa (RP) is the condition of visual impairment which has most feared impact on blind individuals and family. As the cheerful life is made possible by illumination of sight similarly on opposite, blindness snatch this beauty of life and push the sufferers to unending darkness which also has a negative social and economic wellbeing impact on individual's life. In society, the negative impact of visual impairment is the rejection and exclusion from all healthy activities of life. Patients suffering with RP first experience nyctalopia which gradually progress to tunnel vision and ultimately masks with complete blindness. Autosomal recessive mode of inheritance which contributes 20-25% of total known cases of RP, is almost the result of inbreed union or cousin marriages. In this study, a large consanguineous family with 11 affected individuals was recruited from Azad Jammu and Kashmir which was analyzed through linkage mapping and confirmed by Sanger sequencing. This family showed a homozygous c.2536G>A mutation in CRB1 gene as an underlying pathogenic variant for non-syndromic autosomal recessive retinitis pigmentosa.

INTRODUCTION

Eye or ophthalmic anomalies are the heterogeneous group of disorders which show large diversity in phenotype and genetics, and are characterized into different categories. Epidemiological data of retinitis pigmentosa (RP) show that 1/3500 individuals are suffered with this devastating disease and most common incidences been reported from developing countries (Jay, 1982; Ayuso and Millan, 2010). As in these countries people with vision impairment have least approach or facilities to the health services, so undiagnosed or due to failure in treatment or management, these people are snatched from illumination and ultimately push them into permanent darkness (Bittles, 2005; Shintani *et al*., 2009).

Retinitis pigmentosa is a photoreceptors degenerative disorder which manifests with its initial symptoms of nyctalopia and progresses gradually by narrowing down the visual field just looking like through a tunnel. Retina of human eye has rods and cones photoreceptors which are specialized for vision in low light and color vision respectively. Rods are in periphery of retina while cones are concentrated in a small depression called fovea centralis. In typical RP rods are initially malfunctioned following cones and ultimately masks the individual with complete blindness (Travis, 1998). RP manifests in syndromic as well as non-syndromic form. In syndromic form, Usher syndrome, Senior-Loken syndrome, Joubert

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syndrome, Bardet Biedl syndrome and Meckel syndromes are very common while in non-syndromic form RP is the most common cause of vision impairment worldwide (Hildebrandt and Zhou, 2007; Hildebrandt *et al*., 2011) and Pakistan (Adhi and Ahmed, 2002).

RP is characterized on different basis *viz.,* involvement of region (peripheral, sectorial and pericentral), involvement of organ (syndromic and non-syndromic), age of onset (early or late onset) and mode of inheritance (autosomal dominant, autosomal recessive, X-linked and simplex/unknown). Autosomal dominant contributes 15-20%, autosomal recessive 20-25% and X-linked 10-15% while 40-55% is still simplex (Buch *et al*., 2004).

Pakistan having a strong cultural and social (cast and clan) ties bears a highest burden of recessive disorders due to cousin marriages (60%) (Bittles, 2001). Azad Jammu and Kashmir locating at north of Pakistan also has highest proportion of consanguineous marriages, it remained unexplored for analysis of genetic disorders including RP.

RP is clinically and genetically heterogeneous, as it shows a diversity in both patterns. To date, more than 132 loci for different forms of non-syndromic retinal disorders have been known and out of them 35 different genes are reported from Pakistani origin families (Khan *et al*., 2014), and 59% cases accounts just for arRP from total cases of retinal disorders manifested by many genes. The present study has been conducted to identify the causative variant in a large consanguineous Kashmiri family showing early onset typical arRP to explore Kashmiri population against this sever retinal disorder.

MATERIALS AND METHODS

Ethical approval

Prior to the conduction of study and clinical and genetic analysis of the family, informed written consent was taken from all participated members and the guardians of the family. Meanwhile the study was also approved by the ethical committee.

Family ascertained

The family included in this study was searched out through a comprehensive survey at different localities of Azad Jammu and Kashmir. The studied family was recruited from remote and hilly area of AJ&K. There were 11 blind individuals in this pedigree and information about all patients were collected to draw conclusion and study design.

Clinical assessment of affected members

All the participated members including control and patients were clinically well evaluated to exclude the syndromic possibilities of RP. To find a particular type of retinal disorder, the patients were assessed through general ophthalmic examination, fundoscopy, auto-fluorescence fundoscopy (FFA) and optical coherence tomography (OCT) by an expert ophthalmologist at Al-Shifa Trust Eye Hospital, Islamabad.

Pedigree construction and blood sampling

Mode of inheritance of RP, was analyzed through getting information from elders of the studied family and constructing a pedigree of this family using the standard method of Bennett *et al*. (1995). Blood samples from nine members including four affected (IV:4, IV:5, IV:6 and IV:7) and five normal (III:6, III:7, IV:8, IV:9 and IV:10) individuals were taken into K3 containing EDTA tubes which was stored at 4°C in laboratory till further processing (Fig. 1).

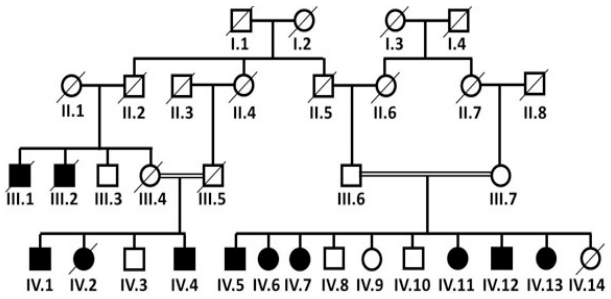

Fig. 1. Pedigree showing autosomal recessive mode of RP in the affected family.

DNA extraction and genotyping

Genomic DNA was extracted from collected blood samples through phenol chloroform protocol. Linkage analysis was carried out by amplifying the genomic DNA with STS markers through homozygosity mapping against the loci, known to cause arRP. Amplified products through PCR were resolved on 8% non-denaturing poly acrylamide gel (PAGE). After staining the gel with ethidium solution, allelic score was assigned to the normal and affected individuals through gels reads in gel documentation system. Haplotype of allelic pattern was drawn to clear the homozygosity and co-segregation with diseased individuals.

Sanger sequencing

Locus showing homozygosity which co-segregated with disease phenotype for disease individuals was confirmed through Sanger sequencing. Identified CRB1 locus showing homozygosity for affected members allelic pattern, was amplified for Sanger sequencing by using forward primer (CCATCAgCCTCTCCATgTTT) and reverse primer (gCACAgCCTTgggTTACATT). Sanger sequencing was performed for one normal (III:7/mother) and three affected (IV:5, IV:6 and IV:7) individuals to confirm the underlying pathogenic mutation as a causative

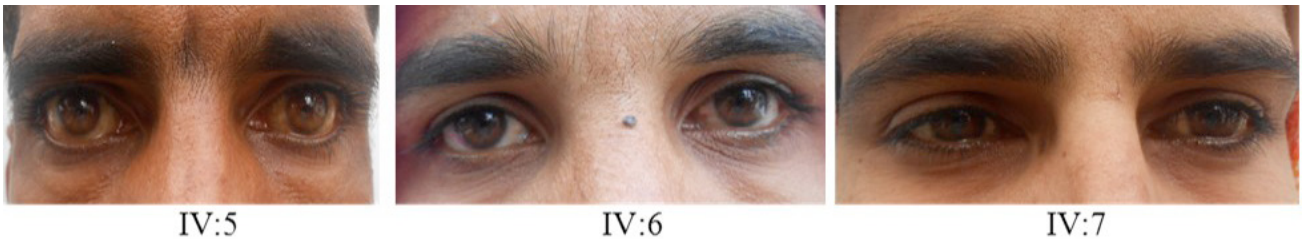

Fig. 2. Clinical presentation of three affected (IV:5, IV:6 and IV:7) individuals shown through close-up photographs of eyes.

player in all affected members with mRNA transcript variant of *CRB1* gene NM_201253.2.

RESULTS

Clinical findings

All affected individuals included in this study presented clear sign and symptoms of arRP. Initially (10-12 years of age), all suffers faced the difficulty to see in dim light or night vision (Fig. 2). Gradually with increasing age, they reduced the visual field and finally reaching at 25 years of age, all affected members were completely snatched from their vision as complete blindness. Fundus findings of patients showed typical signs like, bone spicules clustering in the peripheral region of retina and retinal arteriolar attenuation which clearly classified typical RP.

Locus identification

This family showing a clear pattern of early onset retinitis pigmentosa was checked through linkage analysis against the known genes by using the STS markers. Homozygosity at CRB1 locus ($1q^{31.1}$) with four amplified markers (D1S2816, D1S2840, D1S2816 and D1S1660) shown a clear homozygous pattern for all affected and heterozygous against controlled members which confirmed it as a diseased locus. The locus identified through linkage analysis was checked for underlying pathogenic variant by performing Sanger sequencing in the affected family.

Mutation screening

Results of sanger chromatograms from one control (III:7/mother) and three blind individuals (IV:5, IV:6 and IV:7) pin pointed the exact pathogenic variant c.2536G>A as role player in all blind patients. As the control individual/mother's chromatogram presented the heterozygous state for this mutation confirming the carrier status for arRP while all affected sibs were the homozygote for this mutation (Figs. 3, 4). The identified variant confirmed through Sanger sequencing unravel the underlying player causing retinal pathogenicity by showing a homozygous missense (c.2536G>A mutation) behavior in exon 7 of CRB1 gene. The highlighted sequence change co-segregated with the disease phenotype of this family.

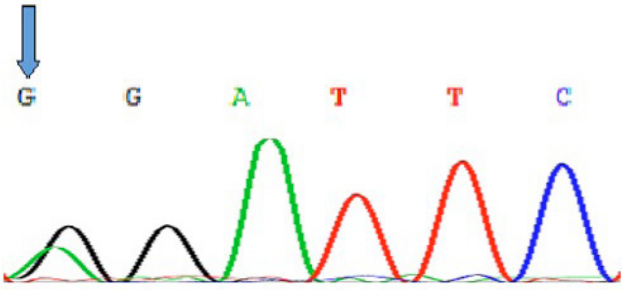

Fig. 3. Sanger sequence chromatogram of CRB1 gene of normal individual (III:7/mother). Chromatogram showed heterozygous carrier status for c.2536G>A mutation indicated with blue arrow.

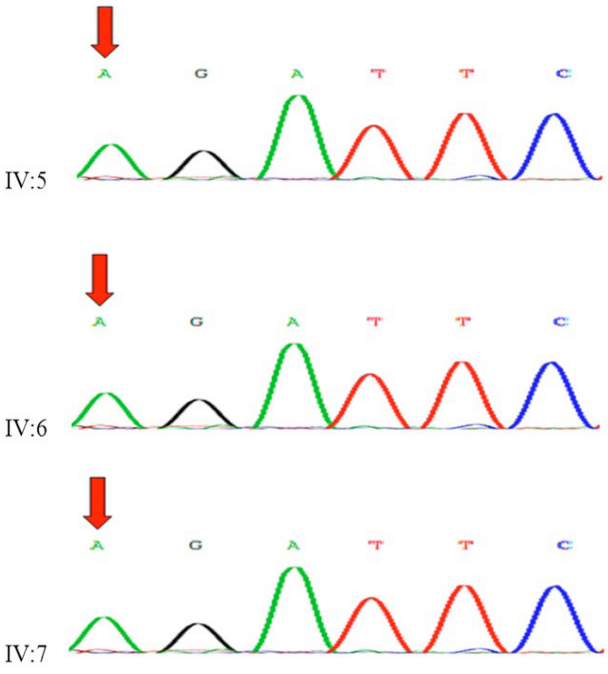

Fig. 4. Sanger sequence chromatogram of CRB1 gene of three blind patients (IV:5, IV:6 and IV:7) showing homozygous c.2536G>A mutation which is indicated with red arrows in all affected individuals.

DISCUSSION

This consanguineous four generations pedigree with eleven affected individuals suffered with early onset arRP, was assesed both at clinical and genetic level. Clinically, this family experienced nyctalopia at start of 2nd decade of age among all affected individuals with uniform pattern which masked the complete vision in progressive age. Genetically, this family was identified a chromosomal change at g.1: 197.396991 and missense mutation of c.2536G>A in exon 7 of *CRB1* gene, consequently with protein variation of p.G846R (in Lam AG 2 domain). *CRB1* gene located at 1q31.3, have 12 exons, was first time identified in Drosophila for encoding transmembrane protein which is required for embryonic epithelia's junctions adherence and apico-basal cell polarity maintenance. *CRB1* along with its two paralogue (*CRB2* and *CRB3*) are highly conserved in animal (22 species) kingdom. Mutations in *CRB1* gene manifest the light induced degradation of photoreceptors and morphological defects. It causes LCA (9-13%) with 50 known mutations, classic and RP12 caused by 35 known pathogenic mutations. RP caused by *CRB1* mutations is inherited in autosomal recessive mode which accounts for 6.5% (Vallespin *et al*., 2007). The disease caused by *CRB1* gene may overlap RP with LCA as it may commence at first decade of life or much later in life.

Mutations in *CRB1* gene are very well known for varying degrees of retinopathies ranging from LCA to rod-cone dystrophies (Clark *et al*., 2010; Coppieters *et al*., 2010; Azam *et al*., 2011). Crumb homologue 1 gene expresses in brain and retina and encode crumbs (crb) protein (den Hollander *et al*., 1999). Mutations of this gene produce short or abnormal protein and shortage of CRB1 protein halts retinal morphogenesis which manifests partial or complete blindness. *CRB1* gene exhibits alternative splicing on its 3' end which yields two proteins with 1406 and 1376 amino acids (den Hollander *et al*., 2001). These expressed proteins contain 19 EGF like domains, 3 AG (laminin A globular) and a signal peptide sequence. Crumbs as its mouse homologue plays important role in morphogenesis of photoreceptors (Pellikka *et al*., 2002). Mutant mouse with this gene showed developmental defects of retina by presenting outer limiting membrane disruption and retinal folds formation, and similar clinical features were observed in patients with crumbs mutation having thickened retina and altered laminar organization showing as immature normal retina (Jacobson *et al*., 2003; van de Pavert *et al*., 2007). The finding further supports the importance of *CRB1* gene in retina development.

Developmental disorder of retinal organization through interrupting naturally occurring apoptosis was also highlighted by Jacobson *et al*. (2003). The pathogenic variant (c.2536G>A) detected in this family was identified by Khaliq *et al*. (2003) in Pakistani population. The identified substitution of Glycine to Arginine halted the Laminin AG domain 2 of transmembrane crumbs protein and resulted in severe type of visual impairment (arRP). The reported mutation or missense sequence change is new for Kashmiri population and it is very well characterized for its pathogenesis for other populations. It matches with the findings of other ethnically related and non-related groups, as it is strong enough to cause arRP in this family.

CONCLUSION

This study was conducted as pioneer attempt to explore the Kashmiri population against genetically inherited retinal disorders, as Azad Jammu and Kashmir is a small piece of land located at north of Pakistan remained unattempt. Like Pakistan, Kashmiri population is also providing a suitable source for genetic disorders due to a common practice of cousin marriages. Present study identified a homozygous missense mutation in 7th exon of CRB1 gene which is compatible with other studies conducted for different ethnic groups, but this sequence change is new for Kashmiri population. Mutations of this gene are known to cause LCA and arRP by halting the photoreceptors morphogenesis both at early (1st year) age and late onset of RP. Present study confirms the identification of homozygous missense c.2536G>A mutation as causative variant for early onset arRP in Kashmiri population.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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