Human Molecular Genetics, 2014, Vol. 23, No. 21 ***5827–5837***
doi:10.1093/hmg/ddu276
Advance Access published on June 4, 2014

Rare and common variants in extracellular matrix gene Fibrillin 2 (*FBN2*) are associated with macular degeneration

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Received November 11, 2013; Revised May 15, 2014; Accepted June 2, 2014

Neurodegenerative diseases affecting the macula constitute a major cause of incurable vision loss and exhibit considerable clinical and genetic heterogeneity, from early-onset monogenic disease to multifactorial late-onset age-related macular degeneration (AMD). As part of our continued efforts to define genetic causes of macular degeneration, we performed whole exome sequencing in four individuals of a two-generation family with autosomal dominant maculopathy and identified a rare variant p.Glu1144Lys in Fibrillin 2 (FBN2), a glycoprotein of the elastin-rich extracellular matrix (ECM). Sanger sequencing validated the segregation of this variant in the complete pedigree, including two additional affected and one unaffected individual. Sequencing of 192 maculopathy patients revealed additional rare variants, predicted to disrupt FBN2 function. We then undertook additional studies to explore the relationship of FBN2 to macular disease. We show that FBN2 localizes to Bruch′s membrane and its expression appears to be reduced in aging and AMD eyes, prompting us to examine its relationship with AMD. We detect suggestive association of a common FBN2 non-synonymous variant, rs154001 (p.Val965Ile) with AMD in 10 337 cases and 11 174 controls (OR = 1.10; *P*-value = 3.79×10^{-5}). Thus, it appears that rare and common variants in a single gene—*FBN2*—can contribute to Mendelian and complex forms of macular degeneration. Our studies provide genetic evidence for a key role of elastin microfibers and Bruch′s membrane in maintaining blood–retina homeostasis and establish the importance of studying orphan diseases for understanding more common clinical phenotypes.

INTRODUCTION

Macular degenerative diseases are a major cause of untreatable visual impairment and affect the central region of the retina (termed the macula) and the underlying retinal pigment epithelium (RPE). Mutations in genes including *ABCA4*, *TIMP3*, *ELOVL4*, *VMD2* and *EFEMP1* cause relatively rare and early-onset macular degeneration (MD), with diverse clinical manifestations (1,2). In contrast, age-related MD (AMD) represents a common multifactorial neurodegenerative disease, resulting from complex interplay of genetic susceptibility variants, environmental factors and aging-associated alterations in the retina, RPE and choroid (3–6). At least 19 AMD risk loci have been identified by genome-wide association studies (GWAS) using common variants (7,8) and subsequent meta-analysis of large case–control cohorts (9), implicating genes associated with specific cellular pathways, such as complement, high density lipoprotein cholesterol metabolism, extracellular matrix (ECM) and angiogenesis, in AMD pathology (10–12). Despite the genetic variability, inherited maculopathies and AMD have overlapping clinical presentations, pointing to related underlying molecular mechanisms. Identification and functional analysis of genes implicated in early-onset forms have provided important insights into AMD pathogenesis. For example, rare variants in *ABCA4* and *TIMP3* cause Stargardt disease (13) and Sorsby fundus dystrophy (14), respectively, whereas common variants at or near these two genes reportedly contribute to AMD (8,15).

Bruch′s membrane (BrM) is a heterogeneous, pentalaminar ECM structure consisting of collagen and elastin fibers that separates the RPE from choroidal capillaries. BrM represents an important physical barrier to transport of nutrients and waste products between blood circulation and retinal photoreceptors (16,17). Genetic studies of early onset MD have identified mutations in several ECM genes, including *TIMP3* (Sorsby's fundus dystrophy) (14), *EFEMP1* (Malattia leventinese, Doyne

honeycomb retinal dystrophy) (18), and *CTRP5* (late-onset retinal degeneration) (19,20). In model systems, loss of function of these genes leads to increased fundus autofluorescence, aberrant accumulations of deposits between RPE and BrM, and/or ECM abnormalities (21–24). BrM also undergoes significant age-related changes (6,25,26), and structural and molecular changes in BrM are implicated in AMD pathology (26,27). Accumulation of soft drusen on BrM is an early clinical manifestation of disease (28,29). Not surprisingly, GWAS of AMD cohorts has identified susceptibility loci including several ECM genes, such as *TIMP3* (8), *TGFBR1* (9), *COL8A1* (9) and *COL10A1* (9,30).

In this study, we initially focused on identifying the genetic cause of early-onset macular degeneration in a two-generation family by whole exome sequencing that resulted in the discovery of a rare variant in fibrillin 2 (*FBN2*). Fibrillins are ubiquitous glycoproteins that self-polymerize into filamentous microfibrils and are critical for ECM formation and remodeling (31,32). Mutations in *FBN2* and its homolog *FBN1* are associated with related disorders of connective tissues, congenital contractural arachnodactyly (CCA) (33) and Marfan syndrome (34), respectively. We demonstrate that FBN2 is expressed in BrM of fetal and young adult eyes and that its expression is reduced in aging and AMD. Furthermore, we present additional evidence of rare variants in maculopathy patients and association of a common variant in FBN2 with AMD. Taken together, our studies reveal that FBN2 is an attractive candidate to elucidate underlying mechanisms of BrM pathology in macular degeneration.

RESULTS

Clinical characteristics

As part of our ongoing studies, we identified a two-generation family (Fam-979) with five affected members presenting as an autosomal dominant form of early-onset macular dystrophy (Fig. 1A). Clinical features varied among affected members. The proband (II:1) reported a history of distorted vision in his left eye since the age of 46 years. When examined at age 52, his left fundus had pigmentary changes in the macula. The proband's father, I:1, was diagnosed with macular degeneration at age 69; when examined at the age of 75, he showed large areas of pigment epithelial atrophy in the macula of both eyes (Fig. 1Bi). Patient II:4 had a history of choroidal neovascularization in the right eye at age 35 (Fig. 1Bii). Patients II:3 and II:5 had

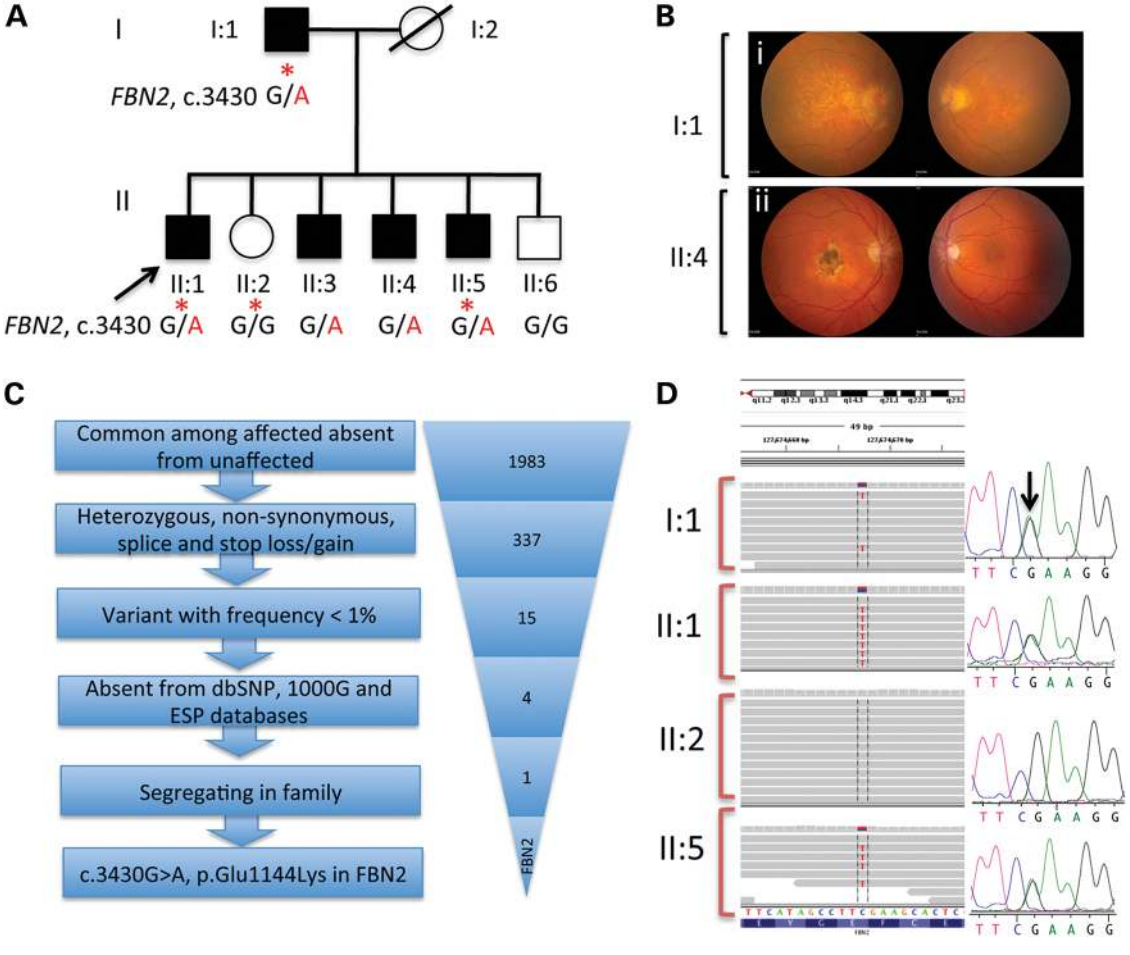

Figure 1. Identification of Glu1144Lys mutation in *FBN2* using whole exome sequencing. (**A**) Pedigree of a two-generation family, Fam-979 with early-onset macular degeneration. Samples used for exome sequencing are marked by red asterisks. Filled symbols represent members affected with early-onset macular dystrophy. (**B**) Fundus images of two affected members. (**C**) Step-wise filtration of variant to identify disease-causing mutation. (**D**) Sequencing reads showing the heterozygous variant, c.3430 G > A, p.Glu1144Lys in exon 26 of *FBN2* gene in IGV viewer and confirmation using Sanger sequencing.

clinical findings consistent with an atrophic macular disease in their 40s. There was no history of skeletal, joint or muscle abnormalities observed in these patients. Unaffected sister II:2 had her dilated exam at age 49, and no retinal abnormalities were observed. Unaffected brother, II:6 also reported no vision problems.

Exome sequencing reveals a rare variant in *FBN2* in Fam-979 family

Whole exome sequencing of three affected members (I:1, II:1 and II:5) and one unaffected member (II:2) from the Fam-979 maculopathy family identified a total of 1983 variants that were common among the affected individuals, but absent from the unaffected family member. After multiple filtering steps (Fig. 1C, see Materials and Methods for details), we identified c.3430 G > A (p.Glu1144Lys) variant in the *FBN2* gene (RefSeq accession number NM_001999) that was validated by Sanger sequencing and segregated in the complete pedigree, including two additional affected (II:3 and II:4) and one unaffected (II:6) individual (Fig. 1A and D). *FBN2* encodes for Fibrillin 2, an extracellular matrix (ECM) protein of microfibrils and elastin fibers. Given the role of ECM remodeling in AMD and some macular dystrophies (35,36), we further explored the relationship of FBN2 to macular degeneration.

FBN2 is expressed in the ECM component of the eye

Fibrillins are known to be an integral part of matrix assemblies, though their expression in the eye is not documented. The analysis of RNA-Seq data from human fetal tissues (18 weeks) revealed high levels of *FBN2* transcripts in RPE and choroid but not in the retina (Fig. 2A). In human fetal eyes, abundant FBN2 immunostaining was observed in BrM, choroid and sclera (Fig. 2Bi–ii). Similar FBN2 expression was obtained in the young adult monkey eye, although the labeling was less intense (Fig. 2Biii–iv). In contrast to human fetal eyes, FBN2 immunostaining was significantly reduced in the choroid and sclera, of aged human donors (over 75 years) (Fig. 2Bv–vi) and human donors with AMD (Fig. 2Bvii–viii) and was undetectable in the BrM. The bright green signal in the RPE (shown as asterisks) of human donor (Fig. 2Biii–vi) represents lipofuscin autofluorescence, a common feature in RPE from aged human eyes. In concordance, FBN2 protein (350 kDa) was detected in the RPE and choroid and was conspicuously absent in the neural retina by immunoblot analysis (Fig. 2C). Based on co-labeling studies using collagen IV or elastin antibodies, FBN2 appears to be localized to the central elastin layer of BrM (2Di–ii). No signal was detected when primary FBN2 antibody was not included (Supplementary Material, Fig. S1).

FBN2 rare variants are identified in additional patients with macular degeneration

The immunolocalization data prompted us to further explore the genetic contribution of *FBN2* to maculopathies. The *FBN2* gene includes 65 exons, yet CCA mutations have been reported to cluster in exons 24–34 (33,37,38). The mutation identified in Fam-979 is located in this mutation cluster as well. We therefore sequenced exons 24–34 (and flanking intronic sequences) in 96 patients with early-onset MD and 96 patients with AMD. Our screen identified two non-synonymous rare variants, [Minor allele frequency, MAF, from 4300 exome sequenced individuals of European ancestry in NHLBI Exome Sequencing Project (ESP)′s Exome Variant Server]: c.3740T > C (p.Met1247Thr; MAF = 0) in a maculopathy patient and c. 4246A > G (p.Thr1416Ala; identified only once in ESP database) in an AMD patient. Another two relatively rare non-synonymous variants, c.4312G > A (p.Glu1438Lys; MAF = 0.008) and c.4141C > A (p.His1381Asn; MAF = 0.004), were also observed in maculopathy patients, but these were detected at similar allelic frequency in control individuals.

Rare variants are predicted to alter FBN2 protein stability

The FBN2 protein is comprised of 2912 residues (39) and contains three epidermal growth factor-like (EGF) domains, 43 calcium-binding consensus sequences (Ca_EGF-like domains) and nine transforming growth factor β1 binding protein-like (TB) domains (Fig. 3A). The native protein fold of each Ca_EGF-like domain is maintained by six conserved cysteine residues, which form three disulfide bridges to support protein stability (Fig. 3B). Ca^{2+} binding in a negatively charged cavity improves the fold stability and helps to secure a relative orientation of two neighboring Ca_EGF domains (Fig. 3C). The p.Glu1144Lys variant introduces a positive charge into the negatively charged Ca^{2+} binding cavity, thereby likely decreasing the calcium-binding affinity. The interaction of Lys1144 with Glu1178 is also predicted to cause a clockwise change in relative orientation of a neighboring Ca_EGF domain (Fig. 3D). Located in a similar position of the Ca_EGF- domain, the p.Met1247Thr change should alter the surface loop formed by hydrophobic residues—Met1247, Ile1248 and Met1249 (Fig. 3E), which are positioned in close proximity to the C1246-C1257 disulfide bridge. The p.Thr1416Ala variant is predicted to modify intramolecular interactions, based on its location on the domain surface as well as affect N-glycosylation sequence which includes Asn1414 and T1416. Computer modeling simulations of the p.Glu1438Lys variant suggest a change in relative orientation of the EGF- like domain and altered calcium binding, whereas p.His1381Asn variant is predicted to be mild, with potential effect on intermolecular interaction (data not shown).

A common variant in *FBN2* exhibits modest association with late AMD

Encouraged by the expression of FBN2 in RPE, BrM and choroid and identification of a rare variant in an AMD patient, we evaluated 255 variants (including three missense) in the region spanning *FBN2* gene (Chr5: 127 642 826–127 668 726 bp) in our AMD-GWAS meta-analysis data (9), which identified suggestive association of a common non-synonymous SNP rs154001 [P-value = 2×10^{-3}, OR = 1.08, effect allele C (frequency 0.31)] (Fig. 4A). None of the known AMD susceptibility locus maps to chromosome 5, and rs154001 was the strongest signal in the FBN2 region when advanced AMD cases were compared with controls. Thus, we performed further meta-analysis of 10 337 cases and 11 174 controls in 15 cohorts of European ancestry using rs154001 and identified the AMD association

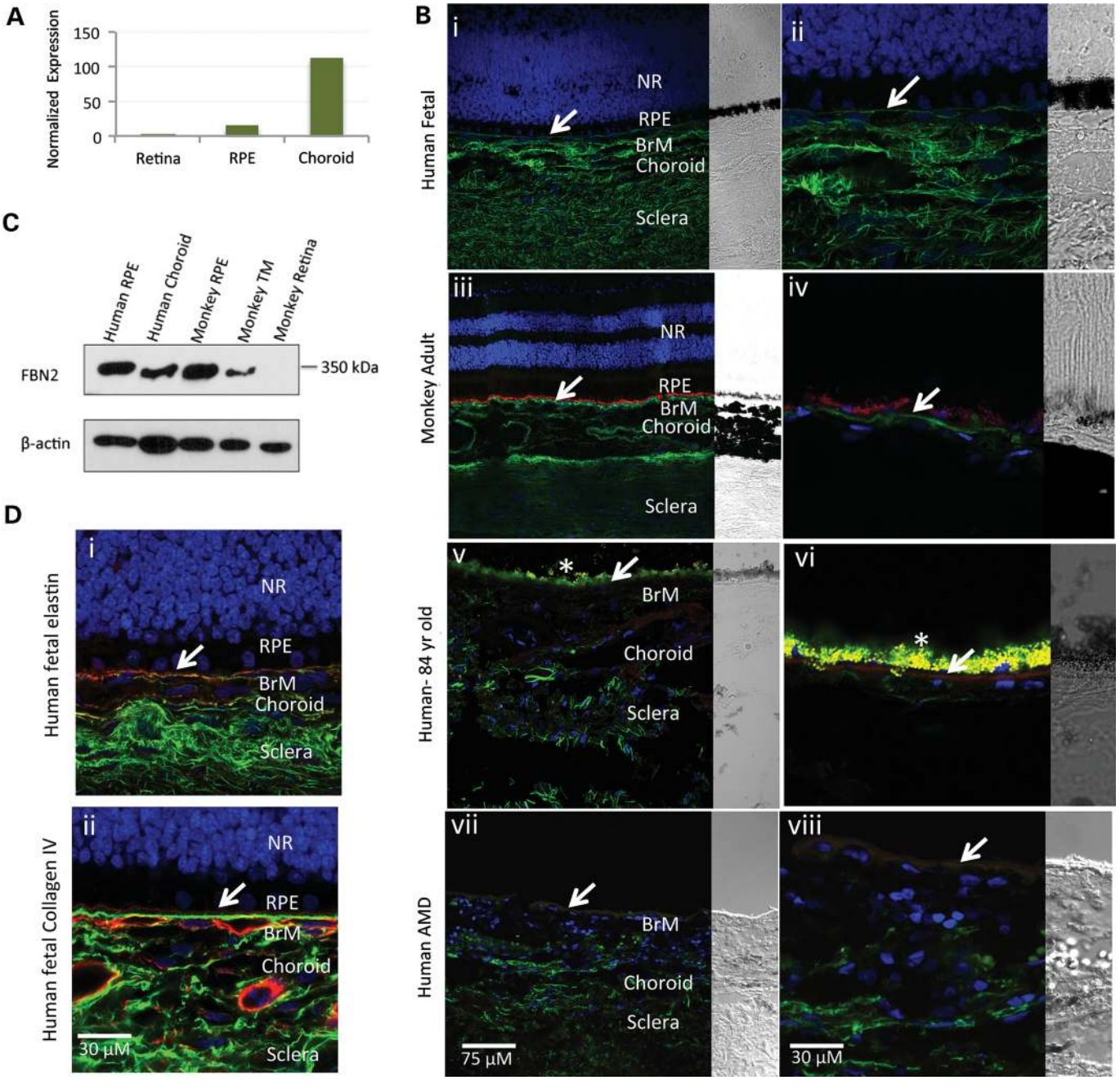

Figure 2. Expression analysis of FBN2 in human and monkey eyes. (**A**) FBN2 transcript level in human fetal retina, RPE and choroid. (**B**) FBN2 is expressed in human fetal, adult monkey and AMD donors. FBN2 signal was abundant in BrM, choroid and sclera in human fetal (i–ii) and adult monkey (iii–iv) (shown in green fluorescence). In aged human (v–vi) and in individuals with AMD (vii–viii), FBN2 signals were seen in choroid and sclera though expression was conspicuously absent in BrM. Although Bruch's membrane is still visible in these sections, RPE is no longer present because of widespread degeneration and cell death, which is a common feature of advanced AMD including geographic atrophy (**C**) Immunoblot analysis of the tissue lysates from normal human (RPE and choroid) and monkey (RPE, TM and retina). (**D**) Co-localization was also observed with elastin and collagen IV antibody in human fetal sections (i–ii). NR, neural retina; RPE, retinal pigment epithelium; BrM, Bruch's membrane; TM, trabecular meshwork. Green signals are for FBN2, red signal is for elastin (Di) and collagen IV (Dii) and blue represent the DAPI stained nuclei. Arrows indicate the location of BrM in sections.

[*P*-value $= 3.79 \times 10^{-5}$, OR = 1.10 (1.05–1.15), effect allele C] (Table 1, Fig. 4B). The contribution of *FBN2* to AMD appears relatively modest. We did not observe significant heterogeneity ($I^2 = 21.56\%$), indicating no evidence for variability in effect size estimates.

DISCUSSION

In this study, we have identified a rare variant in the elastin microfibril gene *FBN2* in an early-onset macular degeneration family (Fam-979), exhibiting a spectrum of phenotypes. As

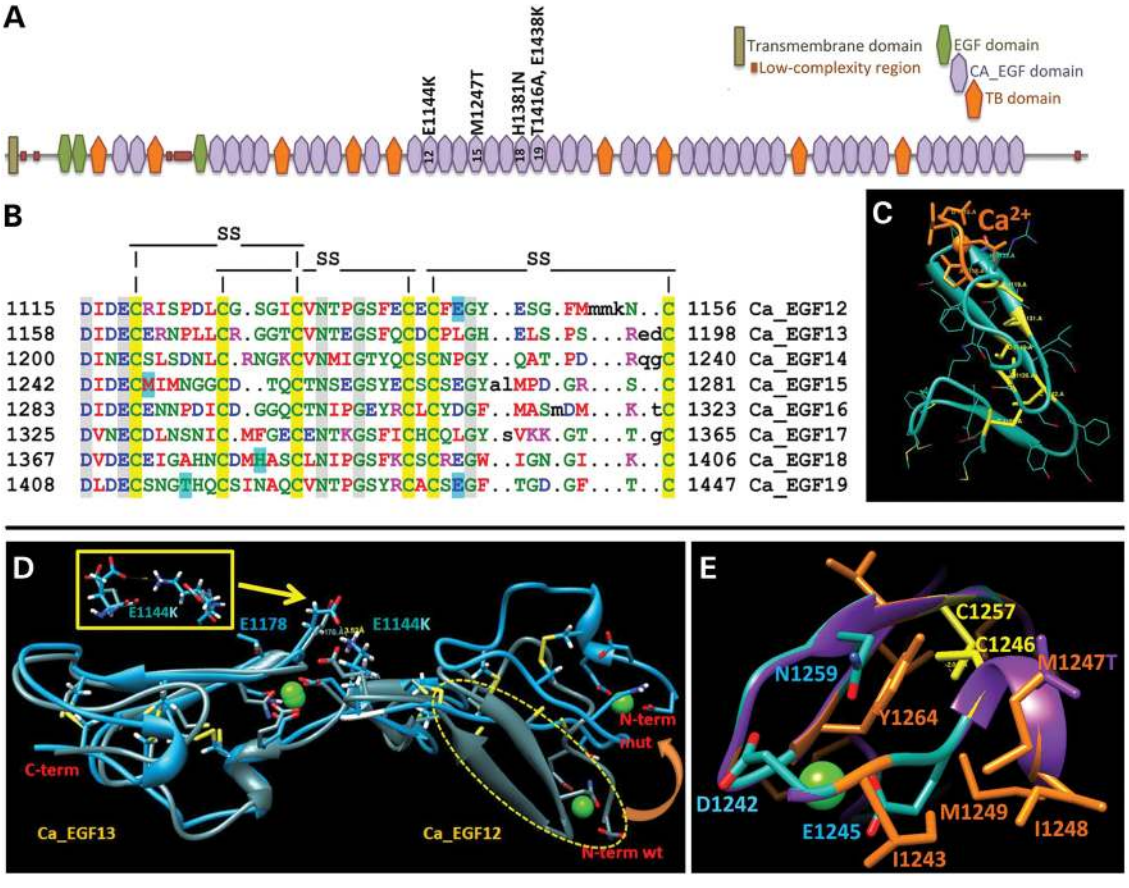

Figure 3. Homology modeling of wild-type and mutant *FBN2* variants. (**A**) Putative structural domains and variants identified are located in Ca_EGF domains 12, 15, 18, and 19, respectively. (**B**) Multiple sequence alignment of Ca_EGF-motifs 12–19. Each of 8 Ca_EGF domains has consensus sequence D*hp*E where *h* and *p* are hydrophobic and polar residues, respectively. Conserved positions of cysteines and disulfide bonds are shown by yellow background and by black lines (SS), respectively. Locations for missense changes are shown by cyan background. (**C**) Homology modeling of Ca_EGF domain structure is shown by cyan ribbon. Each domain contains a short anti-parallel β-sheet and three loops stabilized by disulfide bridges. The motif of first four residues which includes two conserved negatively charged residues (Asp, Glu) involved in Ca^{2+} binding as well as the Ca^{2+} atom are shown by orange. Cysteine residues are yellow. (**D**) The superposition of wild-type (sea green) and mutant (blue) structures for a pair of Ca_EGF domains 12 and 13. p.Glu1144Lys variant causes change in structural orientation of the Ca_EGF12 domain. Calcium atom is shown by green. The effect of the structural change is associated with a salt bridge formation between positively charged lysine 1144 and negatively charged Glu1178 is shown in inset. (**E**) Replacement of hydrophobic methionine with polar threonine in the p.Met1247Thr mutant variant decreases hydrophobic potential of a cluster formed by hydrophobic residues Met1247, Ile1248, Met1249.

maculopathy patients carrying *PRPH2/RDS* mutations (41) can present a similar range of clinical findings, we carefully examined the *RDS* gene, but our whole exome analysis did not reveal a causative mutation in any known inherited maculopathy gene. Affected members in the Fam-979 family did not report a history of skeletal, joint or muscle abnormalities, as identified in CCA, which is also associated with *FBN2* mutations. CCA and Marfan syndrome are related disorders with ocular phenotypes, including bilateral ectopia lentis, myopia and retinal detachment (42,43). Recently, a patient with CCA was reported to exhibit disciform maculopathy that was subsequently treated with ranibizumab to control choroidal neovascularization (44). This finding is consistent with the association of a common *FBN2* variant to AMD, as reported here. High expression of FBN2 in RPE, BrM and choroid and identification of maculopathy-associated rare variants predicted to disrupt protein structure together provide strong evidence for a critical role of FBN2 in maintaining the functional integrity of blood–retina barrier in support of macular retina.

FBN1 and FBN2 polymerize to assemble microfibrils and elastin fibers that provide architectural framework for tissues and scaffolds for physiological functions (31). In BrM, elastin and 10-nm fibrillin containing microfibrils form the central layer that is flanked by two layers of collagen, providing a barrier through which metabolites delivered by the choroidal capillaries reach the RPE. BrM has long been suspected to play significant role in macular disease. For example, changes in thickness and ultrastructure of the elastic layer in BrM at the macula and accumulation of basal linear deposits constitute the early signature of AMD pathology (17,27). Even though FBN1 and FBN2 are building blocks of matrix assemblies, they perform discrete functions and exhibit temporal and tissue-specific expression in developing elastic tissues (45). Thus, localization of FBN2 represents an unambiguous demonstration of a BrM constituent in AMD pathology. We hypothesize that *FBN2* variants observed in maculopathy alter elastin fiber flexibility, packaging, and/or permeability. In aging and AMD eyes, the reduction or loss of FBN2 (see Fig. 2) would likely

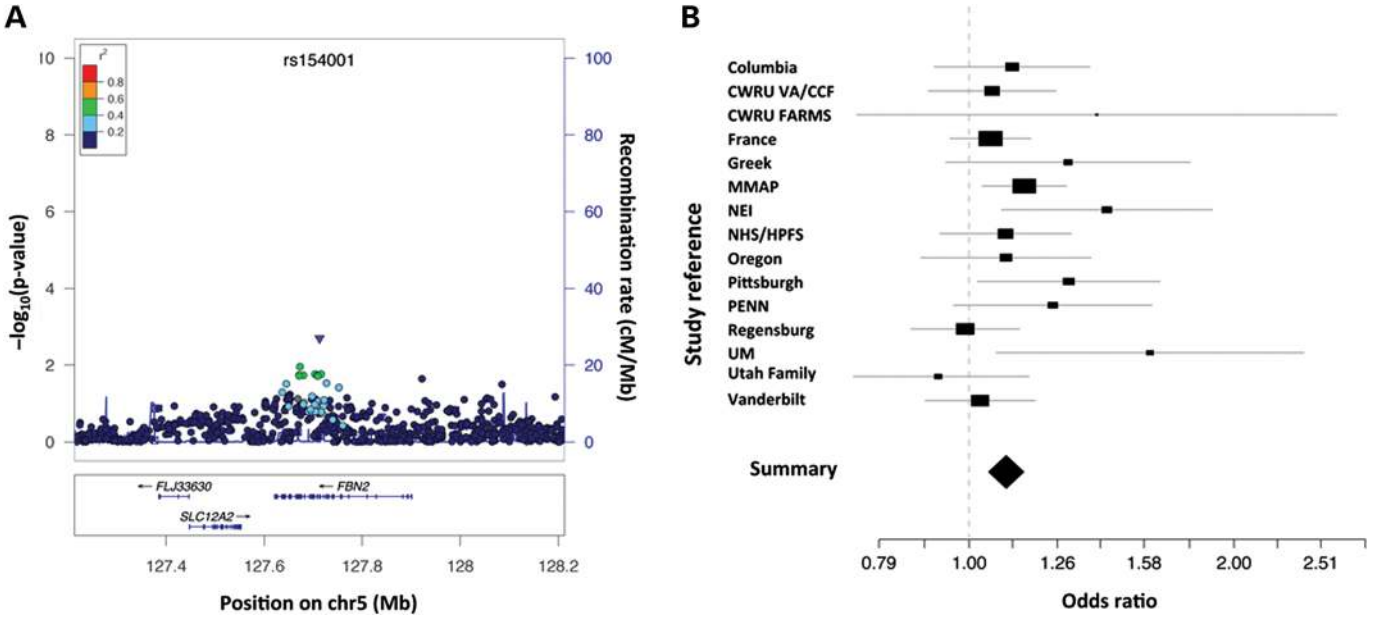

Figure 4. Meta-analysis of a common *FBN2* coding SNP, rs154001. (**A**) LocusZoom plots for association signal in the 200 kb region surrounding the *FBN2* gene (40). The representative non-synonymous SNP (rs154001) for this region is shown by purple inverted triangle. (**B**) Forest plot for rs154001 association in 10 337 cases and 11 174 controls across 15 cohorts.

compromise the barrier properties of BrM and may initiate a cascade of pathophysiological processes, which may accelerate accumulation of lipids, immune complexes and other debris. Further analysis of multiple human eyes with wide age range should be helpful in defining the role of dynamic extracellular environment of RPE and choroid in AMD pathogenesis.

Genetic variants in components of the ECM and associated signaling pathways are implicated in early- (*TIMP3*, *EFEMP1*) (2) and late- (*CTRP5*) (19,20) onset inherited macular degeneration as well as in AMD (*TIMP3*, *TGFBR1*, *COL8A1* and *COL10A1*) (8,9). Our study adds FBN2, an integral component of elastin fibers in BrM matrix, to this list. Fibrillins are demonstrated to sequester cell surface and signaling molecules, including TGFβ, bone morphogenetic protein pro-peptides, syndecans, integrins and control ECM formation and remodeling (31,39). Altered microfibril structure or binding properties caused by *FBN2* variants can weaken the elastin fiber and affect ECM-mediated signaling by changing the microenvironment. Vascular endothelial growth factor plays a major role in choroidal and retinal neovascularization and its secretion is induced by TGFβ in human RPE cells (46). Our studies thus indicate a possible role of TGFβ and related signaling pathways in AMD pathogenesis.

Genetic studies of AMD represent a remarkable success story for understanding a complex disease, with 19 validated susceptibility loci (9). However, as in other complex diseases (47), identification of causality at associated AMD loci as well as the functional consequence of underlying variants are still elusive. Rare causal variants, additional unknown small-effect loci and interaction among known loci (epistasis) and with environment can probably explain additional genetic susceptibility (48–50). Recently, rare high-risk alleles have been identified in genes with common AMD-associated variants: *CFH* (51), *CFI* (52,53), *C3* (52,54,55) and *C9* (52). Our study unravels rare *FBN2* coding variants in patients with early-onset macular degeneration and shows modest association of a common non-synonymous variant with AMD. Thus, rare and common variants in a single gene (e.g. *FBN2*) can both result in clinical findings of macular degeneration and help in elucidating the genetic architecture of common and complex diseases, such as AMD.

MATERIALS AND METHODS

Whole exome sequencing and variant calling

All patients signed informed consent, and the Institutional Review Boards of the University of Michigan and National Eye Institute approved the study. Genomic DNA (3 μg) from three affected (I:1, II:1 and II:5) members and one unaffected (II:2) member from Fam-979 was fragmented by Covaris and subjected to whole exome capture using Agilent SureSelect Human All Exon 50 Mb kit (Agilent Technologies, Santa Clara, CA), following manufacturer′s instructions. Captured libraries were amplified and converted to clusters using Illumina Cluster Station. Single-end sequencing was performed on Illumina GAIIx. Approximately 5 GB of sequence was generated per individual, resulting in ∼90% coverage of targeted Consensus Coding Sequence project (CCDS) exonic bases, with an average depth of 135×. Reads were mapped to the reference human genome (UCSC hg 19; http://genome.ucsc.edu/) using Burrows-Wheeler Alignment (BWA) tool (56). Variants were subsequently called using SAMtools (57) and UMAKE (http://genome.sph.umich.edu/wiki/UMAKE/) and annotations were obtained using ANNOVAR (58). We selected heterozygous variants (non-synonymous, splice-site, stop gain/loss), which were common in three affected (I:1, II:1 and II:5), but were absent from the unaffected member (II:2). Subsequent filtering of common variants was done using frequency

Table 1. Meta-analysis of association of rs154001 (p.Val965Ile) with AMD

Study reference	Cases				Controls				Odds ratio (OR)	*P*-value
	Genotype (%)			Sample size (*N*)	Allele frequency T (%)	Genotype (%)			(Range)	
	C/C	C/T	T/T			C/C	C/T	T/T		
Columbia	57 (7.91)	311 (43.13)	353 (48.96)	721	70.53	24 (6.61)	150 (41.32)	189 (52.07)	363	72.73	1.12 (0.91, 1.37)	0.28
CWRU VA/CCF	62 (10.63)	267 (45.80)	254 (43.57)	583	66.47	77 (11.88)	263 (40.59)	308 (47.53)	648	67.82	1.06 (0.90, 1.25)	0.48
CWRU FARMS	9 (14.06)	32 (50.00)	23 (35.94)	64	60.94	8 (6.56)	64 (52.46)	50 (40.98)	122	67.21	1.40 (0.75, 2.61)	0.3
France	110 (11.42)	411 (42.68)	442 (45.90)	963	67.24	426 (10.04)	1824 (42.98)	1994 (46.98)	4244	68.47	1.06 (0.95, 1.17)	0.3
Greek	13 (6.77)	86 (44.79)	93 (48.44)	192	70.83	13 (6.13)	77 (36.32)	122 (57.55)	212	75.71	1.30 (0.94, 1.78)	0.11
MMAP	222 (10.30)	909 (42.16)	1025 (47.54)	2156	68.62	98 (8.53)	454 (39.51)	597 (51.96)	1149	71.71	1.16 (1.04, 1.29)	0.01
NEI	100 (9.80)	443 (43.43)	477 (46.76)	1020	68.48	8 (5.06)	61 (38.61)	89 (56.33)	158	75.63	1.43 (1.09, 1.89)	0.01
NHS/HPFS	52 (12.04)	181 (41.90)	199 (46.06)	432	67.01	89 (8.70)	455 (44.48)	479 (46.82)	1023	69.06	1.10 (0.93, 1.31)	0.27
Oregon	57 (8.21)	331 (47.69)	306 (44.09)	694	67.94	24 (8.66)	119 (42.96)	134 (48.38)	277	69.86	1.10 (0.88, 1.38)	0.39
Pittsburgh	20 (9.57)	75 (35.89)	114 (54.55)	209	72.49	73 (9.62)	336 (44.27)	350 (46.11)	759	68.25	1.24 (0.96, 1.61)	0.1
PENN	42 (9.79)	186 (43.36)	201 (46.85)	429	68.53	19 (6.76)	109 (38.79)	153 (54.45)	281	73.84	1.30 (1.02, 1.65)	0.03
Regensburg	105 (10.81)	523 (47.81)	466 (42.60)	1094	66.5	84 (10.81)	356 (45.82)	337 (43.37)	777	66.28	0.99 (0.86, 1.14)	0.89
UM	8 (10.00)	47 (58.75)	25 (31.25)	80	60.63	18 (7.79)	102 (44.16)	111 (48.05)	231	70.13	1.61 (1.07, 2.40)	0.02
Utah Family	58 (11.42)	227 (44.69)	223 (43.90)	508	66.24	29 (11.93)	115 (47.33)	99 (40.74)	243	64.4	0.92 (0.73, 1.17)	0.5
Vanderbilt	122 (10.23)	529 (44.38)	541 (45.39)	1192	67.58	68 (9.90)	301 (43.81)	318 (46.29)	687	68.19	1.03 (0.89, 1.19)	0.69
Meta-analysis	**1037 (10.03)**	**4558 (44.09)**	**4742 (45.87)**	**10337**	**67.92**	**1058 (9.47)**	**4786 (42.83)**	**5330 (47.70)**	**11174**	**69.12**	**1.10 (1.05, 1.15)**	**3.79×10^{-5}**

AMD, age-related macular degeneration; BrM, Bruch's membrane; CCA, congenital contractural arachnodactyly; EGF, epidermal growth factor-like; ECM, extracellular matrix; FBN, fibrillin; GWAS, genome-wide association studies; RPE, retinal pigment epithelium.
Logistic regression, with no covariates, was used to compare advanced cases and controls in each study. Bold values represent combined values from meta-analysis data.

information from dbSNP132 (http://www.ncbi.nlm.nih.gov/SNP/), 1000 Genome Project (http://www.1000genomes.org/) and ESP databases (http://eversusgs.washington.edu/EVS/). We confirmed these variants by Sanger sequencing and checked for segregation in the complete pedigree and in 384 matched controls.

Expression analysis in human and monkey eyes

Human donor eyes were obtained from National Disease Research Interchange (Philadelphia, PA), and Rhesus monkey (*Macaca mulatta*) eyes were obtained from the Vaccine Testing Program, Center for Biological Evaluation and Research, US Food and Drug Administration (Bethesda, MD). The donors with a documented history of ocular trauma, ocular infection, diabetic retinopathy or glaucoma were excluded. An Ophthalmologist trained in AMD clinical pathology and an ocular pathologist evaluated the fundi. We noted the presence of confluent soft macular drusen, areas of RPE hypo- or hyperpigmentation and/or regions of RPE atrophy, consistent with GA. These eyes were not screened for presence of pseudodrusen. Cryosections were stained with H&E and evaluated for histological evidence of AMD, including extensive drusen deposits, RPE atrophy or death and corresponding degeneration of adjacent photoreceptors. Eyes used as normal controls were devoid of AMD pathology (both gross and histologic) and showed no evidence of retinal or RPE degeneration.

Fetal eyes were obtained from Advanced Bioscience Resources (Alameda, CA), from unidentified 16–18 week donors. Cryosections of unfixed or fixed (4 h in 4% formaldehyde) human and monkey eyes (8–10 μm) were treated for 5 min in 4% formaldehyde prior to immunolabeling. Sections were blocked briefly in 5% normal goat serum in PBS with 0.5% BSA, 0.2% Tween-20, pH 7.3 and incubated overnight at 4°C with the primary antibody (at indicated dilution): FBN2 polyclonal (Sigma, St Louis, MO)—1:100; FBN2 monoclonal (Millipore, Billerica, MA)—1:100; Collagen IV (Col94-Abcam, Cambridge, MA)—1:50; Elastin (Elastin Products Company, Owensville,MO)—1:100. Following multiple washes, secondary antibodies conjugated to either Alexafluor-488 or Alexafluor-568 (Invitrogen, Carlsbad, CA) were added at 1:400 dilution, and sections were incubated for 1 h at room temperature. DNA binding fluorophore, DAPI (1 μg/ml; Invitrogen) was used to label nuclei. Antigen-retrieval was performed on tissues fixed for >24 h using Target Retrieval Solution, pH 6 (DAKO, Carpinteria, CA). Briefly, a beaker was filled with enough 1× retrieval solution to cover slides and heated to 65°C. Fixed slides were then immersed into preheated target retrieval solution and incubated for 20–30 min at 97°C. Sections were left in the solution to cool to 65°C. The slides were then washed gently with 1× DAKO buffer (DAKO, Carpinteria, CA) for several minutes and stained. Tissue preparations were imaged on Olympus FV1000 confocal microscope (Olympus America, Center Valley, PA).

Immunoblot analysis was performed using lysates from retina, RPE and trabecular meshwork of adult monkey eye and from RPE and choroid of a 66-year-old human donor eye. Protein was quantified using BCA method (Thermo Scientific, Rockford, IL), and 100 μg of protein was resolved on sodium dodecyl sulfate-polyacrylamide gels. After transfer to

polyvinylidene fluoride membrane (Millipore), the blot was incubated with rabbit anti-FBN2 polyclonal antibody (Sigma, MO) and developed using ECL System (Amersham Pharmacia Biotech).

Sanger sequencing

Exons 24–34 of *FBN2* were sequenced in 96 dominant maculopathy patients. Primer sequences are available upon request. DNA sequencing was carried out by cycle sequencing using Big Dye^{TM} Terminator Cycle Sequencing kit v3.0 (Applied Biosystems) and analyzed on ABI3100 Genetic Analyzer (Applied Biosystems). Sequence analysis was performed using SeqMan 5.01 (DNASTAR Inc.).

Molecular modeling of FBN2 protein

The amino acid sequence of FBN2 was retrieved from the UniProtKB database (http://www.uniprot.org/uniprot/P35556), and domain structure was recovered using Simple Modular Research Tool, SMART (59). The search of structural repeats and multiple sequence alignment of calcium-binding epidermal growth factor (Ca_EGF)-motifs 12–19 were performed using the Rapid Automatic Detection and Alignment of Repeats (RADAR; http://www.ebi.ac.uk/Tools/pfa/radar/). The initial mutant variant structures for the Ca-EGF motifs 12–19 (residues 1115–1447) were built by automated protein-homology modeling server (60), using protein structure of a Ca_EGF- like domain pair from the neonatal region of human FBN1 as a structural template (PDB: 1lmj). The sequence identity between human sequences of FBN1 and FBN2 was 67.259%. Structures for five FBN2 variants, p.Glu1144Lys, p.Met1247Thr, p.Thr1416Ala, p.Glu1438Lys and p.His1381Asn, were generated and their effect was simulated using a molecular visualization, modeling and dynamics program, YASARA (61).

Genotyping and statistical analysis

We first extracted all SNPs in the 200 kb region surrounding the *FBN2* gene from our previous GWAS meta-analysis data (9). We then genotyped the only associated coding SNP, rs154001 (Chr5:127685135, C/T), in 10 337 AMD cases and 11 174 matched controls from 15 different cohorts using TaqMan assay, except for Pittsburg samples that were genotyped by exome chips. Odds ratios and 95% confidence intervals were calculated by means of logistic-regression analysis for unrelated samples or generalized disequilibrium tests for familial samples (62), and two-sided *P*-value was obtained using Wald statistics. The inverse variance based method was used to combine statistics across different cohorts (63,64). Heterogeneity between different datasets was evaluated using the I^2 statistic (64).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

Conflict of Interest statement. G.R.A. and A.S. have received royalties from patents held by the University of Michigan. E.H.S is consultant for Novartis, Allergan, and Bayer. D.E.W., Y.P.C and M.B.G. hold patents on AMD risk prediction using specific molecular markers. D.A.S. has financial interest in Mimetogen Pharmaceuticals. No other coauthor indicated conflict of interest.

FUNDING

This study was supported by Intramural Research Program of the National Eye Institute and National Institutes of Health grants (EY016862, EY013435, EY09859, EY017362, EY014458, EY019007), Macula Vision Research Foundation, Foundation Fighting Blindness, Kaplen Foundation, Widgeon Point Charitable Foundation, Harold and Pauline Price Foundation, Elmer and Sylvia Sramek Foundation, Veterans Administration, Harold and Pauline Price Foundation, German Research Foundation (WE 1259/19-2), NIHR supported BRC at Moorfields Eye Hospital (London), Welcome Trust (076169/A), American Health Assistance Foundation (M2007110), Frost Charitable Trust, Brian Mercer Charitable Trust, Macular Society and the Gift of Sight, and Research to Prevent Blindness.

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