**BEST1 sequence variants in Italian patients with vitelliform macular dystrophy**

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**Purpose:** To analyze the spectrum of sequence variants in the *BEST1* gene in a group of Italian patients affected by Best vitelliform macular dystrophy (VMD).

**Methods:** Thirty Italian patients with a diagnosis of VMD and 20 clinically healthy relatives were recruited. They belonged to 19 Italian families predominantly originating from central Italy. They received a standard ophthalmologic examination, OCT scan, and electrophysiological tests (ERG and EOG). Fluorescein and ICG angiographies and fundus autofluorescence imaging were performed in selected cases. DNA samples were analyzed for sequence variants of the *BEST1* gene by direct sequencing techniques.

**Results:** Nine missense variants and one deletion were found in the affected patients; each patient carried one mutation. Five variants [c.73C>T (p.Arg25Trp), c.652C>T (p.Arg218Cys), c.652C>G (p.Arg218Gly), c.728C>T (p.Ala243Val), c.893T>C (p.Phe298Ser)] have already been described in literature while another five variants [c.217A>C (p.Ile73Leu), c.239T>G (p.Phe80Cys), c.883_885del (p.Ile295del), c.907G>A (p.Asp303Asn), c.911A>G (p.Asp304Gly)] had not previously been reported. Affected patients, sometimes even from the same family, occasionally showed variable phenotypes. One heterozygous variant was also found in five clinically healthy relatives with normal fundus, visual acuity and ERG but with abnormal EOG.

**Conclusions:** Ten variants in the *BEST1* gene were detected in a group of individuals with clinically apparent VMD, and in some clinically normal individuals with an abnormal EOG. The high prevalence of novel variants and the frequent report of a specific variant (p.Arg25Trp) that has rarely been described in other ethnic groups suggests a distribution of *BEST1* variants peculiar to Italian VMD patients.

Best vitelliform macular dystrophy (OMIM # 153700; VMD) is a macular disease, which generally appears in childhood with a yellowish yolk-like lesion in the macula [1-3]. The first pedigree was described by Best in 1905 [4].

The disease is associated with the accumulation of lipofuscin at the level of the retinal pigment epithelium (RPE); over time the central yellow lesions progressively disintegrate and macular atrophy or fibrosis often develops [1,2]. The clinical picture evolves over many years producing a gradual decline of visual acuity; according to Gass [1] the macular lesions progress through various well defined stages: vitelliform, pseudohypopyon, vitelliruptive (scrambled egg), atrophic and cicatricial. Some patients develop choroidal neovascularization [1], which can be treated with photodynamic therapy [5,6] or intravitreal antiangiogenic drugs [7,8]. In the large majority of families the electro-oculogram (EOG) is markedly abnormal in all stages of progression and in phenotypically normal carriers [9], even if several studies report some patients with normal EOG [10-12]. Full-field ERG is usually normal [10,11] while mfERG often presents reduced amplitudes [13]. Fundus autofluorescence (FAF) imaging shows an increased autofluorescence of the vitelliform deposits [14] while OCT allows the visualization of the vitelliform substance and the associated alterations of the RPE and of the photoreceptors [15-17].

VMD is inherited as an autosomal dominant trait, with incomplete penetrance and highly variable clinical expression. It has been associated with alterations in the gene *BEST1* (OMIM # 607854; previously known as *VMD2*), mapped to the long arm (q13) of chromosome 11, encoding the 585-amino acid transmembrane protein bestrophin-1 [18,19]. This protein localizes to the basolateral membrane of RPE cells [20] and functions as a chloride channel [21,22] but it may act as an inhibitor of intracellular voltage-dependent Ca2+ channels, too. It has also been involved in pH and cell volume regulation and a possible role as a HCO3− channel has been proposed too [23]. Bestrophin-1 dysfunction results in abnormal fluid and ion transport by the RPE, determining a weakened interface
between RPE and photoreceptors; this may affect retinoid transport and photoreceptors outer segment phagocytosis by the RPE, finally leading to increased lipofuscin deposition [24].

In addition to VMD, BEST1 variants have been associated with several other eye diseases [22] including adult-onset vitelliform macular dystrophy [25], autosomal recessive bestrophinopathy [26-28], autosomal dominant vitreoretino-choroidopathy [29,30], retinitis pigmentosa [31], microcornea, retinal dystrophy, cataract, and posterior staphyloma (MRCS syndrome) [32]. To date more than 150 BEST1 variants have been identified in VMD [22] (mutation-database; Retina international, databases accessed January 2012); the large majority of them are missense variants [22,33,34] resulting in amino acid changes in the N-terminal part of the protein.

BEST1 variants have been investigated in several different ethnic groups and in isolated Italian families [6,12,35] but at present there is no specific study on BEST1 variants in Italian VMD patients. Recently the results were published [36] of a BEST1 molecular analysis performed on a group of 23 patients, 10 of whom were from three Italian families. In the present study sequence variants of the BEST1 gene were determined in a group of Italian patients affected by VMD; these 30 patients were taken from a large sample of 19 Italian families, predominantly originating from central Italy.

METHODS

Clinical evaluation: Nineteen Italian families, of which at least one family member was affected by VMD, were recruited through the Hereditary Retinal Degenerations Referring Center of the Eye Clinic of the University of Florence. Criteria for the Best phenotype included the following: 1) juvenile-to-adult onset of the disease; 2) bilateral macular dystrophy with the typical round lipofuscin lesions at the posterior pole (including patients with different stages of the disease); 3) normal ERG; 4) abnormal EOG with Arden ratio always below 1.50. We also studied some apparently healthy relatives of the patients who agreed to participate to identify possible asymptomatic carriers. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Local Ethics Committee. Moreover, each patient gave written informed consent.

All the subjects included in the study were clinically evaluated by means of a standard ophthalmologic examination, fundus photography, OCT scan (Topcon 3D OCT-1000, Topcon Medical Systems Inc., Oakland, NJ) and electrophysiological tests (EOG, ERG; Electrophysiological Diagnostic Unit Retimax, Roland Consult, Brandenburg, Germany) performed according to the existing ISCEV Guidelines [37,38]. In most of the cases electrophysiological examinations were performed in our Department but in two patients we accepted examinations performed during the previous year in other hospitals and included in the medical documentation of the patient. Fluorescein angiography (FA; Zeiss Retinograph with Image Processing Software Visupac, Carl Zeiss, Dublin, CA) was performed on eight patients: to improve the diagnosis (one case), to investigate the possible presence of choroidal neovascularization (CNV) in patients complaining of reduced visual acuity and/or metamorphopsia (six cases), and on one patient presenting retinal vein occlusion. We also took into consideration three fluorescein angiographies performed in other Hospitals. ICG imaging was performed on six patients for a more refined diagnosis of possible CNV. Fundus Autofluorescence Imaging (FAF; Confocal SLO, HRA Inc., Heidelberg Engineering, Heidelberg, Germany) was performed on all the affected patients who agreed to collaborate (18 patients).

DNA extraction and PCR amplification: Following informed consent and a complete medical history of each family, 10 ml of peripheral blood were obtained from the antecubital vein using EDTA-containing vials. DNA was extracted from 200 μl of peripheral blood using an automated method involving the BioRobot EZ1 workstation (QIAGEN GmbH, Germany).

The PCR amplification of 11 exons and flanking intronic regions of the BEST1 gene was performed using the Core System-Robotic Station (Beckman Coulter Inc., Miami, FL). PCR products were purified by the Biomek NX station (Beckman Coulter).

Mutational analysis: Standard cycle-sequencing reaction with BigDye terminator mix v1.1 (Applied Biosystems, Foster City, CA) contained 3–10 ng purified PCR products in 20 μl and were performed with forward and reverse primers used for initial amplification. The sequencing reactions were precipitated, dried and then sequenced on a sequencer 3730 DNA Analyzer. Finally, data obtained from the Sequence Analysis Software (Applied Biosystems) were aligned with the wild-type BEST1 gene sequence (GenBank Database). DNA samples of the probands were analyzed for mutations in all the 11 exons of the BEST1 gene by direct sequencing [28]; in the other members of the family, either clinically healthy or affected, BEST1 gene sequencing was limited to the exon in which the mutation was detected in the proband. A sequence mismatch was considered a potential disease-causing variant only if absent in 300 healthy controls, associated with amino acid change, and confirmed by a new independent PCR (EMQN Best Practice Guidelines).
The Alamut-1.5 software (Interactive Biosoftware, Rouen, France) was used to predict the impact of unclassified variants on the protein function; Alamut is a software suite dedicated to sequence variants interpretation and the prediction of pathogenicity, assembling the information provided by three scoring systems (PolyPhen, SIFT, Align GVGD) on the basis of several parameters such as biophysical characteristics of amino acids and their conservation across species (EuroGenTest) [39-41].

Figure 1. Different stages of VMD (Fundus photographs and OCT). A, B: Vitellifom disc (Patient R-II-1). C, D: Vitelliruptive stage (Patient C-II-1). E, F: Pseudohypopyon stage (Patient B-II-1). G, H: Macular atrophy (Patient Q-I-1). I, J: Macular fibrosis (Patient I-II-1). OD represents the right eye, OS represents the left eye.
RESULTS

Thirty Italian patients with a diagnosis of VMD (from 19 independent pedigrees) were clinically examined: 19 were male and 11 female. The mean age was 42.5 years (±19.1 years; range 11–82 years). The Snellen visual acuity ranged from 0.1 to 1.0, with an average value of 0.62 (±0.31). In 18/30 patients (60%) best corrected visual acuity (BCVA) was different (2 lines or greater) between the two eyes. In the eyes with the better visual acuity, BCVA was equal to or better than 0.5 in 12/13 (92.3%) of the patients younger than 40 years of age, and in 13/17 (76.4%) of the patients older than 40 years of age. In the eyes with the worse visual acuity, BCVA was equal to or better than 0.5 in 7/13 (53.8%) of the patients younger than 40 years of age and in 6/17 (35.2%) of the patients older than 40 years of age.

All the patients showed fundus lesions according to the typical stages as described by Gass (Figure 1) [1]: 11 eyes could be classified as vitelliform lesions, 8 eyes as pseudohypopyon stage, 16 as vitelliruptive stage, 13 as macular atrophy, and 11 as fibrotic macular scar. Stage classification was the same for both eyes in 18 patients and asymmetric in 12 patients. One patient showed a vitelliform lesion in one eye while the macula of the fellow eye presented only mild RPE dystrophy. Clinical data of our series are summarized in Table 1 and Table 2.

EOG was always abnormal with Arden ratio lower than 1.50 while ERG was always within normal limits.

### Table 1. Clinical Details of the VMD Patients Included in the Study (Families A-G).

| Family | Family member | Sex | Age (years) | Onset (years) | BCVA OD | BCVA OS | Fundus OD | Fundus OS | Notes |
|--------|---------------|-----|-------------|---------------|---------|---------|-----------|-----------|-------|
| A      | III-3         | F   | 11          | 6             | 0.8     | 1.0     | VL        | PV        | Unil  |
| A      | II-5          | M   | 45          | //            | 1.0     | 1.0     | Norm      | Norm      |       |
| A      | II-4          | F   | 42          | //            | 1.0     | 1.0     | Norm      | Norm      |       |
| A      | III-4         | M   | 9           | //            | 1.0     | 1.0     | Norm      | Norm      |       |
| A      | II-3          | F   | 42          | //            | 0.6     | 1.0     | Norm      | Norm      | Ambl OD|
| A      | III-1         | M   | 14          | //            | 1.0     | 1.0     | Norm      | Norm      |       |
| A      | III-2         | F   | 12          | //            | 1.0     | 1.0     | Norm      | Norm      |       |
| A      | I-2           | F   | 78          | //            | 0.5     | 1.0     | GC        | GC        | Glau OU|
| B      | I-1           | M   | 16          | 10            | 0.7     | 0.9     | PH        | PH        |       |
| B      | I-2           | F   | 40          | //            | 1.0     | 1.0     | Norm      | Norm      |       |
| C      | II-1          | M   | 18          | 8             | 0.8     | 0.8     | VR        | VR        |       |
| C      | I-1           | M   | 54          | //            | 1.0     | 1.0     | Norm      | Norm      |       |
| C      | I-2           | F   | 50          | //            | 1.0     | 1.0     | Norm      | Norm      |       |
| D      | II-1          | M   | 22          | 14            | 0.3     | 1.0     | FI        | VL        | CNV; P |
| D      | I-1           | M   | 55          | //            | 1.0     | 1.0     | Norm      | Norm      |       |
| D      | I-2           | F   | 57          | 35            | 0.4     | 0.3     | AT        | AT        |       |
| E      | I-1           | M   | 62          | 25            | 0.2     | 0.2     | AT        | AT        |       |
| E      | II-1          | M   | 42          | //            | 1.0     | 1.0     | Norm      | Norm      |       |
| F      | II-1          | F   | 46          | 10            | 0.2     | 0.2     | AT        | AT        |       |
| G      | I-2           | M   | 60          | 29            | 0.3     | 0.1     | FI        | FI        |       |
| G      | II-1          | M   | 27          | 8             | 1.0     | 1.0     | VL        | VL        |       |
| G      | II-2          | F   | 25          | 7             | 1.0     | 1.0     | VL        | VL        |       |
| G      | I-3           | M   | 68          | 35            | 0.8     | 0.9     | AT        | VL        | Mult OS|
| G      | II-5          | F   | 28          | //            | 1.0     | 1.0     | Norm      | Norm      |       |

For each patient the stage of disease is indicated. (BCVA: Best Corrected Visual Acuity; VL: vitelliform lesion; PV: pre-vitelliform lesion; GC: glaucomatous cupping; PH: pseudohypopyon stage; VR: vitelliruptive stage; AT: atrophic stage; FI: fibrotic stage; CNV: previous choroidal neovascularization; P: treated with photodynamic therapy; Unil: unilateral presentation of the disease; Mult: multifocal presentation of the disease; Ambi: Ambiopia; Glau: Glaucoma; OD: right eye; OS: left eye; //: unknown; OU: both eyes).
Electrophysiological results are summarized in Table 3 and Table 4.

OCT showed the accumulation of hyperreflective material in different stages of disorganization between the neuroretina and the RPE, and allowed the visualization of fibrotic or atrophic macular alterations. None of the patients showed any sign of new vessels on fundus examination and OCT scans, or on FA and ICG when performed. In previous years five patients had been diagnosed with CNV; four patients showed sudden visual loss and macular hemorrhage while another patient presented a more intriguing clinical picture with sudden visual loss, very mild ophthalmoscopic changes and subtle FA and ICG leakage. One patient (who developed CNV in 2001) was not treated, three patients received photodynamic therapy (PDT), and one patient was treated with PDT and intravitreal bevacizumab; the three patients treated with PDT showed first a stabilization of the clinical picture and then a slow improvement of visual acuity.

All the 20 asymptomatic relatives showed a normal fundus appearance, normal visual acuity, and ERG response. Ten subjects showed a normal EOG response while five subjects showed an abnormal EOG with reduced Arden ratio. Five subjects were not available for electrophysiological testing.

Ten different BEST1 sequence variants were identified in the 30 VMD patients; each family was found to have a specific BEST1 variant that segregated with the disease. Five of these variants [c.73C>T (p.Arg25Trp), c.652C>T (p.Arg218Cys),...
c.652C>G (p.Arg218Gly), c.728C>T (p.Ala243Val), c.893T>C (p.Phe298Ser) have already been described in literature (mutation-database; Retina international, databases accessed January 2012), while five variants [c.217A>C (p.Ile73Leu), c.239T>G (p.Phe80Cys), c.883_885del (p.Ile295del), c.907G>A (p.Asp303Asn), c.911A>G (p.Asp304Gly)] have not previously been reported, and were not detected in 150 unaffected control individuals (300 chromosomes) of Italian origin. The variant p.Ile295del has already been reported [11,18] but the variant detected in our series shows a slight difference to the sequence change already described in literature; in fact in our patient the deleted codon is ATC while in the previous reports it is TCA.

Nine variants were missense, and only one was a deletion. All the patients carried a single variant on one allele. One heterozygous variant was also identified in all of the five clinically healthy relatives with normal fundus, visual acuity and ERG, but with abnormal EOG; they were members of three different families and were heterozygous for one of two variants already previously described: p.Arg218Gly was detected in two members of the same family, while p.Arg25Trp was found in three subjects from two different families. Another four clinically unaffected subjects carried one BEST1 variant but were not available for EOG examination.

All the other family members who showed a completely normal phenotype presented a wild-type genotype on both BEST1 alleles. The pedigrees of the families included in the study are shown in Figure 2. The BEST1 sequence variants detected in all the subjects who were available for genetic testing are summarized in Table 3 and Table 4.

p.Arg218Cys and p.Arg25Trp were the most common allelic variants among our VMD patients of Italian origin. p.Arg218Cys was found in 5/19 families (26.3%) and in 12/30 (40%) patients; p.Arg25Trp was found in 7/19 families (36.8%) and in 10/30 patients (33.3%). Table 5 shows the
theoretical consequences calculated by the Alamut software (Interactive Biosoftware, database accessed January 2012) of four of the five novel variants; note that its scoring systems provide a predictive evaluation only for missense variants.

### DISCUSSION

We screened a group of 30 Italian VMD patients and some of their relatives for BEST1 sequence variants. In the affected patients BCVA was variable (ranging from 0.1 to 1.0) showing a significant difference between the two eyes in the majority of cases, and was more severely reduced in older subjects. These results are consistent with those of a previous study investigating visual impairment in VMD [42].

We reported one BEST1 variant in all the VMD patients. This high detection rate is in agreement with other studies performed on different ethnic groups [22,36,43-45] and confirms the strong association between VMD phenotype and BEST1 sequence variants.

Most of the variants (9/10) were missense and only one was a deletion (p.Ile295del). The high incidence of BEST1 missense variants in VMD is consistent with the mutation spectrum of the gene databases (mutation-database; Retina international). These findings have already been reported in previous studies [22,33,34,44,46] raising the hypothesis of a possible dominant negative effect of the abnormal protein to determine the VMD phenotype. As in other ethnic groups, the majority (8/10) of the identified variants are clustered in four regions frequently affected by variants and probably associated with a relevant functional role [33,34]. Four out of ten variants were located in the protein region between 289 and 310 aa, recently reported to harbor a significant number of sequence variants [47].
In our series of Italian patients, mainly originating from central Italy, five of the 10 reported variants have never been previously described. All the five variants occurred in moderately to highly conserved regions of the protein. Four are missense and one (Ile295del) is a deletion.

Three of the missense variants (p.Phe80Cys, p.Asp303Asn, p.Asp304Gly) lead to an amino acid group change with different physicochemical properties and are labeled as affecting protein function by the SIFT algorithm of the Alamut software. p.Asp303Asn and p.Asp304Gly lie very close to one another in a region where other variants
have already been reported [18,35], suggesting a significant pathogenic impact for the alterations located in this region of the protein.

The physiopathological effect is more questionable for the other missense variant (p.Ile73Leu) because it is associated with a change between amino acids that are chemically

| cNomen | Exon | pNomen     | Amino acid conservation | AGVGD class | SIFT (score)       | POLYPHEN (score) |
|--------|------|------------|-------------------------|-------------|--------------------|------------------|
| c.217A>C | 3    | p.Ile73Leu | Moderately              | C0          | Tolerated (0.93)   | Benign (1.274)   |
| c.239T>G | 3    | p.Phe80Cys | Highly                  | C65         | Affected protein function (0.00) | Benign (2.771)   |
| c.907G>A | 8    | p.Asp303Asn| Highly                  | C15         | Affected protein function (0.00) | Possibly damaging (1.960) |
| c.911A>G | 8    | p.Asp304Gly| Highly                  | C65         | Affected protein function (0.00) | Probably damaging (2.530) |

Predictions of the scoring methods (Align GVGD, SIFT, Polyphen) are shown.

Figure 3. Fundus photographs, OCT and EOG of Patient M-II-1 carrying the novel sequence variant p.Ile295del. A, B: Vitelliform lesion with partial re-absorption of the vitelliform material. C, D: Macular detachment of the neurosensory retina partially occupied by hyperreflective material. E, F: Reduced Arden Test. OD represents the right eye, OS represents the left eye.
similar, and it is interpreted as a benign or tolerated substitution by the software; moreover, p.Ile73Leu is located outside the four regions frequently affected by mutations. However, its association with a typical VMD phenotype (Patient F-II-1) suggests a pathogenic role.

The p.Ile295del deletion found in our series is the deletion of codon ATC, different from codon TCA whose deletion has already been reported [11,18]. However both nucleotide variants lead to the same variation of the protein, with the elimination of an isoleucine and an alteration of the protein sequence that is likely to impair its function. This variant does not lead to a premature stop codon in the downstream sequence of the protein, in agreement with the in vitro study showing a dominant negative effect of p.Ile295del on Cl\(^{-}\) channel function [48]. In a previous paper [11] the variant p.Ile295del with the deletion of codon TCA was associated with reduced penetrance and normal EOG in the early stages of the disease; in our series the patient (patient M-II-1)
families (31%) and in 11/30 patients (36%); it has already been reported [22,36], and may be due to the influence of environmental factors or unknown modifier genes [22]; genetic results must be interpreted with caution especially when providing genetic counseling. We found one of the two variants p.Arg25Trp and p.Arg218Gly in all of the five asymptomatic carriers with EOG abnormalities without overt clinical alterations; however, our series is too small to establish a different penetrance of the various genotypes. In conclusion, we identified five novel and five previously reported BEST1 sequence variants in a series of 30 VMD patients from 19 independent Italian families; to our knowledge this is the largest study in the literature investigating BEST1 variants in an Italian population. The high number of novel variants and the high prevalence in our patients of a variant that is uncommon in other groups of different origin (p.Arg25Trp) suggest a difference in the spectrum of BEST1 sequence variants between Italian VMD patients and other ethnic groups. A better knowledge of the spectrum of BEST1 sequence variants in specific populations may help to improve molecular diagnostic approaches and select patients for future therapeutic options.

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