and proteomic techniques, the materials in drusen were found to be composed of molecules that mediate inflammatory and immune processes (Russell, Mullins, Schneider, and Hageman 2000; Mullins, Russell, Anderson, and Hageman 2000). These molecules include components of the complement pathway and modulators of complement activation, viz., vitronectin, clusterin, membrane cofactor protein, and complement receptor-1. In addition, molecules triggering inflammation, amyloid P component, α1-antitrypsin, and apolipoprotein E, were identified in drusen. Cellular debris from macrophages, RPE cells, and choroidal dendritic cells has also been identified in drusen. Additional proteins such as crystallins, EEFMP1, and amyloid-beta have been also found in drusen. The presence of immunoreactive proteins and the oxidative modifications of many proteins in drusen imply that both oxidation and immune functions are involved in the pathogenesis of AMD. These findings suggest that complement activation triggers innate immune responses in the subretinal space. The co-distribution of IgG and terminal complement complexes in drusen indicate that immune responses that directly target antigens in retinal cells might also be occurring. Anti-retinal autoantibodies have been reported in a number of ocular disorders, e.g., macular degeneration in an aged monkey model.

## 5 Primate Model for AMD

Over the past few years, genetic engineering techniques have generated a number of animal models of AMD in mice, rats, rabbits, pigs, and dogs (Chader 2002). However in mammals, a well-defined fovea is found only in primates (humans and monkeys), and a search for a monkey line affected with macular degeneration has been persistent for a long time. A monkey with macular degeneration was first described by Stafford et al in 1974. They reported that 6.6 % of the elderly monkeys they examined showed pigmentary disorders and drusen-like spots (Stafford, Anness, and Fine 1984). El-Mofty et al reported that the incidence of maculopathy was 50% in a colony of rhesus monkeys at the Caribbean Primate Research Center of the University of Puerto Rico (El-Mofty, Gouras, Eisner, and Balazs 1978). At the Tsukuba Primate Research Center (Tsukuba City, Japan), Suzuki et al found a single cynomolgus monkey (*Macaca fascicularis*) (Suzuki Monkeys) in 1986 with a large number of small drusen around the macular region (Nicolas, Fujiki, Murayama, Suzuki, Mineki, Hayakawa, Yoshikawa, Cho, Kanai 1996; Nicolas, Fujiki, Murayama, Suzuki, Shindo, Hotta, Iwata, Fujimura, Yoshikawa, Cho, Kanai 1996; Suzuki, Terao, and Yoshikawa 2003). This single affected monkey has multiplied to a large pedigree of more than 65 affect and 210 unaffected monkeys. Drusen were observed in the macular region as early as one year after birth, and the numbers increased and spread toward the peripheral retina throughout life. No histological abnormalities have been found in the retina, retinal vessels, or choroidal vasculatures of the eyes with drusen. However, abnormality in electroretinogram (ERG) were observed in sever case showing dysfunction of the macula.

Immunohistochemical and proteomic analyses of the drusen from these monkeys showed that the drusen were very similar to those in other monkeys with aged

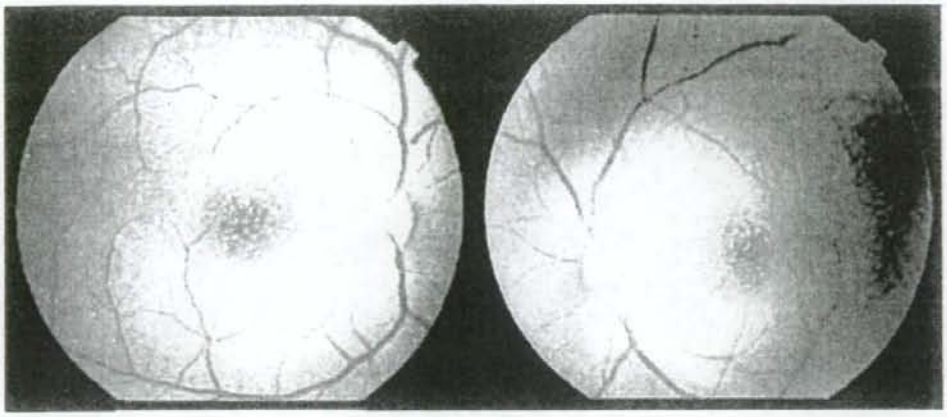

**Fig. 1.** Fundus photograph of both eyes of Suzuki Monkey showing accumulation of drusen (white spot) around the macular region.

macular degeneration sporadically found in older monkeys and also with human drusen (Umeda, Ayyagari, Allikmets, Suzuki, Karoukis, Ambasudhan, Zernant, Okamoto, Ono, Terao, Mizota, Yoshikawa, Tanaka, and Iwata 2005; Umeda, Suzuki, Okamoto, Ono, Mizota, Terao, Yoshikawa, Tanaka, and Iwata 2005; Ambati, Anand, Fernandez, Sakurai, Lynn, Kuziel, Rollins, and Ambati 2003). These observations have shown that the Suzuki Monkeys produce drusen that are biochemically similar to those in human AMD patients, but the development of the drusen occurs at an accelerated rate. More than 240 loci are being investigated to try to identify the disease causing gene and to understand the biological pathways leading to complement activation. Simultaneously, we have been studying a colony of aged monkeys which develop drusen after 15 years of birth.

Drusen components of these sporadically found affected monkeys were compared with human and Suzuki Monkeys by classical immunohistochemical techniques and by proteome analysis using mass spectrometer. Significant finding was that drusen contained protein molecules that mediate inflammatory and immune processes. These include immunoglobulins, components of complement pathway, and modulators for complement activation (e.g., vitronectin, clusterin, membrane cofactor protein, and complement receptor-1), molecules involved in the acute-phase response to inflammation (e.g., amyloid P component, α1-antitrypsin, and apolipoprotein E), major histocompatibility complex class II antigens, and HLA-DR antigens (Umeda et al. 2005). Cellular components have also been identified in drusen, including RPE debris, lipofuscin, and melanin, as well as processes of choroidal dendritic cells, which are felt to contribute to the inflammatory response. In addition to immune components, a number of other proteins were found in drusen. These appear to be vitronectin, clusterin, TIMP-3, serum amyloid P component, apolipoprotein E, IgG, Factor X, crystallins, EEFMP1, and amyloid-beta. The presence of immunoreactive proteins and oxidative modified proteins implicate both oxidation and immune functions in the pathogenesis of AMD.

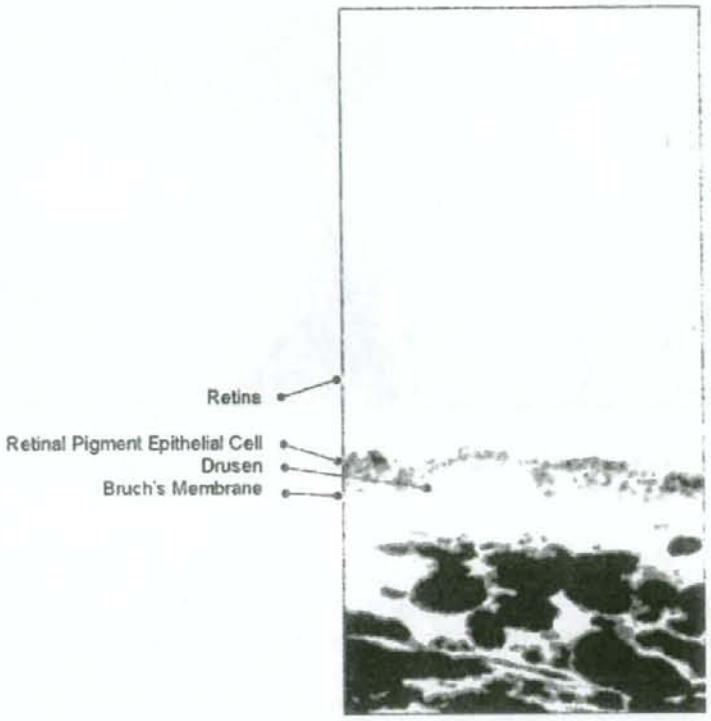


Fig. 2. Retinal histological section of affected Suzuki Monkey showing the accumulation of drusen between the retinal pigment epithelium and Bruch's membrane.

The eyes of monkey are structurally similar to human eyes which make them extremely valuable for AMD studies. However, there are limitations in using this species over other laboratory animals. Monkeys have a relatively longer life span, have a longer gestation period, have a lower birth numbers resulting in a slower expansion of the pedigree, more difficult to genetically manipulate, and the maintenance cost is high. In the other laboratory animals, the differences in the eye structure, lack of a fovea, and a low cone/rod ratio compared to humans have been considered to be a disadvantage for using them as AMD models. However, they are easier to manipulate genetically and easier and less expensive to maintain. This has made the development of a mouse model of AMD very attractive, and a number of mouse AMD models have been reported recently.

## 6 Mouse Model for AMD

The mouse model described by Ambati et al is deficient either in monocyte chemoattractant protein-1 or its cognate C-C chemokine receptor-2. These mice were found to develop the cardinal features of AMD including accumulation of lipofuscin

in drusen beneath the RPE, photoreceptor atrophy, and CNV (Ambati et al. 2003). An impairment of macrophage recruitment allowed the accumulation of C5a and IgG, which leads to the production of vascular endothelial growth factor by the RPE cells and the development of CNVs. Another mouse model that has three known AMD risk factors: age, high fat cholesterol rich diet, and expression of human apolipoprotein E (apoE2, apoE3, apoE4) has been developed (Malek, Johnson, Mace, Saloupis, Schmechel, Rickman, Toth, Sullivan, and Bowes Rickman 2005). ApoE4-deficient mice are severely affected showing diffuse subretinal pigment epithelial deposits, drusen, thickened Bruch's membrane, and atrophy, hypopigmentation, and hyperpigmentation of the RPE.

Oxidative stress has long been linked to the pathogenesis of AMD. Imamura et al reported a Cu, Zn-superoxide dismutase (SOD1)-deficient mice that had features typical of AMD in human. Senescent Sod1 (-/-) mice had drusen, thickened Bruch's membrane, and choroidal neovascularization (Imamura, Noda, Hashizume, Shinoda, Yamaguchi, Uchiyama, Shimizu, Mizushima, Shirasawa, and Tsubota 2006). The number of drusen increased with age and also after exposure of young Sod1 (-/-) mice to excess light. The retinal pigment epithelial cells of Sod1 (-/-) mice showed oxidative damage, and their beta-catenin-mediated cellular integrity was disrupted. These findings suggested that oxidative stress may affect the junctional proteins necessary for the barrier integrity of the RPE. These observations strongly suggested that oxidative stress may play a major role in AMD.

The complement components, C3a and C5a, are present in drusen, and were observed in Bruch's membrane of a laser-induced CNV mice model. Neutralization of C3a or C5a by antibody or by blockade of their receptors by a complement inhibitor significantly reduced the CNV (Nozaki, Raisler, Sakurai, Sarma, Barnum, Lambris, Chen, Zhang, Ambati, Baffi, and Ambati 2006). These observations revealed a role for immunological mechanisms for the angiogenesis and provided evidence for future therapeutic strategies for AMD. Although the pathology of AMD is pronounced in the macula area, it is not confined to this region. Characteristics of human AMD such as thickening of Bruch's membrane, accumulation of drusen, and CNV have been observed in mouse models. Nevertheless, the primate model will still be the choice for AMD studies, especially at the stage when new therapeutic methods are tested and evaluated for the first time. However, it would be wise and more productive to study both primate and mouse models in AMD research. This will be necessary to learn the mechanisms underlying the disease and to identify clinical and molecular markers for the early stages of AMD. The findings from these studies will provide critical information needed to develop therapies for AMD.

## 7 Acknowledgements

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図説　感覚器疾患シリーズ　No. 9

特集；視覚障害(3)

# 眼疾患バイオマーカーの探索

国立病院機構東京医療センター　臨床研究センター　分子細胞生物学研究部

岩田　岳

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# 眼疾患バイオマーカーの探索

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キーワード：眼，遺伝子，遺伝子多型，シークエンサー，予防医学

## はじめに

近年ゲノム配列や体内のタンパク質を分析して疾患の発症を予測する研究が行われている．この背景には飛躍的な技術革新によるゲノム解析技術や質量分析を用いた微量タンパク質の網羅的解析技術などの進歩がある．これらの技術はすでに多くの疾患で成果を上げており，眼科分野でも応用されている．患者の眼球において，まだ生理機能が保持されている環境では再生医療を，そして環境が破綻（はたん）している場合には人工網膜等による視力回復が検討されている．本章ではこれらの研究に加え，未発症の患者予備軍を選別し，病気の予防あるいは遅延を目的とした予防医学について，その選別部分について最近の研究を紹介したい．

## 感覚器症例情報収集システムの構築

眼球内部は透明な組織で構成されていることから容易に組織内部を観察できる一方，生検することがきわめて困難な組織でもある．したがって早期診断に利用できるサンプルは涙液，血液，尿に限られ，必然的に研究対象は白血球のDNAと血漿成分に絞られる．また，手術中に破棄される房水，硝子体の一部についても倫理委員会の承認と患者の同意があれば研究材料として利用できる．われわれはこれらのDNAや体液から疾患と相関する疾患バイオマーカーの探索を行っている．平成15年，国立病院機構および厚生労働省の支援を受けて，オンラインによる症例情報の収集とDNA・血漿バンクの設立を目的とした「感覚器ネットワーク」を東京医療センター臨床研究センター（感覚器センター）に構築した（図1）．関連施設の国立病院および大学病院から白内障，緑内障，加齢黄斑変性，網膜色素変性の症例情報をウェッブ画面上から登録し，同時に検体を受け取る仕組みである．症例情報には個人情報を除く患者の臨床所見が詳細に登録できるようになっており，眼底写真の添付も可能である．遺伝子やタンパク質の解析結果は症例情報と一体化され，個々の患者についてデータベースが構築されることによって，疾患バイオマーカーの探索をより容易にしている．

## 遺伝子解析による加齢性眼疾患の感受性遺伝子の探索

これまでの研究から加齢性眼疾患の多くは多因子疾患（遺伝，習慣，環境）であると考えられている．

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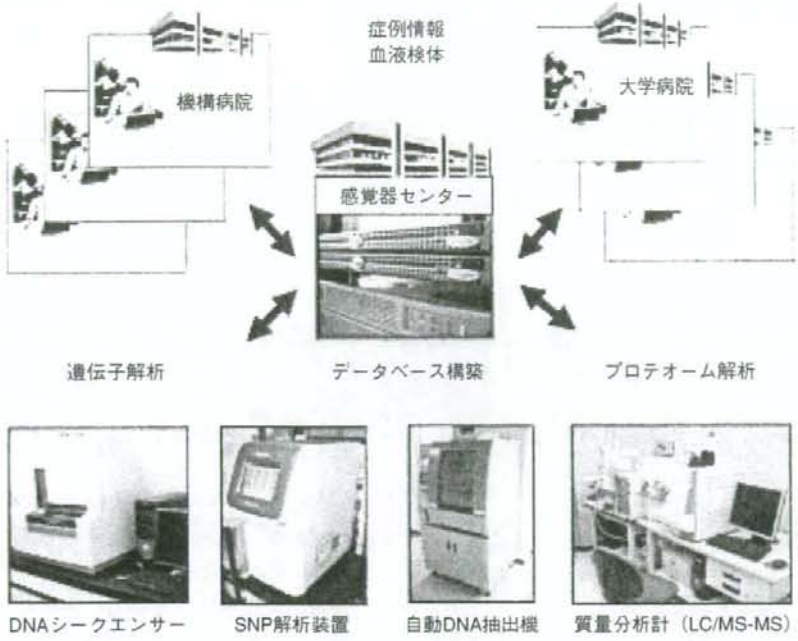


図1　感覚器センターに設置された症例情報システムと各種分析装置

加齢黄斑変性の場合，加齢，遺伝，喫煙，肥満，青い光などがリスク因子として報告されている．とくに遺伝因子（感受性遺伝子）については技術的な進歩によって全ゲノムを対象とした患者と健常者の比較が可能になっている．ヒトゲノムプロジェクトによってヒトゲノムには約22,000個の遺伝子が存在し，平均で1,000塩基に1つの割合で配列が個々に異なることが明らかになった．この一塩基配列の変化である遺伝子多型：Single Nucleotide Polymorphism（SNP）を組み合わせてブロックにし，比較することによって感受性遺伝子の染色体上の位置を調べることができる．SNPの組み合わせをハプロタイプと呼ぶが，複数の国が参加して，すべてのハプロタイプを明らかにする国際ハップマッププロジェクト（International HapMap Project, http://www.hapmap.org/）が進行中である．

ゲノム上には1千万個のSNPが存在すると計算されるが，これだけのSNPを患者個々に解析することは困難であった．しかし，最近SNPアレーの開発が進み，50万-100万種類のSNPを同時に検出することができるようになっている．このチップを利用しても全SNPの5-10%しかカバーできないが，感受性遺伝子の位置情報，病気との相関（頻度，オッズ比）について有用な情報が得られる．われわれはこの方法によって白内障，緑内障，加齢黄斑変性，偽落屑症候群の感受性遺伝子について解析している．疾患と相関するSNPは疾患を発症する塩基配列と物理的に近い位置に存在すると考えられるが，そのものではないことが多い．これはハップマッププロジェクトが健常者を対象にして作成されたデータベースであり，患者のSNPは登録されていないからである．発症原因の塩基配列を知るためにはSNP間の塩基配列をDNAシークエンサーで読むしかない．近年この塩基配列解読についても驚異的な技術革新が進行している（図2）．1990年にアメリカエネルギー省と厚労省によって30億ドルの予算で開始されたヒトゲノムプロジェクトが10年間かけて解読した30億塩基を1日で解読するDNAシークエンサーがアメリカPacific Bioscience社を含む複数の会社から発表される予定である．アメリカではヒトゲノムを1,000ドルで完全解読する1,000ドルゲノムプロジェクトが進行しており，今後SNPアレーからゲノム配列を患者と健常者で直接比較することによってより詳細な情報が得られると考えられる．また，DNAシークエンサーの感度が上がり，必要なDNA量も年々減少している．このことから採血は不要と

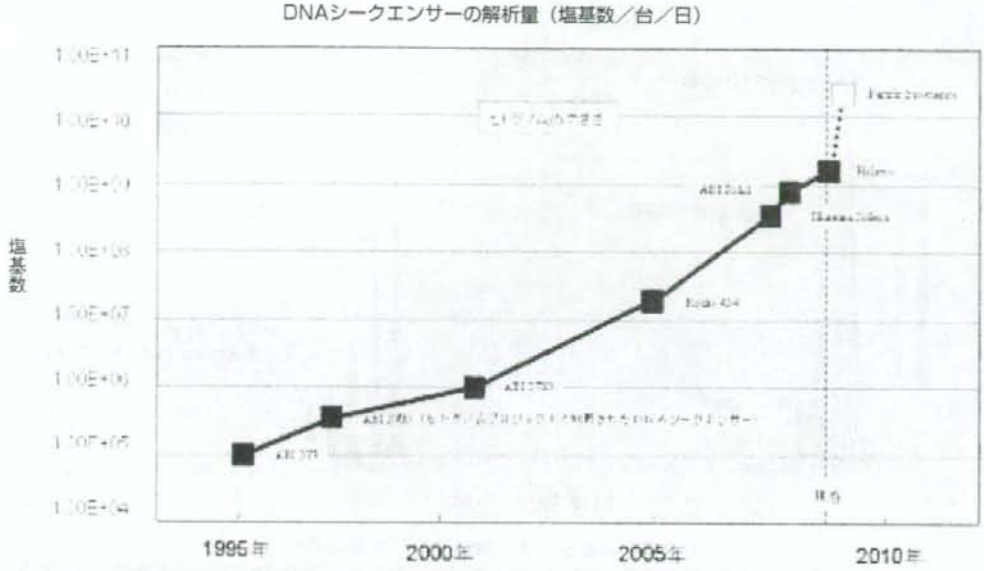


**図2　DNAシークエンサー1台が1日に解読できるゲノム塩基数**
1人分のゲノムが1日で解読できるDNAシークエンサーが発表される予定である．

なり，爪や髪の毛から抽出されるDNA量でも十分な解析が可能になると予測される．

近年の研究によってゲノム上の2％の配列が約22,000遺伝子をコードし，残り70％の配列からRNAが生成され，このRNAが遺伝子発現やタンパク質合成の制御に関係していることが明らかになってきた．このようなタンパク質を合成しないRNA（ncRNA：non-coding RNA）が眼疾患にも関わっている可能性は高く，感受性遺伝子に加え，感受性ncRNAについても積極的に研究を進めている．

## 眼疾患のためのプロテオミクスとバイオマーカーの探索

感受性遺伝子が明らかにされ，発症の有無が判断できるようになっても，個々の患者の発症時期を予測することは困難である．体内を循環する血液や排泄される尿の成分を分析することによって発症時期を予測する研究が行われている．近年，質量分析計が飛躍的に進歩し，コンピューターソフトによる機械の操作性も改善されたことから，専門の技官でなくても操作できるようになってきた．発現しているタンパク質の総称をプロテーム，その分析方法をプロテオミクスというが，今回われわれは白内障，緑内障，加齢黄斑変性の患者から血漿を集め，質量分析計を用いた血漿プロテオミクスを行った．血漿にはアルブミン，グロブリンなど20種類のタンパク質が重量換算で99％を占めており，これらを除去しなければ疾患によって変動する微量タンパク質を質量分析計の検出できる感度で捕捉することが困難である．そこでわれわれは東レ株式会社との共同研究によって低分子のタンパク質だけを分離・濃縮する分画装置を開発し，患者血漿の分画を試みた（図3）．分画成分はさらに逆相クロマトグラフィー，トリプシン処理，2次元クロマトグラフィーによる分離を経て，イオントラップ型質量分析計よってタンパク質の同定が行われた．その結果，白内障，緑内障，加齢黄斑変性からそれぞれ固有に17，15，21種類のタンパク質が同定され，現在これらのタンパク質についてELISAやウエスタンブロットによって確認作業を進めている．同様な方法によって涙液，房水，硝子体液など眼由来の体液についても解析を行う予定である．また，今回同定されたタンパク質情報の確認作業と遺伝子多型情報と症例情報に結びつけるための作業を行っている．

## おわりに

眼疾患のバイオマーカー探索として遺伝子解析と質量分析計を用いた眼疾患プロテオミクスについて

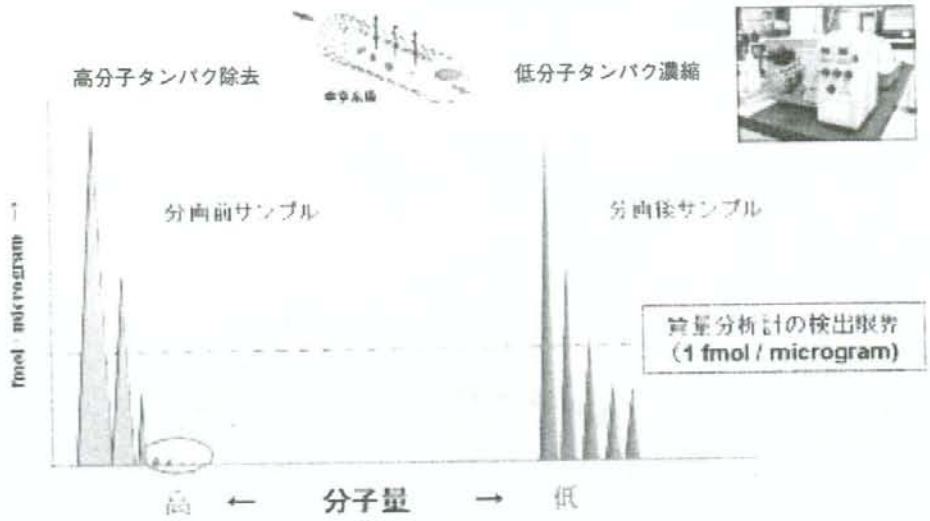


**図3　中空糸膜を用いた低分子分画装置による微量タンパク質の濃縮**
東レ株式会社が開発した分画装置によって，選択的に低分子（50KDa以下）の血漿タンパクを質量分析計で検出できる濃度に調製できるようになった．

紹介した．これまでに日本人と欧米人との間で疾患と相関する遺伝子多型が一致しないことがたびたび報告されている．しかし，日本人と体質がより近いと考えられるアジアの人々と連携しながら研究が進めば，日本人で発見された疾患バイオマーカーを利用して，広くアジアの患者の早期診断にも利用できる可能性がある．

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Letter to the Editor

# Immunohistochemical analysis of aldehyde-modified proteins in drusen in cynomolgus monkeys (*Macaca fascicularis*)☆

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**Abstract**

Protein modifications resulting from reactive aldehydes are thought to be involved in the pathogenesis of various degenerative diseases. Aged cynomolgus monkey (*Macaca fascicularis*) spontaneously develop drusen in the macula, consistent with the phenotype observed in early-stage age-related macular degeneration (AMD), indicating that this animal is an optimum model for AMD. In retinal sections from three monkeys with macular degeneration, regardless of their size, drusen were consistently positive with immunohistochemical labeling against protein modifications by 4-hydroxynonenal and 4-hydroxyhexenal, end products of non-enzymatic oxidation of n-6 and n-3 polyunsaturated fatty acids, respectively. Positive labeling for both modifications was observed in the ganglion cell layer, the inner nuclear layer, the outer nuclear layer, and the retinal pigment epithelium. However, no consistent differences in location or intensity of the labeling were observed between monkeys with normal macula and macular degeneration. The results suggest a possible association between drusen formation and protein modifications by aldehydes in the pathogenesis of AMD.


*Keywords:* aldehydes; 4-hydroxynonenal; 4-hydroxyhexenal; cynomolgus monkey; drusen; age-related macular degeneration; protein modification

Age-related macular degeneration (AMD) is the leading cause of legal blindness in elderly individuals in industrialized countries (Fine et al., 2000). Accumulation of extracellular deposits between the retinal pigment epithelium (RPE) and Bruch's membrane, referred to as drusen, is regarded as a hallmark risk factor for development of AMD (de Jong, 2006). Various lipids, polysaccharides, and glycosaminoglycans have been identified as constituents of drusen (Hageman et al., 2001). Recent studies have revealed that drusen contains various protein molecules that are related to inflammation, immune responses, and oxidative stresses (Mullins et al., 2000; Crabb et al., 2002); yet the mechanism of formation is not fully understood.

Aged monkeys spontaneously develop macular degenerative changes such as pigment mottling, hyperpigmentation or hypopigmentation, and drusen in the macula, consistent with the phenotype observed in early-stage AMD (Stafford et al., 1984; Ishibashi et al., 1986). Previous proteomic analysis indicated that a number of protein components are common in drusen from monkeys and humans (Crabb et al., 2002; Umeda et al., 2005). Thus, these animals are thought to be an optimum animal model for AMD.

4-Hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE) are α,β-unsaturated aldehydes that are end products of non-enzymatic oxidation of n-6 and n-3 polyunsaturated fatty acids, respectively (Esterbauer, 1993). These highly reactive aldehydes can react readily with histidine, cysteine, or lysine

☆ The authors have no proprietary interest in any aspect of this report.

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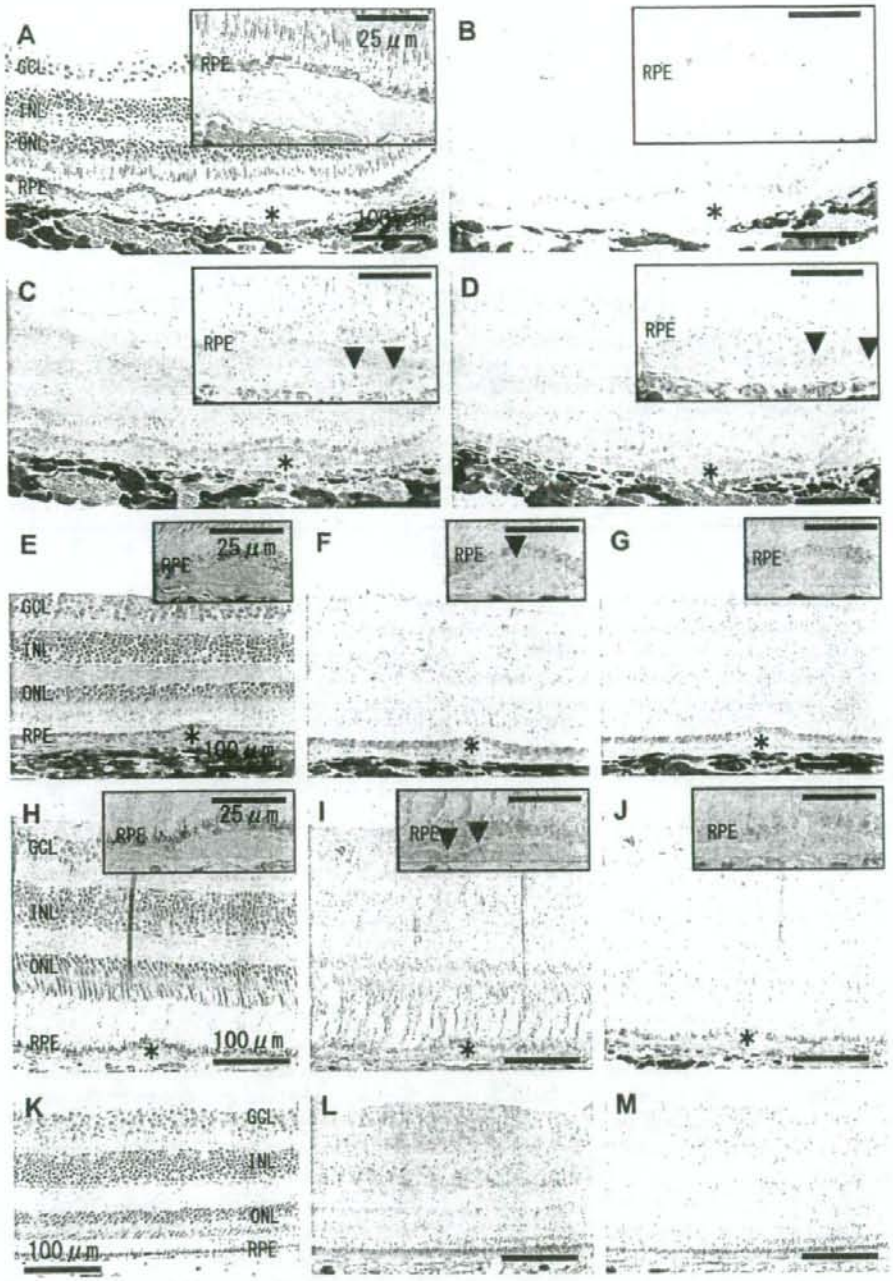


Fig. 1. Retinal sections from monkeys with macular degeneration and normal macula. Representative retinal sections from three monkeys with macular degeneration (panels A–D, E–G, and H–J, respectively) and those from a monkey with a normal macula (panels K–M) are shown. Representative sections stained with hematoxylin and eosin (panels A, E, H, and K) labeled with normal mouse IgG (panel B), 4-HNE-modified proteins (panels C, F, I, and L) and 4-HHE-modified proteins (panels D, G, J, and M). Asterisks and arrowheads indicate drusen and granular labeling, respectively. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; and RPE, retinal pigment epithelium.

residues of proteins, leading to formation of stable Michael adducts with a hemiacetal structure (Uchida and Stadtman, 1992). Formation of these adducts leads to a variety of cytopathological effects such as inhibition of enzyme activity, inhibition of protein, RNA, and DNA synthesis, cell cycle arrest, and apoptosis (Awasthi et al., 2004). The use of specific antibodies to recognize the hemiacetal structure of Michael adducts enables their detection in tissues (Uchida et al., 1993). Previous studies have suggested that modifications by these aldehydes of proteins common to drusen components in humans are molecular events preceding light-induced retinal degeneration in rats (Tanito et al., 2005, 2006). Recently, proteins modified by 4-HNE were detected in the neural retina of patients with AMD (Ethen et al., 2007). We evaluated immunohistochemically the localization of proteins modified by these aldehydes on retinal sections from aged monkeys.

Cynomolgus monkeys (*Macaca fascicularis*) were reared in large-scale breeding facilities at the Simian Conservation Breeding and Research Center, Inc., Manila, the Philippines. Maintenance of monkeys and preparation of paraffin-embedded retinal sections have been described previously (Umeda et al., 2005). At the breeding facilities, 278 aged female monkeys (age range, 13–25 years; average age, 16.9 years) were funduscopically examined three times from 2001 to 2004. Of the 278 animals, 67.6% had a normal macula with no detectable pigmentary abnormalities, 10.8% were diagnosed with mild macular degeneration (<5 drusen), 11.2% with moderate macular degeneration (5–20 drusen), and 10.4% with severe macular degeneration (>20 drusen) (Umeda et al., 2005).

In the current study, retinal sections from three monkeys with normal macula and three monkeys with severe macular degeneration were examined. The immunohistochemistry methods were described previously (Tanito et al., 2005). Mouse anti-4-HNE- and anti-4-HHE-modified protein antibodies were purchased from NOF Corporation (Tokyo, Japan). Hematoxylin and eosin staining was performed to observe accumulation of drusen of various sizes between the RPE and choriocapillaries in the macular region in retinal sections from the three monkeys with severe disease (Fig. 1A, E, and H), whereas no drusen accumulated in any retinal sections from three monkeys with normal macula (Fig. 1K). By immunohistochemistry, regardless of size, drusen were consistently positive with diffuse labeling against both protein modifications by 4-HNE (Fig. 1C, F, and I) and 4-HHE (Fig. 1D, G, and J) in retinal sections from three monkeys with macular degeneration. Using high magnification, drusen frequently contained granular labeling against both protein modifications by 4-HNE (insets, Fig. 1C, F, and I) and 4-HHE (inset, Fig. 1D). Positive labeling for both antibodies was observed in the ganglion cell layer, the inner nuclear layer, the outer nuclear layer, and the RPE. However, no consistent differences in localization or intensity of labeling were observed between monkeys with normal macula and those with macular degeneration. Retinal sections incubated with normal mouse IgG as a negative control showed no positive staining over all retinal layers. The results clearly showed that the drusen contained proteins modified by the reactive aldehydes, 4-HNE and 4-HHE.

AMD progression can be slowed with antioxidant and zinc (The AREDS Research Group, 2001), thus oxidative stress has been suspected of contributing to the pathogenesis of AMD (Beatty et al., 2000). The relationship between abnormal protein oxidation/modification and macular degeneration has been reported, including detection of cross-linked species of tissue metalloproteinase inhibitor 3 and vitronectin, docosahexaenoic acid-derived carboxyethylpyrrole protein adducts in drusen from patients with AMD (Crabb et al., 2002), and protein modifications from oxidized carbohydrate such as carboxymethyl-lysine and pentosidine in ocular tissues from aged donors and those with AMD (Ishibashi et al., 1998; Handa et al., 1999). In addition to the previous evidence, we identified possible involvement of protein modifications by 4-HNE and 4-HHE in the formation of drusen in AMD. Protein modification by biologically active molecules including reactive aldehydes may be a critical process in drusen formation and the development of AMD.

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# *LOXL1* variants in elderly Japanese patients with exfoliation syndrome/glaucoma, primary open-angle glaucoma, normal tension glaucoma, and cataract

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**Purpose:** To evaluate the association of lysyl oxidase like 1 (*LOXL1*) gene variants in Japanese patients with open-angle glaucoma.

**Methods:** We evaluated the association of three *LOXL1* variants (rs1048661, rs3825942, and rs2165241) in 142 Japanese patients with exfoliation syndrome (EX; n=59) and exfoliation glaucoma (EG; n=83) as well as in 251 control patients aged 70 years or older with primary open-angle glaucoma (PG; n=40), normal tension glaucoma (NG; n=54), and cataract (CT; n=157).

**Results:** In comparison with the CT group, the single nucleotide polymorphisms (SNPs) showed significant association with EX, EG, and EX+EG. The odds ratio (OR)=19.71–28.23 and $p=1.69\times10^{-23}$-$3.00\times10^{-45}$ for allele T of rs1048661; OR=28.21–39.78 and $p=1.77\times10^{-8}$-$2.42\times10^{-22}$ for allele G of rs3825942; and OR=16.59–23.40 and $p=4.79\times10^{-5}$-$1.08\times10^{-9}$ for allele C of rs2165241. In comparison with the controls (CT+PG+NG), the haplotype rs1048661/rs3825942 (T/G) was significantly associated with EX+EG ($p=8.27\times10^{-44}$), and haplotype G/A had a significant protective effect ($p=2.25\times10^{-14}$). None of the three SNPs showed significant differences between the EX and EG groups or between the PG and NG groups.

**Conclusions:** These SNPs are associated with exfoliation syndrome/glaucoma in the Japanese population. The risk alleles in rs1048661 and rs2165241 are different from other populations. Additional genetic or environmental risk factors other than these *LOXL1* SNPs could be associated with the development of exfoliation syndrome as well as exfoliation glaucoma among exfoliation syndrome patients.

Exfoliation syndrome is the most common identifiable cause of open-angle glaucoma worldwide. It is an age-related, generalized disorder of the extracellular matrix that is characterized by the production and progressive accumulation of a fibrillar extracellular material in many ocular tissues [1]. A recent genome-wide association study demonstrated that one intronic single nucleotide polymorphism (SNP, rs2165241) and two exonic SNPs (rs1048661 [R141L] and rs3825942 [G153D]), which are located in the first exon of the lysyl oxidase like1 (*LOXL1*) gene on chromosome 15q24.1, are highly associated with exfoliation syndrome in Icelandic and Swedish populations while none of these SNPs were associated with primary open-angle glaucoma in either of the two populations [2].

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Until now, several lines of studies have reported significant associations of these *LOXL1* SNPs with exfoliation syndrome [3-16] or the lack of associations of these SNPs with primary open-angle glaucoma [16-18] in various populations. In these studies, subjects in their 40s and 50s are sometimes recruited as controls. The prevalence of clinical exfoliation syndrome increases with age, particularly after the age of 60 years [1]. However, preclinical exfoliation syndromes in younger generations are able to distinguish from normal subjects until patients become older. Accordingly, the level of statistical significance between exfoliation syndrome and controls could be underestimated in studies that include younger patients in the control group. In this study, we evaluated the association of three *LOXL1* SNPs in Japanese patients with exfoliation syndrome/glaucoma, primary open angle glaucoma, normal tension glaucoma, and cataract. To reduce the chance of misclassifying latent syndromes within the control group, only patients aged 70 years or older were recruited as primary open-angle glaucoma, normal tension glaucoma, or cataract subjects in this study. To the best of our knowledge, this is the first study reporting an association of

Table 1. Summary of study populations.

| | Exfoliation syndrome or glaucoma | | | Primary glaucoma | | | | |
|---|---|---|---|---|---|---|---|---|
| Parameter | EX | EG | EX+EG | PG | NG | PG+NG | CT | p value |
| No. of subjects | 59 | 83 | 142 | 40 | 54 | 94 | 157 | |
| Male:Female | | | | | | | | |
| N | 12:47 | 42:41 | 54:88 | 14:26 | 18:36 | 32:62 | 45:112 | 0.2264* |
| % | 20.3:79.7 | 50.6:49.4 | 50.6:49.4 | 35.0:65.0 | 33.3:66.7 | 34.0:66.0 | 28.7:71.3 | |
| Age (years) | | | | | | | | |
| mean±SD | 78.2±8.0 | 78.8±8.5 | 78.5±8.2 | 75.6±5.3 | 78.3±4.8 | 77.2±5.1 | 77.2±5.0 | 0.1271** |
| Range | 55–95 | 57–95 | 55–95 | 70–87 | 70–91 | 70–91 | 70–90 | |

The asterisk and double asterisk indicate that the p values were obtained by Pearson's $\chi^2$ test and one-way ANOVA, respectively, among three (EX+EG, PG+NG, and CT) groups. EX, exfoliation syndrome; EG, exfoliation glaucoma; PG, primary open-angle glaucoma aged 70 years or older; NG, normal tension glaucoma aged 70 years or older; CT, cataract aged 70 years or older.

*LOXL1* SNPs with exfoliation syndrome/glaucoma using age-controlled subjects. This is also the first study reporting the association of *LOXL1* SNPs with normal tension glaucoma.

## METHODS

*Subjects:* Three hundred and ninety-three unrelated Japanese subjects presenting exfoliation syndrome without glaucoma (EX), exfoliation syndrome with glaucoma (EG), primary open-angle glaucoma (PG), normal tension glaucoma (NG), and cataract (CT) were recruited at the Shimane University Hospital and Iinan Hospital in Shimane, Japan. This study adhered to the tenets of the Declaration of Helsinki. The research was reviewed and approved by the Institutional Review Boards of both hospitals. Written informed consent was obtained from all of the subjects. All of the subjects underwent a dilated pupil examination of the anterior segments, ocular media, and fundus using a slit-lamp and a funduscope. The subjects with EX and EG exhibited the typical pattern of exfoliation material on the anterior lens surface and/or pupillary margin during slit-lamp examination. The subjects with EG and PG had a history of intraocular pressure (IOP) greater than or equal to 21 mmHg, and the subjects with EX, NG, and CT had a history of IOP never exceeding 20 mmHg. The subjects with PG, NG, and EG presented a typical glaucomatous optic disc cupping or rim thinning and visual field loss. The patients who had a history of IOP greater than or equal to 21 mmHg but no glaucomatous optic disc changes nor visual field loss were assigned as EG in this study. To avoid possible misclassification of latent exfoliation syndromes as PG, NG, or CT, patients younger than 70 years old were not recruited. The number of subjects, each gender, and the mean and range of ages in each group are summarized in Table 1.

*DNA genotyping:* Genomic DNA was extracted from the peripheral white blood cells of each subject. Polymerase chain reaction (PCR) was performed using primers designed to amplify the genomic region containing both rs1048661 and rs3825942 (forward primer: 5'-AGG TGT ACA GCT TGC TCA ACT C-3' and reverse primer: 5'-TAG TAC ACG AAA CCC TGG TCG T-3') or just rs2165241 (forward primer: 5'-AGA ATG CAA GAC CTC AGC ATG AG-3' and reverse primer: 5'-TAG TGG CCA GAG GTC TGC TAA G-3'). The sequence was determined based on the dideoxy terminator method using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. We used SeqScape Software version 2.5 (Applied Biosystems) to analyze the sequence alignment.

*Statistical analysis:* The deviation of the genotype distributions from the Hardy–Weinberg equilibrium was assessed in the case and control samples using HAPLOVIEW version 4.0 [19]. Statistical analysis was performed using R version 2.6.2. Fisher's exact test was used to compare the allele or genotype frequencies of each case group with the controls. The odds ratio (OR) and 95% confidence intervals (CIs) were calculated by the logistic regression method. Individual haplotypes and their estimated population frequencies were inferred using HAPLOVIEW version 4.0 [19] with all of the parameters set at the default values.

## RESULTS

The allelic and genotypic counts and frequencies of SNPs rs1048661, rs3825942, and rs2165241 within *LOXL1* are shown in Table 2. The ORs and p values for the allelic and genotypic frequencies of the three SNPs in comparison between the cases (EX, EG, and EX+EG) and controls (CT, PG, NG, and PG+NG) are shown in Table 3. In comparison with the CT group, the SNPs showed a significant association with EX, EG, and EX+EG for the T allele of rs1048661 at OR=19.71–28.23 and $p=1.69\times10^{-23}$-$3.00\times10^{-45}$, the G allele of rs3825942 at OR=28.21–39.78 and $p=1.77\times10^{-8}$-$2.42\times10^{-22}$, and the C allele of rs2165241 at OR=16.59–23.40 and $p=4.79\times10^{-5}$-$1.08\times10^{-9}$ (Table 3). The genotypes, TT of rs1048661 ($p=4.11\times10^{-25}$-$3.78\times10^{-43}$), GG of rs3825942 ($p=3.53\times10^{-11}$-$2.10\times10^{-33}$), and CC of rs2165241 ($p=1.95\times10^{-4}$-$1.07\times10^{-8}$), also showed significant