Detailed phenotypic and genotypic characterization of bietti crystalline dystrophy

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Detailed Phenotypic and Genotypic Characterization of Bietti Crystalline Dystrophy

Short title: Phenotypic and Genetic Findings in Bietti Dystrophy

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Abstract

Objective: To provide a detailed phenotype/genotype characterization of Bietti Crystalline Dystrophy (BCD).

Design: Observational case series.

Participants: Twenty patients from 17 families recruited from a multi-ethnic British population.

Methods: Patients underwent color fundus photography, near infra-red (NIR) imaging, fundus autofluorescence (AF) imaging, spectral domain-optical coherence tomography (SD-OCT) and electroretinogram (ERG) assessment. The gene CYP4V2 was sequenced.

Main Outcome Measures: Clinical, imaging, electrophysiological and molecular genetics findings.

Results: Patients ranged in age from 19 to 72 years (median 40), with visual acuity of 6/5 to perception of light (median 6/12). There was wide intra- and inter-familial variability in clinical severity. Fundus AF imaging showed well defined areas of retinal pigment epithelium (RPE) loss which corresponded on SD-OCT to well demarcated areas of outer retinal atrophy. Retinal crystals were not evident on fundus AF imaging, and were best visualized with NIR imaging. SD-OCT showed them to be principally located on or in the RPE/Bruch’s membrane complex. Disappearance of the crystals, revealed by serial recording, was associated with severe disruption and thinning of the RPE/Bruch’s membrane complex. Cases with extensive RPE degeneration (N=5) had ERGs consistent with generalized rod and cone dysfunction, but those with more focal RPE atrophy either showed amplitude reduction without delay (N=3), consistent with restricted loss of function, or were normal (N=2). Likely disease-causing variants were identified in 34 chromosomes from 17 families. Seven were novel including p.Met66Arg, found in all 11 patients from 8 families of South Asian descent. This mutation appears to be associated with earlier onset (median age 30) compared to other substitutions (median age 41). Deletions of exon 7 were associated with more severe disease.

Conclusions: The phenotype is highly variable. Several novel variants are reported, including a highly prevalent substitution in patients of South Asian descent associated with earlier onset disease. AF showed sharply demarcated areas of RPE loss which coincided with abrupt edges of outer retinal atrophy on SD-OCT; crystals were generally situated on or in the RPE/Bruch’s complex but could disappear over time with associated RPE disruption. These results support a role for the RPE in disease pathogenesis.
Introduction

Bietti crystalline corneoretinal dystrophy (BCD, Online Mendelian Inheritance in Man (OMIM) 210370) is a rare autosomal recessive disease that is characterized by yellow-white crystalline retinal deposits, progressive atrophy of the retinal pigment epithelium (RPE) and loss of choriocapillaris. In some patients, but not all, this is accompanied by crystalline deposits at the corneal limbus. BCD typically presents between the second and fourth decade with progressive night blindness, reduced vision and visual field constriction, often with legal blindness in the fifth or sixth decades of life. In 2000 Jiao et al mapped BCD to chromosome 4q35.1 and subsequently Li and colleagues identified disease-causing variants in CYP4V2, a novel member of the cytochrome P450 gene family (family 4, subfamily V, polypeptide 2).

The majority of phenotypic data are derived from a small number of case reports and case series, with the largest series to date describing 21 patients. The main characteristics and natural history of the disease are reasonably well characterized, but there remain areas of uncertainty, including the precise intraretinal location of the crystals and primary pathology, the degree of variability and its association, if any, with the underlying mutations, the pattern of cell loss and its retinal distribution. There is limited information on the time course of crystal formation within the retina and few studies have described the retinal characteristics using newer imaging modalities such as near infra-red (NIR) imaging and spectral domain optical coherence tomography (SD-OCT).

This report describes the detailed clinical features and molecular pathology in a cohort of 20 patients from 17 families affected by BCD from a British multi-ethnic population, and examines the relationship between disease-causing variants and disease severity.

Methods

Twenty patients with a clinical diagnosis of BCD were ascertained from Moorfields Eye Hospital, London, United Kingdom over a 13-year period from 1999 to 2012. Patients were recruited from one of three ophthalmologists’ clinics (ARW, ATM, MM) and included in the study only if all clinical data were consistent with the diagnosis. This was later confirmed with CYP4V2 sequencing. Patients provided informed consent as part of a research project approved by the local research ethics committee, and all investigations were...
conducted in accordance with the principles of the Declaration of Helsinki. One patient (case 19) has been described previously.  

Clinical studies:

All patients had their clinical history documented and underwent eye examinations which included best-corrected visual acuity, slit lamp biomicroscopy, dilated fundus examination and digital fundus photography (TRC-501A; Topcon, Tokyo, Japan). The severity of fundus appearance was graded according to the scale proposed by Yuzawa et al.\(^\text{10}\) and simplified by Weleber and Wilson\(^\text{11}\) which describes 3 stages of increasing severity – stage 1: RPE atrophy only; stage 2: RPE and choroidal atrophy localized to the macular region; stage 3: RPE and choroidal atrophy extending beyond the macula. Severity grading was performed by one investigator (GL) and independently checked by another (AGR). Disagreements were adjudicated through discussion and the consensus grading was used. Imaging studies were addressed in a similar fashion.

Imaging:

The Spectralis HRA + OCT with viewing module version 5.1.2.0 (Heidelberg Engineering, Heidelberg, Germany) was used to acquire autofluorescence (AF) images over 30x30° and 55x55° fields as well as SD-OCT images. Fundus autofluorescence imaging (FAF) was available in 17 patients; SD-OCT in fifteen patients; 11 patients had longitudinal data available. The SD-OCT protocol included a dense horizontal linear scan centered on the fovea and the HEYEX software interface (version 1.6.2.0; Heidelberg Engineering) was used to correlate position markers on NIR and SD-OCT scanning to localize lesions. The same program also allows registration of blood vessels from the NIR images to facilitate longitudinal analysis of crystal evolution. The location of crystals was correlated across color photographs, NIR, AF and SD-OCT imaging using side-by-side comparisons, and where available, HEYEX position markers. Observations had to be replicated in at least two patients before they were accepted as valid and described in this study.

Electrophysiology:

Eleven patients underwent electrophysiological assessment using full field electroretinography (ERG) and pattern ERG. All protocols incorporated the recommendations of the International Society for Clinical Electrophysiology of Vision (ISCEV) standards.\(^\text{12-14}\)
Blood samples were collected and DNA was extracted using the Puregene blood extraction kit (Invitrogen, Paisley, UK) following manufacturer’s instructions. Each of the 11 exons of CYP4V2 was amplified from patient genomic DNA using primers located in the flanking intron and untranslated regions. The primers and conditions were as described previously\(^3\) and allowed each exon, including splice junctions to be amplified.

The products were purified using Qiagen polymerase chain reaction (PCR) purification columns (Qiagen, UK) and sequenced using the fluorescently-labeled dideoxy-terminator method on an ABI 3100 Automated DNA Sequencer (Applied Biosystems, UK). Electropherograms were analyzed for sequence changes using DNASTar computational software (DNASTar, Inc., USA). Sequencing data obtained from PCR products were analyzed using SeqMan, a program designed to detect potential alterations in the sequence. Any sequence changes identified were checked visually. When family samples were available the segregation of potentially disease-causing variants was investigated. Missense mutations were analyzed using three software prediction programs:

- SIFT (Sorting Intolerant from Tolerance) (J. Craig Venter Institute; Available at: http://sift.jcvi.org/. Accessed August 1, 2013.),
- PolyPhen2 (Available at: http://genetics.bwh.harvard.edu/pph/index.html. Accessed August 1, 2013) and
- pMUT (University of Barcelona Molecular Recognition & Bioinformatics Group; Available at: http://mmb.pcb.ub.es/PMut/. August 1, 2013) splice mutations were analysed using
- NetGene2 (Technical University of Denmark Center for Biological Sequence Analysis; Available at: http://www.cbs.dtu.dk/services/NetGene2/ Accessed October 15, 2013).

Fluorescence in situ hybridization (FISH)

Chromosome slides for FISH analysis were obtained from peripheral blood lymphocyte cultures following standard cytogenetic procedures. Prior to the hybridization the slides were denatured in 70% formamide at 70°C for 2 minutes, quenched in 2xSSC at 4°C and then dehydrated in an ethanol series. The FITC-labeled BAC probe 173M11 (Empire Genomics) was co-hybridized to the slides with a TRITC-labeled chromosome 4 specific “paint” (Cambio). Hybridization and post-hybridization washes were carried out following the manufacturers’ instructions. The slides were mounted with Vectashield (Vector Laboratories) containing 4',
6-diamidino-2-phenylindole (DAPI) for chromosome counterstaining. Image capture and analysis were carried out on a CytoVysion system (Genetix) consisting of an Olympus BX-51 epifluorescence microscope coupled to a JAI CVM4+ CCD camera. At least 30 informative metaphases per sample were captured and analyzed.

**Comparative genomic hybridization (CGH)**

Array CGH was used to evaluate DNA copy number differences on chromosome 4 (performed by NimbleGen Systems Inc). Test samples (patients 3 and 7) were labeled with Cy-3 and the reference sample was labeled with Cy-5. The data were visualized using SignalMap software (NimbleGen). The array was designed, and labeling, hybridization and normalization were performed at NimbleGen. Subsequent analysis was performed in-house.

**Long range PCR**

Potential breakpoints for a CNV of interest 5’ of CYP4V2 were identified by visual inspection of array CGH data. Primers were designed approximately 2kb 5’ and 500bp 3’ of the maximal predicted CNV region, with the following sequences: CYP4V2 F2 5’-CAAGGACTCATCCTGATCAC-3’ and CYP4V2 R1 5’-AGCCTAGTGAGTTGTCACA-3’. Long range PCR was performed using the Bio-X-Act Long kit (Bioline) following manufacturer’s protocols, with >10ng template DNA, 1.5mM Mg²⁺ and cycling conditions of 95°C 2 minutes, 60°C 1 minute, followed by 35 cycles of 95°C 30 seconds, 60°C 30 seconds and 68°C 20 minutes, with a final extension step of 68°C for 20 minutes.

**Quantitative PCR (qPCR)**

Nine pairs of primers were designed at regular intervals across the CYP4V2 region, from approximately 25kb 5’ of the gene to approximately 15kb 3’, using Primer3 (http://frodo.wi.mit.edu/. Accessed March 13, 2012), sequences and PCR conditions are given in Table 1 (available at http://aaojournal.org). Quantitative PCR (qPCR) was performed using QuantiFast (Qiagen) following manufacturer’s protocols. Reactions were performed on a Step One Plus real time PCR system (Applied Biosystems) in 25µl total volume, using 10ng diluted DNA as template and with final concentrations of 1µM of each primer. PCR efficiencies were determined using serial dilutions of a control DNA sample. Data was analyzed in Microsoft Excel using the
Comparative Ct Method (ΔΔCt), normalizing against the 5’ most primer pair, and a pair located in TMEM107 (chr17:8,076,297-8,079,714).

Statistical analyses

Kaplan Meier survival analysis was used to compare the age of onset of symptoms for different mutations. Median survival time to onset of symptoms was derived from the Kaplan Meier product limit estimators. Visual acuity was converted from Snellen acuity into LogMAR units (Logarithm of the Minimum Angle of Resolution) and analyzed as the algebraic mean of two eyes, the better seeing eye, and the worse seeing eye. LogMAR was grouped into 4 categories as <0.3, 0.3≤LogMAR<0.5, 0.5≤LogMAR<1 and ≥1 and analyzed as an ordinal variable. Severity of disease and electrophysiological abnormalities (no, mild and severe generalized retinal dysfunction) were also analyzed as ordinal variables using Fisher’s exact test (univariate analyses) and ordinal logistic regression (adjusting for age and gender). Additional patient data was extracted from a publication by Lai et al. and used for pooled analyses. SAS version 9.2 (SAS Institute, Cary NC) was used for analyses.

Results

Clinical presentation

The clinical phenotypes are summarized in Table 2 (available at http://aaojournal.org). There was marked intra- and inter-familial variability. The 20 patients, 10 male and 10 female, ranged in age from 19 to 72 years (median 40 years). Visual acuity varied from 6/5 to perception of light (median 0.3 logMAR, 6/12). The onset of symptoms varied from 22 -45 years, with most patients (n=15, 75%) presenting in the third or fourth decade of life with reduced central vision (n=12) and/or nyctalopia (n=6); 2 patients were asymptomatic at the time of presentation and detected through presentation for unrelated conditions (e.g. corneal foreign body). Visual acuity was highly variable and was not age-related (Table 2, available at http://aaojournal.org). For example, patient 13 had 6/6 vision in both eyes aged 40 years, whereas patient 6 had only perception of light vision in both eyes at a similar age. Five patients (25%) had moderate myopia (-3 to -5 dioptres) while the others were emmetropic or had low myopia. Family GC19455 showed a pseudodominant mode of inheritance with an affected father and two affected sons from a consanguineous family. All other patients were simplex cases, with 1 pair of affected dizygotic twins
patients 1 and 2, GC5048), and an affected sibling of patient 8 (not included in this study as not a patient of this center). A sample of pedigrees is provided in Figure 1 (available at http://aaojournal.org).

On clinical examination all patients had retinal crystals at the posterior pole with varying degrees of RPE and inner choroidal atrophy. Four patients from different families (20%) had crystalline deposits at the corneal limbus, and all had either stage 2 or 3 disease. Patients 1 and 2 (GC5048) were dizygotic twin sisters from a non-consanguineous pedigree with a somewhat discordant phenotype; patient 1 had an earlier onset of disease (age 29), vision of 6/60 in both eyes, stage 3 disease and undetectable PERGs in keeping with severe macular dysfunction and ERG evidence of generalized rod and cone photoreceptor dysfunction. Her sibling had onset of symptoms 4 years later (age 33), vision of 6/12 in both eyes, a similar appearance of sparse retinal crystals and widespread atrophy (stage 3) but with less severe macular and generalized retinal dysfunction than her sister (Table 2, available at http://aaojournal.org). In GC19455 both the older son (proband, patient 13) and father (patient 12) had stage 1 disease localized to the macula, while the younger son (patient 14) had stage 3 disease with a much greater extent of involvement despite being the youngest affected in the pedigree. However, in general, older patients had fewer crystals and more severe retinal atrophy.

Retinal Imaging

Color, AF and SD-OCT scanning of patients with different presentations is shown in Figure 2. Areas of RPE and choroidal atrophy tend to develop at the posterior pole, become confluent and expand centrifugally to involve peripheral retina. Areas of normal looking retina anterior to the macula showed normal AF (Figure 2B), while involved areas showed abnormal AF which progressed to become sharply demarcated areas of hypo-AF (Figures 2E, 2K, 2N). These sharply demarcated areas correspond to relatively abrupt loss of outer retinal layers including the outer plexiform and outer nuclear layers on SD-OCT (Figure 2F, 2L).

Crystals seen on color photographs were not visualized on FAF but were highly reflective on NIR imaging (Figures 3A-C; 3E-G). SD-OCT scans revealed hyperreflective spots located in or on the RPE/Bruch’s membrane complex (Figure 3D, 3H up arrows) corresponding to these crystals on color and NIR images. The majority of crystals in all patients were located in or on the RPE/Bruch’s membrane complex. However, not all hyperreflective lesions seen on SD-OCT spatially associated with crystalline deposits, as shown in
Figure 3H (down arrow), where the hyperreflective spot in the inner plexiform layer did not correspond to a crystal on either color or NIR images. Outer retinal tubulations were observed in all patients, except patient 19, who was also the youngest patient (19 years) in the case series. There were no hyperreflective spots suggestive of crystals in the choroid in any patient.

The appearance of a new crystal in the RPE/Bruch’s membrane layer of patient 11 in 2012 which was not present in corresponding scans in 2010 is shown in Figure 4. The overlying external limiting membrane remained intact above the new crystal. Crystal disappearance was associated with thinning of the RPE/Bruch’s membrane layer, loss of the ellipsoid and external limiting membrane layers, and the formation of what is likely to be an early retinal tubulation.

Electrophysiology

Electrophysiology was performed on 11 patients and revealed a range of abnormalities (Table 2 and Figure 5, both available at http://aaojournal.org). The ERGs in five of 5 patients with extensive RPE/choroidal atrophy (patients 1, 2, 6, 11 and 17) showed evidence of generalized rod and cone photoreceptor dysfunction whereas 3 patients with atrophic lesions confined to the maculae (patients 3, 5 and 10) showed restricted loss of cone and/or rod system function (ERG amplitude reduction without delay). Two further patients with focal macular atrophy had normal full-field ERGs (patients 7 and 13). One patient without atrophy had normal ERGs (patient 19).

The pattern ERG P50 component, reflecting macular function, was subnormal bilaterally in 9 patients (1-3, 6, 7, 10, 11, 13 & 17); unilaterally in 1 (patient 5). Six of 10 patients showed significant P50 component delay bilaterally (patients 2, 3, 5, 6, 10 and 17). There was no clear correlation between the electrophysiological abnormality and age although the youngest patient with macular crystals without atrophy had normal pattern and full-field ERGs (patient 19; age 15 years).

Molecular analysis

Twenty patients from 17 families were screened. Likely disease-causing variants were found in all 34 chromosomes from the 17 families and comprised 11 distinct variants in CYP4V2 (Table 2, available at http://aaojournal.org, and Table 3). The majority (7/11) of the variants were missense mutations. Interestingly, the most common mutation in our cohort was a novel variant c.197T>G (p.Met66Arg) which
was seen in 16/34 chromosomes (47%). This variant was identified in a homozygous state in all 11 patients in 8 families of South Asian ancestry (Table 2, available at http://aaojournal.org), but not in other patients of European, East Asian or Middle Eastern ancestry. Seven further missense mutations were also identified, 4 (6/34 chromosomes, 18%) were novel and 3 have been previously described (4/34 chromosomes, 12%).

The next most common mutation detected in our cohort was an indel spanning a splice acceptor site, previously shown to cause the in-frame deletion of exon 7 and this occurred in 6/34 (18%) chromosomes. One instance each of two potential novel splice site mutations and one nonsense mutation completed the spectrum of mutations. The mutations published to date, including the novel variants reported in this study, are represented schematically in Figure 6 (available at http://aaojournal.org) and suggests that there is no significant clustering of the variants, which have been found throughout the entire coding sequence of the gene.

In silico analysis of these missense variants using three methods, SIFT, Polyphen 2 and pMUT, showed that they are all predicted to be disease-causing by all three methods with the exception of p.Gly26Asp and p.Met66Arg. The p.Gly26Asp change is only reported by Polyphen 2 to be probably damaging, whereas SIFT and pMUT predict it is a tolerated change (Table 3). This substitution was only seen in one patient who also had a putative splice-site mutation, c.985+3A>G in intron 7. Analysis of this potential splicing mutation using NetGene2 showed that the donor splice site at the 3’ end of exon 7 is abolished when the A at position +3 is changed to a G. This would result in an mRNA that does not splice to exon 8 but adds on 10 amino acids before a stop codon truncates it. This patient (patient 5) had relatively mild disease given her age (stage 2, mild full field ERG abnormalities, 55 years) but otherwise had typical BCD with corneal and retinal crystals (Table 2, available at http://aaojournal.org). The p.Met66Arg substitution which is reported for the first time here is predicted by Polyphen 2 to be benign but SIFT and pMUT both predict it to be a pathological change with good reliability in pMUT. The other predicted splice mutation, c.327+11G>C needs further analysis.

Fifteen of the 17 families harbored homozygous mutations. Multiple single nucleotide polymorphisms (SNPs) were also detected (Table 4). The most common mutation (16/34 chromosomes) in the present cohort was the novel missense mutation p.Met66Arg. This was identified in the homozygous state in all 8
families of South Asian descent. Analysis of 11 SNPs across CYP4V2 (shown in Table 3 but excluding rs35200327) showed that there is an associated haplotype (G-C-C-G-A-C-G-G-C-A-G). This haplotype also occurs in a homozygous state in these patients suggesting a founder effect.

Examining copy number variation (CNV) of CYP4V2

The finding that the majority of our patients had homozygous mutations and were not reported as being from consanguineous families raised the possibility that there may be a common deletion in this region of chromosome 4, making the individuals appear homozygous, when they are really hemizygous for the reported variant. To test this hypothesis, patient 4, a Turkish woman diagnosed in her early 20’s, was initially examined. Metaphase spreads were prepared from peripheral blood lymphocytes and a fluorescently labeled probe from a PAC clone (173M11) containing CYP4V2 and a chromosome 4 paint were used to examine the region. This fluorescent in situ hybridization (FISH) showed that patient 4 has two copies of the gene (Figure 7a, available at http://aaojournal.org). Subsequently samples were obtained from the family (GC17557) and analysis showed each parent had one wild type CYP4V2 and one mutated copy (c.677T>A, p.Met226Lys), and that her 2 siblings harbored two wild type copies (Figure 7b, available at http://aaojournal.org). Patient 8 was used as a control as he was a compound heterozygote and FISH confirmed he also had 2 copies of the gene as expected (data not shown). Analysis of this family (GC4795) demonstrated that his father was a carrier of a missense mutation (c.1503G>A, p.Arg400His) and his mother a carrier of the splice mutation c.802-8_810del17insGC (Figure 1, available at http://aaojournal.org).

In order to determine whether other members of the cohort exhibiting apparent homozygosity across the gene might instead have a CNV, DNA was sent for array comparative genomic hybridization (aCGH) analysis. DNA from patients 3 and 7 (European and of South Asian descent respectively) were analyzed using a custom array designed to cover this region of chromosome 4. Probes were 20bp apart for the region of interest on chromosome 4 and 250bp apart for the rest of chromosome 4. Nimblegen analysis indicated a deletion in patient 7 and a duplication in patient 3, spanning ~4.8kb in the region chr4:187,093,500-187,098,300 (Hg19), approximately 15kb 5’ of CYP4V2 (Figure 8, available at http://aaojournal.org). This is a region of known copy number variability (Database of Genomic Variants; http://projects.tcag.ca/variation/).
Accessed March 12, 2012, and is mainly non-genic, with the exception of ~300bp of the 3’ untranslated region on FAM149A, the gene immediately flanking CYP4V2 upstream. To further explore the role of CNVs in CYP4V2, qPCR analysis of the region spanning chr4:187,088,471-187,149,502 (from ~25kb 5’ of CYP4V2 to ~15kb 3’) was performed on three patients (patients 3, 5, and 7). The placement of the primers was designed to detect CNVs within CYP4V2, rather than in the potential 5’ CNV indicated by the Nimblegen data. The results provided no convincing evidence of CNVs in CYP4V2 in any of these three patients (data not shown). These data support the assertion that these probands were homozygotes rather than hemizygotes for mutations in CYP4V2.

Genotype-phenotype correlations

The patients displayed wide phenotypic variability; the oldest patient (patient 12, 72 years, homozygous p.Met66Arg) had one of the mildest phenotypes (stage 1), while one of the younger patients (patient 6, 39 years, heterozygous deletion exon 7) had one of most severe phenotypes (stage 3).

Mutations were grouped into 3 categories, those homozygous for p.Met66Arg (n=11), homozygous or heterozygous for other substitutions (n=5) and homozygous or heterozygous for deletion of exon 7 (n=4) and tested for associations with clinical features (Table 5, available at http://aaojournal.org). Survival curves for age to first onset of symptoms suggested those with the p.Met66Arg mutation or deletions had an earlier onset of symptoms (median age of onset 30 years for both groups) compared to those with other substitutions (median age 41 years), although these differences did not reach statistical significance (p log rank=0.17, Table 5, available at http://aaojournal.org, Figure 9A). Combining the present data with published results from Lai et al.15 (12 patients heterozygous or homozygous for deletions, 6 heterozygous or homozygous for other substitutions) strengthened the impression of earlier onset of symptoms for p.Met66Arg homozygotes (Figure 9B), and the log rank test reached statistical significance (p=0.03). Mean visual acuity was worse for patients with deletions (6/60) compared to those with p.Met66Arg (6/18) or other substitutions (6/15) in the present cohort but these differences did not reach statistical significance. Including patients from Lai et al.15 and adjusting for age and gender reinforced these impressions but the differences remained non-significant (p>0.05, data not shown). The analyses were repeated for visual acuity in the better, and in the worse seeing eye, with similar results. All (4/4, 100%) patients with deletion...
of exon 7 had stage 3 disease compared with 5/11 (45%) for those with p.Met66Arg and 3/5 (60%) for those with other substitutions. Similarly, all (3/3, 100%) with deletions had severe generalized full field ERG abnormalities compared with 2/5 (40%) patients with p.Met66Arg and none with other substitutions. These differences did not reach statistical significance both before and after adjustment for age and gender.

Discussion

To date, most patients with BCD that have been reported are of East Asian descent where the prevalence of BCD seems to be higher. The present study of British multi-ethnic patients shows significant variability in both phenotypic presentation and severity of disease consistent with prior reports in Asian cohorts and suggests other gene and environmental interactions.

Few previous studies to date have utilized FAF imaging in BCD. The present study found that areas of uninvolved peripheral retina on color images correspond to areas of normal FAF, while areas of involved retina start out as areas of abnormal FAF and progress to become sharply demarcated areas of hypo-AF. These hypo-AF areas represent areas of RPE cell loss and their well defined edges correspond to areas of abrupt outer retinal atrophy on SD-OCT. Similar SD-OCT appearances occur in other diseases with a postulated primary RPE pathology such as gyrate atrophy and choroideremia. The lack of AF associated with the crystals themselves is consistent with suggestions that the crystals in BCD may represent collections of cholesterol esters from abnormal lipid metabolism, although the precise composition of the crystals remains unclear.

There has been controversy as to the location within the retina of the crystalline deposits in BCD. Some report hyperreflective spots on the RPE/Bruch’s membrane complex on SD-OCT, while others have reported similar looking hyperreflective spots throughout the neurosensory retina and choroid which has been interpreted as crystal deposition occurring throughout the retina and choroid. Using SD-OCT with co-registration of lesions to NIR images, the vast majority of crystals in the present series were either on or in the RPE/Bruch’s membrane complex with a small number of crystals located elsewhere in the retina. There were none in the choroid. Many hyperreflective spots on SD-OCT did not correspond to crystals or any other visible abnormality on either NIR or color images; these may be related to clusters of inflammatory cells, a gliotic response to retinal degeneration protein deposits or simply artefacts. In
patient 19 it is clear that the crystals are an early manifestation of the disorder, and need not be
accompanied by altered retinal function.\(^5\)

The evolution of crystals documented using serial SD-OCT imaging showed that in the early stages crystals
appear in the RPE/Bruch’s membrane complex with preservation of the overlying external limiting
membrane. Atrophy and thinning of the RPE/Bruch’s membrane complex was associated with
disappearance of the crystals, as well as loss of the photoreceptor ellipsoid and external limiting membrane
layers and formation of outer retinal tubulations. These findings are consistent with the clinical
observations that despite the presence of numerous crystals there can be relatively preserved visual acuity
(cases 10 and 17) or normal macular function (patient 19), while the loss of crystals over time is associated
with expanding RPE and inner choroidal atrophy and loss of macular function (patients 1, 3, 7). It can be
speculated that crystals may be a visible phenotype of metabolic dysfunction in RPE or photoreceptor cells
and their disappearance associated with cell death. It has not been possible to determine if crystals are
intra- or extracellular deposits, although histopathological studies report the presence of crystalline
intracellular inclusions in extraretinal lymphocytes and fibroblasts in patients with BCD.\(^28, 29\) Tubulations,
believed to be photoreceptor rosettes, have previously been reported in BCD, as well as multiple other
conditions, including age-related macular degeneration, pseudoxanthoma elasticum and gyrate atrophy\(^30, 31\)
and suggests that the site of initial dysfunction is likely to be the RPE, with photoreceptor degeneration a
secondary consequence.

Delayed and subnormal ERGs consistent with generalized rod and cone dysfunction were present in all
cases with extensive RPE and choroidal atrophy (Table 2, available at http://aaojournal.org). Mildly reduced
rod- and/or cone-mediated ERGs without delay suggest restricted loss of retinal function and occurred in
those with focal areas of macular atrophy. Other patients showed no full-field ERG abnormality. Previous
studies report full-field ERGs ranging from normal to undetectable, with most having generalized rod and
cone system involvement.\(^5, 15, 28, 32-34\) Serial ERG data are not available for the present cohort, but
progressive ERG worsening has been documented by others.\(^32, 33\) Multifocal ERG reduction and delay have
also been reported in keeping with macular dysfunction\(^5, 15\) and it is of interest that a high proportion of the
present series showed significant PERG P50 delay, with or without amplitude reduction; in general, delays
in P50 occur less frequently in genetically determined macular dysfunction than amplitude reduction, and rarely occur unaccompanied by amplitude reduction.

Eleven sequence variants were identified, 7 of which are novel and the majority of which are missense mutations (accounting for 74% of chromosomes in this cohort), in contrast to previous observations indicating the most common variant is the deletion c.802-8_810del17insGC.\(^{24,35}\) A review of the literature by Xiao et al. shows that this deletion accounts for 63% of the mutated alleles reported to date in patients of Chinese or Japanese origin.\(^{24}\) The most common mutation (16/34 alleles) in the present cohort was a novel missense mutation p.Met66Arg. This was identified in the homozygous state in all 8 families of South Asian descent, suggesting that exon 1 should be screened first in such patients; just as patients of East Asian descent should have exon 7 screened first to exclude the indel c.802-8_810del17insGC. Interestingly, this novel substitution appeared to be associated with an earlier onset of symptoms. This finding should be interpreted with caution as it may be related to biases such as recall bias (i.e. the two sons in family GC19455 with an affected parent may be more aware of symptoms and hence report earlier onset) and issues with pooling data from another centre in a different country and health system.\(^{15}\) Nonetheless, this substitution still appears to be related to earlier onset of symptoms even after excluding data from family GC19455 and Lai et al.\(^{15}\) The specific amino acid sequence and surrounding region is highly conserved in mammals but not in non-mammalian animals or invertebrates.

In terms of clinical severity, patients with deletions seemed to have more severe disease and worse visual acuity. The 17bp deletion (c.802-8_810del17insGC) includes the exon 7 splice acceptor site and so causes an in-frame deletion of exon 7 that would result in expression of a truncated 463 amino acid protein.\(^{3}\) This deletion has previously been reported to be associated with a more severe ERG phenotype.\(^{15}\) Since 2004 there have been 20 reports, including this one, documenting over 50 different mutations in CYP4V2.\(^{3,5,6,8,15,36-39}\) The missense and nonsense mutations reported to date are summarized in Figure 6, available at http://aaojournal.org. CYP4V2 is an 11 exon gene encoding a predicted protein of 525 amino acids, with mutations identified across the entire gene (Figure 6, available at http://aaojournal.org). A recent study by Nakano and colleagues reported p.H331P as the most common missense mutation, accounting for 7% of all mutated alleles, compared to the 63% of alleles with the c.802-8_810del17insGC
deletion.⁴⁰ They went on to demonstrate that the p.H331P mutant encodes an unstable protein. This is the only report showing that a change in CYP4V2 results in a non-functional protein. Further studies need to be undertaken to determine the effect of the other described missense and nonsense mutations on the function of CYP4V2.

A number of biochemical findings indicate systemic abnormalities of lipid metabolism in patients with BCD.⁴¹, ⁴² CYP4V2 is the most distinct of the human CYP4 family with only ~35% sequence identity to other family members.⁴³ The encoded protein is a microsomal omega (ω)-hydroxylase that functions together with mitochondrial and peroxisomal β-oxidation enzymes to degrade cellular lipids, with a preference for ω-3 polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA).⁴⁴ These PUFA are important constituents of photoreceptor outer segments that are recycled by RPE cells. In addition to being structural components, some PUFA derived metabolites e.g. resolvin and protectin possess anti-inflammatory and immunoregulatory signaling properties which may be disrupted by mutations in CYP4V2.⁴³ The protein is expressed in human RPE, retina, cornea and many other tissues including kidney, liver, lung and lymphocytes.³, ⁴⁰ Its phenotypic effects may be most prominent in the RPE where it appears to be the primary CYP4 protein expressed.⁴⁰ Together with the presented imaging data, such evidence further implicates the RPE as the cellular site of dysfunction in BCD. Given that the therapeutic window from onset of symptoms to severe vision loss is a decade or more, this offers the possibility of pharmacologic intervention to delay progression.

This study was not prospective and although all available data are presented for all patients, it is inevitable that patients seen earlier before the introduction of new imaging modalities did not have these performed. Six patients had complete imaging and ERG assessment. It is possible that this may have introduced a bias but we believe this is unlikely as the six patients with complete assessments are a representative sample that spans a large age range (15-40 years at time of ERGs) and the full spectrum of severity on both ERG and clinical severity.

In conclusion, this report expands and refines the phenotypic characteristics of Bietti Crystalline Dystrophy. FAF showed sharply demarcated areas of RPE loss which coincided with abrupt edges of outer retinal atrophy on SD-OCT. The crystals, which are best visualized using NIR imaging and which are not evident on
FAF imaging, are shown by SD-OCT to be located on or in the RPE/Bruch’s membrane complex; this combined with other data suggests that RPE dysfunction underlies disease pathogenesis. The mutation spectrum in this multi-ethnic British cohort, including novel mutations, differs to that previously described in other populations.
Acknowledgments

We also wish to thank Professor Alison Hardcastle, Institute of Ophthalmology for useful discussions on the array CGH data and Dr. Alex Morris, Imperial College for help with the haplotype analysis.
References


Table 3. *In silico* analysis of missense mutations in CYP4V2

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutation</th>
<th>SIFT</th>
<th>Polyphen 2</th>
<th>pMUT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prediction</td>
<td>Prediction</td>
<td>NN output</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tolerance</td>
<td>Score</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>c.77G&gt;A, p.Gly26Asp</td>
<td>Tolerated</td>
<td>PRD</td>
<td>0.3834</td>
</tr>
<tr>
<td>1</td>
<td>c.197T&gt;G p.Met66Arg</td>
<td>Intolerant</td>
<td>Benign</td>
<td>0.8042</td>
</tr>
<tr>
<td>2</td>
<td>c.283G&gt;A, p.Gly95Arg</td>
<td>Intolerant</td>
<td>PRD</td>
<td>0.8878</td>
</tr>
<tr>
<td>6</td>
<td>c.677T&gt;A, Met226Lys</td>
<td>Intolerant</td>
<td>PRD</td>
<td>0.6214</td>
</tr>
<tr>
<td>8</td>
<td>c.998C&gt;A, p.Thr333Lys</td>
<td>Intolerant</td>
<td>PRD</td>
<td>0.8480</td>
</tr>
<tr>
<td>9</td>
<td>c.1503G&gt;A, p.Arg400His</td>
<td>Intolerant</td>
<td>PRD</td>
<td>0.5805</td>
</tr>
<tr>
<td>10</td>
<td>c.1393A&gt;G, p.Arg465Gly</td>
<td>Intolerant</td>
<td>PRD</td>
<td>0.6607</td>
</tr>
</tbody>
</table>

SIFT\(^{15}\) results are reported to be tolerant if tolerance index ≥0.05 or intolerant if tolerance index <0.05.

Polyphen 2\(^{16}\) appraises mutations qualitatively as Benign, PossiblyDamaging (POS) or Probably damaging (PRD) based on the model's false positive rate.

pMUT\(^{17}\) is based on the use of different kinds of sequence information to label mutations, and neural networks to process this information. NN=neural network values from 0 to 1. >0.5 is predicted as a disease associated mutation. Reliability=values 0–9. >5 is the best prediction.
Table 4. SNPs identified in CYP4V2

<table>
<thead>
<tr>
<th>rs number</th>
<th>Exon/intron</th>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>dbSNP MAF (minor allele frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1055138</td>
<td>Exon 1</td>
<td>c.64C&gt;G</td>
<td>p.Leu22Val</td>
<td>G=0.4390</td>
</tr>
<tr>
<td>rs10013653</td>
<td>Intron 1</td>
<td>c.215-22C→A</td>
<td></td>
<td>A=0.4222</td>
</tr>
<tr>
<td>rs7682918</td>
<td>Intron 2</td>
<td>c.327+75C→T</td>
<td>T=0.3201</td>
<td></td>
</tr>
<tr>
<td>rs35200327</td>
<td>Intron 3</td>
<td>c.412_414delCT</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>rs4862662</td>
<td>Intron 4</td>
<td>c.605-25G→T</td>
<td>T=0.3512</td>
<td></td>
</tr>
<tr>
<td>rs13146272</td>
<td>Exon 6</td>
<td>c.775C&gt;A</td>
<td>p.Gln259Lys</td>
<td>C=0.4547</td>
</tr>
<tr>
<td>rs3817184</td>
<td>Intron 6</td>
<td>c.802-7C→T</td>
<td>0.424</td>
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<tr>
<td>rs34745240</td>
<td>Exon 7</td>
<td>c.823G&gt;A</td>
<td>p.Glu275Lys</td>
<td>A=0.0298</td>
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<tr>
<td>rs3736455</td>
<td>Exon 7</td>
<td>c.810T&gt;G</td>
<td>p.Ala270Ala</td>
<td>T=0.4492</td>
</tr>
<tr>
<td>rs3216724</td>
<td>Intron 8</td>
<td>c.1090+99delC</td>
<td>-=-0.326</td>
<td></td>
</tr>
<tr>
<td>rs2276918</td>
<td>Intron 9</td>
<td>IVS9+45A→G</td>
<td>G=0.3365</td>
<td></td>
</tr>
<tr>
<td>rs72646291</td>
<td>Exon 10</td>
<td>c.1328G&gt;A</td>
<td>p.Arg443Gln</td>
<td>A=0.0018</td>
</tr>
</tbody>
</table>
Figure 2
A. Survival curves of time to onset of symptoms by mutation, patients from this sample (n=19)

B. Survival curves of time to onset of symptoms by mutation, patients from this sample plus those of Lai et al (n=37)
Figure Legends

Figure 2. Color imaging, fundus autofluorescence (AF) and Spectral domain Optical Coherence Tomography (SD-OCT) imaging.

Color, AF and SD-OCT scanning of patients with different presentations, patient 13 panels A-C; patient 14 panels D-F; patient 15 panels G-I; patient 8 panels J-L and patient 11 panels M-O. Panels A-C from patient 13 show localized disease confined to the macula. Areas of normal looking retina on color images (A) show normal AF (B). Affected areas (B) show disrupted outer retinal layers on SD-OCT (C). Panels D-F from patient 14 (younger brother of patient 13) show more severe disease with more widespread abnormalities on AF (E). The sharp transition from abnormal AF to hypo-AF (E) corresponds on OCT (F) to a fairly abrupt transition from relatively intact retinal layers to loss of outer retinal layers including the outer plexiform and nuclear layers (up arrow). Panels G-O show patients with more severe, generalized disease and low AF suggesting widespread loss of retinal pigment epithelium (RPE). In patient 8, the central island of relatively preserved retina (J) on AF (K) corresponds to relatively preserved outer retinal layers on OCT (L) and highlights again the sharp demarcation (up arrows) between this preserved island and the surrounding hypo-AF retina (K) and atrophic outer retinal layers (L). Patient 11 (M) illustrates the usefulness of AF in demonstrating the clearly defined areas of RPE loss with a small island of preserved central retina (N), that is also visible on SD-OCT (O).

Figure 3. Color photographs of crystals and corresponding appearances on autofluorescence (AF), near infra-red and spectral domain optical coherence tomography (SD-OCT).

Patient 14 panels A-D; patient 19 panels E-H. Color photograph showing crystals (A, arrows, arrowheads, patient 14). Crystals are neither hyper nor hypo-AF (B) but show up clearly on near infra-red imaging (C). SD-OCT shows that the crystals are located in or on the retinal pigment epithelium/Bruch’s membrane layer (D, arrow). A similar appearance is shown for crystals in a different patient (E-H, up arrow, patient 19); however in that case a hyperreflective spot in the inner
plexiform layer on SD-OCT (H, down arrow) does not correspond to a crystal on either color photography, AF or near infra-red (E-G).

**Figure 4.** Longitudinal evolution of crystals over 2-3 years.

Comparative scans from Patient 11 are shown from 2010 and 2012 (A-H) and from patient 10 from 2009 and 2012 (I-L). Panels A-D show the appearance of a new crystal in the retinal pigment epithelium (RPE) /Bruch’s membrane layer in 2012 (C, D arrows), which was not present in 2010 (A, B patient 11). Panels E and F from the same patient in 2010 show a crystal in the RPE/Bruch’s membrane complex with a granular appearance to the overlying ellipsoid line and external limiting membrane. Between 2010 and 2012, this crystal disappeared on both near infra-red and Spectral domain Optical Coherence Tomography (SD-OCT) scanning, with disruption and loss of the RPE/Bruch’s membrane and overlying ellipsoid (photoreceptor) and external limiting membrane (G,H). Figures I-L compare images obtained in 2009 with those obtained in 2012 for patient 10. In panel I there is a crystal at the margin of an atrophic zone, which on SD-OCT imaging (J) has a present ellipsoid line and to a lesser extent, external limiting membrane line. Over 3 years, the area of atrophy expanded (K) with disappearance of the crystal on near infra-red and appearance of a new crystal (black arrow, K). The disappearance of the crystal corresponds to thinning of the RPE/Bruch’s membrane layer, loss of ellipsoid and external limiting membrane lines, and the formation of possible early tubulation (L).

**Figure 9.** Kaplan Meier survival curves for survival time to first onset of symptoms by mutation type.

Panel A includes patients from this study; Panel B also incorporates data from Lai et al. Patients with p.Met66Arg mutations had earlier onset of symptoms than those with other substitutions (p logrank=0.03). Patient 12 was excluded from analyses due to uncertain age of onset of symptoms.
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