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若年性黄斑変性カニクイザルの病理学的 および分子生物学的解析

平成１６年度　総括・分担研究報告書

平成１７年３月

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若年性黄斑変性カニクイザルの病理学的及び分子生物学的解析

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Ｉ Ｉ． 総括研究報告書

厚生省科学研究費補助金(難治性疾患克服研究事業)

総括研究報告書

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研究要旨: 今年度は疾患サルのデータベースの構築、ドルーゼンの質量分析計による網羅的プロテオーム解析、若年性黄斑変性カニクイザルの遺伝子連鎖解析、加齢性黄斑変性カニクイザルの自己免疫疾患抗原分子の検索、そしてヒト加齢黄斑変性患者の血漿解析などを中心に研究が行われ、大きな成果が得られた。

キーワード: カニクイザル、加齢黄斑変性、連鎖解析、プロテオーム解析、自己免疫疾患

A. 研究目的

黄斑は角膜と水晶体によって収束した光が網膜上で結像する領域で、視覚を司る視細胞が最も密に集中しており、視力を決定する重要な部位である。ここが障害されると著しい視力低下、ひいては法的失明に至り、その代表的な難治性眼疾患に加齢黄斑変性などがある。加齢黄斑変性は米国では65歳以上の失明原因として最も多い眼疾患である。日本でも高齢化が進み、生活の欧米化にともない、また診断装置の普及にしたがって、加齢黄斑変性の患者数は増加の一途をたどっており、その原因解明と治療法の開発は急務である。

黄斑は解像度の高い視力を獲得した霊長類や鳥類で発達しており、通常の実験動物として利用されているラットやマウスなどの夜行性のゲツ歯類には存在しない。加齢黄斑変性はその詳細な発症過程が明らかになっておらず、その原因の一つとして黄斑を持つ動物(サル)に黄斑変性が発症していて、これを研究できるケースがまれであるからである。今回我々が取り組んでいる、若年性黄斑変性カニクイザルは世界で唯一の大型家系として存在する黄斑変性動物モデルであり、家系に属する個体数から、連鎖解析をはじめ、複数の研究が展開可能である。

筑波霊長類センターで維持されているこの疾患家系のサルはこれまでに野田、溝田等の観察によって若年性ありながら加齢性の黄斑変性カニクイザルと類似する臨床所見が観察されている。黄斑部及び周辺部にドルーゼン(網膜色素上皮細胞とブルッフ膜間の蓄積物)が観察され、免疫染色法によってヒトと類似した組成を持つことが明らかとなった。ドルーゼンの生成は加齢黄斑変性の特徴で、ヒトでは50歳以上で蓄積が観察されているが、若年黄斑変性カニクイザルの場合には生後2年でこれが観察される。短い期間で病態の進行を解析できるこのモデル動物は、国立感染症研究所霊長類センターにのみ存在し、世界的に見ても貴重である。現在の診断法や治療法は主に病気の末期で観察される新生血管にターゲットが絞られており、ドルーゼンが観察される初期における情報はきわめて乏しい。新生血管が増殖するはるか以前に黄斑部周辺でどのような異変が生じるのか、疾患の原因解明と有効な治療法・診断法の確立をめざす。

今年度の研究ではドルーゼンの網羅的プロテオーム解析、連鎖解析による原因遺伝子の染色体座位の絞り込み、加齢性黄斑変

性カニクイザルの自己免疫疾患抗原分子の同定、さらにこれを検証するためにヒト加齢黄斑変性患者の血液検体の収集と血漿解析を行った。

B. 研究方法

(1)若年黄斑変性カニクイザルのデータベースの構築

これまでに行われてきた眼底観察、ERG(網膜電図)、FAG(蛍光眼底撮影)、IG(蛍光眼底造影)などの観察結果(テキスト、画像)を個体別にファイルメーカーのフォーマットに変換し、データベース化して、サーバーを立ち上げた。ID、パスワード、家系図があればインターネット上から共同研究者が個体番号を入力することにより、疾患個体の解析状況を把握することができる。

(2)ドルーゼンのプロテオーム解析

疾患サルのドルーゼンをトリプシン消化した後、2次元液体クロマトグラフィーイオントラップ型質量分析計を用いて解析を行った。生データーはタンパク質検索ソフトを用いてNCBIの最新データベースを検索した。

(3)若年性黄斑変性カニクイザルの連鎖解析

連鎖解析んはELOVL4, TIMP, ABCA4, RDS, VMD2, EFEMP1に加え7つの遺伝子マーカー(ABI, linkage mareker set)を用いて、DNAシークエンサーABI 3100で解析を行った。

(4)加齢黄斑変性カニクイザルの自己免疫抗原体の解明

カニクイザルの網膜抽出液、RPE細胞抽出液をゲルに展開し、加齢性黄斑変性カニクイザルの血清中を使ってウエスタンブロットを行った。抗原タンパク質はクローニングした後にHisタグを付けて大腸菌で発現し、ニッケルアフィニティーカラムによって精製した。精製タンパク質を96穴ニッケルプレートの底に固定し、ELISAを行った。

(5)加齢黄斑変性患者の血液検体の収集と質量分析計による血漿解析による疾患マーカーの検索

東京医療センター倫理委員会、順天堂大学浦安病院倫理委員会の承認を得て、加齢黄斑変性患者の採血を行った。血漿を遠心分離して、ドルーゼンの解析と同様にトリプシン処理の後、2次元液体クロマトグラフィーイオントラップ型質量分析計を用いて解析を行った。生データーはタンパク質検索ソフトを用いてNCBIの最新データベースを検索した。

C. 研究結果

(1)若年黄斑変性カニクイザルのデータベースの構築

これまでに行われてきた眼底観察、ERG(網膜電図)、FAG(蛍光眼底撮影)、IG(蛍光眼底造影)などの観察結果(テキスト、画像)を個体別にファイルメーカーのフォーマットに変換し、データベース化して、サーバーを立ち上げた。ID、パスワード、家系図があればインターネット上から共同研究者が個体番号を入力することにより、疾患個体の解析状況を把握することができる。

(2)ドルーゼンのプロテオーム解析

疾患サルのドルーゼン組成を質量分析計によって解析し、ヒトとの比較を行った結果、疾患サルのドルーゼンにはヒトと同様に補体活性因子、補体活性抑制因子、クリスタリンなどが存在することが明らかになった(論文投稿中)。若年性黄斑変性カニクイザルは生後2年でヒトと同様なドルーゼンを黄斑周辺に蓄積していることが直接的な方法で確認された。

(3)若年性黄斑変性カニクイザルの連鎖解析

若年性疾患サルの原因遺伝子検索についてはこれまでヒト黄斑変性(加齢性でない)の原因遺伝子であるELOVL4, TIMP, ABCA4, RDS, VMD2, EFEMP1について、対応するカニクイザルの遺伝子をクローニングして遺伝

子変異の検索を行ったが、疾患との相関性はなかった(Umeda, Iwata et al. IOVS 2005)。さらに、これらの遺伝子ローカスを含む13の候補ローカスについて連鎖解析を行った結果、染色体6qに位置するマーカーと連鎖することがわかった。この領域にはCONE DYSTROPHY 3やINTERPHOTORECEPTOR MATRIX PROTEOGLYCAN 1など眼疾患遺伝子が存在し、これらと疾患との関係を解析中である。

(4)加齢黄斑変性カニクイザルの自己免疫抗原体の解明

加齢性黄斑変性カニクイザルの血清中に含まれる自己免疫抗体の検索を行った結果、網膜に含まれる2種類のタンパク質について自己免疫抗体が存在することが明らかとなった。このタンパク質はアネキシンIIAとミュークリスタリンである。このタンパク質を大腸菌で発現・精製し、ELISAによって個体別に抗体量を比較した結果、抗体量の増加が疾患個体に優位な結果が得られた(論文投稿中)。

(5)加齢黄斑変性患者の血液検体の収集と質量分析計による血漿解析による疾患マーカーの検索

疾患サルの自己免疫抗体の検索に加え、加齢黄斑変性患者と正常者の血漿プロテオームの比較を開始したが、アルブミンや免疫グロブリン分子などの多量タンパク質が妨害して少量分子の検出がこれまでできなかった。これらの多量分子を除くためのアフィニィティーカラムなどを複数検討し、またサンプルの前処理の検討を行った結果、1000－1500のタンパク質の同定が可能なシステムを構築中である。今後解析条件が整えば、疾患と正常のサル、ヒトの血漿解析を行い、比較検討を行う。

D. 考察

今年度は最大の成果は若年性黄斑変性カニクイザルのドルーゼン組成がヒト加齢黄斑変性患者のドルーゼンと一致したことである。すなわちヒトで50年かかる現象が2年で観察できることが証明されたことである。これまでの研究からこの家系は1遺伝子の変異よって発症し、これが遺伝していることが示されている。この遺伝子の発見によってヒト加齢黄斑変性のドルーゼン生成に関わる重要な情報が得られ、それは加齢黄斑変性の発症機序を知る上でもきわめて重要である。この情報はドルーゼン生成の阻害剤や疾患そのものを遅延させる薬の開発にも役立つと考えられる。

第2の大きな成果は疾患家系の連鎖解析によって染色体6qに原因遺伝子が絞り込まれたことである。この領域には候補となる遺伝子が複数存在することから平成17年度は、これらを粒さに調べ、本研究課題の最大の目的である原因遺伝子のクローニングにつなげたい。

最近の加齢黄斑研究から疾患の原因として網膜色素上皮細胞(RPE細胞)周辺における補体の活性化が重要視されている。しかしながら、何がきっかけとなって活性化が起こるのかわかっていない。可能性として考えられるのは自己免疫抗体によるRPE細胞への攻撃である。今回我々が発見した、アネキシンIIAはRPE細胞表面で発現しており、このタンパク質に対しての自己免疫抗体が加齢性黄斑変性カニクイザルに優位に高いことから、平成17年度はヒト加齢黄斑変性患者の血清について同様な自己抗体が存在するか調査したい。

E. 結論

今年度は疾患サルのデータベースの構築を行い、ドルーゼンの質量分析計による網羅的プロテオーム解析によって、その組成が明らかとなった。また、若年性黄斑変性カニクイザルの遺伝子連鎖解析によって染色体6qに原因遺伝子が存在する可能性がでてきた。加齢性黄斑変性カニクイザルの自己免疫疾

患抗原分子としてアネキシンIIAとミュークリスタリンが発見された。ヒト加齢黄斑変性患者の血漿解析が進行中で多量タンパク質の除去が今後の課題となった。

F. 健康危険情報

特になし

G. 研究発表

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H. 知的所有権の出願・取得状況

1 特許取得　　なし
2 実用新案登録　なし
3 その他　　なし

研究成果の刊行に関する一覧表

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Early-Onset Macular Degeneration with Drusen in a Cynomolgus Monkey (*Macaca fascicularis*) Pedigree: Exclusion of 13 Candidate Genes and Loci

Shinsuke Umeda,[1,2] Radha Ayyagari,[3] Rando Allikmets,[4] Michihiro T. Suzuki,[5] Athancios J. Karoukis,[3] Rajesh Ambasudhan,[3] Jana Zernant,[4] Haru Okamoto,[1] Fumiko Ono,[5] Keiji Terao,[6] Atsushi Mizota,[7] Yasuhiro Yoshikawa,[2] Yasuhiko Tanaka,[1] and Takeshi Iwata[1]

Purpose. To describe hereditary macular degeneration observed in the cynomolgus monkey (*Macaca fascicularis*), which shares phenotypic features with age-related macular degeneration in humans, and to test the involvement of candidate gene loci by mutation screening and linkage analysis.

Methods. Ophthalmic examinations with fundus photography, fluorescein angiography (FA), indocyanine green angiography (IA), electroretinography (ERG), and histologic studies were performed on both affected and unaffected monkeys in the pedigree. The monkey orthologues of the human *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* genes were cloned and screened for mutations by single-strand conformation polymorphism (SSCP) analysis or denaturing high-performance liquid chromatography (DHPLC) and direct sequencing in six affected and five unaffected monkeys from the pedigree and in six unrelated, unaffected monkeys. Subsequently, 13 human macular degeneration loci including these five genes were analyzed to test for linkage with the disease. Nineteen affected and seven unaffected monkeys in the pedigree were analyzed by using human microsatellite markers linked to the 13 loci.

Results. Yellowish white spots were observed in the macula and fovea centralis, and in some cases the spots scattered to the peripheral retina along the blood vessels. FA showed hyperfluorescence corresponding to the dots except in the foveola. No anomalies were found by IA and ERG. Histologic studies demonstrated that the spots were drusen. Mutation analysis of the *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* genes identified a few sequence variants, but none of them segregated with the disease. Linkage analysis with markers linked to these five genes and an additional eight human macular degeneration loci failed to establish linkage. Haplotype analysis excluded the involvement of the 13 candidate loci for harboring the gene associated with macular degeneration in the monkeys.

Conclusions. Significant homology was identified between monkey and human orthologues of the five macular degeneration genes. Thirteen loci associated with macular degeneration in humans or harboring macular degeneration genes were excluded as causal of early-onset macular degeneration in the monkeys. It is likely that none of these loci, but rather a novel gene, is involved in causing the observed phenotype in this monkey pedigree. (*Invest Ophthalmol Vis Sci.* 2005;46:683–691) DOI:10.1167/iovs.04-1031

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The inherited macular dystrophies comprise a heterogeneous group of blinding disorders characterized by central visual loss and atrophy of the macula and underlying retinal pigment epithelium (RPE).[1] The complexity of the molecular basis of monogenic macular disease is being elucidated through identification of many of the disease-causing genes.[2-8] Because of limitations associated with studies in humans, nonhuman species with phenotypes similar to human macular degeneration have been used as model systems to study these diseases. Rodent models generated by altering the genes homologous to the disease-causing genes in humans are most extensively used in such studies; however, rodents do not have a defined macula and, hence, the clinical symptoms observed in humans with macular degeneration cannot be fully replicated.[9-11] Because the macula is found only in primates and birds, a monkey model of macular degeneration would be extremely valuable for studies elucidating the mechanism and etiology underlying these diseases. A primate model for macular degeneration is much needed to develop sensitive diagnostic techniques and potential therapeutic strategies to cure or prevent the disease. Furthermore, such models are of particular value if their genetic basis is understood.

Macular degeneration in monkeys was first described by Stafford in 1974.[12] He reported that 31 (6.6%) of eyes of elderly monkeys showed pigmentary disorders and/or drusen-like spots. In 1978, El-Mofty et al.[13] reported a high incidence (50%) of maculopathy in a closed rhesus monkey colony at the

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Caribbean Primate Research Center of the University of Puerto Rico. The latest report from the center states that specific maternal lineages have a statistically significant higher prevalence of drusen.[14] Although they suspected the involvement of hereditary factors, genetic analysis of the macaque population has not been reported.

We have reported a high incidence of macular degeneration in one of the cynomolgus monkey (*Macaca fascicularis*) colonies at the Tsukuba Primate Center.[15,16] This macular degeneration originated from one affected male monkey, which showed phenotypic characterization of macular degeneration. The disease affects the central retina specifically, with yellowish white dots in the macula and lipofuscin deposits in the RPE, consistent with the phenotype observed in the early stages of age-related macular degeneration (AMD). These symptoms appear at the age of ~2 years and progress slowly throughout life. Mating experiments have demonstrated that this familial macular degeneration is segregating as an autosomal dominant trait.[17]

AMD is currently considered a multifactorial disorder involving both environmental and genetic factors. Recent studies have substantiated the evidence for AMD as a complex genetic disorder in which one or more genes contribute to an individual's susceptibility to the development of the disease.[18-20] To date, full-genome scan studies have indicated that some regions of the genome harbor AMD-predisposing genes.[21,22] However, most genes associated with susceptibility to AMD have not been identified, presumably because of a complex pattern of inheritance, late age of onset, and difficulties in obtaining large pedigrees for standard linkage analysis. Genes implicated in monogenic macular dystrophies that occur earlier in life with a clear pattern of inheritance have been considered as good candidates for susceptibility to AMD.[23-26] To date, 15 macular degeneration genes have been linked or cloned for human macular degeneration (RetNet; http://www.sph.uth.tmc.edu/Retnet/home.htm; provided in the public domain by University of Texas Houston Health Science Center, Houston, TX). However, with the exception of *ABCA4*, none of these genes has shown a convincing association with AMD.

Because the monkey macular degeneration model we present here shares phenotypic similarities with the early stages of AMD, the identification of the gene involved in this monkey pedigree may provide critical clues to the understanding of the mechanism of AMD. In this study, monkey orthologues of the human genes responsible for Stargardt macular degeneration 1 (*ABCA4*),[2] Best macular degeneration (*VMD2*),[3,7] Doyn honeycomb dystrophy (*EFEMP1*),[4] Sorsby fundus dystrophy (*TIMP3*),[5] and Stargardt macular degeneration 3 (*ELOVL4*)[6,8] were cloned and screened for mutations in the affected monkeys. Subsequently, 13 human macular degeneration loci, including these five genes, were analyzed to test for linkage with the disease in the pedigree. During this process, we evaluated the nature and utility of human microsatellite markers in the cynomolgus monkey for linkage studies. This article also describes the gene structure and evolutionary conservation of the five human macular degeneration genes in the cynomolgus monkey.

Materials and Methods

Maintenance of Monkeys

The cynomolgus monkeys in the pedigree with macular degeneration were reared at the Tsukuba Primate Center for Medical Science (National Institute of Infectious Diseases; Tokyo, Japan). All monkeys were treated in accordance with the rules for care and management of animals at the Tsukuba Primate Center[27] under the Guiding Principles for Animal Experiments using Non-Human Primates formulated and enforced by the Primate Society of Japan (1986). All experimental procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases of Japan. These animal protocols fulfill the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Clinical Studies

Fundus photographs, fluorescein angiography (FA), and indocyanine green angiography (IA) were performed with a fundus camera (TRC50; Topcon, Tokyo, Japan) in animals under anesthesia. Electroretinography (ERG) was recorded in four affected and six normal monkeys with a white/color LED stimulator and contact lens electrode (LS-W; Mayo, Aichi, Japan). After 20 minutes of dark adaptation, rod ERG, combined ERG, and oscillatory responses were recorded, and single-flash cone response and 30-Hz flicker ERG were recorded after 10 minutes of light adaptation. The stimulus and recording conditions conformed to the standards for clinical electroretinography recommended by the International Society for Clinical Electrophysiology of Vision.[28]

Genomic DNA and RNA Isolation

Peripheral blood was collected from 19 affected and 11 unaffected monkeys from the pedigree (Fig. 1, asterisks, pound signs) and an

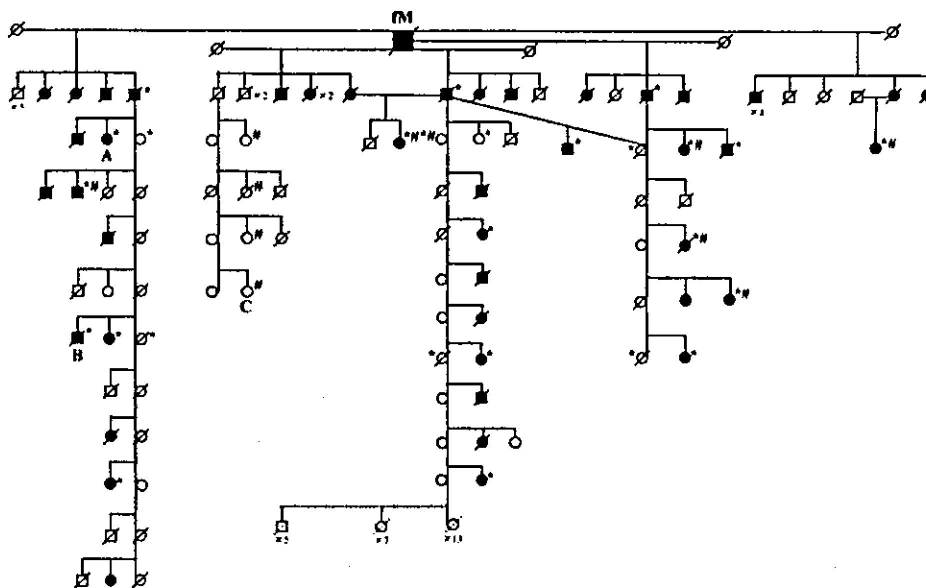

Figure 1. Edited version of the monkey pedigree with macular degeneration: fM, the founder breeding male monkey with typical macular degeneration, is shown with five healthy mates arrayed horizontally. The first-generation offspring are also arrayed horizontally. The breeding members from each branch of the first generation offspring are arrayed vertically with their mates and progeny. Monkeys used for *linkage analysis and #mutation screening are marked.

Table 1. Primer Sets Used for Cloning of the Monkey Homologues

Gene	Amplified Region	Amplified Name	Forward Primer	Position	Name	Reverse Primer	Position	Size (kb)
VMD2	Exon 1	P1F	GACCAGAAACCAGGACTGTTGA	Intron	P1R	GAACTCGCCATATAGCAGCTT	Exon 2	2.1
	Exon 2	P2F	GCTCTGACCAGGGTCTCTGA	Intron	P3R	CCGCACCTTTCCCTGAACTA	Intron	4.5
	Exon 3	P3F	CTAGACCTGGGGACAGTCTCA	Intron	P3R	CCGCACCTTTCCCTGAACTA	Intron	0.3
	Exon 4-5	P4F	CACGGAAGAACAACAGCTGA	Exon 3	P5R	ACACCAGTGGGATACTAATCCAG	Exon 6	2.3
	Exon 6	P6F	GCCAGGAATGGACCATGAGTA	Intron	P6R	GAGCCACTTAGCCTCTAGGTGA	Intron	0.3
	Exon 7-8	P7F	CCTGGAGCATCCTGATTTCA	Intron	P8R	TGAGGCCTCCCTACAGAACA	Intron	2.3
	Exon 9	P9F	TGGCAGAGCAGCTCATCA	Exon 8	P9R	AGCTTCCAGGCCTTGTTG	Exon 10	3.0
	Exon 10	P10F	AAGGGAGAAGGCCAGGTGTT	Intron	P10R	TTTCCTGTAGTGCTTGGGTACTA	Intron	1.2
	Exon 11	P11F	TGCCCTCCTACTGCAACATT	Intron	P11R	ATGCAATGGAGTGTGCATTA	Intron	1.1
EFEMP1	Exon 1	P1F	TTCTAGAACCCTCTGGTCTCTGA	Intron	P1R	CCCTTTCTTAACAGCAAGCTAAC	Intron	0.9
	Exon 2	P2F	GATTGGAAGTTGAGTATGGTGGA	Intron	P2R	CATTCTAGGGATAATGTGGTACCAA	Intron	1.3
	Exon 3-4	P3F	AAGATGGTACTGGGCAACTGTAC	Intron	P4R	ACATCTGTAGAGTAGCTTGACAGCA	Intron	1.4
	Exon 5	P5F	CTACACAGGCTAGAGGAATATGATCA	Intron	P5R	GACACAGGATTTAAGTAACTTGCTCA	Intron	1.3
	Exon 6-7	P6F	CACTGAATGGCATGAACATTG	Intron	P7R	TAGAACAGAATTCCCATGGGTAA	Intron	1.6
	Exon 8	P8F	AATAGGACAAGAAGCCAGATCTCT	Intron	P8R	TTCCTGGTTAAAACTAAATACCTAACA	Intron	0.4
	Exon 9-10	P9F	AACAGATGAACAATAGGTGCTTGA	Intron	P10R	TATCTATCTGGCAGTGTTACCAAGA	Intron	0.9
	Exon 11	P11F	GTATTAGACAAGGGATAAGAGCCAA	Intron	P11R	CAGAGGTTATGCATATATGCTGTGA	Intron	1.7
TIMP3	Exon 1	P1F	CCCAGCGCTATATCACTCG	Intron	P1R	AGCCACTGTGAGTTTCCTCTG	Intron	0.7
	Exon 2	P2F	CAATGGCTCTAACAGGAGAAGTAG	Intron	P2R	CTTGACCAAGGTCTCATGGTTA	Intron	0.8
	Exon 3-4	P3F	TCCAGTTCCAGCTGCATTG	Intron	P4R	AGTTAGTGTCCAAGGGAAGCT	Exon 5	2.6
	Exon 5	P5F	ATGTACCGAGGCTTCACCAA	Exon 3	P5R	AGGTGAGCTAAACACTATTCTGGA	Intron	3.5

additional six unrelated normal monkeys, and genomic DNA was extracted (QIAamp DNA Blood Maxi Kit; Qiagen, Valencia, CA). A normal monkey outside the pedigree was killed for bilateral eye enucleation, and enucleated eyes were immersed and stored in RNA-stabilization solution (RNAlater; Ambion, Austin, TX) at −80°C until RNA isolation. After thawing on ice, the eyeballs were dissected to separate the neural retina and choroid followed by extraction of total RNA.

Histologic Studies

An affected 14-year-old male monkey (Fig. 1, monkey B) was killed for histologic studies. Enucleated eyes were fixed in 10% neutralized formaldehyde solution at 4°C overnight, dehydrated, and embedded in paraffin. Four-micrometer-thick sections were prepared and stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS). Serial sections were used for immunohistochemical analysis with anti-complement 5 (C5) antibody. After pretreatment with 0.4 mg/mL proteinase K in phosphate-buffered saline (PBS) for 5 minutes and blocking with 5% skim milk in PBS for 20 minutes at room temperature, the sections were incubated with rabbit anti-human C5 polyclonal antibody (Dako, Glostrup, Denmark) diluted to 1:200 dilution in PBS for 2 hours at room temperature. Alexa 488 - conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR), diluted to 1:200 in PBS, was used as the secondary antibody. The negative control experiments were performed using normal rabbit immunoglobulin fraction (Dako) instead of anti-C5 antibody.

Characterization of the Genomic Organization and cDNA Sequence of the Monkey *ABCA4*, *VMD2*, *EFEMP1*, and *TIMP3* Genes

Gene-specific primers of the human macular degeneration genes *ABCA4*, *VMD2*, *EFEMP1*, and *TIMP3* were designed based on the human genomic DNA sequence to amplify exons of monkey genes (Table 1). Amplified products were directly sequenced. For all genes except *ABCA4*, the 5′/3′-rapid amplification of cDNA ends (5′/3′-RACE) was performed using total RNA isolated from the monkey retina. Amplification of partial cDNAs by both 5′- and 3′-RACE was designed to generate overlapping PCR products to obtain a full-length cDNA sequence. Primers were initially designed based on the exonic sequences obtained by genomic sequence (Table 2). RACE products were subcloned into the pCRII cloning vector (TA Cloning Kit Dual Promoter; Invitrogen, Carlsbad, CA) and sequenced directly. The obtained nucleotide sequence data have been submitted to GenBank, and assigned accession numbers: *TIMP3*: AY207381-207385, AH012631; *EFEMP1*: AY312407-312415, AH012997; *VMD2*: AY357925-357936, AH013172; *ELOVL4*: AF461182-461187, AH012403; *ABCA4*; AY793687 (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

Mutation Analysis

Coding regions and adjacent intronic sequences of the monkey *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* genes were analyzed for sequence variants by single-strand conformation polymorphism (SSCP) or denaturing (D)HPLC (for the *ABCA4* gene) analysis in parallel with direct sequencing. Genomic DNA from six affected and five unaffected monkeys from the pedigree (Fig. 1, pound signs) and six unrelated normal subjects were used for mutation analysis. Primers located in the intronic regions were designed to amplify coding sequences of individual genes (Table 3). Large exons were divided into smaller segments to obtain amplification products suitable for SSCP analysis. The purified amplicons were analyzed by SSCP or DHPLC analysis, as previously described.[29,30] All the samples were also analyzed by bidirectional sequencing with the PCR primers. Exons 2, 7, and 10 of the *VMD2* gene were screened for sequence variants only by direct sequencing.

Table 2. Primers for 5′-3′-RACE

Gene	5′-RACE	Position	3′-RACE	Position
VMD2	GTATACACCAGTGGGATA	Exon 6	AGAGCAACAGCTGATGTTTGAGAA	Exon 3
EFEMP1	GGATGGTACATTCATCTA	Exon 7	GATCCTGTGAGACAGCAATGCA	Exon 3
TIMP3	ATCATCTGGGAAGAGTTA	Exon 5	GATGAAGATGTACCGAGGCTTCA	Exon 2-3

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TABLE 3. Primer Sets Used for Mutation Screening

Gene	Exon No.	Length (bp)	Name	Forward Primer	Name	Reverse Primer	Size (bp)
ABCA4	1	66	01F	TCTTCGTGTGGTCATTAGC	01R	ACCCCACACTTCCAACCTG	152
	2	91	02F	AAGTCCTACTGCACACATGG	02R	CTAGACAAAAGGCCCAGACC	266
	3	142	03F	TTCCCAAAAAGGCCAACTC	03R	CACGCACGTGTGCATTTCAG	301
	4	139	04F	GCTATTTCCTTATTAATGAGGC	04R	GGGAAATGATGCTTGAGAGC	212
	5	128	05F	CCCTTCAACACCCTGTTCTT	05R	TTCTTGCCTTTCTCAGGCTGG	237
	6	198	06F	GTATTCCCAGGTTCTGTGG	06R	TACCCCAGGAATCACCTTG	330
	7	88	07F	AGCATATAGGAGATCAGACTG	07R	GGCATAAGAGGGGTAAATGG	241
	8	238	08F	GAGCATTGGCCTCACAGCAG	08R	CCCCAGGTTTGGTTTCACC	397
	9	139	09F	AGACATGTGATGTGGATACAC	09R	GTGGGAGGTCCAGGGTACAC	271
	10	117	10F	AACACTAAGTGATAGGGGCAGAA	10R	GGCCTGCTTGTTGTATTTTGAT	344
	11	198	11F	AGCTCACTCGCTCTTTAGGG	11R	TTCAAGACCACTTGACTTGC	406
	12	206	12F	TGGGACAGCAGCCCTTATC	12R	CCAAATGTAATTTCCCACTGAC	362
	13	177	13F	AATGAGTTCCGAGTCACCCTG	13R	CCCATTAGCGTGTCATGG	308
	14	223	14F	TCCATCTGGGCTTTGTTCTC	14R	AATCCAGGCACATGAACAGG	407
	15	222	15F	AGACAGTAACTAACAGGCTCGTG	15R	GGACTGCTACAGACCCTTCC	386
	16	205	16F	CTGTTGCATTGGATAAAAGGC	16R	GATGAATGGAGAGGGCTGG	330
	17	65	17F	CTGCGGTAAGGTAGGATAGGG	17R	CACACCGTTTACATAGAGGGC	232
	18	90	18F	CAGCTCCCGGTGGTAGAGTA	18R	CCCTTGCCATGAGATGTTTT	222
	19	175	19F	TGGGGCCATGTAATTAGGC	19R	TGGGAAAGAGTAGACAGCCG	322
	20	132	20F	GCATGTTGCTAAAGGCCATC	20R	TATCTCTGCCTGTGCCCAG	293
	21	140	21F	GTAAGATCAGCTGCTGGAAG	21R	GAAGCTCTCCTGCTCCAAGC	301
	22	138	22F	CCCTCCACAGTCCCTTAACTC	22R	GAGAGTGGGGACCACAGGTA	244
	23	194	23F	TTTTGCAACTATGTAGCCAGGA	23R	AGCCTGTGTGAGTAGCCATG	384
	24	85	24F	GCATCAGGGAGAGGCTGTC	24R	CCCAGCAATATTGGGAGATG	212
	25	206	IVS24F	GTAAGGACTGGACGGGCCATACTTGG	IVS24R	TCCAGCTCTCTGAAAAGGCTGGCATA	2 kb
			IVS25F	AAAGCTGGTGGAGTGCATTGGTCAAG	IVS25R	CCTGAATCAGAATCCTCCGTGACCTTC	500
	26	49	26F	TCCCATTATGAAGCAATACC	26R	ACCCAGCCCCTTAGACTTTC	228
	27	266	IVS26F	GGATTCTGATTCAGGACCTCTGTTTGC	IVS26R	CTGCGGATGGTGTGTTGGAATCTCTT	2 kb
			IVS27F	TCCCAGAGAGAAGGCTGGACAGACAC	IVS27R	CCCATATATCCAGGGGTGAAGGGTCA	1 kb
	28	125	28F	TGCACGCGCACGTGTGAC	28R	TGAAGGTCCCAGTGAAGTGGG	291
	29	99	29F	CAGCAGCTATCCAGTAAAGG	29R	AACGCCTGCCATCTTGAAC	263
	30	187	30F	GTTGGGCACAATTTCTTATGC	30R	ACTCAGGAGATACCAGGGAC	347
	31	95	IVS30F	GAGAAGCTCACCATGCTGCCAGAGT	IVS30R	GAGATGTTCCTGTCCGTCAGGTCTTG	2 kb
			IVS31F	CGCAGCACGGAAATTCTACAAGACCT	IVS31R	CCTCTGTTCATTGACCCAGAATTTGCT	700
	32	33	32F	ACGGCACTGCTGTACTTGTG	32R	TCAACATGGCTGTGAGGTGT	182
	33	106	IVS32F	GAGCAAATTCTGGGTCAATGAACAGAGG	IVS32R	CGCTTAAAAACCCAACAAGTGCTTCC	1.2 kb
			IVS33F	AGGTATGGAGGAATTTCCATTGGAGGA	IVS33R	CTTTAGAGGCCTCTCTAGTGATAGG	300
	34	75	34F	AAACCGTCTTGTTTGTTTGTTT	34R	AGGAGGGAGGGAATTCAATG	208
	35	170	IVS34F	GGCCCTATCACTAGAGAGGCCTCTAAAG	IVS34R	GGTTGGCTAATGACGGTGATTCCATAC	550
			IVS35F	CATGCCCTGGTCAGCTTTCTCAATGT	IVS35R	GAGAAAATCACGCAGATGGCAACCAC	2 kb
	36	178	36F	TGTAAGGCCTTCCCAAAGC	36R	TGGTCCTTCAGAGCACACAC	346
	37	116	37F	CATTTTGCAGAGCTGGCAGC	37R	CTTCTGTCAGGAGATGATCC	260
	38	158	38F	GGAGTGCATTATATCCAGACG	38R	CCTGGCTCTGCTTGACCAAC	302
	39	125	39F	TGCTGTCCTGTGAGAGCATC	39R	CTTCCAGCCCAACAAGGTC	344
	40	130	IVS39F	CTGCTCATTGTCTTCCCCCACTTCTG	IVS39R	CAGCAGGGTCAGGAGGAAGTACACCA	700
			IVS40F	GTGAGGAGCACTCTGCAAATCCGTTC	IVS40R	AGATGAGGAAAAGGGGTCAGGATTGG	3.5 kb
	41	121	41F	GAAGAGAGGTCCCATGGAAAGG	41R	GCTTGCATAAGCATATCAATTG	299
	42	63	42F	CTCCTAAACCATCCTTTGCTC	42R	AGGCAGGCACAAGAGCTG	214
	43	107	43F	GGTCTCTAGGGCCAGGCTA	43R	CACATCTTTCAGGGCCTCAG	271
	44	142	44F	GAAGCTTCTCCAGCCCTAGC	44R	TGCACTCTCATGAAACAGGC	277
	45	135	IVS44F	ACATCTTTACCTTTATGCCCGGCTTCG	IVS44R	AATGAGTGCGATGGCTGTGGAGAGTT	4 kb
			IVS45F	TTAAGAGCCTGGGCCTGACTGTCTACG	IVS45R	GAATCTCTTGCCTGTGGGATGTGAGG	1 kb
	46	104	48F	GAAGCAGTAATCAGAAGGGC	46R	GCCTCACATTCTTCCATGCTG	257
	47	93	47F	TCACATCCCACAGGCAAGAG	47R	TTCCAAGTGTCAATGGAGAAC	258
	48	250	48F	ATTACCTTAGGCCCAACCAC	48R	ACACTGGGTGTTCTGGACC	365
	49	87	49F	GGTGTAGGGTGGTGTTTTCC	49R	ACTGCCTCAAGCTGTGGACT	187
VMD2	2*	152	P2F	GCTCTGACCAGGGTCTCTGA	P3R	CCGCACCTTTCCCTGAACTA	4.5 kb
	3	95	P3F	CTAGACCTGGGGACAGTCTCA	P3R	CCGCACCTTTCCCTGAACTA	325
	4	234	MP4aF	TGGGAGACAGAACCCTTGGA	MP4aF	GTCCTTGCCTTCCACGAA	302
			MP4bF	TGGTGGAACCAGTACGAGAA	MP4bF	TCCACCCATCTTCCATTGTT	286
	5	155	MP5F	AAAGGAGTGCTGAGGTTCCTATA	MP5R	CTTGTTTCCTGTGAACCACAA	330
	6	78	P6F	GCCAGGAATGGACCATGAGTA	P6R	GAGCCACTTAGCCTCTAGGTGA	292
	7*	153	P7F	CCTGGAGCATCCTGATTTCA	P8R	TGAGGCCTCCCTACAGAACA	2.3 kb
	8	81	MP8F	GCATCATGTGGTGTGGAAAT	P8R	TGAGGCCTCCCTACAGAACA	270
	9	152	MP9F	CAAGTCATCAGGCACGTACAA	MP9R	CTAGGCAGACCCCTGCTACTA	286
	10*	639	P10F	AAGGGAGAAGGCCAGGTGTT	P10R	TTTCCTGTAGTGCTTGGGTACTA	1.2 kb
	11	19	P11F	TGCCCTCCTACTGCAACATT	MP11R	AAGTAGTCCTGGACTGCTGATTT	270
EFEMP1	2	81	MP2F	CCGCAGCAGATACTAAATATCAG	MP2R	CCGCTGAACCGTACTTATTTC	173
	3	49	MP3F	CTTAGGGAATGGACACACCAA	MP3R	ACAGAAGGCCAAAGATCACAT	155

(*continues*)

TABLE 3. (*continued*).

Gene	Exon No.	Length (bp)	Name	Forward Primer	Name	Reverse Primer	Size (bp)
	4	387	MP4aF	CCCTCTTAGAAGATTCCTGACTTA	MP4aR	ACACTCCACTGGTTGCCAT	249
			MP4bF	ATGAACAGCCTCAGCAGGA	MP4bR	GCAAAAGCTTTCGATGGTTA	316
	5	123	MP5F	GGAGGCAATATCAACATCTTCA	MP5R	TGCTTGAGGTTGAAACAGTTAAG	248
	6	120	MP6F	GCAAACAGCAATGCTAATTCA	MP6R	GAAATACTGCAACATGGCATG	250
	7	120	MP7F	CAGCTAGGGAATTATTTATCAGCA	MP7R	CAGGGATTGGACTTTATTCCA	279
	8	120	MP8F	ATATCCAAAGTAGTGGTGCACAA	P8R	TTCCTGGTTAAAACTAAATACCTAACA	235
	9	124	MP9F	TGCAAACAGAATCTGCCAGTA	MP9R	TTTGGCTTGGTAAGACCAGAA	265
	10	196	MP10F	CTTACCAAGCCAAACTGCTAACTA	MP10R	AACAAACTCCCATCTTTCTCAATAG	289
	11	162	MP11F	AAAGCATAGAAACTCCAATGCA	MP11R	AGGTAACAATATTCTTTGGCTGACT	281
ELOVL4	1	100	MP1F	CCGCGGTTAGAGGTGTTC	MP1R	GAGACCAGGGGTCGGTGAC	281
	2	188	MP2aF	TTGAGACATCTTGATTCCTAGAAAG	MP2aR	AAGTTAAGCAAAACCATCCCA	252
			MP2bF	CTGGGTCCAAAGTGGATGAA	MP2bR	AGCTAACAGTTATGTCTGGGTACAA	213
	3	81	MP3F	GCAATTGGAATGCATGACA	MP3R	TTTCACAGATTGGGGCCTATA	304
	4	172	MP4aF	AAATGATTCCATGCCTTGTACA	MP4aR	AACGCAAGCAGTATATTCCTGA	330
			MP4b	TGGTGTTTATAACACGCTTTCC	MP4bR	CTCATTGCTTTCCACTGAACA	271
	5	128	MP5F	ATCTCGGTGGCTTACTGCTTA	MP5R	AATAAGTCGGCTGGAGTCAACT	356
	6	276	MP6aF	TTGGGCCTGTGATAGCTATG	MP6aR	TTAGGCTCTTTGTATGTCCGAA	247
			MP6bF	CTCTAATTGCCTACGCAATCAG	MP6bR	GGGAGTTTTTCCTCACTGTCA	242
TIMP3	1	121	MP1F	AACTTTGGAGAGGCGAGCA	MP1R	CCTAAGCAGCGCTGCAGTC	233
	2	83	MP2F	TGAGATGCTGTTCCTGATGTG	MP2R	GGCTGGTGCTTAGACACACA	266
	3	112	MP3F	AGCAGTGGGATTATGGATCATAC	MP3R	ACATTTGGTGAGTCAGCTACTCA	267
	4	122	MP4F	TGGGCTAAGTGGGAACATAGTA	MP4R	GTTTCTAGGGCTGCAAGTCA	274
	5	198	MP5F	TACCATGGCAGATTCCATCA	MP5R	AGTTAGTGTCCGAGGGAAGCT	306

* Exon 2, 7, and 10 of the *VMD2* gene were screened for sequence variants only by direct sequencing.

Linkage Analysis

Linkage analysis was performed on DNA from 19 affected and 7 unaffected members of the pedigree. Individuals used for the analysis are indicated by asterisks in Figure 1. Human microsatellite markers linked to human macular degeneration loci were analyzed with monkey genomic DNA used as the template. Details of microsatellite markers and their primer sequences were obtained from the genome database. Microsatellite marker analysis was performed by two methods: Markers linked to candidate gene loci and included in a linkage mapping set (ver. 2.5MD10; Applied Biosystems, Inc. [ABI], Foster City, CA) were analyzed on the a DNA sequencer (model 3100; ABI) with fluorescence-labeled primers. Additional microsatellite markers were analyzed by ^{32}P dCTP incorporation into the amplified product.[31] Two-point linkage analysis was performed between the disease locus and microsatellite markers with the MLINK program of the LINKAGE package, as described elsewhere.[32,33] Linkage was assessed under the conditions of autosomal dominant inheritance of the disease trait with a frequency of 0.001 for the disease-causing allele, by using the affecteds-only model, as published earlier.[34] Linkage analysis was performed assuming equal frequencies for marker alleles. Haplotypes were constructed with genotypes of microsatellite markers according to their order on human chromosomes.

RESULTS

Clinical and Histologic Findings

Fundus photographs and FA of a 14-year-old female affected monkey (Fig. 1, monkey A) are shown in Figure 2. Fine, yellowish white dots were observed in the maculae (Figs. 2a–d), scattered in the peripheral retina along blood vessels in this monkey (Figs. 2a, 2b). However, in most cases, the locations of the lesions fell within the region centered on the fovea centralis with the same diameter as one optic disc. FA showed hyperfluorescence corresponding to these dots, except foveola (Figs. 2e, 2f). No abnormalities were found in the optic disc, retinal blood vessels, or choroidal vasculatures in any eyes examined. The amplitude and peak latency of both dark- and light-adapted ERG showed no alteration compared with normal control eyes, indicating that global rod or cone degeneration was absent. Histologic studies demonstrated that there were various-sized drusen, weakly stained by PAS (light purple), between the RPE and choriocapillaris in the macular region (Figs. 3a, 3b, asterisk). These drusen were strongly reactive with antibodies against complement C5 (Figs. 3c, 3d). This finding was consistent with the property of drusen reported in patients with AMD.[35] Accumulation of lipofuscin in RPE cells was also obvious by PAS (Figs. 3a, 3b, deep purple, arrows).

Mutation Analysis of the *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* Genes

To evaluate the involvement of the *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* genes in disease, we first determined the genomic sequence and the complete cDNA sequence of the orthologous genes in the monkey. Subsequently, these genes were screened for sequence variants in affected and unaffected monkeys in the pedigree, in addition to unrelated, unaffected animals by SSCP, or by DHPLC for the *ABCA4* gene, analysis and direct sequencing.

***ABCA4*.** The monkey *ABCA4* gene consists of 50 exons, with its translation stop codon in exon 50, similar to the human gene. The complete 6819-bp cDNA encodes a protein of 2273 amino acids. *ABCA4* is a member of the superfamily of ATP-binding cassette (ABC) transporters, which are associated with membranes and transport various molecules across extra- and intracellular membranes of all cell types. ABC genes typically encode four domains that include two conserved ATP-binding domains and two domains with multiple transmembrane segments. Comparative sequence analysis revealed that the monkey ABCA4 protein was only 1.8% (41 amino acids) different from the human orthologue, whereas the sequence was identical in the two adenosine triphosphate (ATP)-binding domains. Five of the 41 nonconserved amino acids in the monkey protein (codons 223, 423, 1300, 1817, and 2255) involve polymorphisms in the human. Surprisingly, the Lys223Gln and Arg1300Gln changes reported to be associated with Stargardt disease in humans were observed in the homozygous state in

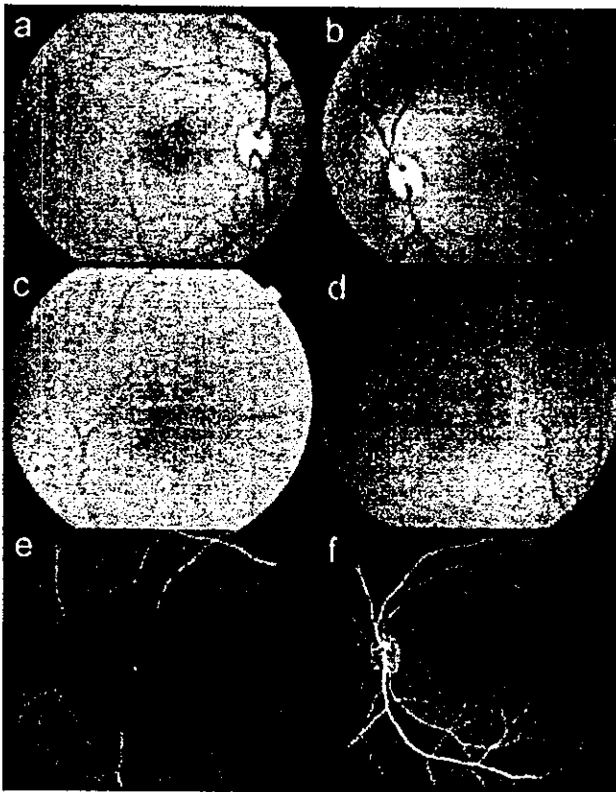

FIGURE 2. Fundus photographs and fluorescein angiogram (FA) of a 14-year-old female cynomolgus monkey (Fig. 1, monkey A) with macular degeneration, showing the right (a, c, e) and left (b, d, f) posterior poles. Fine *grayish white* or *yellowish white* dots were visible in the macula (a–d). The dots were observed in the peripheral retina along blood vessels in this monkey (a, b). These dots showed hyperfluorescence in FA except in the foveola (e, f). High-magnification of the macular region (c, d, e).

one normal control monkey (Fig. 1, monkey C). In addition, the mutation analysis revealed heterozygous amino acid changes at five positions—Leu424Val, Arg1017His, Val1114Ile, Ile1615Val, and Pro2238Gln—in both affected and normal monkeys. However, these missense variants did not segregate with the disease phenotype.

VMD2. The monkey *VMD2* gene consists of 11 exons, with its translation initiation codon in exon 2, as observed in its human orthologue. The complete cDNA was 2187 bp, encoding 585 amino acids. The *VMD2* gene encodes the bestrophin protein, which localizes to the basolateral plasma membrane of the RPE with the postulated function as an oligomeric chloride channel.[36,37] The hydropathy profile predicted that bestrophin contains four stretches of hydrophobic amino acids that function as transmembrane domains. Comparative sequence analysis demonstrated that monkey bestrophin had 19 amino acids different from its human homologue, and the four putative transmembrane domains are highly conserved. To date, 72 disease-associated nucleotide substitutions of the *VMD2* gene have been identified in patients with Best disease.[3,7,26] The mutation analysis of the *VMD2* gene in the monkey pedigree detected six amino acid sequence variants. A polymorphism (Val/Ile) was detected at codon 275 in the fourth transmembrane domain, which has also been reported in humans.[26] Four polymorphisms (Tyr465His, Thr542Met, Glu557Gln, and Thr566Ala) were detected in exon 10. These changes did not segregate with the disease. In addition, one nonsense mutation at codon 582 (Glu→Stop) in exon 11 was detected in two normal monkeys, whereas none of the examined six affected monkeys showed the change.

EFEMP1. The exon-intron gene structure of the monkey *EFEMP1* gene was also similar to the human *EFEMP1* gene. It was composed of 11 exons with its translation initiation codon in exon 2. The complete cDNA was 2034 bp, encoding 493 amino acids. Although the function of this gene remains unclear, this class of proteins is known to have characteristic sequence of repeated calcium-binding EGF-like domains.[4] The monkey *EFEMP1* cDNA was found to have six EGF repeats. Four EGF repeats (numbers 2–5) are encoded by single exons (exons 5–8), one EGF repeat (number 1) is encoded by three exons (exons 2–4), and EGF repeat number 6 is encoded by two exons (exons 9, 10). This finding is in agreement with one of the two transcriptional variants with a distinct 5′ untranslated region (UTR) described in its human homologue. Comparative sequence analysis demonstrated that the monkey *EFEMP1* has three amino acids different from that of the human, but the sequence in the entire region of six EGF repeats is completely conserved. In humans, a single mutation (Arg345Trp) that disrupts one of these domains is known to cause Malattia Leventinese.[4] No amino acid–changing polymorphisms were found in all the monkeys tested. Three single nucleotide polymorphisms (SNPs), that did not alter the amino acid sequence, were detected in exons 4, 5, and 10.

TIMP3. The monkey *TIMP3* gene consisted of five exons, similar to its human orthologue. The complete cDNA was 1887 bp in length, encoding 211 amino acids. *TIMP3* is the third member of the tissue inhibitors of metalloproteinase family, a group of zinc-binding endopeptidases involved in the degradation of the extracellular matrix. *TIMP3* has 12 cysteines characteristic of the TIMP family, which are proposed to form intramolecular disulfide bonds and tertiary structure for the functional properties of the mature protein. The predicted amino acid sequence of the monkey *TIMP3* gene was identical with the human orthologue, including the 12 cysteine residues. Mutations in the *TIMP3* gene are known to cause Sorsby's fundus dystrophy.[5] With a few exceptions,[38,39] most previously described mutations disrupt the disulfide bonds by changing residues into cysteines, leading to misfolding of the protein.[5,40] No coding sequence changes were detected in the *TIMP3* gene in monkeys by mutation screening.

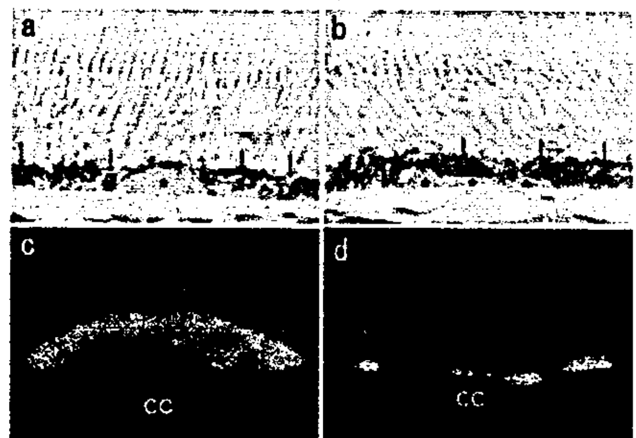

FIGURE 3. Drusen in the affected monkey retina. An affected 14-year-old male monkey (Fig. 1, monkey B). There were various-sized drusen, which were weakly stained by PAS (✱), between the RPE and choriocapillaris (CC) (a, b). These drusen were strongly reactive with antibodies against complement C5 (*green* channel). Lipofuscin autofluorescence is shown (*red*) in the RPE (c, d). Accumulation of lipofuscin in RPE cells was also obvious by PAS (a, b, *arrows*).

Table 4. Two-Point Lod Scores between the Monkey Macular Degeneration Locus and Markers at the Human Macular Degeneration Loci

Markers	Distance from the Gene (CM)	Order on the Chromosome (M)	Lod Scores at θ									Exclusion ($Z = -2$)
			0	0.001	0.005	0.01	0.05	0.1	0.2	0.3	0.4	
CORD8		154.28										
D1S431	10.5	165	$-\varepsilon$	−2.116	−1.422	−1.128	−0.483	−0.248	−0.071	−0.01	0.006	0.001
D1S2635	0	154.28	$-\varepsilon$	−11.078	−7.598	−6.112	−2.773	−1.469	−0.392	0.019	0.119	0.075
D1S2715	−6.9	147.01	$-\varepsilon$	−7.7	−4.925	−3.747	−1.162	−0.232	0.388	0.464	0.299	0.03
D1S498	−10.6	144.94	$-\varepsilon$	−1.124	−0.439	−0.154	0.416	0.564	0.567	0.433	0.227	0.0001
ABCA4		94.1										
D1S188	−2.3	91.7	$-\varepsilon$	−6.139	−4.058	−3.175	−1.24	−0.541	−0.05	0.074	0.066	0.01
D1S2849	−1.2	92.9	$-\varepsilon$	−1.766	−1.075	−0.784	−0.166	0.032	0.133	0.119	0.067	
D1S2868	0.1	94	$-\varepsilon$	−14.824	−10.623	−8.809	−4.599	−2.846	−1.264	−0.522	−0.146	0.1
STGD3		80.5										
D6S1662	−2.67	77.83	$-\varepsilon$	−1.232	−0.544	−0.257	0.324	0.476	0.472	0.34	0.17	0.0
D6S1048	0.28	80.78	$-\varepsilon$	−0.063	0.614	0.889	1.38	1.416	1.172	0.79	0.362	0.0
D6S1596	7.1	87.6	$-\varepsilon$	−8.746	−5.965	−4.78	−2.138	−1.127	−0.319	−0.025	0.049	0.05
D6S1609	12.08	92.58	$-\varepsilon$	−7.326	−5.235	−4.34	−2.302	−1.475	−0.724	−0.349	0.131	0.05
DHRD		56.1										
D2S2230	3.9	60	$-\varepsilon$	−11.691	−8.209	−6.719	−3.349	−2.006	−0.842	−0.325	−0.084	0.1
D2S378	1.1	57.2	$-\varepsilon$	−9.268	−6.482	−5.29	−2.593	−1.517	−0.588	−0.186	−0.019	0.05
ARMD1		192.2										
D1S384	−2.11	190.09	$-\varepsilon$	−5.565	−3.486	−2.606	−0.696	−0.032	0.375	0.389	0.236	0.01
D1S413	2.1	194.1	$-\varepsilon$	−11.068	−7.59	−6.106	−2.784	−1.501	−0.46	−0.067	0.047	0.05
D1S2622	3.7	195.9	$-\varepsilon$	−1.961	−1.271	−0.982	−0.375	−0.185	−0.084	−0.066	−0.047	0.0
VMD2		61.5										
D11S1993	−2.3	59.2	$-\varepsilon$	−1.615	−0.925	−0.636	−0.032	0.151	0.224	0.181	0.1	0.0
D11S4174	1.4	62.9	$-\varepsilon$	−7.132	−5.026	−4.112	−1.979	−1.102	−0.368	−0.087	0.003	0.01
D11S4076	7.3	66.8	$-\varepsilon$	−5.617	−3.537	−2.656	−0.736	−0.061	0.364	0.385	0.231	0.01
Rhodopsin		130.6										
D3S3515	−4.01	126.59	$-\varepsilon$	−2.756	−1.379	−0.803	0.383	0.717	0.775	0.584	0.302	0.001
D3S3720	−2.8	127.8	$-\varepsilon$	−2.626	−1.247	−0.67	0.531	0.879	0.945	0.729	0.389	0.001
D3S1269	0.3	130.9	$-\varepsilon$	−11.566	−8.081	−6.588	−3.2	−1.846	−0.7	−0.238	−0.062	0.05
Timp3		31.5										
D22S1162	7.05	38.55	$-\varepsilon$	−3.587	−2.203	−1.619	−0.365	0.055	0.291	0.276	0.159	0.005
D22S280	0	31.5	$-\varepsilon$	−4.051	−2.664	−2.075	−0.785	−0.321	−0.002	0.065	0.044	0.01
D22S273	−1	30.5	$-\varepsilon$	−1.878	−1.187	−0.896	−0.278	−0.078	0.026	0.025	0.004	0.0
CTRP5		118.7										
D11S4127	−1.6	117.1	$-\varepsilon$	−0.771	−0.088	0.192	0.73	0.827	0.719	0.495	0.244	0.0
D11S924	0.2	118.9	$-\varepsilon$	−1.424	−0.736	−0.449	0.137	0.298	0.322	0.232	0.113	0.0
D11S4129	4.48	121.58	$-\varepsilon$	−9.057	−6.275	−5.089	−2.435	−1.41	−0.566	−0.214	−0.054	0.05
STGD4		26.1										
D4S403	0	26.1	$-\varepsilon$	−16.798	−11.919	−9.83	−5.081	−3.159	−1.445	−0.633	−0.206	0.1
D4S391	1.2	27.3	$-\varepsilon$	−3.615	−2.231	−1.647	−0.392	0.026	0.255	0.234	0.13	0.005
CORD5	(Interval)	64.5										
D17S938	0	64.5	$-\varepsilon$	−16.296	−11.422	−9.339	−4.638	−2.776	−1.176	−0.466	−0.125	0.1
D17S796	0	64.5	$-\varepsilon$	−3.594	−2.209	−1.624	−0.358	0.075	0.324	0.305	0.176	0.0
MCDR1	(Interval)	98.1										
D6S434	4.3	102.4	$-\varepsilon$	−4.496	−3.103	−2.507	−1.163	−0.632	−0.183	−0.005	0.043	0.0
CORD9	(Interval)	47.6										
D8S1820	0	47.6	$-\varepsilon$	−11.981	−8.501	−7.014	−3.65	−2.277	−1.002	−0.385	−0.092	0.1

ELOVL4. We have reported cloning and characterization of the *ELOVL4* gene in the cynomolgus monkey.[41] Three mutations leading to truncation of the *ELOVL4* protein were reported in humans with Stargardt-like macular dystrophy[23,42] (Karen G, et al. *IOVS* 2004;45:ARVO E-Abstract 1766). Mutation analysis of monkeys with macular degeneration did not detect any amino acid-altering sequence changes. Silent polymorphisms were observed in exons 1, 3, and 4 of the *ELOVL4* gene.

Linkage Analysis of Candidate Gene Loci

The methodology we used to screen for mutations in the candidate genes could miss disease-associated changes that may be present in the promoter or intronic regions; therefore, linkage analysis was performed to exclude the five genes further. Moreover, the macular degeneration phenotype in the monkey pedigree could be caused by a single gene defect. In these cases, linkage analysis would be a comprehensive approach to confirm or exclude a particular gene locus. Microsatellite markers linked to the five candidate gene loci in addition to eight human macular degeneration loci—*ABCA4*, *VMD2*, *DHRD* (*EFEMP1*), *TIMP3*, *STGD3* (*ELOVL4*), Cone rod dystrophy-8 (*CORD-8*), age-related macular degeneration 1 (*ARMD1*, gene Hemicentin1), rhodopsin, *STGD4*, North Carolina macular degeneration (*MCDR1*), *CORD9*, late-onset retinal degeneration (*CTRP5*), and *CORD5* loci—were analyzed to test for linkage with the macular degeneration in the monkey pedigree. None of the tested loci gave significant positive lod scores (Table 4). We also constructed haplotypes using the genotype data of markers at the 13 loci. This analysis further supported the exclusion of these loci from being among those that might harbor the gene associated with macular degeneration in these monkeys.

Discussion

We report a detailed description of early-onset macular degeneration in cynomolgus monkeys and the exclusion of known genes responsible for macular degeneration in humans as a disease-associated gene in this animal model. Several forms of macular degeneration have been described in humans, including autosomal dominant, autosomal recessive, and X-linked modes of inheritance. The most common form of macular disease in humans is AMD. Major clinical characteristics of AMD are loss of central vision with RPE atrophy or exudation. The presence of subretinal deposits known as drusen is one of the early signs observed in AMD and several other macular degenerations. Recent studies suggest that the process of drusen formation includes inflammatory and immune-mediated events.[35] Immunohistochemical examinations have revealed that drusen contains activated complement factors. These molecules include C5, the cleavage product of C3 (C3b, iC3b, and C3dg), and the terminal complement complex C5b-9. Clinical and histologic studies of the affected monkeys showed the presence of drusen (Figs. 2, 3). Immunologic analysis demonstrated that drusen in monkeys had C5 as a component, suggesting that the nature of monkey drusen was similar to that reported in human AMD. At the same time, the onset of the disease in monkeys is at ~2 years of age; therefore, the monkey macular degeneration resembles early-onset human macular degeneration with drusen.

Comparison of the gene maps and chromosome painting data revealed a high degree of synteny and genome conservation between human and Macaque genomes.[43,44] Amplification of cynomolgus monkey DNA with human microsatellite marker primers and sequence analysis revealed that not only the sequences flanking the microsatellite repeat regions but also the polymorphic nature of these repeats is conserved between human and monkey genomes (data not shown). Comparative studies on human and chimpanzee genomes have shown the same average heterozygosity at microsatellite marker loci and conserved genetic distance between markers.[45] Molecular cloning of monkey orthologues of the human *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* genes further demonstrated the high conservation between the human and macaque genomes not only in the organization of the gene structure, but also at the sequence level. Considering the high conservation between human and macaque genomes, human macular degeneration loci can be considered plausible candidates for identification of the gene associated with macular degeneration in the monkeys. We tested this hypothesis using microsatellite markers linked to human macular degeneration loci and successfully amplified microsatellites in the monkey DNA with human primers. However, we failed to establish linkage with the tested loci, and the subsequent haplotype analysis further confirmed this finding. Therefore, the macular degeneration locus in the monkey pedigree is not likely to be associated with the regions of the monkey genome that are syntenic to human genomic regions comprising the 13 macular disease loci tested. Mutation analysis of candidate genes also supported the exclusion of the *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* genes. The analyses detected five- and six-amino-acid substitutions in the *ABCA4* and *VMD2* genes, respectively. Some silent nucleotide substitutions or intronic sequences changes, such as small insertions/deletions, SNPs, and variations of short tandem repeats were observed in the *EFEMP1*, *TIMP3*, and *ELOVL4* genes. All these sequence variants did not segregate with the disease phenotype in the extended pedigree. Hence, these changes were interpreted as benign polymorphisms.

In the *ABCA4* sequence of a normal monkey, we found two amino acid replacements (K223Q and R1300Q) that are associated with Stargardt disease in humans. Because of the extensive conservation between the monkey and human gene sequences, one would expect these amino acid changes to have similar disease-associated effects in monkeys. One explanation of this discrepancy could be that K223Q and R1300Q are not causing the disease phenotype in humans, but rather represent markers linked to disease-causing mutations somewhere else in the gene. Alternatively, the disease-causing effect of these amino acid changes on the function of the human ABCA4 protein could be eliminated or compensated for by other differences in the monkey protein. Comparative analysis of the monkey and human genes may provide clues for understanding the molecular pathogenesis caused by *ABCA4* variation. In the *VMD2* gene sequence of normal monkeys, we found a nonsense mutation at codon 582. The change is located at the fourth residue from the C terminus. Bestrophin was shown to form oligomeric chloride channels in cell membranes.[37] The C-terminal cytosolic tail, encoded by exons 10 and 11, has been reported not to be essential for the protein's function. Moreover, although 72 nucleotide substitutions have been identified in Best disease to date,[3,7,26] none of them is reported in exons 10 and 11. Hence, the deletion of four amino acids from the C-terminal end of the protein could be considered not to be associated with the disease.

In summary, we demonstrated that none of the 13 human macular degeneration loci tested were involved in causing the macular degeneration phenotype observed in the monkey pedigree. These results demonstrate the need for additional studies to identify the genetic locus associated with the phenotype in these monkeys and to understand the genetic defect underlying the disease. Identification of the gene responsible for this specific macular degeneration phenotype not only defines a new candidate locus for human macular degeneration, but also provides a primate animal model that can be extensively studied for elucidation of the mechanisms, diagnosis, prophylaxis, and treatment of macular degenerations, including AMD.

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