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MUTATION IN BRIEF

Ten Novel Mutations in *VMD2* Associated With Best Macular Dystrophy (BMD)

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Mutations in the vitelliform macular dystrophy 2 (*VMD2*) gene encoding besrtophin are responsible for Best macular dystrophy (BMD), a juvenile-onset autosomal dominant disorder of the central retina. Here, we report ten novel *VMD2* mutations identified in clinically diagnosed BMD patients. The heterozygous alterations include nine missense mutations (c.32A>T, c.76G>C, c.85T>C, c.122T>C, c.122T>C, c.310G>C, c.722C>A, c.880C>G, c.893T>C) resulting in amino acid changes (respectively: Asn11Ile, Gly26Arg, Tyr29His, Leu41Pro, Trp102Arg, Asp104His, Thr241Asn, Leu294Val and Phe298Ser) located within four previously defined hotspot regions of the gene. In addition, a silent exonic mutation (c.624G>A) was identified in a two generation BMD pedigree. To determine a possible pathogenic effect of this variant, the consequences on splicing behaviour and potential exonic splice enhancer (ESE) motifs were analyzed. Finally, a 1-bp deletion (c.779delC) resulting in a frameshift mutation (Pro260fsX288) was found in exon 7, representing the first case of a potential frameshift mutation that affects the N-terminal half of the VMD2 protein. Besides a dominant negative effect which is likely attributable to the identified missense mutations, the deletion mutation suggests haploinsufficiency as an infrequent disease-causing mechanism in BMD. © 2003 Wiley-Liss, Inc.

KEY WORDS: Best macular dystrophy; Best disease; *VMD2;* bestrophin; mutation analysis; retinal pigment epithelium

INTRODUCTION

Best macular dystrophy (BMD; MIM# 153700) is an early-onset autosomal dominant maculopathy typically characterized by bilateral yellowish lesions in the central area of the retina (Best, 1905). In advanced stages, atrophy of the sensory neuroretina and the underlying retinal pigment epithelium (RPE) may result in progressive loss of central vision. The molecular basis of BMD has been elucidated by positional cloning of the disease-causing gene, *VMD2*, encoding a 585-amino-acid-(aa)-residue protein, termed bestrophin (Marquardt et al. 1998; Petrukhin et al., 1998). Bestrophin together with three closely related VMD2-like proteins (VMD2L1-L3) share an invariant tripeptide motif of arginine (R), phenylalanine (F) and proline (P) as well as four putative transmembrane (TM) domains in the N-terminal half of the proteins (Stöhr et al., 2002). While the cellular functions of the VMD2-

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like family members (VMD2L1-L3) are still unknown, bestrophin was shown to act as a Ca^{2+}-sensitive heterooligomeric or homooligomeric chloride channel (Sun et al., 2002). In addition, an interaction between the C-terminal cytoplasmic domain of bestrophin and protein phosphatase 2A was established suggesting that phosphorylation/dephosphorylation mechanisms may play a role in the fine tuning of light responses (Marmorstein et al., 2002).

To date, 83 distinct mutations are associated with BMD which are publicly-accessible in the *VMD2* Mutation Database (http://www.uni-wuerzburg.de/humangenetics/vmd2.html). Of these, the majority are missense mutations (94 %) primarily residing in four hotspot regions within the evolutionary highly conserved N-terminal part of the protein in close proximity to the four putative membrane-spanning domains. In addition, three in-frame deletions (4 %) and two frameshift mutations (2 %), one due to a single splice site mutation and another due to a two-base pair (bp) deletion have been associated with BMD.

Here, we report the finding of ten novel likely disease-associated mutations in the *VMD2* gene, including nine missense mutations and a 1-bp deletion. We also identified a number of *VMD2* sequence alterations that are probably of benign nature. To gain insight into the functional consequences of a *VMD2* sequence change with uncertain pathogenicity (c.624G>A), we utilized an *in vitro* heterologous expression assay to ascertain the possible effect of this sequence variation on splicing behaviour.

MATERIALS AND METHODS

Patient sample and molecular analysis of *VMD2*

Molecular screening of *VMD2* (GenBank: NM_004183.1) was carried out in a total number of 58 unrelated individuals affected with BMD (51 persons), unspecified vitelliform macular degeneration (3), adult vitelliform macular dystrophy (2), pattern dystrophy (1), and unclassified macular degeneration (1). The patients were evaluated by ocular examination, fundus photography, fluorescein angiography and/or electrophysiological testing. Informed consent was obtained from all participants prior to blood withdrawal. 50 individuals of German descent were selected as a population-based control sample.

Genomic DNA was extracted from blood lymphocytes by standard protocol. The coding exons of the *VMD2* gene (exons two to eleven) and the flanking splice junctions were analyzed by PCR amplification and direct sequencing as described in Marquardt et al. (1998). Fluorescence-labelling of PCR products was achieved by using the BigDye terminator kit (Applied Biosystems, Weiterstadt, Germany). The sequencing products were analyzed on an automated capillary sequencer (ABI Prism 310) and evaluated with software package Sequencing Analysis version 3.6.1 and Sequencing Navigator (Applied Biosystems, Weiterstadt, Germany).

Functional analysis of a putative splicing mutation

The expression vector pSPL3b (Invitrogen™, Karlsruhe, Germany) was used to evaluate the consequences of a mutation (c.624G>A) possibly affecting splicing behaviour. Using genomic DNA from the affected patient and oligonucleotide primers VMD2-*Eco*RI Ex5F (5'-CCGGAATTCGCCATCCCTTCTGCAGGTT) located in intron four and VMD2-*Bam*HI-Ex6R (5'-CGACGGATCCGGCAGCCTCACCAGCCTAG) located in intron six of *VMD2*, a genomic region of 720 bp including the G>A substitution at nucleotide position c.624 was PCR-amplified. Addition of the *Eco*RI and *Bam*HI recognition sites to the oligonucleotide primers enabled directional cloning of the PCR product into the *Eco*RI/*Bam*HI-predigested pSPL3b vector. Wild-type (WT) and mutant (MUT) clones were selected by direct sequencing. Then, the wild-type and mutant constructs (pSPL3B-WT and pSPL3B-MUT) were transiently transfected into COS7 cells, incubated for a period of 48 hours and total RNA was isolated from the cells. A vector-/vector-specific set of primers (SD6 5'-TCTGAGTCACCTGGACAACC and SA2 5'-ATCTCAGTGGTATTTGTGAGC) was used for RT-PCR analyses. After electrophoretically separating the RT-PCR products in a 1 %-agarose gel, differently-sized fragments were excised from the gel. The various splicing products of the wild-type and mutant constructs were determined by direct sequencing.

Electronic Database Information

Genomic DNA and cDNA sequences of the human *VMD2* gene are available at the GenBank database (http://www.ncbi.nlm.nih.gov:80/): see Refseq NM_004183.1 (mRNA). *VMD2* mutations are collected in the

VMD2 Mutation Database (http://www.uni-wuerzburg.de/humangenetics/vmd2.html). The ESEfinder© program (http://exon.cshl.edu/ESE/) was used to search for putative ESE motifs (Cartegni et al. 2003).

RESULTS AND DISCUSSION

Mutations

Mutation analysis in the *VMD2* gene of 58 unrelated patients revealed a total of 27 disease-causing mutations in 26 patients affected with BMD and in one patient (ID# 011259) affected with pattern dystrophy. 17 out of 27 mutations are non-recurrent, while two out of 27 are known recurrent mutations that were found five times each. Of the 19 distinct mutations, ten are novel and nine have previously been reported (Table 1).

Table 1. Novel and Known Mutations Identified in *VMD2*

Exon	Nucleotide Change[a]	Amino Acid Change[a]	Interspecies conservation[b]	Hotspot[c]	Number of Patients	Patient ID Number	Literature
2	**c.32A>T**	**Asn11Ile**	**yes**	**yes (I)**	**1**	**991200**	**novel**
2	**c.76G>C**	**Gly26Arg**	**yes**	**yes (I)**	**1**	**010251**	**novel**
2	**c.85T>C**	**Tyr29His**	**yes**	**yes (I)**	**1**	**000041**	**novel**
2	**c.122T>C**	**Leu41Pro**	**no**	**no**	**1**	**022431**	**novel**
4	c.299T>G	Leu100Arg	yes	yes (II)	1	990723	Krämer et al. (2000)
4	**c.304T>C**	**Trp102Arg**	**yes**	**yes (II)**	**1**	**011102**	**novel**
4	**c.310G>C**	**Asp104His**	**yes**	**yes (II)**	**1**	**990489**	**novel**
5	c.584C>T	Ala195Val	yes	yes (III)	1	011456	Lotery et al. (2000)
6	c.652C>A	Arg218Ser	yes	yes (III)	1	001843	Bakall et al. (1999) Krämer et al. (2000)
6	c.652C>T	Arg218Cys	yes	yes (III)	1	021384	Marquardt et al. (1998) Caldwell et al. (1999) Bakall et al. (1999) Lotery et al. (2000) Marchant et al. (2001)
6	c.710C>G	Thr237Arg	yes	yes (III)	1	990335	Krämer et al. (2000)
7	**c.722C>A**	**Thr241Asn**	**yes**	**yes (III)**	**1**	**022492**	**novel**
7	c.728C>T	Ala243Val	yes	yes (III)	5	991228, 011259[d] 000144, 010185 990700	Krämer et al. (2000)
7	**c.779delC**	**Pro260fsX288**	**-**	**no**	**1**	**020482**	**novel**
8	**c.880C>G**	**Leu294Val**	**yes**	**yes (IV)**	**1**	**000433**	**novel**
8	c.884delTCA	Ile295del	yes	yes (IV)	5	990803, 990551 020177, 000989 022367	Marquardt et al. (1998) Krämer et al. (2000)
8	c.884T>C	Ile295Thr	yes	yes (IV)	1	022292	Yanagi et al. (2002)
8	**c.893T>C**	**Phe298Ser**	**yes**	**yes (IV)**	**1**	**990273**	**novel**
8	c.903G>C	Asp301Glu	yes	yes (IV)	1	031810	Caldwell et al. (1999) Krämer et al. (2000)

[a] The “A” of start codon ATG is numbered +1 and ATG coding for start-methionine is numbered residue +1. Refseq NM_004183.1

[b] Alignment to bovine and mouse *Vmd2* sequences

[c] The location of hotspot regions I – IV are shown in Figure 1

[d] Patient diagnosed with pattern dystrophy

The novel mutations were classified as disease-associated based on several criteria. Firstly, the novel mutations are located within regions that are known to be affected by frequent mutational changes (“hotspot” regions). Secondly, the affect amino acid residues display a high degree of conservation within phylogenetically distant orthologues of bestrophin. Thirdly, in two cases (ID# 991200 and ID# 022492) further affected family members were available and tested for segregation in two or more generations. Finally, in the case of Asp104His functional

analysis of a variant also affecting codon 104 (Asp104Glu) was carried out previously and shown to significantly reduce whole-cell current (Sun et al., 2002).

The ten novel disease-associated mutations represent nine exonic missense mutations (Asn11Ile, Gly26Arg, Tyr29His, Leu41Pro, Trp102Arg, Asp104His, Thr241Asn, Leu294Val and Phe298Ser) and a single 1-bp deletion (Pro260fsX288). Similar to previous reports (summarized in the *VMD2* Mutation Database), the missense mutations are mainly located in exons two, four, six and eight while the 1-bp deletion occurred in exon 7 of the *VMD2* gene (Fig. 1).

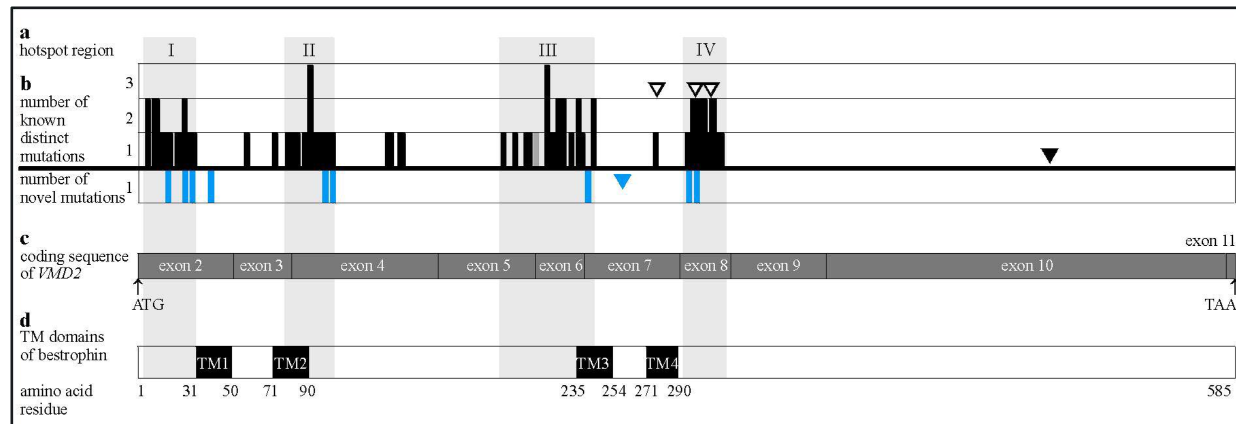

Figure 1. Distribution of known and novel distinct mutations identified in the *VMD2* gene. (a) The location of hotspot region I – IV (light-gray vertical bars) is shown relative to (c) the coding sequence of the *VMD2* gene and (d) the translated protein sequence. (b) Distinct known mutations are indicated by black bar (missense mutations), grey bar (splice site mutation), filled triangles (out-of-frame deletions) and open triangles (3-bp in-frame deletions). Novel mutations reported herein are depicted in blue bars (missense mutation) and in a blue triangle (out-of-frame deletion). The sizes of the coding exons of *VMD2* as well as the bestrophin amino acid sequence with its four putative transmembrane (TM) domains are given to scale.

In the second exon of *VMD2*, three out of four novel mutations (Asn11Ile, Gly26Arg and Tyr29His) are located in the first defined hotspot region prior to the first putative transmembrane (TM1) domain of bestrophin (Fig. 1). It should be noted that Gly26 and Tyr29 are invariant residues in human VMD2L1 to L3 (Stöhr et al., 2002) and Asn11 is conserved in both VMD2L1 and VMD2L3. The potential functional importance of residue Gly26 is underscored by its species-conservation in the *Vmd2* gene of the fruit fly and the worm (Marquardt et al., 1998). The missense mutation Leu41Pro is located within the putative TM1. The introduction of a proline residue at position 41 is likely to cause a major effect on the conformational properties of the membrane-spanning sequence. Thus, Leu41Pro is considered disease-causing.

Two novel missense mutations were found in exon four (Trp102Arg and Asp104His). According to the “four TM domain model” of bestrophin suggested by Bakall et al. (1999), the residues Trp102 and Asp104 are located between TM2 and TM3 (Fig. 1). While conservation of Try102 is limited to human VMD2L3, Asp104 represents an invariant residue within the entire VMD2-like protein family (Stöhr et al. 2002). Moreover, the Asp104 residue is highly conserved among other species, including nematode and fruit fly (Marquardt et al., 1998). Finally, codon 104 has previously been shown to be affected by a mutational change which alters the asparagine residue to a glutamic residue (Asp104Glu) (Petrukhin et al., 1998). Taken together, Trp102Arg and Asp104His are likely to be causative of BMD.

The novel missense mutation Thr241Asn in exon seven is located within the third putative TM domain of bestrophin. Threonine is highly conserved at residue position 241 in the bestrophin-related family members (VMD2L1, VMD2L2 and VMD2L3) suggesting an important functional or structural role of this residue.

The heterozygous 1-bp deletion of cytosine at nucleotide postion 779 (c.779delC) in exon seven is the first finding of an out-of-frame micro-deletion affecting the N-terminal half of bestrophin. This frameshift mutation is thought to lead to a premature stop codon after 27 altered amino acid residues (Pro260fsX288). The hypothetical Pro260fsX288 peptide most likely represents a non-functional protein consisting of 287 amino acid residue with the entire C-terminal half as well as the TM4 domain missing. Therefore, this mutation could represent a true null allele suggesting that haploinsufficiency may be a disease mechanism in BMD on rare occasions.

In exon eight, the novel mutations Leu294Val and Phe298Ser are located within a highly conserved sequence motif of 26 residues, encompassing codons 289 to 314 (Stöhr et al., 2002). This sequence motif, likely located intracellularly and on the C-terminal side of the fourth putative TM domain, bears the highest density of reported distinct mutations (22 distinct mutations; *VMD2* Mutation Database). Within the human VMD2-like protein family, Leu294 and Phe298 are invariant residues.

Variants and Polymorphisms

We identified nine common and previously described polymorphisms as well as two novel exonic changes (c.624G>A and c.720G>C) in exons five and seven and four novel intronic sequence alterations in intervening sequence three (c.IVS3-12C>T, c.IVS3-17C>T, c.IVS3-26C>T and c.IVS3-32C>T) in our cohort of 58 unrelated patients (Table 2). Except for c.IVS3-12C>T which was found in two unrelated patients and c.IVS3-26C>T which was identified as homozygous sequence variation in one patient, the remaining four silent variants were only found once in our patient sample. Sequencing of these variants in the control individuals revealed no significant deviation from the findings in the patients (Table 2) although the number of patients and controls may be too small to accurately determine the frequencies of these rare variants.

Table 2. Novel and Known Rare Variants and Common Polymorphisms Identified in *VMD2*

Exon	Intron	Nucleotide Change[a]	Affected Amino Acid Residue[a]	Allele frequency of minor allele in patients	Allele frequency of minor allele in controls	Novel/known
2		c.109T>C	Leu37	36/110 (32.7 %)	12/44 (27.3 %)[b]	known
3		c.201 G>C	Leu67	1/110 (0.9 %)	10/392 (2.6 %)[c]	known
3		c.219 C>A	Ile73	8/110 (7.3 %)	42/584 (7.2 %)[c, d]	known
	3	c.IVS3-12C>T	none	2/116 (1.7 %)	1/94 (1.1 %)	novel
	3	**c.IVS3-17C>T**	**none**	**1/116 (0.8 %)**	**0/94 (0 %)**	**novel**
	3	**c.IVS3-26C>T**	**none**	**2/116 (1.7 %)**	**0/94 (0 %)**	**novel**
	3	**c.IVS3-32C>T**	**none**	**1/116 (0.8 %)**	**0/94 (0 %)**	**novel**
	4	c.IVS4-24 C>T	none	15/110 (13.6 %)	94/486 (19.3%)[c, d]	known
5		**c.624G>A**	**silent**	**1/116 (0.8 %)**	**0/96 (0 %)**	**novel**
7		**c.720G>C**	**silent**	**1/116 (0.8 %)**	**0/98 (0 %)**	**novel**
9		c.1023 C>T	Pro341	4/110 (3.6 %)	6/584 (1.0 %)[c, d]	known
10		c.1410 A>G	Thr470	48/110 (43.6 %)	111/400 (27.8 %)[c, d]	known
10		c.1557 C>T	Ser519	20/110 (18.2 %)	72/298 (24.2 %)[c]	known
10		c.1608 T>C	Thr536	31/110 (28.2 %)	103/294 (35.0 %)[c]	known

[a]The “A” of start codon ATG is numbered +1 and ATG coding for start-methionine is numbered residue +1. Refseq NM_004183.1.

[b]Marquardt et al., 1998; [c]Allikmets et al., 1999; [d]Krämer et al., 2000.

Five out of the six novel silent variants are unlikely to be disease-causative, as they occur together with likely disease-associated mutations (Ala243Val/c.IVS3-12C>T, Gly26Arg/c.IVS3-17C>T, Ala243Val/c.IVS3-26C>T, Thr241Asn/c.720G>C) or lie outside the consensus acceptor splice sequence (c.IVS3-32C>T). In the case of the c.624G>A mutation no other sequence variation was identified in the patient (ID# 011378, not listed in Table 1) who displays typical features of BMD and, most importantly, reveals a positive family history of the disease. Therefore, a putative pathogenic effect of c.624G>A was analyzed in more detail. We established a functional assay to evaluate the c.624G>A sequence change on mRNA splicing. Heterologous expression of pSPL3b constructs containing wild-type and mutant genomic *VMD2* sequences result in RT-PCR products of either 496 bp (corresponding to normally spliced transcripts) or 263 bp (corresponding to an unspliced transcript) (Fig. 2). Although no marked quantitative differences were observed between wild-type and mutant RT-PCR products for the 496 bp fragment (primer pair SD6/SA2), the mutant RT-PCR products reveal an additional faint fragment of 341 bp in size (primer pair SD6/SA2). Direct sequencing of the 341 bp product demonstrates skipping of exon five in the mutant construct (Fig. 2). This finding suggests the c.624G>A mutation to exert a weak but defined effect on exon definition. Analysis of a possible effect of the c.624G>A sequence change on exon splicing enhancer (ESE) elements (Hastings and Krainer, 2001; Berget, 1996) demonstrates that c.624G>A lies within the putative binding site for SC35 (Cartegni et al., 2003). While the wild-type 8 bp sequence motif for SC35 (5’-TGCTCCAG) results

in a score value of 3.148, the value of the mutant SC35 element (5'-TGCTCCAA) was markedly reduced (2.698) although still above the threshold score of 2.383. At present it remains unclear whether these *in silico* findings are related to functional consequences *in vivo*.

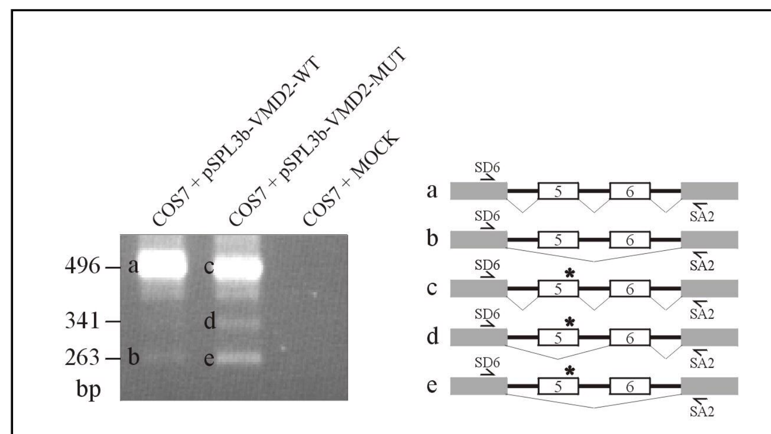

Figure 2. Evaluation of sequence variant c.624G>A on splicing. The splicing behaviour of the wild-type (pSPL3b-VMD2-WT) and mutant (pSPL3b-VMD2-MUT) constructs were evaluated by RT-PCR resulting in products of 496 and 263 bp in size in both wild-type and mutant. In addition, an aberrant fragment of 341 bp is observed in the mutant RT-PCR product which is shown to be the result of skipping of exon five due to the c.624G>A sequence change.

CONCLUSION

Our data expand the spectrum of distinct disease-causing *VMD2* mutations from 83 to 93. Nine out of ten novel mutations reported herein are located within known hotspot regions of *VMD2* further underscoring a functional/structural significance of these regions for the biological activity of bestrophin. We also describe a first nonsense mutation (c.779delC) likely leading to haploinsufficieny of bestrophin. One nucleotide change identified in familial BMD (c.624G>A) emphasizes the difficulty in establishing an effect of a sequence variant on protein properties without testing this variant in appropriate functional assays (e.g. as developped by Sun et al., 2002).

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