

A V235L Mutation of the Human BEST1 Gene Associated with Best Macular Dystrophy with Intra-familial Clinical Variations

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Abstract

Introduction: Best vitelliform macular dystrophy (BVMD) accounts for 1% of all cases of macular degeneration resulting in progressive loss of central vision. In this work we sought to evaluate the clinical and genetic background in a two generation pedigree of autosomal dominant BVMD for clinical management and follow up. To our knowledge this is the first report on association of Bestrophin 1 (BEST1) mutation with BVMD in an Indian family.

Case report: Complete ophthalmic examination done in a family with a complaint of impaired vision revealed the presence of yellow-orange yolk like lesions in the macula upon fundus examination. Further investigations through EOG revealed decreased Arden ratio. Besides the proband, two other members namely, mother and siblings were also affected in the family. Follow up clinical examination was done after three years to clinically document the progression of the disease, OCT examination was done in addition during follow up studies. Genomic DNA samples of the affected family members showed a sequence variation, c.703 G>T transversion in exon # 6 which results in substitution of valine by polar leucine as V235L. This variant was not observed in the unaffected father and 65 ethnically matched controls. Follow up clinical examination of the proband and his sib showed rapid progression of the disease.

Conclusions: We report for the first time an attempt on genetic diagnosis in a case of Best disease from Indian ethnicity. The disease condition revealed a rapid progression in the proband as compared to his mother, depicting severity of the disease in successive generation. The study prompts the need for genetic prognosis to identify predisposed/ susceptible individuals to enable proper counseling to the family members about the disease.

Keywords: Best disease; *Bestrophin*; CERES; Retinal pigment epithelium; Optical coherence tomography; Electrooculography

Introduction

Critical central vision is mandatory for detailed tasks such as reading, driving, and recognizing faces.

Best vitelliform macular dystrophy (*BEST1* Gene (OMIM-ID*607854), Best disease (OMIM-ID* 153700)) [1] is a bilateral, dominantly inherited macular dystrophy that causes progressive loss of central vision. Best disease is the second most common form of juvenile macular degeneration, with a bimodal onset with two peaks, one around 3-9 years and in the twenties [2], but there is a great variability in onset from infants to over 50 years of age. This disorder is characterized by variable deposition of yellowish pigments (lipofuscin) in the sub retinal space. Fundus examination of the central macula reveals a transient “egg yolk”-like lesion depicting an abnormal accumulation of lipofuscin-like material within and beneath the retinal pigment epithelium (RPE). The lesion progresses through several stages over the years, as follows. i. the previtelliform stage (20/20), ii. the vitelliform stage (20/20 to 20/50), iii. the pseudohypopyon stage (20/20 to 20/50). The vitelli-ruptive stage (20/20 to 20/100) and the atrophic stage arises when acuity may be reduced to less than 20/200, due to disintegration of the yellowish material (scrambled egg stage)

[3]. The Arden ratio determined by electrooculography (EOG) is usually <1.5 for the affected as well as for the phenotypically normal carriers, however there are reports as well of normal Arden ratio in family with Best disease [4].

It is now recognized that several distinct phenotypes/diseases are associated with *BEST1* mutations. Amongst them, the most common is the typical Best vitelliform macular dystrophy which is inherited either as an autosomal dominant trait or as an autosomal recessive bestrophinopathy (ARB). There are also distinct cases, with homozygous mutation in the *BEST1* gene, but the phenotype however resembles more or less BVMD and not ARB [5]. Hence it is clear from the literature that different mutations in the *BEST1* gene can cause distinct phenotypes [6]. The degree of central vision impairment and the age of onset vary widely amongst members of the same family [3,7]. The molecular basis of BVMD has been elucidated by positional cloning of the disease causing gene, *BEST1*, encoding the 585-amino acid transmembrane protein bestrophin-1 (hBest1), located in the basolateral plasma membrane of the retinal pigment epithelium (RPE). While the cellular functions of bestrophin is still unclear, this protein likely acts as a channel that controls the flow of chloride ions into or out of the retinal cells or may regulate voltage-gated L-type calcium-ion channels that act as a Ca²⁺-sensitive hetero- or homo-oligomeric chloride channel [8-10]. While more than 100 disease-causing mutations in *BEST1* vitelliform macular dystrophy (BVMD) are

reported [11] most (>90%) are point mutations [12] and none have been reported from Indian ethnicity. In this study, we performed molecular screening of a family with Best disease, who otherwise would have remained undiagnosed until its progression. This was the first report of an Indian family with BVMD to have a V235L mutation in the *BEST1* gene. Through an *in-silico* approach, an attempt was made to explain the pathogenicity of the V235L mutation, which predicts an alteration in CERES (Composite Exonic Regulatory Elements of Splicing) function.

Materials and Methods

Clinical examination

A two generation familial case (SEB1) (Figure 1), suffering from Best disease (Vitelliform macular dystrophy type 2, BEST1) was registered from Regional Institute of Ophthalmology, Egmore, Chennai. Ophthalmic examination of the anterior segment of the eye was carried out using slit-lamp biomicroscopy after pupil dilatation. Fundus examination was performed using an ophthalmoscope and photodocumented. The proband attended the clinic at the age of 9 years for long sight problems and underwent examination of the fundus. At the time of recruitment only his right eye was affected, but later he developed the disease in both eyes. Other family members were also clinically examined. The mother was found to be affected and the other sibling (7 year old brother) was normal at the time of case registration. EOG was performed on the proband and his mother at Sankara Nethralaya, Chennai. The proband's brother was not co-operative for examination by EOG. Standard protocols recommended by International Society for Clinical Electrophysiology of Vision Standard (ISCEV), were adopted for both ERG and EOG analyses [13].

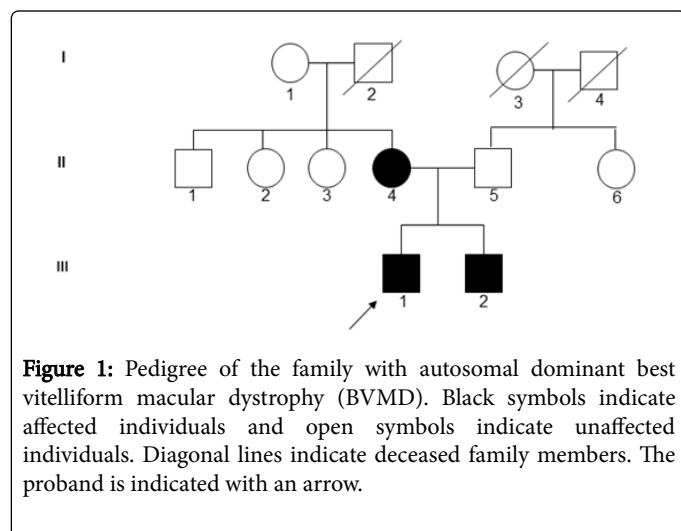


Figure 1: Pedigree of the family with autosomal dominant best vitelliform macular dystrophy (BVMD). Black symbols indicate affected individuals and open symbols indicate unaffected individuals. Diagonal lines indicate deceased family members. The proband is indicated with an arrow.

Follow up clinical examination by funduscopy and optical coherence tomography (OCT) was performed three years later for all three family members (the proband, brother and mother). The study was approved by the Institutional Ethical Committee of Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Chennai and Madras Medical College, Chennai. Upon informed consent, blood samples were collected from affected /unaffected family members for genetic analysis. We certify that all applicable institutional and governmental regulations concerning the ethical use

of human volunteers were followed during this research and the entire study adhered to the tenets of the Declaration of Helsinki.

Mutation analysis of the *Best1* gene

Genomic DNA was extracted from venous blood of study participants as previously described [14]. All the coding exons (#2 to #11) were amplified in a PTC-200 DNA engine (Bio-Rad, USA), in 60 μ l reaction volumes [30 μ l-Master mix Red; Forward/Reverse Primer - 6 μ l each (5 pm); Template DNA - 6 μ l (100 ng) ; water - 12 μ l] using suitable primers as described by Marquardt et al. [15] with some modification (primer sequence available upon request). After purification, PCR products (BIO Basic Inc., NY, USA) were commercially sequenced (1st BASE Pte Ltd., Singapore) and were checked for homology through NCBI-BLASTN analysis in comparison with that of the wild type sequence (NM_004183.3). Restriction site analysis was performed with 100 ng of the PCR product following the manufacturer's guidelines (NEB, USA) for all the affected and unaffected family members and in 65 unrelated control persons, and was resolved in 3% Biozyme agarose gels to rule out polymorphism.

Bioinformatic analysis

The altered protein sequence was analyzed using Polyphen2, SIFT and MutationTaster to know the effect of this mutation at the protein level. *In silico* analysis of putative exonic splicing enhancers and control elements in the wild type and mutant were done employing the RESCUE-ESE (<http://genes.mit.edu/burgelab/rescueese/>) [39] and ESEFinder program (<http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home>) [40].

Results

Clinical evaluation

The proband (SEB1 III1) had oval lesions (Figure 2c) seen just infero nasal to the macula of the right eye, while his left eye appeared to be normal at the time of case registration. EOG and ERG (Electro retinogram) (Table 1, Figure 3) investigated for all affected family members presented decreased Arden ratios (<1.5) and normal ERG. The proband and his brother were not co-operative for EOG and ERG examination, hence their results shows unreliable response. Fundus examination upon follow up of the proband and his brother were observed to be of the typical pseudohypopyon stage with yellowish vitelliform deposits in the macular lesion. Both the left and right eyes of the proband are found to be affected during the follow up clinical examination (Figure 4). OCT imaging (Figure 5) of the affected patient depicted a convex foveal contour with hyper-reflective lesions in the RPE chorio capillary complex and an invasion into the subfoveal retinal region. Further, a hyporeflective space seen in the subfoveal region along with serous detachment depicts a characteristic feature of neurosensory retinal detachment.

Molecular characterization

DNA sequence analyses of all the three affected members of the family revealed a c.703 G>T transversion as variation in exon 6 (Figure 7), however, the same was not seen in the unaffected father. This base change creates a restriction site for the enzyme HpyCH4III (Figure 6a). The affected members were heterozygous (G/T) at position c.703. Restriction digestion with HpyCH4III (NEB, USA) results in four fragments of 481 bp, 442 bp, 220 bp & 39 bp in size for all affected

individuals (Figure 6b), while the unaffected father and the unrelated controls (n=65) had only two fragments of 481 bp and 220 bp (Figure 6b). Two variations in the intronic region (c.636+44 C>T (Homozygous) & c.636+113 T>G (Heterozygous)) were also documented.

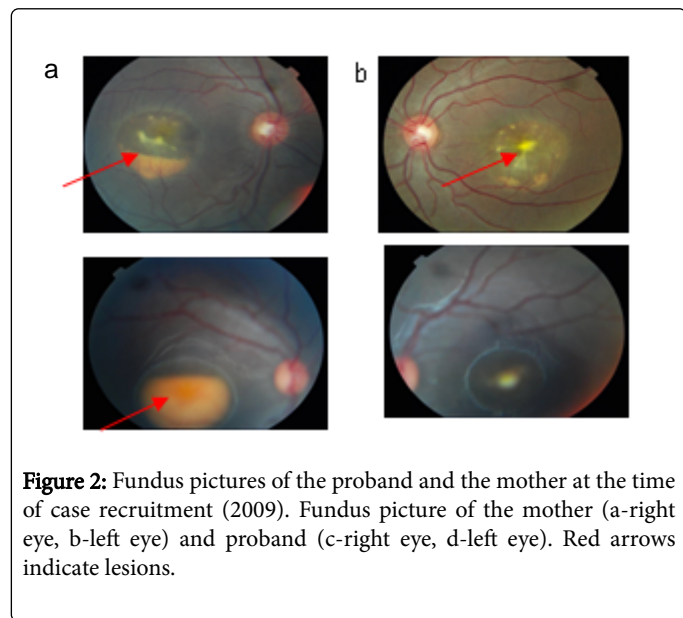


Figure 2: Fundus pictures of the proband and the mother at the time of case recruitment (2009). Fundus picture of the mother (a-right eye, b-left eye) and proband (c-right eye, d-left eye). Red arrows indicate lesions.

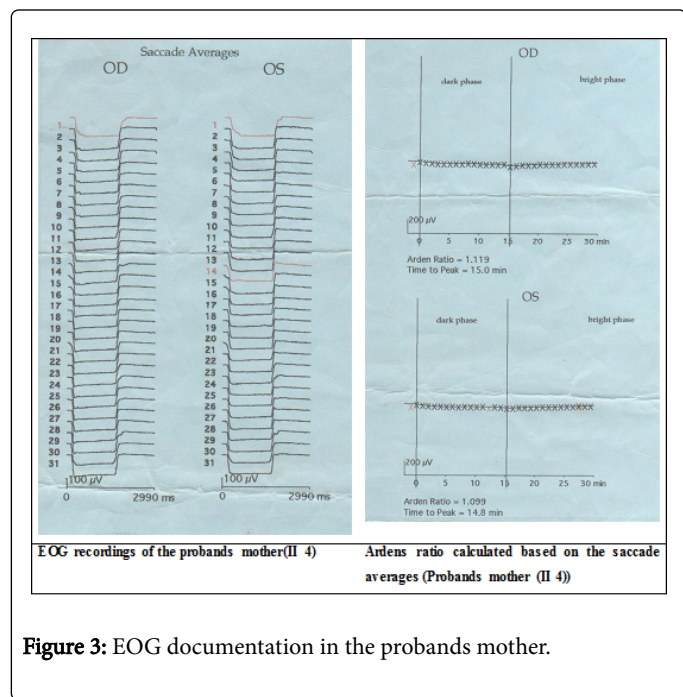


Figure 3: EOG documentation in the proband's mother.

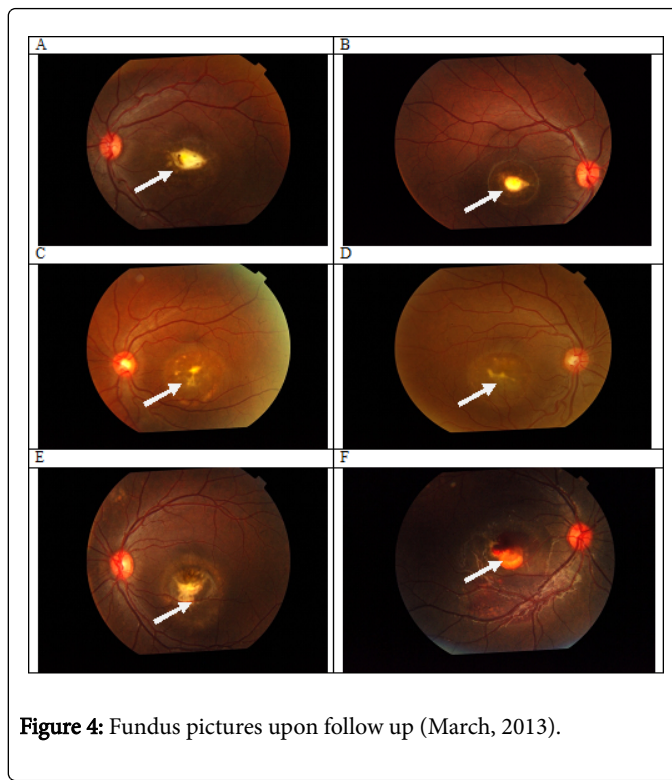


Figure 4: Fundus pictures upon follow up (March, 2013).

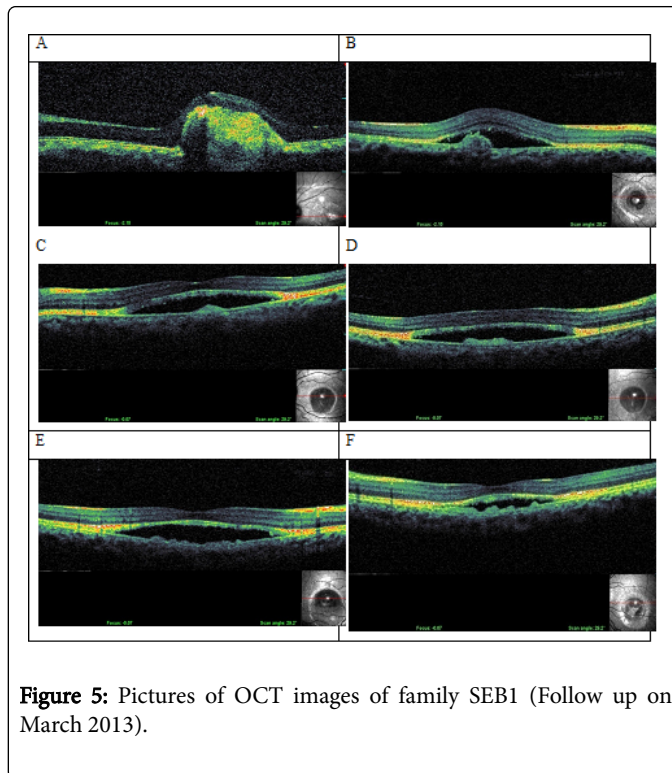
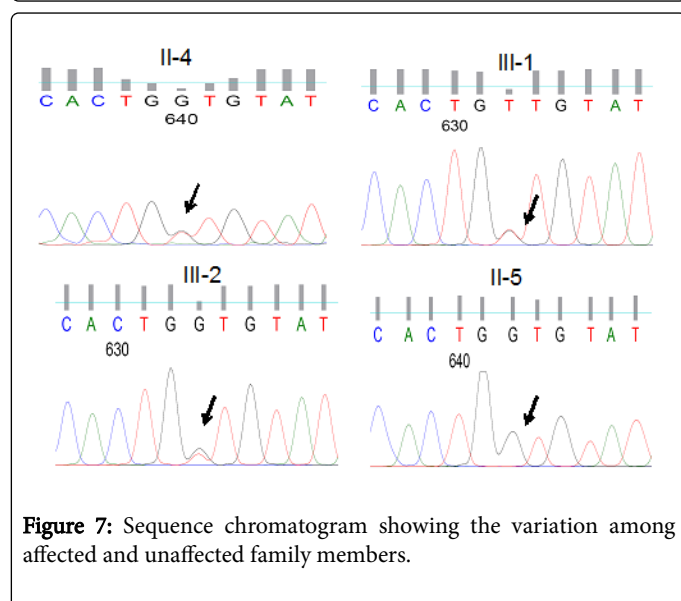
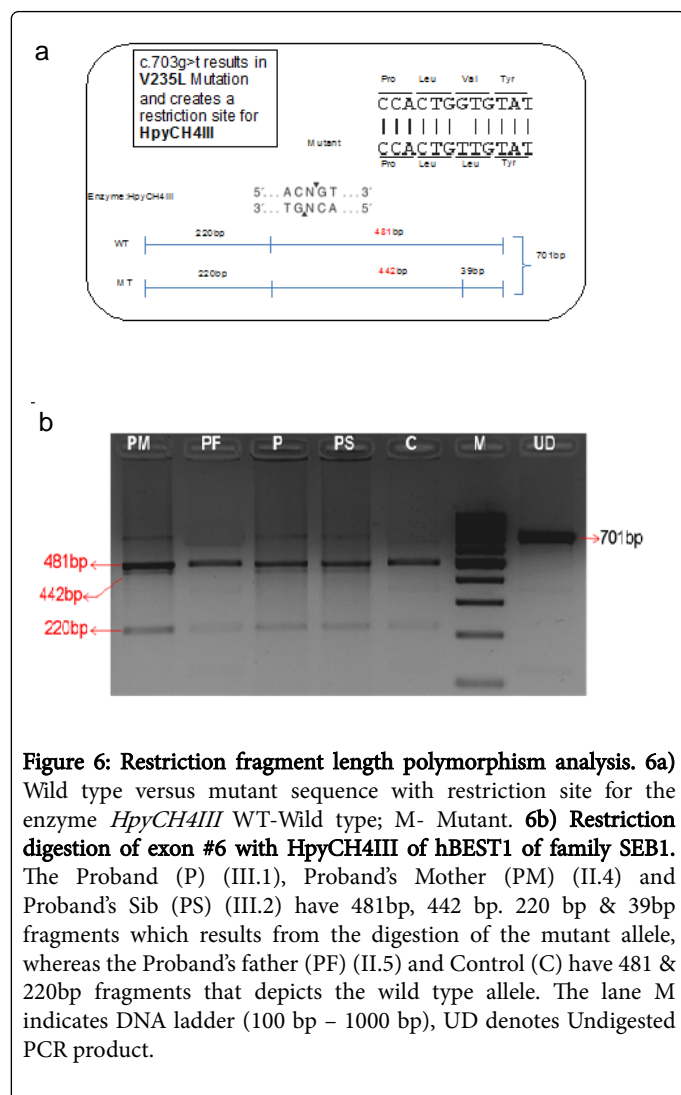


Figure 5: Pictures of OCT images of family SEB1 (Follow up on March 2013).



Individual	Age at the time of examination	Arden ratio	Visual Acuity	Fundus examination
II4	38 Y	LE- 1.119; Time to peak-15.0 min RE- 1.099; Time to peak-14.8 min	BE- 6/12 NIP	Macula - Egg yolk like appearance of macula with pseudohypopyon stage
III1	9 Y	LE- 1.398; Time to peak-1.0 min RE- 1.480; Time to peak-2.2 min	LE- 6/12 RE- 6/18 (Visual field- Vision with pin hole)	LE- Macula- Normal RE- Macula- Oval lesions seen just infero nasal to macula
III 2	7 Y	(non-co-operative)	BE- 6/6	BE- Macula - Normal

LE: Left Eye; RE: Right Eye; BE: Both Eyes; NIP: Not Improved with Pinhole

Table 1: Clinical documentation of the individuals with Best disease – SEB1 (at the time of recruitment-2009).

Bioinformatic analysis

Sift analysis shows that this variation as damaging with SIFT score 0.01. Polyphen analysis also showed that this mutation is probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00). Mutation taster predicted this variation as disease causing, and might affect protein features and also indicate change in splice site.

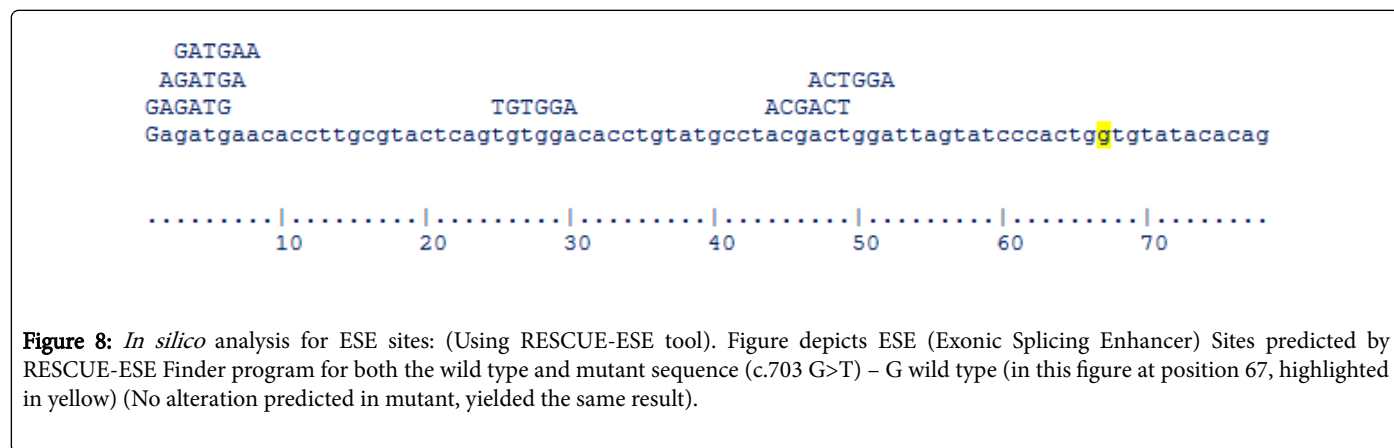
RESCUEESE predicted six common ESE sites (GAGATG, AGATGA, GATGAA, TGTGGA, ACTGGA, ACGACT) in both the wild type and mutant exon #6 sequences (Figure 8). On the contrary, as per ESEFinder analysis, two common (GTATCCCA, TGCGTA), as well as two variant enhancers (CCACTGG, CACTGGT) are predicted at the specific site of transversion in the wild type, two variant enhancers (CACCTGT and CGACTGG) in the mutant respectively (Table 2).

Discussion

Mammalian *bestrophin* gene was identified as the candidate responsible for autosomal dominant best vitelliform macular dystrophy (BVMD) [1], a relatively rare devastating form of macular degeneration. To date, more than 100 different *BEST1* mutations have been reported in BVMD. An overview of *BEST1* mutations could be seen in the VMD2 database (http://www-huge.uni-regensburg.de/VMD2_database) and on the Retina International website (<http://www.retina-international.com/sci-news/vmd2mut.htm>). BVMD is characterized by the following symptoms: (i) a diminution or loss of the late light peak of the electro-oculogram, caused by a defect within the RPE; (ii) a progressive loss of central vision; and (iii) an accumulation of debris within and beneath the RPE. The site of occurrence of these lesions implicate enrichment of bestrophin mRNA and protein in the RPE.

Wild Mutant	type/ SRSF1 threshold: 1.956	SRSF1 (IgM-BRCA1) threshold: 1.867	SRSF2 threshold: 2.383	SRSF5 threshold: 2.67	SRSF6 threshold: 2.676
	Position*/Site/Score	Position*/Site/Score	Position*/Site/Score	Position*/Site/Score	Position*/Site/Score
Wild type	62 (-17) cactggt 3.18086	62 (-17) cactggt 3.30473	56 (-23) gtatccca 3.46812	61 (-18) ccactgg 5.72198	14 (-65) tgcgta 5.77064
Mutant	31 (-48) cacctgt 2.71857	31 (-48) cacctgt 2.95562	56 (-23) gtatccca 3.46812	45 (-34) cgactgg 4.90044	14 (-65) tgcgta 5.77064

Table 2: ESE sites predicted for wild type/mutant sequence using ESE Finder program.



BEST1 is a member of the RFP-TM family of proteins, so named for their highly conserved arginine, phenylalanine, proline (RFP) motif [16]. They most likely function as chloride channel in regulating the ionic milieu of the sub retinal space. Majority of the mutations (92%) identified in BVMD are missense mutations and are mapped in the highly conserved N-terminal half of the protein, while the C-termini differ substantially amongst paralogs. Mutations tend to cluster within or close to the four predicted transmembrane domains in the families reported. This features the importance of specific regions spanning the amino acids 6-30, 80-104, 221-243, and 293-312 for bestrophin function [17,18]. These regions are situated at or near the plasma membrane of the RPE, and may therefore be of particular significance for *BEST-1* Cl⁻ channel function and/or CaV channel modulation [18].

In SEB1 family, the affected family members (II2, III1 & III2) show a distinct variation, G>T at position c.703 as V235L in exon #6 of *BEST1* that occurs in the Transmembrane Domain 5 of the protein Bestrophin-1. Both the residues are of aliphatic nature that confers flexibility to the polypeptide chains. Earlier a similar mutation (c.703G>C; V235L) (c.703G>A; V235M) at a phylogenetically conserved site in different ethnicities has been reported in a family and a sporadic case leading to a similar phenotype [15,19]. BVMD features a markedly variable penetrance and expressivity even within a family [20-22]. This has been documented earlier in some of the patients who had no accompanying changes in fundus morphology, although with normal EOG even in genetically confirmed BVMD cases [7,23]. In our proband, the disease had apparently progressed later. Eventually in some of the individuals who present late-onset symptoms with *BEST1* mutations may be misdiagnosed of having AMD [24-26].

OCT in general elaborates features about the topography of sub-/intra- retinal lesions of a subretinal scar, its position, atrophic areas, hyper reflective material, and the location and amount of sub-/intra-retinal fluid. OCT imaging also provides further insight into lesions not resolved through either FFA or FAF (Fundus Fluorescein

Angiography, Fundus Auto Fluorescence). In the subjects described here, both the yellowish vitelliform and the subretinal fibrosis appeared hyper reflective. In the present family study, the OCT examination, clearly elucidate the fact that, the progression of the disease in the proband is severe compared to that of the affected mother and the sib. The left eye OCT shows medium to hyper reflective lesion in the RPE suggestive of a CNVM (Choroidal NeoVascular Membrane), it has to be an inactive scarred membrane because of the absence of intra retinal edema or cystoid macular edema or subretinal fluid and consequently, the normal foveal contour is altered. In the right eye, medium reflective lumpy lesion from the RPE suggests presence of lipofuscin deposits between the RPE and the photoreceptor layer (IS_OS junction). The black area around probably denotes the sub retinal fluid collection. The initial onset of the disease in the proband was only in the right eye, but later developed in both the eyes. The OCT picture of the sibling, shows disintegration of the material, which is quite unique from the other affected members. In the affected mother, deposits are seen in the right eye, while in the left eye only the subretinal fluid could be seen. This may be interpreted as those deposits beyond the scan limit of imaging.

The decline of visual acuity tends to vary in cases of BVMD [27]. Apart from possible detrimental influences of mutant bestrophin-1 on RPE cell function, a progressive build-up of toxic fluorophores in the subretinal space might cause damage to the photoreceptors and the RPE [28,29]. Oxidized A2E precursors could damage the adjacent photoreceptors [30,31], and its derivatives are known to exert a wide range of damaging effects on the RPE [32,33].

With the aforesaid clinical background, the mutation V235L is predicted to have certain pathological implications. Exonic regions might harbour elements that influence splicing, as exonic splicing enhancer (ESE) and silencer (ESS) sites [34-37] that in the event of an overlap is termed as composite exonic regulatory elements of splicing (CERES) [38]. This prompted us to search for the presence of ESE/ESS

sites at this region in precise to check if the said variation, G>T c.703 has any effect on the splicing of the mutant *BEST1* transcript. The same was tested through *in silico* analysis. RESCUEese predictions do not show any alteration either in the wild type or in the mutant sequence (Figure 8). On the contrary, as per ESEFinder analysis, the mutant revealed two variant enhancers (CACCTGT and CGACTGG) that might influence alternate splicing of the mutant *BEST1* transcript in patients (Figure 9). However such a probability remains to be experimentally proven. In general five nucleotides between c.701 and c.710 are conserved from human to fly genome which provides further evidence of occurrence of a CERES site around nucleotide positions c.701 and c.707.

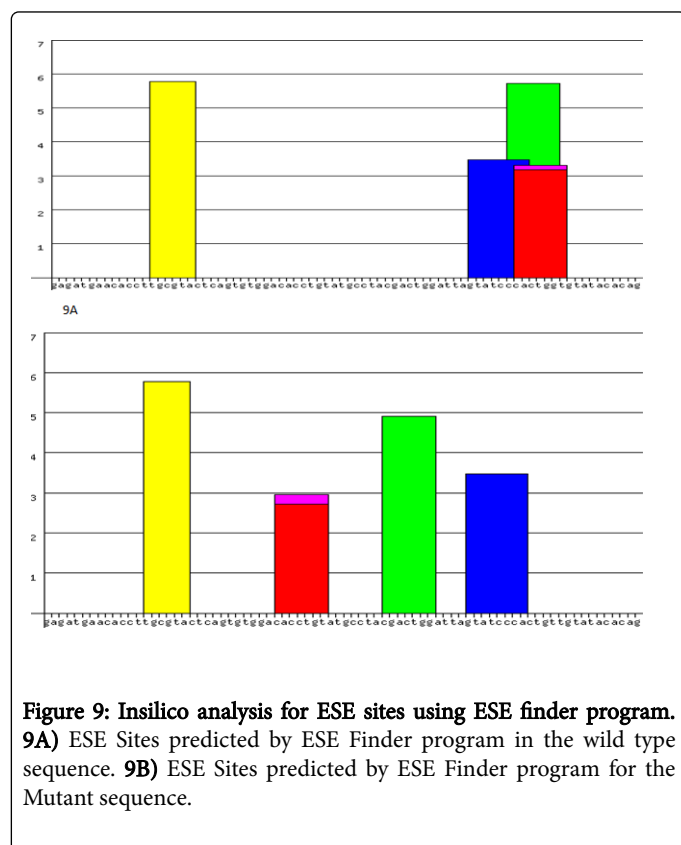


Figure 9: In silico analysis for ESE sites using ESE finder program. 9A) ESE Sites predicted by ESE Finder program in the wild type sequence. 9B) ESE Sites predicted by ESE Finder program for the Mutant sequence.

Alternative splicing has important physiological role in regulating gene expression in multicellular organisms. Alternate splicing changes the structure of the mRNAs and their encoded proteins. As a consequence, almost all aspects of protein function, such as binding properties, enzymatic activity, intracellular localization, protein stability, phosphorylation and glycosylation patterns are influenced there by playing a critical role in deciphering the complex human proteome [39,41]. Interestingly, any aberrant alternative splicing due to mutation in ESE/ESS regions could be pathological as evidenced in certain diseases like ADVIRC (Autosomal Dominant Vitreo Retino Choroidopathy) and the MRCS (Microcornea, Rod-cone dystrophy, early-onset Cataract, posterior Staphyloma) syndrome [42].

In vitro experimental validation of the *BEST1* p.Val235Ala (c.704T>C) and p.Tyr236Cys (c.707A>G) mutations through *Ex vivo* splice assays on HEK293 cell lines has indicated that the specific region c.704 to c.709 in *BEST1* has a CERES function [42]. Such mutations may influence binding of modulating proteins (so-called serine-arginine-rich RNA binding proteins, shortly named SR proteins) of the

spliceosome to CERES, thereby altering exonic splicing that results in an in-frame alteration of bestrophin -1 leading to an mRNA that either lacks exon # 4, #6, or #7, or with a duplication of exon #6 [42]. In ADVIRC patients, three isoforms of the protein may thus be encountered: the wild-type protein, the protein containing a missense substitution, and the protein with an in-frame deletion or duplication. The authors reported that the variation G>C at 703, did not alter CERES function.

However the variation that we observed (c.703 G>T) in the family (SEB1) appears to alter the CERES function through *in silico* analysis. Both the substitutions lead to the same change at the amino acid level (V235L), this indicates that alteration of amino acid at this position is pathogenic, as confirmed through *in silico* predictions indicating alteration at this position might be pathogenic. Together with our prediction of altered CERES functions, further substantiates that the variation at nucleotide level influences the severity of the disease, as evidenced by the rapid progression of the disease in the proband. We therefore speculate that such a mechanism could be in operation in our patients to explain the clinical severity. However, experimental validation of the same is needed to validate the proposed mechanism. This is the first case report of Best disease with intra familial clinical variations of the same genotype, from Indian ethnicity.

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All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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