眼科インストラクションコース
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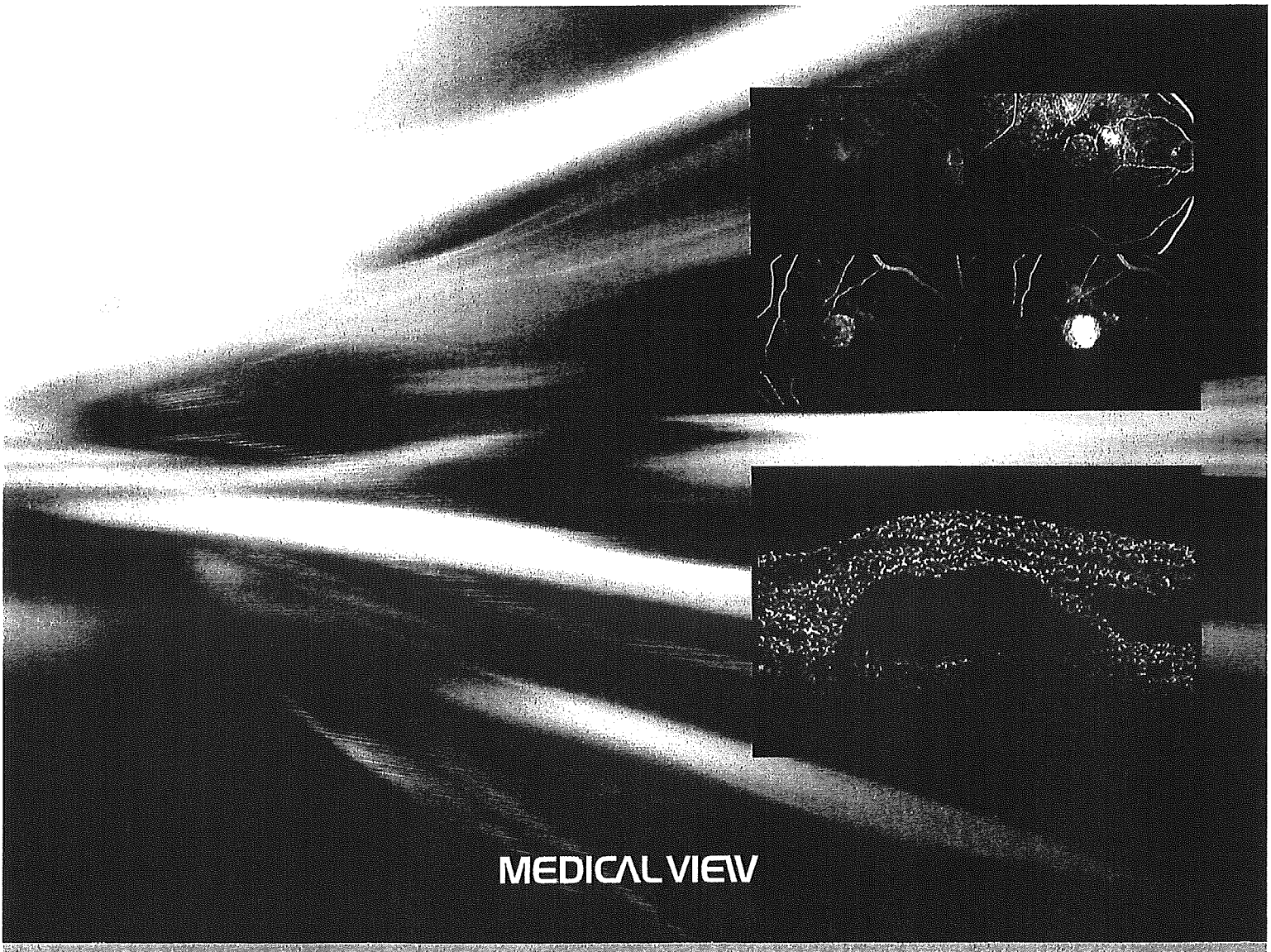
打倒！加齢黄斑変性

速報―眼科クリニックIT化の現状



MEDICAL VIEW

眼科インストラクションコース 5

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No.5　打倒！加齢黄斑変性

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まず敵をよく知ろう

My Opinion 遺伝子解析の現状，またその行方は？

●インストラクションポイント

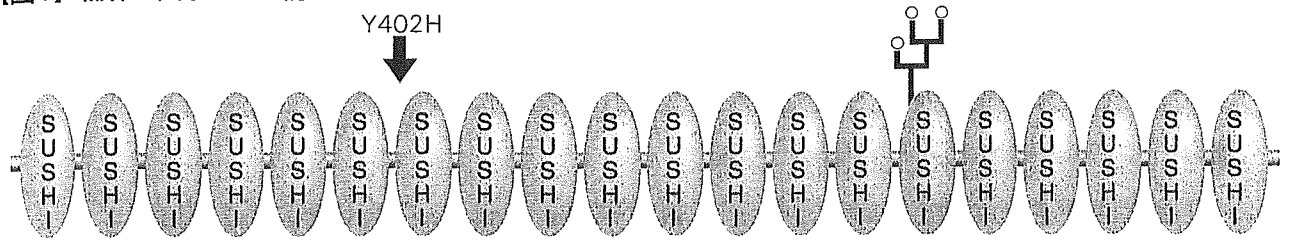
- 加齢黄斑変性と相関性のある遺伝子にはABCA4，ApoE，Fibulin5などがある
- 加齢黄斑変性のリスク遺伝子は11の染色体の13の遺伝子座位が散在する
- 補体H因子の遺伝子多型と加齢黄斑変性の高い相関性が最近発表されたが日本人では認められなかった
- 感覚器センターでは加齢黄斑変性の症例登録システムと血液収集を開始した

加齢黄斑変性(age-related macular degeneration；AMD)は多因子疾患と考えられており，遺伝的な背景に環境因子が加わって初めて発症すると考えられている[1]。そのために単一遺伝子の変異によって発症する黄斑ジストロフィのような発展の著しい研究とは異なり，AMDの遺伝子解析は思うように進んでいない。これまでの研究からAMDの原因の約25%が遺伝的要因と推測されているが，その遺伝因子も単一ではなく複数存在することが示唆されている[2]。これまでに発見された黄斑ジストロフィの原因遺伝子のなかからAMDとの相関性が報告されている遺伝子としてはStargardt病の原因遺伝子であるABCA4[3]やApoE[4]，そしてFibulin5[5]があるが，その関係を疑う研究者も多い。

近年，ヒトゲノムプロジェクトによる全染色体の塩基配列が決定され，平均で1千塩基に1つ発見される1塩基配列の違い(例：アデニン(A)がチミン(T)と置き換わる)，いわゆる遺伝子多型(single nucleotide polymorphism；SNP)が注目されてきた。ゲノム上に散在するSNPを組み合わせて，これまで未知遺伝子の探索に利用されてきた連鎖解析マーカーと同様に利用することが可能になってきたからである[6]。これらの連鎖解析技術を用いたAMDのリスク遺伝子座位(リスク遺伝子が存在する染色体上の領域)が最近報告された。その結果，11の染色体(1，2，4，5，9，10，12，15，16，18，20)の13の座位にAMDのリスク遺伝子が存在することが明らかとなり，これらのすべてあるいはいくつかの遺伝子多型と環境因子が組み合わさって発症すると推測されている[7,8]。

補体H因子に関する報告

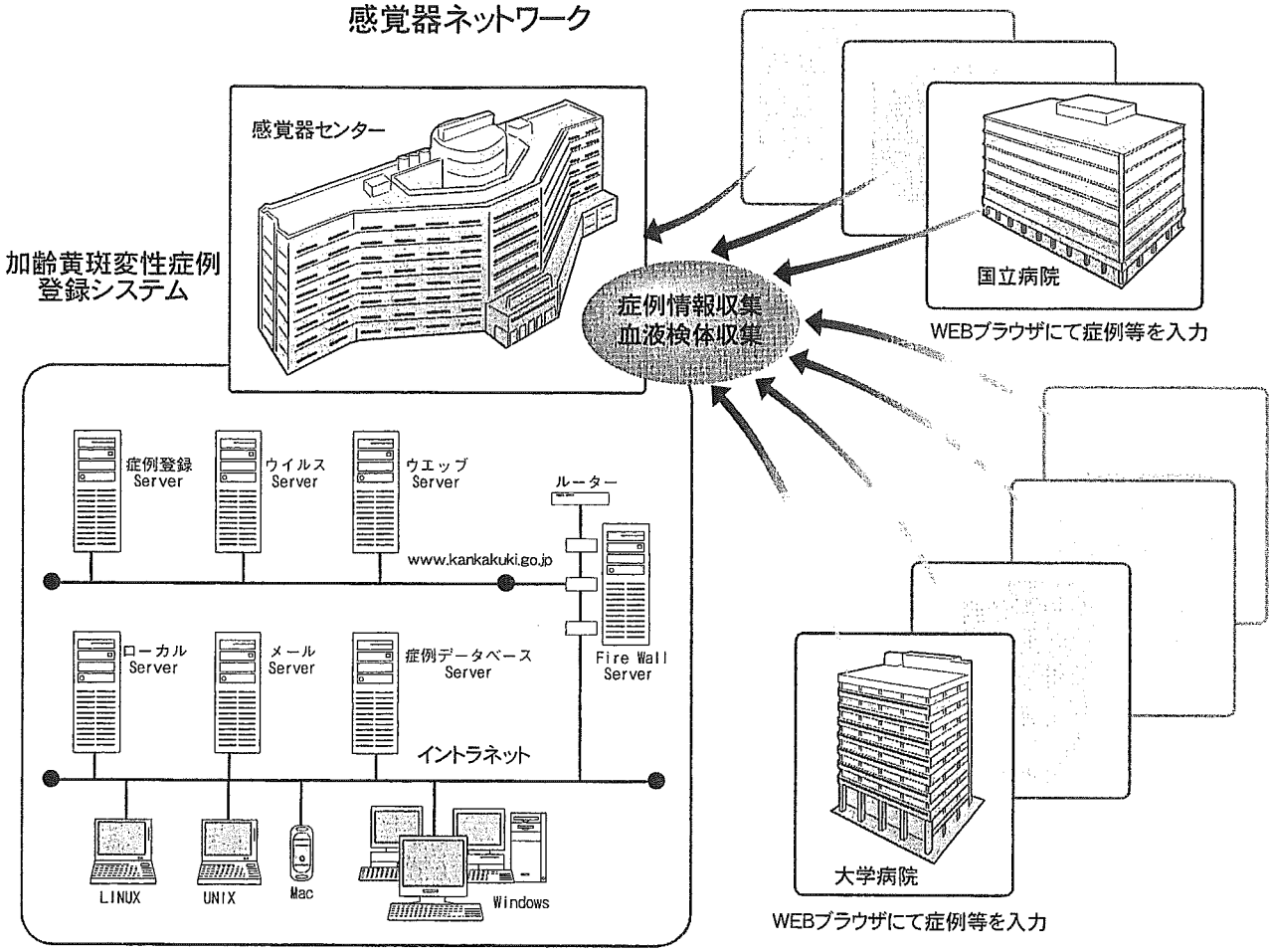
【図1】補体H因子の2次構造



AMDとの相関が報告された補体H因子はSUSHIドメインが20回反復される細長い構造をしている。矢印は遺伝子多型Y402Hが発見された第7SUSHIドメインの位置を示す。

今年4月にScience誌に3つの論文が連続で報告され[9,10,11]，新聞などでも記事として取り上げられた。さらにその直後にその内容に類似する2つの論文が別の科学誌にも報告されている[12,13]。5つの異なるグループがほぼ同時に発表したこの研究内容とは，染色体1番のAMDリスク遺伝子の同定の報告であった。この遺伝子は自然免疫システムの古典経路と2次経路からなる補体活性経路に対してこれを抑制する補体H因子(complement factor H)である【図1】。5つの論文は402番のアミノ酸がヒスチジンからトリプトファンに変異する多型がAMDと強く連鎖することを報告した。しかしこの多型が患者および健常者に現れる頻度については5つの論文で数字が異なっている。Hainesらの論文[10]ではH402Tは健常者(185人)で46%，患者(495人)では96%の頻度で現れると書かれているが，Zareparsiらの論文では健常者(275人)で34%，患者(616人)で61%と大きく数字が異なる。さらに著者らが独自に日本人だけを対象に行った調査では，健常者(89人)で5%，患者(96人)で8%とさらに大きく異なることが明らかとなった。これほど大きく数字に隔たりがある理由は今後の国際的な研究によって解明されるであろう。2005年6月14日に米国国立眼研究所(National Eye Institute；NEI)で補体H因子に関するシンポジウムが開かれ，この研究に携わる代表的な研究者が集まってこれまでの研究経過と今後の方向性が話し合われた。この模様はインターネット上で同時配信され，録画映像もウェブサイト(http://videocast.nih.gov)で見ることができる[14]。

【図2】感覚器センター症例登録システム



感覚器センターがAMDの情報と血液検体を収集するために構築した感覚器ネットワークシステム。全国の大学病院や国立病院機構病院がネットワークに参加している。

AMDは発症初期に網膜色素上皮細胞とBruch膜の間にドルーゼンといわれる蛋白質や細胞断片からなる複合体の蓄積が観察される。AMDがアルツハイマー，糸球体腎炎そして粥状動脈硬化症など局所的な補体活性化と炎症反応による沈着物を特徴とする疾患に類似すると考えたHagemanとAndersonらの研究グループは，免疫染色法という方法で患者の網膜切片を使ってこれを証明した。Hollyfieldらも質量分析計を使って直接ドルーゼンの組成成分を分析したところ，前者と同様な蛋白質が含まれていることを明らかにした。どのようなきっかけで炎症反応が起こるのか，ドルーゼンは網膜やその周辺にどのような悪影響を及ぼすのか，そしてドルーゼンの蓄積を防ぐことがAMDを未然に防ぐ方法なのか，今後数年間の研究によってこの回答が得られる可能性が高い。AMDの最大の環境危険因子として喫煙があるが，喫煙によって補体H因子の量が減少することが報告されている。すなわち，喫煙者は補体の活性化を抑制する能力が低いことを意味する。著者らの研究室ではサルを使って補体の活性化を網膜色素上皮細胞下で誘導し，人工的にドルーゼンの蓄積を促す実験に取り組んでいる。

AMDの研究はこのように遺伝学と病理学の2本柱がうまく協調して進行しているが，遺伝子解析技術の進歩によって遺伝的多因子の同定がさらに加速されると考えられる。今回発見された補体H因子は11の染色体に散在する13の遺伝子の1つであり，今後同様な遺伝子が次々と発見され，検証されると考えられる。日本での今後の課題として，日本人AMD患者の遺伝情報が欠落していることである。これまでに福岡県久山町でAMDの疫学調査などが行われてきたが，遺伝学的解析には至っていない。今回の遺伝子多型についても日本人では有意な差が観察できなかったことから，この疾患に対する日本人と欧米人の遺伝的素因は異なっていると考えられ，米国主導の研究結果をそのまま日本人に当てることが難しい。感覚器センターでは加齢黄斑変性DNAバンクを設立して，全国の大学および国立病院機構の病院から患者DNAをプールして独自に日本人のための大規模な遺伝子解析を開始した【図2】。

ヒトが得る情報の9割は感覚器(視聴覚)を通じて入ってくると考えられており，世界最速で高齢化が進行する国民のquality of life(QOL)を維持するためにも高齢化に伴って発症するAMDに対する国家レベルの対策が求められている。

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(岩田　岳)

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Molecular Cloning of ELOVL4 Gene from Cynomolgus Monkey (Macaca fascicularis)

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Abstract: ELOVL4, *elongation factor of very long chain fatty acids-4, is known to be responsible for autosomal dominant macular degeneration and Stargardt-like macular degeneration. In this study, we cloned the monkey homologue of* ELOVL4 *and determined the cellular and tissue distribution of the gene product. Sequence analysis of the monkey* ELOVL4 *gene revealed a high degree of homology between human and monkey. The cloned full-length cDNA of monkey* ELOVL4 *encoded 314 amino acids, the same length as human and two amino acids longer than mouse. The monkey ELOVL4 conserved the characteristics typical of the super family of ELO enzymes involved in the metabolism of membrane-bound fatty acid elongation. Real-time quantitative PCR demonstrated that the monkey* ELOVL4 *gene was highly expressed in restricted tissue-specific fashion, not only in the retina but also in the skin (90% of retina) and thymus (111% of retina). Immunohistochemical analysis detected signals predominantly in the photoreceptor layer of the monkey retina.*

Key words: *cynomolgus monkey,* ELOVL4, *fatty acid, macular degeneration, photoreceptor metabolism*

Introduction

Stargardt-like macular dystrophy (STGD3) and autosomal dominant macular dystrophy (adMD) are inherited forms of macular degeneration characterized by decreased visual acuity, central macular atrophy and extensive fundus flecks, resulting in loss of central vision between 5 and 23 years of age [1–4]. The disease locus for adMD/STGD3 was localized to 6q14 by linkage analysis [4]. A novel gene, an elongation factor of

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very long chain fatty acids-4 (*ELOVL4*), encoding a protein of 314 amino acids and homologous to the ELO gene family, was found to be associated with adMD/STGD3. The human *ELOVL4* gene is composed of 6 exons, and a 5 bp deletion in exon 6 that resulted in frame shift and the truncation of the protein was found in patients with adMD/STGD3 [5]. In addition, two 1-bp deletions separated by four nucleotides were detected in a family with macular dystrophy [6].

The *ELOVL4* gene is the fourth gene to be included in the *ELO* family which is involved in the membrane-bound fatty acid elongation system [7]. *ELO1 (Ssc1)* is required for microsomal fatty acid chain elongation between C14 and C16 [8]. *ELO2 (Ssc2), ELO3 (Cig30)*, and other members of the *ELO* gene family are necessary for producing 26-carbon, very long chain fatty acids (VLCFA), which become precursors for ceramide and sphingolipids [9, 10]. *ELO1* is ubiquitously expressed in major tissues, while *ELO2* and *ELO3* show restricted tissue-specific expression [10]. The human *ELOVL4* gene is expressed in the brain, testis and specifically in the photoreceptor layer of the retina. Northern blot analysis showed the presence of a 2.9 kb transcript of *ELOVL4* mRNA in the brain, testis and retina where it was found abundantly. *In situ* hybridization of rhesus monkey and mouse retinal sections demonstrated that *ELOVL4* is expressed exclusively in photoreceptor cells [5]. However, in other major tissues, the expression levels of the *ELOVL4* gene have not yet been thoroughly examined.

Macula is found only in primates and birds, and the animal model for macular degeneration is currently limited to monkeys [11–14]. Thus the monkey model is extremely important for understanding the mechanisms and etiology underlying macular degenerative diseases in humans [15, 16]. We have reported a monkey model, which manifested early onset macular degeneration [17, 18]. The cynomolgus monkeys (*Macaca fascicularis*) show signs, such as drusen in the macula and lipofuscin deposit in the retinal pigment epithelium (RPE), consistent with the phenotype observed in age-related macular degeneration in humans. The pattern of inheritance is autosomal dominance.

Monkey homologs of genes responsible for macular degeneration in humans will serve as good candidates for the hereditary macular degeneration in these cynomolgus monkeys. In this study, we describe cloning and characterization of the monkey *ELOVL4* gene and its expression in various tissues.

Materials and Methods

Animal and Tissue Collection

All experimental procedures including maintenance and care of monkeys were conducted in accordance with the Guidelines for Animal Experimentation established by the Japanese Association for Laboratory Animal Science (1987). Eyes and other tissues of cynomolgus monkeys (*Macaca fascicularis*) including heart, liver, pancreas, ileum, kidney, thymus, spleen, lymph node, skin, skeleton muscle, cerebrum and cerebellum were obtained from Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases. These tissues were collected from the age group between 4–6 years, and stored for 1 day at 4°C in RNA stabilization solution (RNAlater, Ambion, Austin, TX) until RNA extraction.

Extraction of Genomic DNA and Total RNA

Genomic DNA was prepared from minced sclera using a DNA Extraction Kit (QIAamp DNA Mini Kit, QIAGEN, Valencia, CA) and total RNA was extracted using a RNA extraction reagent (TRIzol, Invitrogen, Carlsbad, CA) as directed by the manufacturer.

Analysis of the Monkey ELOVL4 *Gene Structure*

PCR of monkey genomic DNA using gene-specific primers (Table 1) and LA-Taq polymerase (TAKARA, Ohtsu, Japan) was performed. Primers were designed according to the human genomic DNA sequence submitted in a public database (GenBank accession number NT_017020). Amplified products were sequenced directly, or after being subcloned into pCRII cloning vector (TA Cloning Kit Dual Promoter, Invitrogen, Carlsbad, CA) using CEQ2000XL DNA analysis system and dye terminator sequencing kit (Beckman Coulter, Fullerton, CA). The 5'/3'-rapid amplification of cDNA ends (5'/3'-RACE) was performed using total RNA isolated from monkey retina and RPE. Primers were designed based on the obtained exon sequence in such a way that both 5'- and 3'-RACE products overlapped to cover full length cDNA. The primers used in this experiment were 5'-AATGAGCACTAGACGCATCTGA-3' and 5'-ATGCAGTCTCCTTGGCCTAC-3' for 5'-RACE

Table 1. Primer sets for analysis of *ELOVL4* gene structure

Forward primer			Reverse primer			Product
No.		Position	No.		Position	Size (kb)
P1	GGGTAGTGTCCTAAACGTAGTGTC	Exon 1	P2	ATCCCAGAGGCTTCAAGGA	Intron	4.5
P3	AACATCACCAGCTGGTAACATG	Intron	P4	AATGCTGCCAATGAACTCAA	Intron	4.7
P5	ATGCGATATTTAGCCTGAGAAGA	Intron	P6	ACTCTGAGGACAGTTGAAGATGAG	Intron	4.4
P7	AGAGAATAGGCTGCAGCAGAA	Intron	P8	ATCATGGAAGCTACAGTAACTGTCA	Intron	4.2
P9	ATTTGCATCTCATCAGAAGCAC	Intron	P10	AATGAGCACTAGACGCATCTGA	Exon 2	4.3
P11	ATGCAGTCTCCTTGGCCTAC	Exon 2	P12	ACATCGTACAGTGATGATACACATG	Exon 4	4.7
P13	ATAGCTGCTGCTCTGTGGTG	Exon 4	P14	AGTTGCAACATAGTCAGGTATCG	Exon 5	2.4
P15	AGCCCAGTTGAATTCCTTTATC	Exon 5	P16	AGGTAGCAGGGAATGAATAGCT	Exon 6	4.7

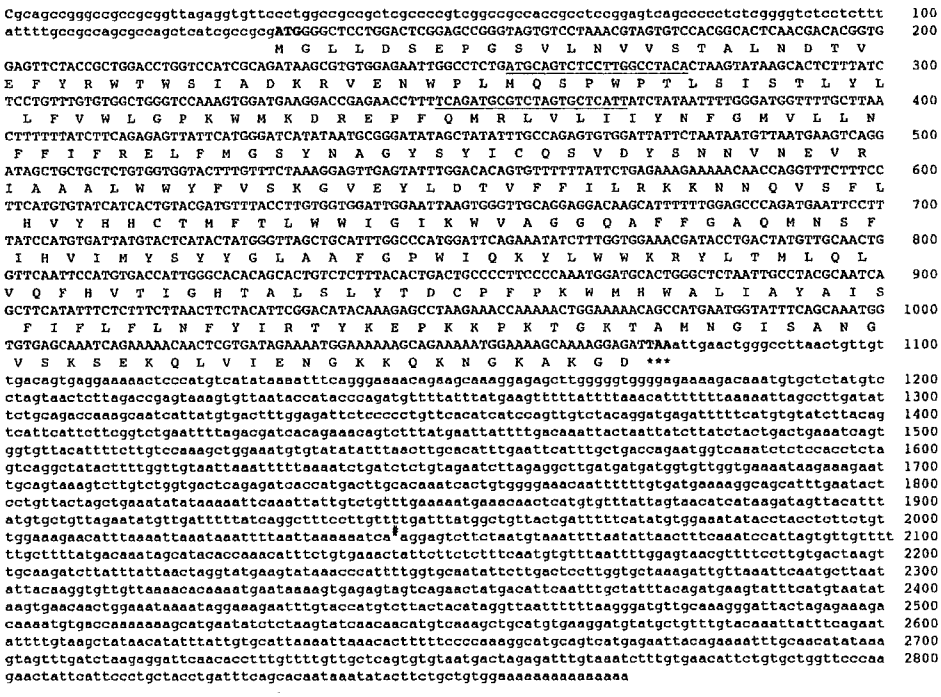

Fig. 1. Nucleotide sequence and encoded amino acid sequence of full-length monkey *ELOVL4* cDNA. The nucleotide sequence from the transcription initiation site to the poly(A) tail, encoding 314 amino acids, were determined by 5'- and 3'-RACE. Primers are also indicated (underlined). The numbering to the right refers to nucleotides and the capital single-letter below represents the corresponding amino acid. Asterisks and pound indicate stop codon and polyadenylation variant, respectively.

and 3'-RACE, respectively (Fig. 1). All procedures were carried out following the manufacturer's protocols (Invitrogen). Amplified PCR products were sequenced as described above.

Real-time Quantitative PCR

Total RNA from tissues was used to make cDNA with a first-strand synthesis system for RT-PCR (Superscript II, Invitrogen) as described by the manufacturer. In order to evaluate expression level in each tissue, we carried out real-time quantitative PCR with an ABI5700 system and SYBR Green reagent (PE Applied Biosystems, Foster, CA). The expression level was standardized with *GAPDH* as an internal control.

Table 2. The gene structure of monkey *ELOVL4*

Intron		Exon		Length	Intron		Approx. size
	CGCAGCCGGG ·····	1:	100 bp	TCCATCGCAG	gtaaagccgc ·····		20 kb
····· ttctctatag	ATAAGCGTGT ·····	2:	188 bp	····· CTTCAGAGAG	gtatgttttt ·····		1kb
····· ctttttccag	TTATTCATGG ·····	3:	81 bp	····· TGAAGTCAGG	gtaagtacat ·····		3 kb
····· cttttacag	ATAGCTGCTG ·····	4:	172 bp	····· GGAGGACAAG	gtgagcattt ·····		2 kb
····· ttttctaaag	CATTTTTTGG ·····	5:	128 bp	····· GTTGCAACTG	gtgagttaaa ·····		2.5 kb
····· tgtttttcag	GTTCAATTCC ·····	6:	276 bp	····· TCTGCTGTGG			

Exon-intron organization of the monkey *ELOVL4* gene is shown. Ten nucleotides on both sides from the exon-intron boundaries are indicated. Open reading frame (ORF) length and approximate intron size are also indicated.

The primers P14 and P15 used for *ELOVL4* are shown in Table 1, and for *GAPDH*, forward primer 5'-CAGCCTCAAGATCATCAGCAAT-3', and reverse primer 5'-GGTCATGAGTCCTTCCACGATAC-3' were used. The PCR products were directly sequenced in order to confirm that the target cDNAs were precisely amplified.

Immunohistochemical Study for ELOVL4

Antibodies were raised by immunizing rabbits with peptide containing residues between 31 and 46 amino acids of human ELOVL4. Antiserum was used for immunohistochemistry on aldehyde-fixed frozen sections of monkey retina. In these experiments, preimmune serum was used for negative control.

Results

Sequence Analysis of the Monkey ELOVL4 *Gene*

Sequence analysis revealed that monkey *ELOVL4* cDNA and gene structures are very similar to those of humans (Table 2). The complete cDNA sequence of the monkey *ELOVL4* gene was 2,856 bp in length. The result from 3'-RACE indicated that an alternative splicing variant existed. This variant was 813 bp shorter and polyadenylated at the 3' non-coding region of exon 6 (Fig. 1).

The cDNA included 945 bp of open reading frame encoding 314 amino acids, the same length as human cDNA, and two amino acids longer than the mouse homologue (Fig. 2). There was a seven amino acid difference between monkey and human ELOVL4, but the monkey ELOVL4 also conserved features typical for members of the *ELO* gene family: a HXXHH motif

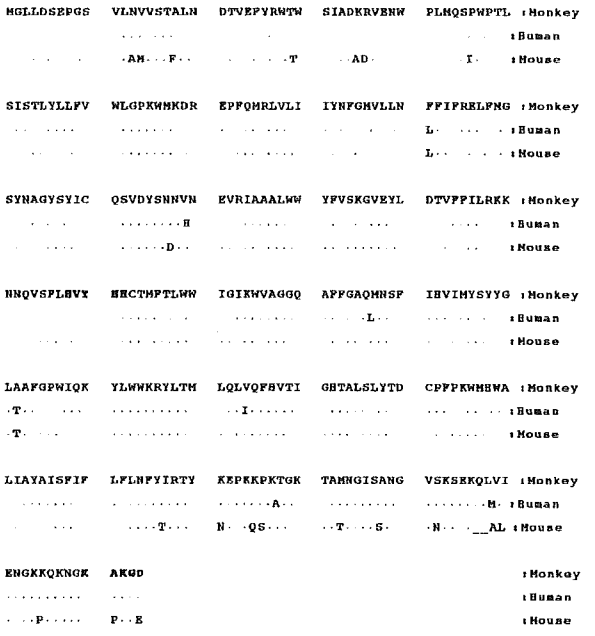

Fig. 2. Comparison of monkey amino acid sequence of *ELOVL4* protein with human and mouse. Monkey *ELOVL4* protein has a seven residue difference from human *ELOVL4* protein and is two residues longer than the mouse homologue. Dioxy iron-binding HXXHH motif and the carboxy-terminal dilysine signal, responsible for retention in endoplasmic reticulum, are also present in the monkey *ELOVL4* protein (shown bold).

identified in fatty acid desaturase and other dioxy iron cluster proteins [19]; and a KXKXX motif shown to be a strong signal for the retention of proteins in endoplasmic reticulum [20], where VLCFA are synthesized (Fig. 2) [21].

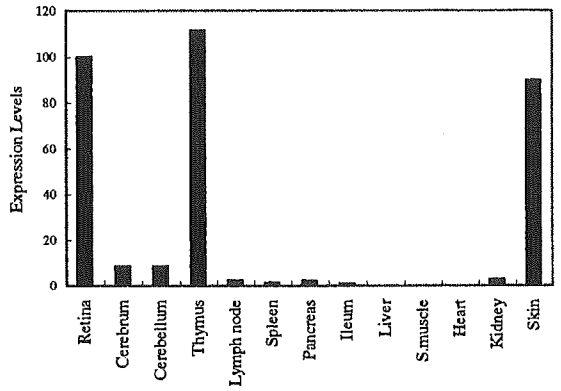

Fig. 3. Real-time quantitative PCR demonstrating tissue-restricted expression of *ELOVL4*. Abundant expression was observed not only in the retina but also in the thymus and skin at the same expression level. Expression level for each tissue is shown as a relative ratio compared to the retina.

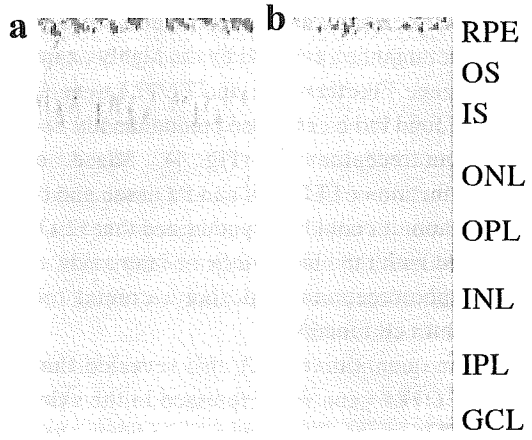

Fig. 4. Immunohistochemical analysis of monkey retina. a. Predominant signal is detected in the photoreceptor layer. b. Control hybridized with pre-immune serum, showing no signal. RPE, retinal pigment epithelium; OS, outer segments of photoreceptors; IS, inner segments of photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Expression of the Monkey ELOVL4 *Gene in Various Tissues*

Real-time quantitative PCR was performed on cDNA synthesized from total RNA from monkey retina, heart, liver, pancreas, ileum, kidney, thymus, spleen, lymph node, skin, skeletal muscle, cerebrum and cerebellum. The expression level in each tissue was calculated based on the standard curve created by PCR using a diluted plasmid containing *ELOVL4* cDNA fragment. The calculated values were evaluated after standardization with the expression level of *GAPDH* obtained in the same way.

The highest level of the *ELOVL4* gene expression was observed in the retina. Remarkable expressions were also detected in the thymus and skin. The expression levels in the thymus and skin were 111% and 90% of that in the retina, respectively. On the other hand, expression in the brain was shown to be less than 9% of that in the retina. In the other tissues, very low expression levels, just under 3% of that in the retina, were observed. In particular, in the liver and skeletal muscles, expression of the *ELOVL4* gene could not be detected (Fig. 3).

ELOVL4 *Expression in Monkey Retina*

Immunohistochemical analysis of monkey retinal sections showed prominent staining in photoreceptor inner segments (Fig. 4). These results are consistent with the observations made on the localization of the *ELOVL4* expression using *in situ* hybridization [5]. The presence of ELOVL4 was detected in both rod and cone photoreceptor cells.

Discussion

In this study, the genomic sequence and complete cDNA of the monkey *ELOVL4* gene, the fourth known homologue of the *ELO* gene family which encode enzymes involved in membrane-bound fatty acid elongation systems, were determined. The structure of the monkey *ELOVL4* gene is similar to the human *ELOVL4* gene (Table 2). These two genes share 97% homology in cDNA sequence and 98% similarity in predicted amino acid sequence. Although the function of the human *ELOVL4* gene still remains unclear, the monkey *ELOVL4* gene also conserves features typical of members of the *ELO* gene family (Fig. 2). In addition, remarkable expression of the *ELOVL4* gene was detected in the monkey retina (Fig. 3). These findings suggest that the monkey *ELOVL4* gene also has an important role in biosynthesis of VLCFA in the retina. The retina is known to have the ability to synthesize

fatty acid with carbon chain lengths up to 36 [22, 23], and this fact might be explained by the highly expressed *ELOVL4* gene. Furthermore, the *ELOVL4* gene in the retina was found to be expressed predominantly in cone and rod photoreceptor cells (Fig. 4). Based on the predicted function of ELOVL4 and its tissue and cellular localization, it could be hypothesized that ELOVL4 is associated with the biosynthesis of fatty acids, especially in photoreceptors, and play a crucial role in photoreceptor cell functions.

Real-time quantitative PCR also revealed that the monkey *ELOVL4* gene was expressed in the skin and thymus. This is the first report to indicate that the *ELOVL4* gene is expressed abundantly not only in the retina but also in the skin and thymus. The expression level in the brain, previously reported [5], was comparatively high among the other tissues, but was only about 9% of that in the retina. No significant expression was detected in the other tissue tested (Fig. 3). Tissue-restricted expression patterns have been reported for the *ELO2* and *ELO3* genes in rodents [10]. In rodents, the *ELO2* gene is reported to be expressed in the liver and testis, on the other hand, the expression of the *ELO3* gene is observed in the liver, skin and the brown fat of cold-exposed mice. These findings strongly suggest that different components of membrane-bound fatty acid elongation systems are involved in each tissue, and that the *ELOVL4* gene may have a particularly important role in the systems of brain, skin, thymus and retina.

It is reported that VLCFA are found at especially high levels in the brain and skin [24]. In the brain, sphingolipids, which contain VLCFA are considered to have an important role in the enhancement of electric insulation of myelin [25]. Sphingolipids are also believed to contribute unique structural properties to the epidermal water barrier in the skin [26]. Although the functional role of VLCFA and membrane-bound fatty acid elongation systems in the retina remains to be determined, growing evidence indicates that the lipid environment influences photoreceptor function [27–29]. This study does not give any insight into the function of the *ELOVL4* gene in the retina. However, the obtained information may be useful for evaluating the normal function of *ELOVL4* in the fatty acid chain elongation systems in the retina using non-human primates. Furthermore, the obtained gene sequence makes it possible to investigate the involvement of the *ELOVL4* gene in non-human primate macular degeneration.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession numbers AF461182, AF461183, AF461184, AF461185, AF461186 and AF461187.

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

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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

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

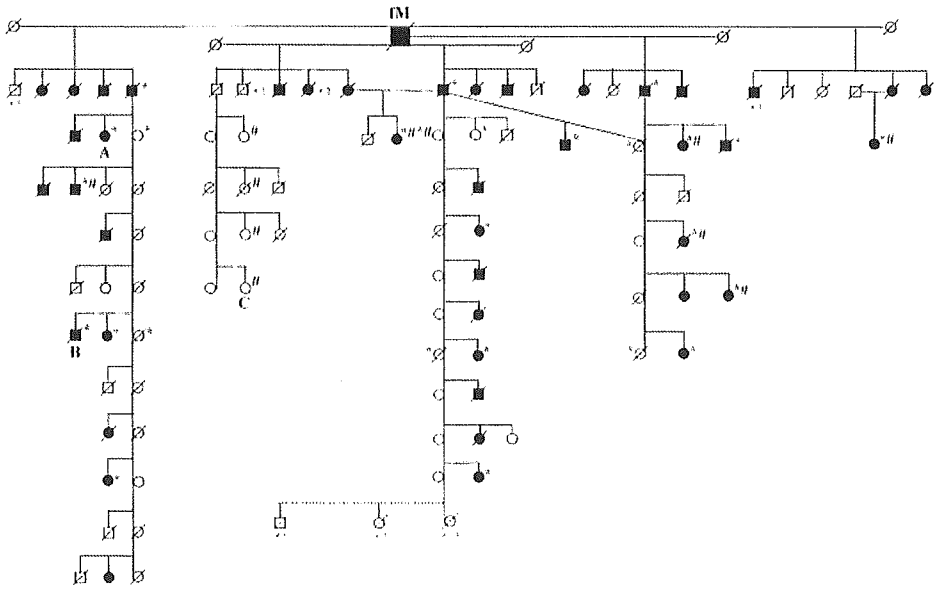

FIGURE 1. Edited version of the monkey pedigree with macular degeneration: fM, the founder breeding male monkey with typical macular degeneration, is shown with five healthy mates arrayed horizontally. The first-generation offspring are also arrayed horizontally. The breeding members from each branch 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	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

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	94	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	AGACAGTAACTAACAGGCTGGTG	15R	GGAGTGCTACAGACCCTTCC	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	CCTGAATCAGAATGCTCCGTGACCTTC	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	TTTCAGAGATTGGGGCCTATA	304
	4	172	MP4aF	AAATGATTCCATGCCTTGTACA	MP4aR	AACGCAAGCAGTATATTCCTGA	330
			MP4b	TGGTGTTTATAACACGCTTTCC	MP4bR	CTCATTGCTTTCCAGTGAACA	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