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EFEMP1 is not associated with sporadic early onset drusen

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Abstract The early onset of multiple drusen in the posterior pole of the retina is characteristic of a group of macular dystrophies often referred to as dominant or radial drusen. At least two forms, Doyne honeycomb retinal dystrophy (DHRD) and Malattia Leventinese (MLVT), are associated with a single missense mutation (R345W) in the gene encoding the EGF-containing fibulin-like extracellular matrix protein-1 (EFEMP1) and are now thought to represent a single entity. Here, we present a further evaluation of the role of EFEMP1 in the pathogenesis of sporadic forms of early onset drusen. We analyzed all coding exons of the EFEMP1 gene by SSCP analysis in 14 unrelated individuals with early onset of multiple drusen and no apparent family history of the disease. In this patient group, we did not detect the R345W mutation or any other disease-associated mutation. Three different polymorphisms and two intragenic polymorphic repeats were present in similar frequencies in the patients and control individuals. We conclude that EFEMP1 is unlikely to be involved in the disease in this patient group. This suggests that mutations in a different as yet unknown gene or genes may lead to the early onset drusen phenotype.

Key words Early onset drusen; EFEMP1; macular degeneration; chromosome 2p16

Introduction Drusen represent extracellular deposits between the retinal pigment epithelium (RPE) and Bruch's membrane and are a feature of a variety of human chorioretinal diseases. The familial forms

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of drusen are typically characterized by the symmetrical development of numerous macular and/or peripapillary drusen, early onset, and an autosomal dominant pattern of inheritance. The drusen generally appear in the second to fourth decade of life but often remain asymptomatic, only being diagnosed after geographic atrophy, choroidal neovascularization, or exudative detachment lead to a noticeable loss of vision, thus making it difficult to establish a family history.[1] Although the significant variability in the size, shape, appearance, and distribution of the drusen has led to the use of an assortment of designations including Hutchinson-Tay choroiditis, Holthouse-Batten chorioretinitis, Doyne honeycomb retinal dystrophy, Malattia Leventinese, crystalline retinal degeneration, guttate choroiditis, and dominant or radial drusen, it has been proposed that all of these conditions may share a common underlying pathogenesis.[2–4] An autosomal dominant inheritance pattern has been definitively demonstrated for Doyne honeycomb retinal dystrophy (DHRD) and Malattia Leventinese (MLVT), two disorders which have historically been distinguished by a honeycomb-like pattern of macular drusen in the former and small, fine, radially oriented drusen in the latter.[4] The genetic linkage analysis mapping of both disorders to overlapping loci on chromosome 2p16 provided the first evidence that they may constitute a single nosologic entity.[5,6] Recently, a single missense mutation, Arg345Trp (R345W), in the EGF-containing fibulin-like extracellular matrix protein-1 (EFEMP1) gene was identified in affected individuals from DHRD and MLVT families.[7] The mutation was consistently found on a common haplotype, implying that all affected individuals descended from a common ancestor.

To further assess the role of EFEMP1 in the pathogenesis of early onset drusen, we identified a cohort of unrelated patients with the same clinical phenotype as that observed in patients with dominant drusen. We selected sporadic cases with no clear family history of the disease in an attempt to investigate individuals not descending from the same common ancestor as the DHRD/MLVT families studied by Stone et al.,[7] thereby increasing the likelihood of finding novel EFEMP1 mutations. We used single-stranded conformational polymorphism (SSCP) analysis and direct DNA sequencing to analyze the entire coding region of the EFEMP1 gene in the affected individuals and present results that strongly suggest that EFEMP1 is not associated with the disease in this patient group.

Patients and methods

PATIENTS We studied 14 individuals with an early onset of multiple drusen from Canada and Germany. Diagnostic evaluation included the assessment of family history of eye disease, measurement of visual acuity, fundus examination, and, in most cases, fluorescein angiography and electroretinography (Figure 1). We attempted to exclude patients with age-related macular degeneration (AMD) from this study by limiting the study group to patients with an early onset of symmetrical disease, centered on the macula and/or nasal to the optic disc. The mean age at evaluation was 46 years (range: 27–67 years) and a wide pheno-

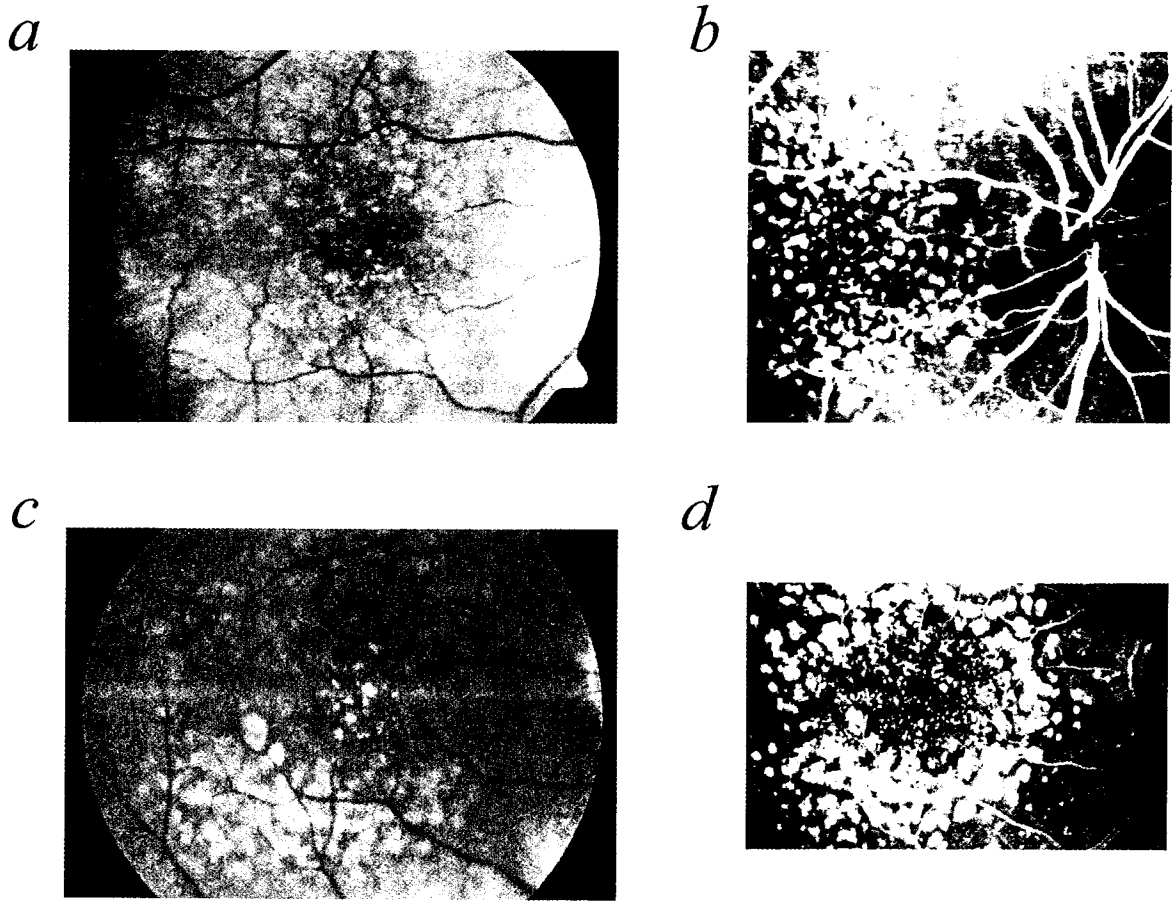

Fig. 1. Fundus photographs showing multiple drusen and some hyperpigmentation in (a) patient #22 at age 57 and (c) patient #9 at age 44. Fluorescein angiogram demonstrating the extent of multiple drusen at the posterior pole in (b) patient #22 and (d) patient #9.

typic spectrum was represented, ranging from drusen only to marked late-stage RPE damage accompanied by significant loss of visual acuity (Table 1). None of the individuals had a family history of confirmed early onset drusen. In two families (those of patients #4 and #10), the possible presence of drusen in other first-degree relatives was raised; however, formal evaluations were not available. A population-based control group of 70 individuals was also studied. As a positive control for the R345W mutation, DNA was obtained from individuals with Malattia Leventinese belonging to a large Leventine valley pedigree.[8] This investigation was performed according to the guidelines of the Declaration of Helsinki.

SINGLE-STRANDED CONFORMATION POLYMORPHISM (SSCP) ANALYSIS

Genomic DNA of the 14 patients and 70 control individuals was isolated from peripheral blood using standard techniques. For each individual, exons 3 to 12, representing the entire coding region of the EFEMP1 gene[9,10] (GenBank Accession Nos. AY004321-AY004330) (Figure 2), were PCR-amplified using previously published oligonucleotide primers.[7] Amplification was carried out with *Taq*-Polymerase (Gibco BRL) in a 25-µl volume with a 1 × PCR buffer supplied by the manufacturer. PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 5 min. The PCR products were electrophoretically separated in a 6% nonde-

ID#	*Age at evaluation*	*Visual acuity (OD/OS)*	*Clinical findings*	*Family history*
4	42	1.0/1.0	Minor visual field defects; small >100 nodular perifoveal drusen; no electrophysiology	Normal fundus in parents
7	27	1.0/1.0	On routine check-up: multiple drusen with pigment epithelial defects symmetrically in both eyes; no metamorphopsia; no electrophysiology	Negative
8	33	1.0/1.0	Visual field defects; hard drusen temporal to the fovea, strongly autofluorescent with fluorescein multiple pigment epithelium defects; no electrophysiology	Negative
9	44	0.7/0.8	On routine check-up: multiple, partly confluent, nonexudative drusen covering the entire posterior pole; no electrophysiology	Negative
10	37	1.0/0.2	Multiple drusen at the posterior pole; ERG[a] slightly reduced	Atrophic AMD in mother
12	54	0.25/0.7	Multiple distinct drusen at the posterior pole, hyperfluorescent on angiography; pigment epithelial defects; central and paracentral responses on multifocal ERG reduced (OD > OS)	3 sibs with normal fundus
13	55	0.1/0.1	Multiple drusen, hyperfluorescent on angiography with pigment epithelial defects but no leakage; central scotomas; central amplitude loss on multifocal ERG	Negative
14	44	1.0/1.0	'Foggy' vision; multiple drusen; small pigment epithelial defect (OD): no electrophysiology	Negative
15	48	0.1/0.6	Several drusen temporal of the fovea, hyperfluorescent on angiography; disciform scar (OD); occult neovascular membrane (OS)	Negative
16	67	0.02/0.8	Numerous drusen, hyperfluorescent on angiography; peripheral pigment epithelial defects and clumping; central scar (OD); central scotoma (OS)	Negative
18	38	1.0/1.0	Metamorphopsia (OS > OD); multiple drusen at the posterior pole showing early hyperfluorescence on angiography; no electrophysiology	2 sibs with normal fundus
19	55	0.8/0.1	Metamorphopsia in both eyes; multiple subconfluent drusen extending to the periphery and hyperfluorescent on angiography; disciform scar (OS); no electrophysiology	Negative
20	46	0.4/1.2	Confluent macular RPE atrophy, pericentral and peripapillary honeycomb-like RPE atrophy with multiple drusen; central scotoma, partly relative, partly absolute	Negative
22	57	0.8/0.6	On angiography: multiple hyperfluorescent soft drusen at the posterior pole; some hard drusen; drusen also nasally and in the midperiphery; ERG slightly abnormal; pigment epithelial abnormalities in the fovea	Negative

[a]ERG refers to Ganzfeld ERG if not specified otherwise.

TABLE 1. Clinical findings in affected individuals.

naturing polyacrylamide gel with 5% glycerol at 4°C. DNA and fragments displaying mobility shifts were directly sequenced using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham, Life Science).

Results and discussion Each of the 10 coding exons of EFEMP1 in addition to flanking acceptor and donor splice sites and the 3′-untranslated region (UTR) were studied by SSCP analysis in the 14 individuals with sporadic early onset drusen as well as in the 70 control individuals. The R345W mutation associated previously with DHRD and MLVT was present in one positive control (individual 7573), but

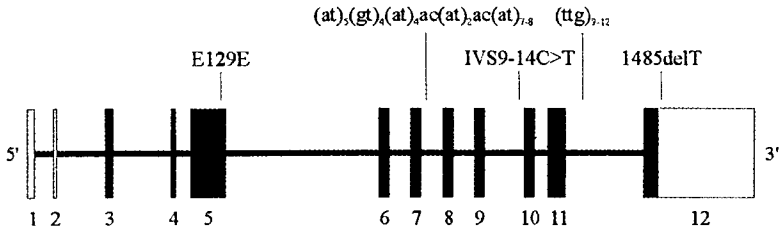

Fig. 2. The genomic organization of EFEMP1 and distribution of detected sequence alterations. The exon-intron organization of the gene is shown to scale.[10] The untranslated regions are indicated by gray boxes, the coding sequence by black boxes, and the intervening sequence by a black bar.

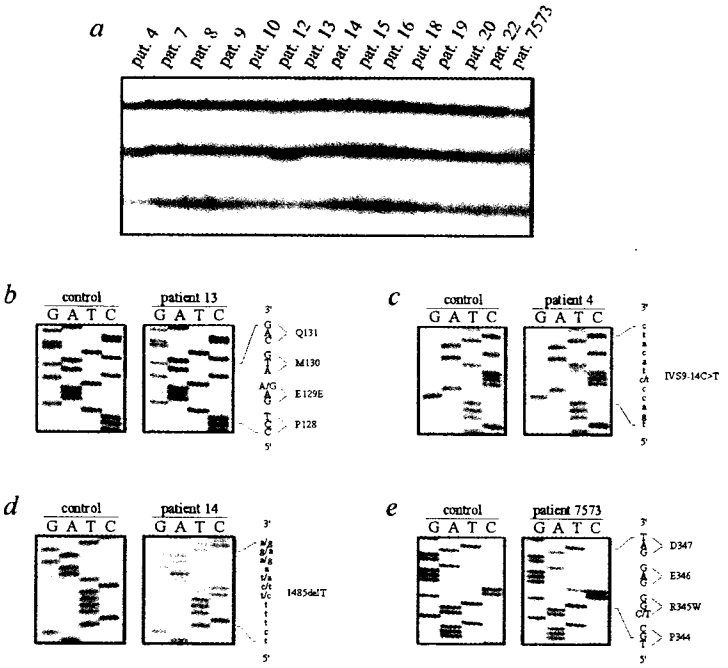

Fig. 3. Mutation analysis in the EFEMP1 gene. (a) Using SSCP analysis, the R345W mutation resulted in a distinct mobility shift in exon 10 for a positive control #7573, which was not present in any of the patients analyzed. Direct sequencing of aberrant mobility shifts revealed (b) an A-to-G transition at nucleotide position 387 in patient #13, (c) an IVS9-14C > T transition in patient #4, (d) a 1-basepair deletion in the 3′-UTR (cDNA position 1485) in patient #14, and (e) confirmed the R345W mutation in the positive control #7573.

not in any of the patients in this study (Figure 3a,e). No other disease-associated mutations were identified. Three different nucleotide alterations were detected in the EFEMP1 gene in both patients and control individuals and are likely to represent polymorphic changes (Table 2; Figure 2). These included an A-to-G transition at nucleotide position 387 (Figure 3b) causing no amino acid change (patients #13, 14), a C-to-T alteration in the intervening sequence 9 (patients #4, 12) (Figure 3c), and a single base-pair deletion in the 3′-UTR (patients #13, 14) (Figure 3d). Two intragenic polymorphic repeats, a complex repeat $(at)_5(gt)_4(at)_4ac(at)_2ac(at)_{7-8}$ in the intervening sequence 7, and a simple $(ttg)_{9-12}$ repeat in the intervening sequence 11 were identified (Figure 2). Comparison of the allele frequencies at these repeats in the patient and control groups revealed no significant differences (Table 3).

The findings in this group of patients with sporadic disease present a contrast to the observations made previously in DHRD and MLVT families where a single EFEMP1 missense mutation was present in all affected members of the 37 families studied.[7] One possible explanation for the lack of mutations in this cohort is that certain types of mutations are not detectable because of the inherent limitations of PCR-based analysis. Specifically, our methodology would not detect alterations in the promoter or other regulatory sequences, large inser-

TABLE 2. Sequence variants in EFEMP1.

Nucleotide change	*Location*	*Allele frequency in patients (n = 28)*	*Allele frequency in controls (n = 140)*
387A > G	exon 5	0.07	0.02
IVS9-14C > T	IVS 9	0.07	0.08
1485delT	3'UTR	0.06	0.02

TABLE 3. Intragenic polymorphic repeats in EFEMP1.

Allele	*Repetitive element*	*Allele frequency in patients (n = 28)*	*Allele frequency in controls (n = 140)*
1	$(at)_7$	0.96	1.00
2	$(at)_8$	0.04	0.00
1	$(ttg)_9$	0.04	0.00
2	$(ttg)_{10}$	0.93	0.96
3	$(ttg)_{11}$	0.00	0.01
4	$(ttg)_{12}$	0.04	0.02

tions or deletions, gene rearrangements, and intronic alterations outside the acceptor and donor splice site sequences. While this explanation could be invoked to explain a few cases, it appears unlikely that it is true for each of the 14 unrelated patients analyzed unless one assumes the presence of an undetected founder mutation in these sporadic cases. More likely, the absence of any detectable mutations suggests that the disease in this patient group is not associated with mutations in EFEMP1.

One important consideration is the differentiation between the phenotype of these patients and that of age-related macular degeneration (AMD), a complex multifactorial condition which, in industrialized countries, accounts for the majority of vision loss in persons over the age of 60.[11] Epidemiological studies have provided evidence for a genetic component in the pathogenesis of AMD,[12,13] but the major causative genes remain to be identified (reviewed in Yates & Moore[14]). Due to the similarities in clinical phenotype, dominant drusen has been considered as a possible genetic model for AMD; however, screening the entire coding sequence of EFEMP1 in 494 AMD patients yielded negative results.[7] Although drusen and loss of visual acuity as a result of geographic atrophy, choroidal neovascularization, or pigment epithelial detachment are features of both conditions, the onset of AMD is generally much later, the drusen are fewer in the early stages of the disease, and the pattern is less likely to be symmetrical. All subjects in this study showed bilateral symmetrical involvement. One half of our patients was under the age of 45 years at evaluation, at which time large numbers of drusen were observed (Table 1; Figure 1c,d). Patients #13, #16, #19, and #22, who were aged 55 years or older at evaluation, showed not only significant numbers of drusen (Figure 1a,b), but also severe retinal damage including extensive atrophy, pigment epithelial defects, and scarring (Table 1), a picture not typical for AMD in this age range.

Although the phenotype of our patients is consistent with that of dominant drusen, one major factor that characterizes this patient group and distinguishes it from the DHRD and MLVT families studied by Stone et al.[7] is the general absence of a positive family history of disease. The importance of family history as a diagnostic criterion has been demonstrated by molecular analyses in other macular dystrophies such as Best disease, in which the likelihood of detecting a mutation in the VMD2 gene is considerably lower when nonfamilial cases are included.[15,16] In Best disease, it thus appears that sporadic phenocopies may be produced by different underlying mechanisms. Possible mechanisms could include mutations in an additional gene (or genes) with a lower penetrance obscuring a family history of disease. Alternately, complex etiologies such as polygenic or multifactorial inheritance could be involved.

Historical difficulties in the classification of the diseases known as dominant drusen may be due in large part to a significant amount of clinical heterogeneity.[4,17,18] Our findings demonstrating the absence of EFEMP1 mutations in a group of patients with the early onset drusen phenotype provide an indication that there may also be some level of underlying genetic heterogeneity. Further investigation in larger numbers of familial cases will be required to determine if this genetic heterogeneity is limited to sporadic occurrences, as seems to be the case in Best disease, or is also reflected in families with demonstrated autosomal dominant inheritance.

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