6. 裂孔原性網膜剥離における

High mobility group box 1の病態形成への関与

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研究要旨 High mobility group box 1 (HMGB1)は、細胞の壊死やTNF-αなどの刺激により細胞外に放出され、好炎症、免疫アジュバント、組織修復などの活性をもつことが報告された。裂孔原性網膜剥離(RD)における HMGB1 の病態形成への関与について検討した。裂孔原性網膜剥離患者の硝子体液中 HMGB1 濃度はコントロールに比べ有意に高値であった。剥離網膜においてはコントロールに比べ抗 HMGB1 抗体に対する強い染色性を示した。HMGB1 は ARPE19 細胞に VEGF の産生を誘導し、細胞死を誘導した。HMGB1 は裂孔原性網膜剥離における細胞死、増殖性変化などの病態形成に深く関わり、炎症、細胞壊死を伴う硝子体疾患の新しい治療ターゲット分子になりうる。

A. 研究目的

HMGB1 は 1973 年 Goodwin らにより遺伝子の転写調節あるいはヌクレオソーム安定化作用を有する核内 DNA 結合タンパク質として発見されたが、細胞の壊死や TNF-α などの刺激により細胞外に放出され、好炎症、免疫アジュバント、組織修復などの活性をもつことが報告された。裂孔原性網膜剥離における HMGB1 の病態形成への関与について検討した。

B. 研究方法

裂孔原性網膜剥離患者 (n=11)およびコントロールとして黄斑円孔(MH)・黄斑上膜(ERM)患者 (n=11)の硝子体液中における HMGB1 濃度を ELISA 法にて測定した。網膜剥離患者の網膜における HMGB1 の発現を抗 HMGB1 抗体による免疫組織化学染色にて検討した。in vitro において recombinant HMGB1 刺激に対する ARPE19 細胞（網膜色素上皮細胞株）の応答様式を検討した。

（倫理面への配慮）

本臨床研究は、ヘルシンキ宣言の勧告に従って行われ、鹿児島大学病院の倫理委員会の承認または倫理規定に基づいて行われた。

C. 研究結果

裂孔原性網膜剥離患者の硝子体液中 HMGB1 濃度 (4.75±4.51 ng/ml)はコントロール(0.28±0.50 ng/ml)に比べ有意に高値であった。剥離網膜においてはコントロールに比べ抗 HMGB1 抗体に対する強い染色性を示した。HMGB1 は 5 μg/ml<の濃度において ARPE19 細胞に VEGF の産生を誘導し、2.5 μg/ml<の濃度にて細胞死を誘導した。

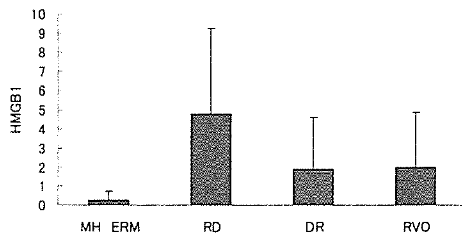
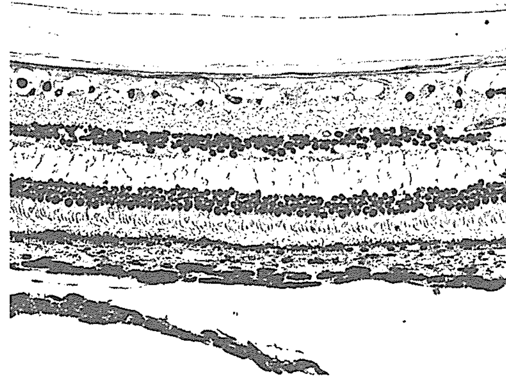
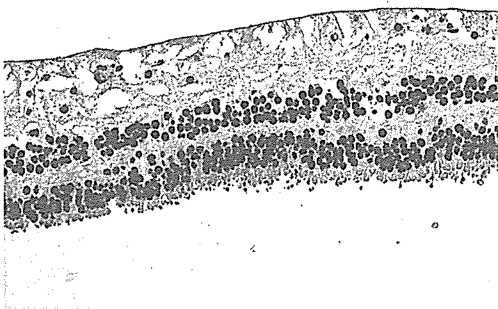


図 1. 硝子体液中の HMGB1 濃度
(DR:糖尿病網膜症、RVO:網膜静脈閉塞症、縦軸単位:ng/ml)

C. 非網膜剥離（抗 HMGB1 抗体）



A. 網膜剥離（抗 HMGB1 抗体）



D. 非網膜剥離（コントロール IgG 抗体）

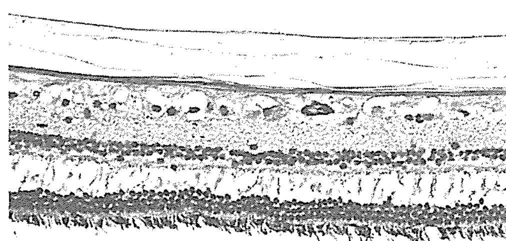
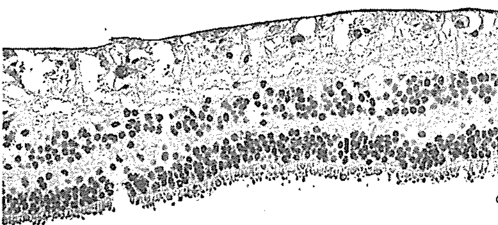


図 2. 免疫組織化学染色（A. B. C. D）

B. 網膜剥離（コントロール IgG 抗体）



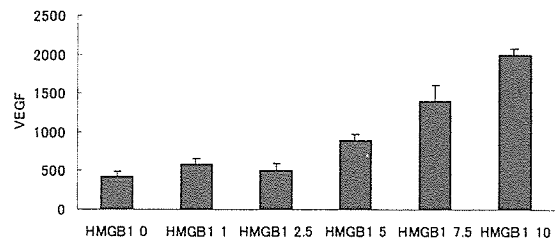


図 3. Recombinant HMGB1 刺激による ARPE19 細胞からの VEGF 発現
（横軸単位:μg/ml、縦軸単位:pg/ml）

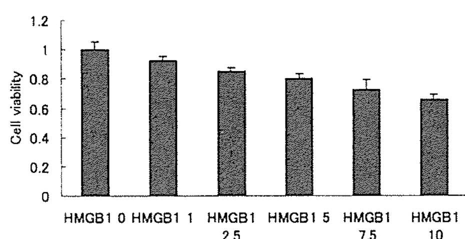


図4. Recombinant HMGB1 刺激による
ARPE19 細胞の生存率
（横軸単位：μg/ml、縦軸：HMGB1 0μg/ml 比）

D．考察

HMGB1 は裂孔原性網膜剥離における細胞死、増殖性変化などの病態形成に深く関わり、炎症、細胞壊死を伴う硝子体疾患の新しい治療ターゲット分子になりうる。

E．結論

HMGB1 は裂孔原性網膜剥離における細胞死、増殖性変化などの病態形成に深く関わり、炎症、細胞壊死を伴う硝子体疾患の新しい治療ターゲット分子になりうる。

F．健康危険情報

裂孔原性網膜剥離は視力障害をきたす疾患であり、手術を行っても高度の視力低下を来すこともある。HMGB1 は、新しい治療ターゲット分子になりうる。

G．研究発表

１．論文発表　　　未定
２．学会発表
第 111 回眼科学会で発表予定である。

H．知的財産権の出願・登録状況

１．特許取得　　　なし
２．実用新案登録　　　なし
３．その他　　　なし

I．参考文献

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7. 網膜前組織のexpressed sequence tag解析による遺伝子発現データベース作成

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小倉　淳[2]、池尾一穂[2]、五條堀孝[2]、山本　健[3]
([1]九州大眼科、[2]国立遺伝学研究所・日本DNAデータバンク、
[3]九州大・生医研・ゲノム機能制御学)

研究要旨　網膜前組織は網膜硝子体界面に生じる極小組織であるが、その生成の分子機序は不明な点が多い。今回網膜前組織のexpressed sequence tag解析による遺伝子発現データベースを作成した。硝子体手術の際に切除した特発性網膜前膜、糖尿病網膜症および増殖性硝子体網膜症に伴う増殖組織から各々totalRNAを抽出し、Switching Mechanism At 5' end of RNA Transcript (SMART)法を用いて全転写産物を増幅後、5'末端から約7000クローンの塩基配列を決定した。低質シークエンスおよびくり返し配列を除去し、アセンブルとクラスタリング後にnon-redundantシークエンスのBLASTサーチを行い、データベース化した。本解析により、網膜前組織において多数の機能遺伝子が発現しており、これらがコードする分子群の複雑な相互作用によって生じていると想定された。患者網膜前組織の遺伝子レベルの知見を蓄積することにより、診断、治療と病態の新しい理解につながる可能性があると考えた。

A．研究目的

網膜硝子体界面の細胞増殖を生じる代表的な疾患として、血管新生を伴わない特発性網膜上膜 (idiopathic epiretinal membrane;iERM)と増殖性硝子体網膜症(proliferative vitreoretinopathy;PVR)および血管新生を伴う増殖糖尿病網膜症(proliferative diabetic retinopathy;PDR)などがある。これらの疾患では網膜光凝固術や硝子体手術などの治療を行っても視機能を保持できない場合が少なくない。また現在までこれらの疾患に対する薬物で明らかな有効性が確認されたものはない。

増殖組織の形態学的観察では、網膜グリア細胞、マクロファージ、硝子体細胞、線維芽細胞や新生血管内皮細胞が認められる。また、PDR患者の硝子体液やモデルシステムで、線維芽細胞増殖因子(basic fibroblast growth factor;bFGF)、 血管内皮細胞増殖因子 (vascular endothelial growth factor;VEGF)、腫瘍壊死因子-α (tumor necrosis factor-α;TNF-α)[1]、インターロイキン-8 (interleukin-8;IL-8)[2-5]、単球遊走因子 (monocyte chemoattractant protein-1 ;MCP1)[6, 7]などのサイトカインや増殖因子の関与が示唆されている。しかし、これまで蓄積されてきた知見は、仮説駆動型、要素還元型の微小環境研究による

ものが大部分で、実際の患者においては、少数の因子だけではなく、より複雑な分子間の相互作用の結果、増殖組織が生成されていることが予想される。従って、増殖組織におけるより包括的な遺伝子発現ネットワークを具体化し、確実な分子標的を抽出することが必要であると思われる。

近年のゲノムプロジェクトの終了とそれに引き続くHapMapプロジェクトなどの国際的な進展は、糖尿病などの多因子性疾患の疾患感受性遺伝子の同定戦略を加速している。さらに我が国を中心に“もう一つのゲノムプロジェクト”と呼ばれる完全長cDNAプロジェクトが行われており、ヒトやマウスの転写産物の全長配列、構造と機能、スプライスバリアントの情報が加速度的に蓄積され、特定の組織中に発現する各遺伝子の注釈付けが数年前とは比較にならないほど容易になっている。この潮流に沿って、expressed sequence tag(EST)解析、serial analysis of gene expression (SAGE)法や、マイクロアレイ法によって、特定の組織中での相対的な遺伝子発現レベルの包括的な定量が行われている。これまで眼科領域では、米国国立眼研究所（National Eye Institute : NEI）などから、ヒト網膜、網膜色素上皮細胞、毛様体、線維柱帯、角膜上皮、マウス網膜など正常組織のEST 解析が報告されているが、今日まで病的組織である増殖組織の EST 解析は行われていないため、ゲノム情報を基盤とした増殖組織の包括的トランスクリプトーム解析を行うことは時期を得ていると考えられる。われわれは、増殖組織中の包括的な遺伝子発現解析から予想される分子カスケードを把握することで、より詳細な増殖組織の生成機序を明らかにすることができる可能性があると考える。そして、このことにより、増殖組織のよりよい内科的、外科的治療につながることが期待される。

B, C 研究方法・結果

硝子体手術の際に切除した増殖組織からtotalRNA を抽出し、Switching Mechanism At 5' end of RNA Transcript (SMART)法を用いて全転写産物を増幅後、5'末端から約 7000 クローンの塩基配列を決定した。PHRED おおび Cross-match により低質シークエンスおよびくり返し配列を除去し、PHRAP, GRIST を用いてアセンブルとクラスタリング後に non-redundant シークエンスの BLAST サーチを行い、データベース化した (図 1)。

さらに、VisAnt(http://visant.bu.edu/)を用いて抽出遺伝子から想定される分子ネットワークを可視化したところ増殖組織で発現を確認した遺伝子と関連しうる分子を含めたネットワークが作成された(図2)。

(倫理面への配慮)

本研究はヘルシンキ宣言に則り、また九州大学病院倫理委員会の承認を得て行った。全ての患者から術前にインフォームドコンセントを得た。

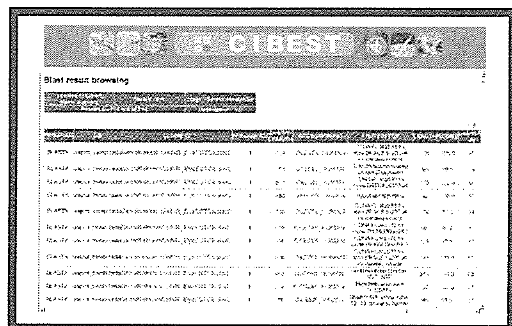

図１ 増殖組織遺伝子ライブラリで発現する遺伝子データベース

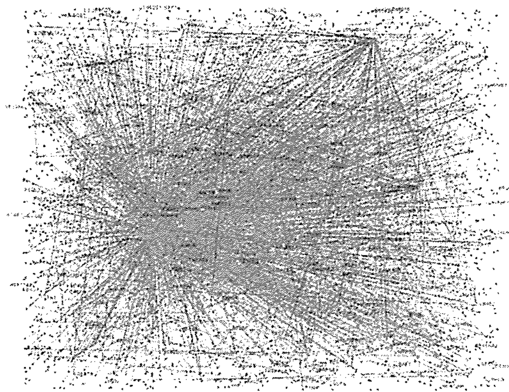

図2 VisANTによる増殖組織における分子ネットワークの視覚化

D．考察

近年の安全な網膜硝子体手術の普及により網膜前組織の生検が以前より容易になっている[8)]。我々は今回初めて網膜前組織からcDNAライブラリを作成することに成功した。EST解析では、網膜前組織において多数の機能遺伝子が発現しており、これらがコードする分子群の複雑な相互作用によって生じていることが想定された。UniGeneデータベースとの照合では、シークエンスしたクローンのうち80%以上が合致した。このことは、近年のゲノム研究進展に伴うデータベースの充実を反映している[9)]。

本研究において、従来の仮説駆動型の研究により想定されたMCP1やCOL2A1などの遺伝子群がEST中に認められており、現在までに想定されているメカニズムは確かに網膜前組織生成の一側面を反映していると考えられる。しかし、同時にこれらの各知見は少数の要素で構成される断片的な事象であり、網膜前組織でのより広範囲かつ多彩なメカニズムの一部にすぎない事も示された。事実、本研究で用いた発見駆動型のアプローチにより、従来その関与が知られていなかった多数の分子の網膜前組織生成への関連の可能性も明らかにできた。

Bioinformaticsを用いて断片化された知見を包括的に捉えることはポストゲノム研究の主要課題である。1つの試みとして我々は、VisANTを用いて抽出遺伝子と関連しうる分子を含めたネットワーク作成を行ってみたところ、EST解析のみでは抽出できないが、関与する可能性のある分子の候補を抽出した。これらの共起因子群が網膜前組織の生成に関わっているか否かは今後の検討課題である。

我々の今回の試みたEST解析は有用で、網膜前組織の診断、治療と病態のさらなる理解につながると考える。現在、網膜前組織のEST解析数を増すことにより、ヒト網膜前組織のさらに正確な分子肖像を把握中である。またiERMとPDRに伴う増殖組織の遺伝子発現を比較検討することで線維血管増殖組織進展の分子スイッチを同定できるかもしれない。さらには発現遺伝子群の網羅的な局在解析、レーザーキャプチャーマイクロディセクション法を用いた網膜前組織中の単一細胞での遺伝子発現解析を併用することにより、より詳細な分子機構を明らかにできる可能性がある。

ゲノム医科学的手法を用いたこれらの研究により、確かなエビデンスに基づいた、ゲノムワイドのRNA干渉法などを用いた次世代の網膜前組織の分子標的療法の開発が期待される。

E．結論

網膜前組織において多数の機能遺伝子が発現しており、これらがコードする分子群の複雑な相互作用によって生じていると想定

された。患者網膜前組織の遺伝子レベルの知見を蓄積することにより、診断、治療と病態の新しい理解につながる可能性があると考えた。

F．健康危険情報 なし

G．研究発表

1．論文発表 なし

2．学会発表 なし

H．知的財産権の出願・登録状況

1．特許取得 なし

2．実用新案登録 なし

3．その他 なし

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Novel triple missense mutations of *GUCY2D* gene in Japanese family with cone-rod dystrophy: Possible use of genotyping microarray

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Purpose: To report a novel mutation in the *GUCY2D* gene in a Japanese family with autosomal dominant cone-rod dystrophy (adCORD), and to examine the possible use of arrayed primer extension (APEX)-based genotyping chip in detecting mutations.

Methods: Genomic DNA was extracted from the peripheral blood of family members with adCORD. It was PCR-amplified, fragmented, and hybridized to APEX-based genotyping microarrays on which known disease-associated sequence variations were arrayed for patients with early-onset retinal dystrophy. All coding exons of the *GUCY2D* gene were directly sequenced. The PCR amplicon carrying a novel mutation was subcloned, and each clone was sequenced.

Results: Five single nucleotide polymorphisms in *AIPL1*, *RPGRIP1*, and *GUCY2D* were detected in the proband by microarray screening, and all were validated by direct sequencing. A novel heterozygous triple missense mutation of c.2540_2542delinsTCC (p.Gln847_Lys848delinsLeuGln amino acid substitutions) was found in both the proband and his father, and the three nucleotide changes were located on the same chromosome. Electroretinography (ERGs) demonstrated a significant reduction in rod function and a complete absence of cone function in both affected individuals.

Conclusions: A novel heterozygous triple consecutive missense mutation in the *GUCY2D* gene has been linked to adCORD. Our study demonstrates that the APEX-based gene screening can be used to identify simultaneously disease-modifying sequence changes as well as disease-causing mutations, once proper and comprehensive sites of sequence variations of the disease are arrayed.

Cone dystrophy (COD) and cone-rod dystrophy (CORD) belong to a subgroup of inherited chorioretinal dystrophies that is characterized by an initial degeneration of cone photoreceptors, causing an early decrease of visual acuity and color vision. The cone degeneration is followed by the degeneration of rod photoreceptors leading to progressive night blindness and peripheral visual field loss [1,2].

At present, COD and CORD are known to be genetically heterogeneous, and dominant, recessive, and X-linked inheritance patterns have been reported. The disease displays phenotypic and genotypic heterogeneity, and recent genetic studies have implicated a number of causative genes for CORD and COD, e.g., the *CRX*, *GUCY2D*, *AIPL1*, *GUCA1A*, *RIMS1*, and *UNC119* genes for autosomal dominant (ad) CORD; the *ABCA4* and *RDH5* genes for autosomal recessive CORD; and the *RPGR* gene for X-linked recessive CORD [3].

Screening for mutations responsible for CORD has yet to become a routine procedure in clinical practice. This is mainly due to the large genetic heterogeneity. Because the current detection technologies are labor-intensive involving a screening of at least 100 amplicons (exons) that encompass the entire open reading frames of several disease-causative genes. Therefore, it would be more convenient if a rapid and efficient method is developed to identify disease-causing genes responsible for CORD.

The capabilities of genotyping microarrays have greatly improved during the past decade [4]. These microarrays display hundreds of specific oligonucleotide probes that are precisely located on a small-formatted solid support. The array-based technologies have both research and potential clinical applications due to their ability to examine multiple genotypes from an individual simultaneously.

Among a number of microarray genotyping devices, the arrayed primer extension (APEX) is a method based on an array of oligonucleotides, immobilized at the 5' end on a glass surface [5]. A patient's DNA is amplified by PCR, digested enzymatically, and annealed to the immobilized primers. This promotes sites for template-dependent DNA polymerase extension reactions using four unique fluorescently-labeled dideoxy nucleotides. This technique for genotyping microarrays has been used to detect different genotypes and mutations, including those for retinal diseases such as Stargardt disease [6] and Leber's congenital amaurosis (LCA) [7].

These microarrays are commercially available, and an LCA chip containing 307 sequence variants previously iden-

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tified in eight genes associated with LCA as well as early-onset retinal degeneration: *AIPL1*, *CRB1*, *CRX*, *GUCY2D*, *RPE65*, *RPGRIP1*, *MERTK*, and *LRAT* is available [8]. Because LCA is considered to be a kind of congenital stationary "cone-rod dystrophy" with high hypermetropia, panretinal degeneration, and greatly reduced visual acuity [9], and because *AIPL1*, *CRX*, and *GUCY2D* are also causative genes for adCORD, we hypothesized that the LCA microarray could be used as an initial screening tool for patients with CORD to identify the disease-causing mutation(s). During our examinations with the LCA chip, we coincidentally identified a novel heterogeneous triple mutation in the *GUCY2D* gene. The possible use of genotyping microarray is discussed.

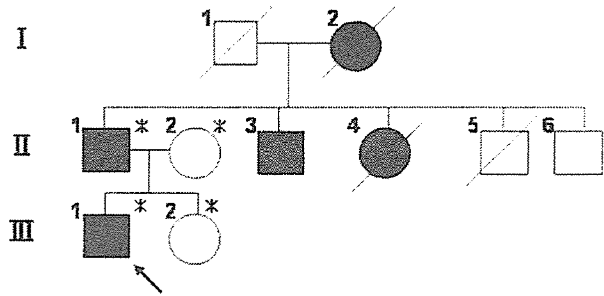

Figure 1. Pedigree of the family. Solid black symbols represent affected members who have autosomal dominant cone-rod dystrophy; white symbols represent unaffected members. Circles and squares indicate women and men, respectively. Those who underwent DNA testing are indicated by asterisk, and the is marked by arrow.

METHODS

Clinical examinations: We examined two affected and two nonaffected members of a family with adCORD (Figure 1). Full medical histories were taken from each individual, and ophthalmologic examinations, including best-corrected visual

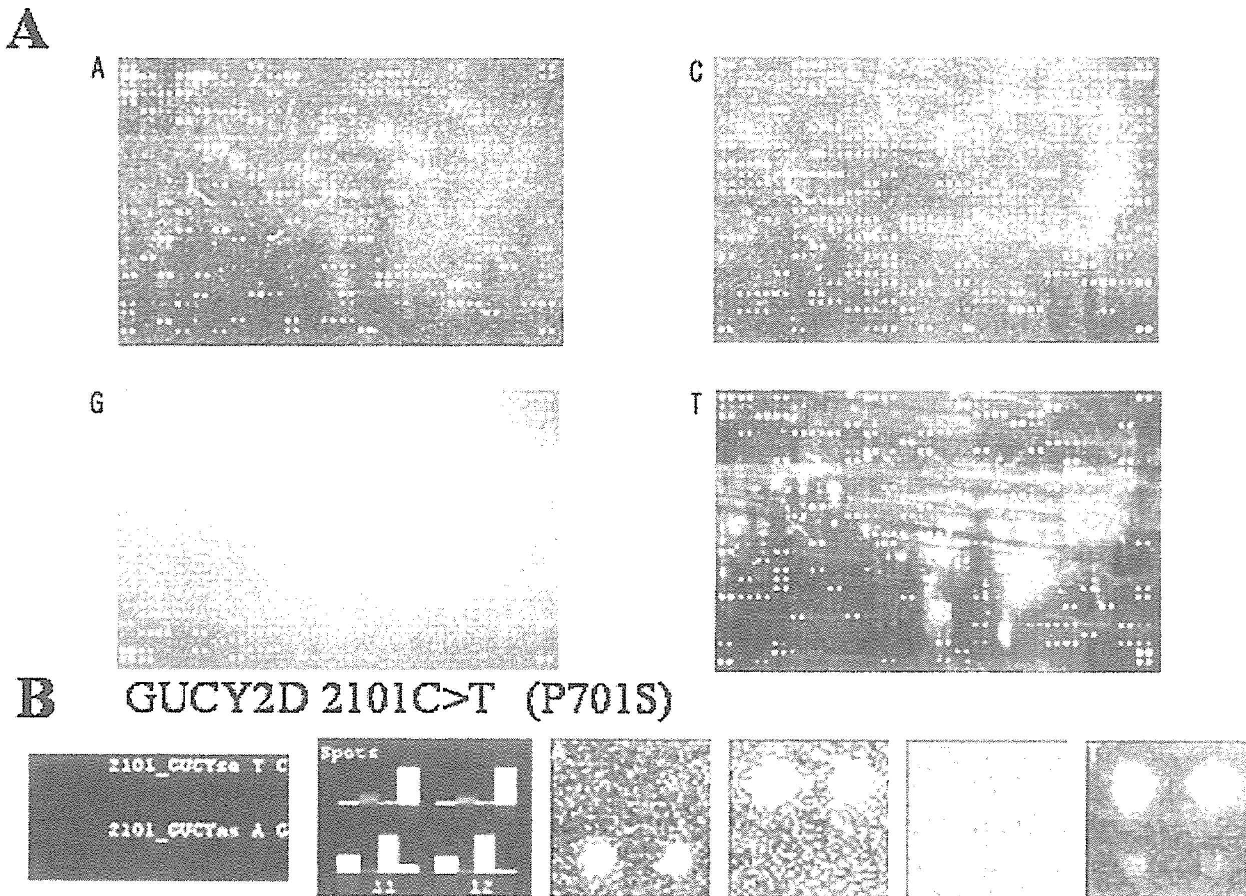

Figure 2. Leber congenital amarousis arrayed primer extension-based microarray assay hybridized to probes generated from proband's genomic DNA. **A**: Grayscale images for each fluorescent dideoxy nucleotide are used for the sequence analysis. **B**: Sequence alteration in the third base of codon 701 of the *GUCY2D* gene, analyzed by the GENORAMA software. Grayscale bitmaps corresponding to all four fluorescent dideoxy nucleotides at the base to be determined are shown enabling visual analysis. T signals in the sense area and A in the antisense area are indicative for a sequence alteration.

acuity, slit-lamp biomicroscopy, kinetic visual field examination, fundus examination, fluorescein angiography, and electroretinography (ERG) were performed on each subject. Color vision testing was performed with the panel D-15 and the Ishihara pseudoisochromatic plates.

Standardized full-field, photopic, flicker, and scotopic ERGs were recorded as recommended by the Standardization Committee of the International Society for Clinical Electrophysiology of Vision (ISCEV). After pupil dilatation with 0.5% tropicamide and 0.5% phenylephrine hydrochloride and 30 min of dark-adaptation, the scotopic ERGs were recorded with a white stimulus at an intensity of 0.12 cd.s/m^2. Rod-cone mixed single-flash ERGs were elicited by a white stimulus at an intensity of 20 cd.s/m^2. The photopic single-flash ERGs and the 30-Hz flicker ERGs were elicited with a white stimulus at an intensity of 1.2 and 0.6 cd.s/m^2, respectively, on a white background of 25 cd/m^2. Ten responses were averaged for the scotopic, the rod-cone mixed, and photopic ERGs, and 20 responses for the 30-Hz flicker ERGs.

Genomic DNA samples: This study was conducted in compliance with tenets of the Declaration of Helsinki, and approved by the Ethics Committee of the Kyushu University Hospital. All patients gave their informed consent prior to their inclusion in the study.

Genomic DNA was extracted from the blood of the patients and from non-affected individuals in the family using standard protocols to screen for genetic mutations [10,11].

Arrayed primer extension-based analysis: A detailed description of the APEX-based analysis is available at www.asperbio.com; Asper Biotech, Ltd. Briefly, selected ex-

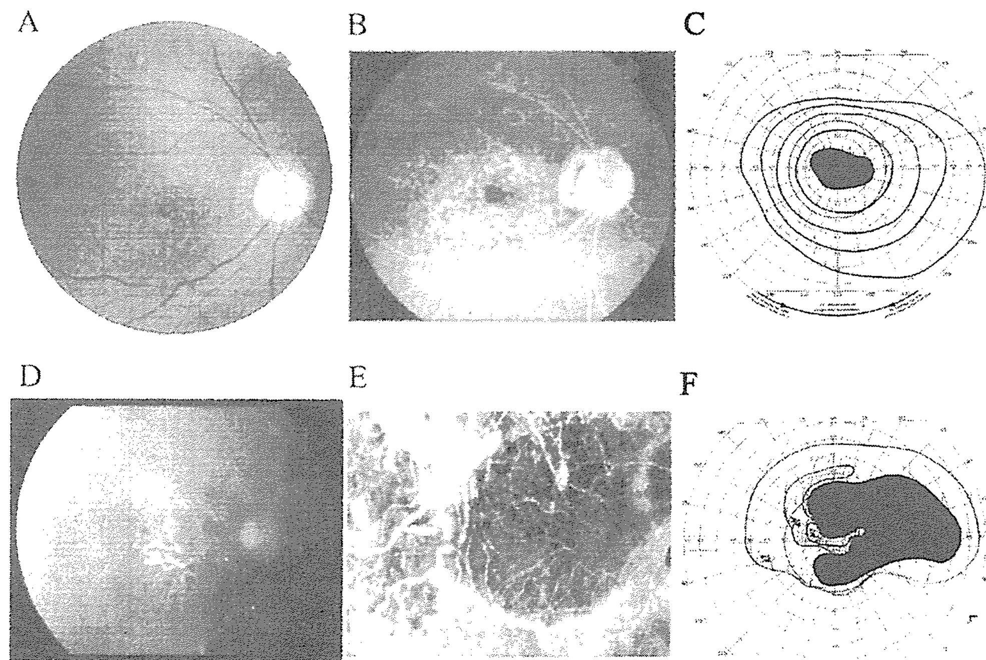

Figure 3. Ophthalmologic and functional analyses. Fundus photographs (**A**, **D**), fluorescein fundus angiograms (**B**, **E**), and Goldmann kinetic perimetric fields (**C**, **F**) of patients with mutations of the *GUCY2D* gene. (**A**, **B**, **C**) Right eye, proband; (**D**, **E**, **F**) Right eye, the proband's father.

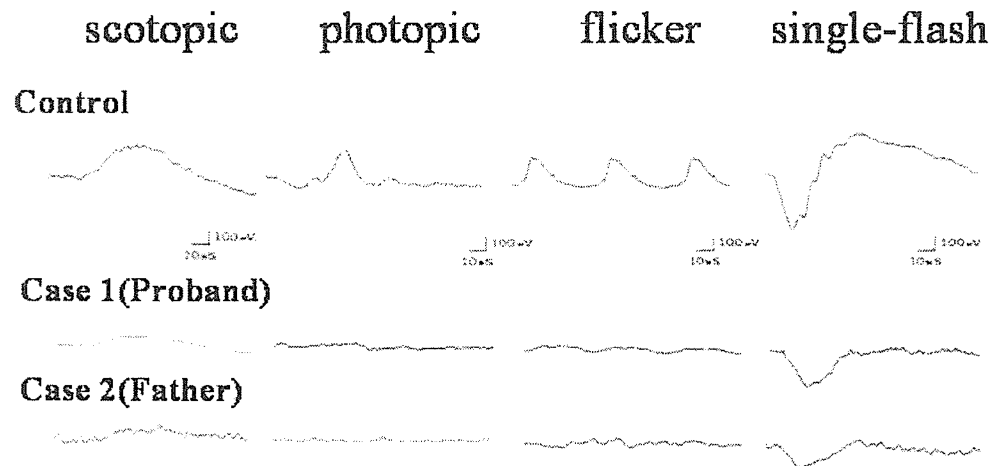

Figure 4. Electrophysiological recordings from the affected individuals and a normal subject. The scotopic rod electroretinogram (ERG), photopic cone ERG, 30-Hz flicker ERG, and bright flash rod cone mixed ERG amplitudes are markedly reduced in the proband. Those of the proband's father exhibited a similar trend, although more severely reduced.

ons of the *AIPL1*, *CRB1*, *CRX*, *GUCY2D*, *RPE65*, *RPGRIP1*, *MERTK*, and *LRAT* genes were amplified by polymerase chain reaction (PCR) following steps previously described [7]. After the amplified products were concentrated and purified they were fragmented by adding thermolabile uracil N-glycosylase (Epicentre Technologies, Madison, WI) with heat of 95 °C [5]. One-sixth of each amplified product was used in the primer extension reaction on the LCA genotyping microarray. The APEX mixture consisted of 10 μl of fragmented products, 4 units of Thermo Sequenase DNA polymerase (Amersham Pharmacia, Pittsburgh, PA), 2 μl of Thermo Sequenase reaction buffer (260 mM Tris HCl, pH 9.5/65 mM $MgCl_2$; Amersham Pharmacia), and 2 μM final concentration of each fluorescein-labeled ddNTP: Texas Red-ddATP, Cy3-ddCTP, fluorescein-ddGTP, Cy5-ddUTP (Amersham Pharmacia) and NEN Life Science Products (Boston, MA). The entire mixture was applied to slides warmed to 58 °C. The reactions were allowed to proceed for 20 min under parafilm and stopped by washing at 95 °C 2 times at 90 s each in MilliQ water. A droplet of SlowFade Light Antifade Reagent (Molecular Probes, Carlsbad, CA) was applied to the microarrays to limit bleaching of the fluorescein. The slides were imaged with the Genorama imaging system (Asper Biotech, Tartu, Estonia) at 20 μm resolution. Gene sequence and mutations were identified by GENORAMA 3.0 genotyping software by using clustered signal patterns from a sequenced control DNA as the statistical reference (Figure 2). The PCR amplification of the samples, hybridization, and image analysis were performed by Asper Biotech (Tartu, Estonia).

The extracted array-identified variants were confirmed by direct sequencing with the Taq Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Sequencing reactions were resolved on an ABI 3100 automated sequencer.

Molecular analysis of GUCY2D *gene:* All 18 coding exons of the *GUCY2D* gene, exon 2 of *AIPL1*, and exons 4 and 7 of *RPGRIP1* genes were amplified by PCR and directly sequenced using the Taq Dyedeoxy Terminator Cycle Sequencing Kit as described in the literature [11,12]. Primer sequences used for amplification of *GUCY2D* were obtained from published sequences [13]. To detect single nucleotide polymorphisms, we directly sequenced exons 10 and 13 of the *GUCY2D* gene from 136 chromosomes (35 men and 33 women) of unrelated normal Japanese individuals.

Subcloning of GUCY2D *alleles:* The PCR fragments of exon 13 of the *GUCY2D* gene in the patient were subcloned into TOPO-2 (Invitrogen, San Diego, CA) according to the manufacturer's protocol [14-16]. The cloned inserts were sequenced using T7 primer.

RESULTS

Case reports: The proband (III-1) was a 50-year-old man who was referred to our hospital because of reduced vision. His best-corrected visual acuity was 0.4 in each eye with mild myopia. He stated that he had noted a decrease in his vision approximately 10 years earlier. Family history revealed that his father, paternal uncle, and grandmother also had depressed central vision (Figure 1). Slit-lamp examination showed that, except for mild cataracts in both eyes, the anterior segments of his eyes were normal. However, fundus examination showed bull's eye maculopathy similar to macular degeneration in both eyes (Figure 3A). There were no apparent abnormalities of the peripheral retina. The proband (and his father) did not have pendular nystagmus.

Fluorescein angiography revealed a granular hyperfluorescence corresponding to the macular degeneration (Figure 3B). He failed all of the Ishihara color plates and also failed the panel D-15 test with a tritan axis. Goldmann kinetic perimetry showed a central scotoma of about 10-20 ° (Figure 3C). The full-field photopic ERGs and the 30 Hz flicker ERGs were almost unrecordable in this proband. The amplitude of the scotopic b-wave was significantly reduced, and the a- and b-waves of the bright-flash mixed rod-cone ERGs, as well as the oscillatory potentials, were reduced (Figure 4).

The proband's 76-year-old father (II-1) showed a more severe clinical phenotype. His best-corrected visual acuity was 0.01 in the right eye and 0.02 in the left. He had chorioretinal atrophy in the macular region with a central scotoma in both eyes (Figure 3D,F). Fluorescein angiography showed a hypofluorescence around the macula, reflecting the retinochoroidal atrophy (Figure 3E). Because of poor visual acuity, the father could not take the Ishihara color vision and panel D-15 tests. His ERGs were similar to those of the proband, although they were more severely reduced (Figure 4).

TABLE 1. SEQUENCE ALTERATIONS DETECTED BY MICROARRAY ANALYSIS

Gene Name	Exon	Nucleotide Change	Protein Change
AIPL1	2	268G>C	Asp90His
GUCY2D	2	154G>T	Ala52Ser
GUCY2D	10	2101C>T	Pro701Ser
RPGRIP1	4	574A>G	Lys192Glu
RPGRIP1	IVS6-17	907-17delTAA	SPLICE

Positions of nucleotide and corresponding protein changes detected by microarray analysis.

Both unaffected family members had good best-corrected visual acuities of >1.0 in both eyes without any ocular abnormalities. All affected and unaffected family members had myopia of approximately -3.0 diopters.

Microarray and genetic analyses: A typical autosomal dominant hereditary pattern through three generations in this family prompted us to screen genes responsible for adCORD. We decided to use LCA genotyping microarray screening first because LCA is considered to be a type of congenital stationary "cone-rod dystrophy" with high hypermetropia, panretinal degeneration, and greatly depressed visual acuity [9]. In addition, the sequences for the *AIPL1*, *CRX*, and *GUCY2D* genes were also represented on the microarray and have been reported to be causative genes for adCORD.

The screening by genotyping the proband revealed five sequence alterations, one of which was c.2101C>T (p.Pro701Ser) in the *GUCY2D* gene (Table 1). This mutation has been called pathogenic by the GENORAMA software (Figure 1B) because it has been reported as a disease-causative gene when the mutation is homozygous in patients with LCA [7]. The other sequence alterations were called polymorphisms by the software. All 5 microarray-identified variants were then confirmed by direct sequencing.

Because a sequence alteration in the *GUCY2D* gene was called pathogenic, we directly sequenced all coding region of the *GUCY2D* gene. Direct sequencing revealed three consecutive novel heterozygous missense mutations of c.2540_2542delinsTCC that would predict p.Gln847_Lys848delinsLeuGln amino acid substitutions. These changes were found in the proband and his affected father (Figure 5A). None of the mutations was detected in the proband's unaffected mother and sister, or in 136 control individuals (data not shown).

To determine whether the three sequence alterations are located on the same chromosome or on two chromosomes, we subcloned the PCR product of exon 13 of the gene from the proband into a TOPO 2 vector and each clone was sequenced. Five of eight clones had the triple sequence changes (Figure 5C), whereas the remaining three showed the wild-type sequence (Figure 5B). Thus, the three consecutive nucle-

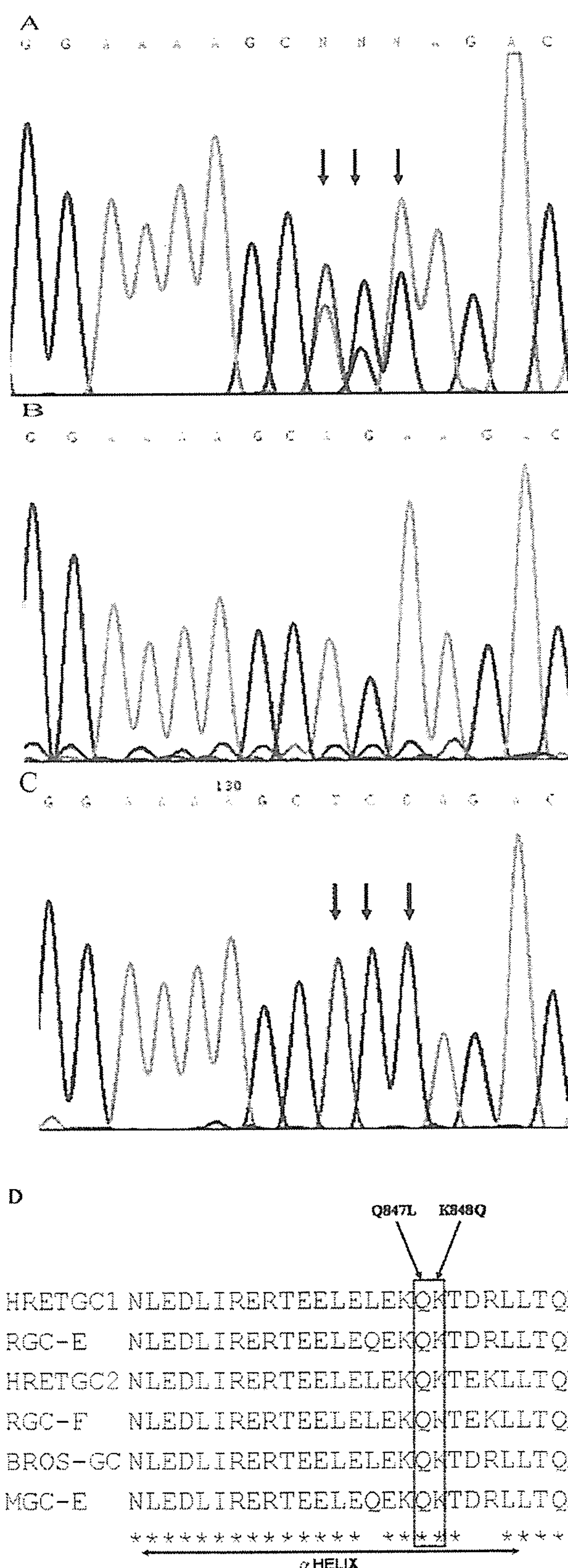

Figure 5. Nucleotide and amino acid sequence of exon 13 of the GUCY2D. **A**: Electropherogram of the sense strand of genomic DNA from the affected proband (III-1) and his father (II-1), showing a novel heterozygous multiple missense mutation of c.2540_2542delinsTCC in exon 13 (p.Gln847_Lys848delinsLeuGln). The arrows indicate the position of the mutation. **B**: The mutation is absent in the proband's unaffected mother and sister (II-2 and III-2). A subcloned sequence of exon 13 of the gene from the proband (III-1) is normal. **C**: Another subcloned sequence of exon 13 of the gene this proband (III-1) demonstrating all three missense sequence alterations are present on the same chromosome. **D**: Amino acid sequence alignment of human RETGC-1, rat GC-E [20], human RETGC-2 [21], rat GC-F [20], bovine ROS-GC [22], and mouse GC-E [20]. Asterisks denote residues of identity, and the [alpha]-helical domain within this region is also indicated. Arrows indicate residues Gln847 and Lys848, which are replaced by Leu and Gln, respectively, in this family.

otide sequence alterations were concluded to be present in one chromosome.

DISCUSSION

We have identified complex novel missense mutations in the GUCY2D gene in two members of a Japanese family with adCORD. The *GUCY2D* gene encodes retinal guanylate cyclase, RetGC-1, which is a photoreceptor-specific enzyme that is involved in recovery during the phototransduction cascade. Its function is to synthesize cyclic guanosine monophosphate (cGMP) from 5'-GMP in the retina and is responsible for increasing the proportion of open cGMP-gated channels in the dark-adapted state.

Our study confirmed the idea that a heterozygous mutation of *GUCY2D* not involving codon 838 can also be linked to CORD [17], although it has been reported that codon 838 appeared to be particularly prone to mutational changes in the heterozygous state.

RetGC-1 is believed to exist in a dimeric state [18], and mutations at sites 847-848, as observed in our case, are located within the putative dimerization domain of the RetGC-1 protein [19]. Moreover, alignment of part of this domain of human RetGC-1 and other members of the subgroup (human RetGC-1 [20], RetGC-2 [21], rat GC-E [20], GC-F [20], mouse GC-E [20], and bovine ROS-GC [22]) showed that 848 and 847 are highly conserved among the sensory cyclase family members (Figure 5D), suggesting that both mutations are critical. In support of this, In Silico analysis using SIFT and PolyPhen, which are Web-based applications that use phylogenetic and structural information from homologous proteins, showed that both sequence alterations might be pathogenic (data not shown).

It appears that the *GUCY2D* gene is prone to complex missense mutations. Thus far, three research groups have reported heterozygous complex missense mutations in the *GUCY2D* gene: a triple mutation of p.Glu837_Arg838_Thr839delinsAspCysMet [23], a complex mutation of p.Glu837_Arg838delinsAspSer [24,25], and a complex missense mutation of p.Ile915Thr and p.Gly917Arg in a Japanese family [26]. It was suggested that the high mutability of the CpG sequences in the *GUCY2D* gene may account for the multiple mutations [25]. However, this is not always the case because the multiple mutations in our case were not located in the CpG sequence. Thus, the mechanism responsible for the generation of multiple mutations in the *GUCY2D* gene remains unclear.

Electrophysiological examination showed that both the proband and his father had significant loss of the scotopic system in addition to the absence of cone responses with the reduction greater in the father. This is consistent with an earlier idea that a moderate to severe loss of rod function was present in families with multiple mutations compared with families with a single mutation showing a marked loss of cone function with only minimal rod involvement [19,24,25,27]. Therefore, different mutations in this dimerization domain of the *GUCY2D* gene can result in differing severities of CORD, especially that of the scotopic system.

Our study demonstrated the potential use of genotyping microarrays for the simultaneous detection of not only the causative but also the modifying sequence alterations in one test. Although the heterozygous c. 2101C>T (p.Pro701Ser) variant was considered to be not causative of the disease because it was detected in normal control individuals heterozygously (data not shown), we assume that the heterozygous c. 2101C>T might modify the disease phenotype as an additive effect, with the c.2540_2542delinsTCC change being the major disease-causing change in our family. This is because homozygous c. 2101C>T can be a causative alteration in LCA. Similarly, it is also possible that the other four silent mutations in the retinal disease causing genes, *AIPL*, *GUCY2D*, and *RPGRIP1*, detected as polymorphisms, could serve as disease modifier genes with another major gene defect occurring simultaneously, even if those changes do not significantly affect healthy individuals. Moreover, the technology that allows the examination of multiple genes simultaneously might also reveal digenic or multigenic inheritance mechanism of the CORD [28].

Because the mutation spectrum of *GUCY2D* in LCA patients is significantly different from that in CORD patients, and because the mutation variations in Japanese patients with CORD possibly may be significantly different from that in Caucasian patients, we were not able to directly detect the disease-causing mutations; however, because all of sequence alterations detected by the microarray were confirmed by independent direct sequencing, it is likely that the APEX-based gene test platform itself provided an accurate and efficient means for detecting genotypes in each individual [7].

Generating a custom-made APEX-based genotyping microarray on which almost all of the CORD disease-causative genes are arrayed by collecting data from the ever growing Web-based mutation database of *GUCY2D*, as well as information on mutations obtained in individual laboratories in ethnically diverse populations, may eventually offer a unique and reliable diagnostic tool. This should then enhance the detection rate of not only disease-causative but also of modifying-sequence changes and may accelerate our understanding of the basic mechanisms underlying CORD and its phenotypic variability which facilitate prospective diagnosis.

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8. 糖尿病網膜症に伴う増殖組織の expressed sequence tag 解析

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研究要旨　糖尿病網膜症に伴う線維血管増殖組織は、その収縮による牽引性網膜剥離が失明の原因となっている。我々は今回、その増殖組織の遺伝子発現を包括的に把握するため、遺伝子ライブラリを作成し、EST 解析を行った。
抽出遺伝子 714 クラスターの機能分類では、細胞生理反応や代謝、細胞間コミュニケーションなどに分類できた。PDR と PVR、iERM 増殖組織間の遺伝子発現比較では、PDR のみに発現が見られる遺伝子は、248 クラスターであった。この中から、新生血管促進因子 IL-8 といった既知の遺伝子に加えて、未知の遺伝子発現の関与が明らかとなった。
今後、抽出遺伝子の構造と機能および増殖組織での局在把握は新規の分子マーカーの同定による、エビデンスに基づいた新しい診断や治療につながる可能性があることが示唆された。

A. 研究目的

糖尿病網膜症に伴い線維血管増殖組織（以下増殖組織）を生じ、その生成にはこれまで VEGF、IL-8 や NF-kB などの関与が明らかになっている。[1] 本研究では、増殖組織の expressed sequence tag (EST)解析による、より包括的な遺伝子発現パターンの同定を試みた。

B. 研究方法

対象は、九州大学眼科を紹介受診、手術目的で入院となった PDR 患者で 33 歳男性、56 歳女性の 2 症例。硝子体手術の際に切除した増殖組織から totalRNA を抽出し、Switching Mechanism At 5' end of RNA Transcript (SMART)法を用いて遺伝子ライブラリを作成後、DDBJ と共同で各クローンの塩基配列を決定、アセンブルとクラスタリングにより遺伝子群を抽出した。遺伝子注釈付けは、オンライン・データマイニングツールである KEGG
(http://www.genome.jp/kegg/)および FatiGO
(http://fatigo.bioinfo.cipf.es/) を用いて、遺伝子シンボルを Gene Ontorogy、GO タームで機能分類を行った。
また、PDR と PVR、iERM 増殖組織間の遺伝子発現比較は、DDBJ の CIBEST データベースを使った。

（倫理面への配慮）
本研究はヘルシンキ宣言に則り、また九州大学病院倫理委員会の承認を得て行った。全ての患者から術前にインフォームドコンセントを得た。

C．研究結果

2人のPDR増殖組織2816クローンから、388の低質クローンを除去した高品質 2448 クローンは、714 の遺伝子群にクラスタリングできた。こうして得られた 714 抽出遺伝子をKEGG、FatiGOで機能分類したところ、biological processのレベル3では、代表的なものとして、細胞生理反応、代謝、細胞間コミュニケーション、ストレス反応などに分類できた（図 1)。PDRとPVR、iERM増殖組織間の遺伝子発現比較では、PDR特異的に発現している遺伝子は、714 群のうち 248 群であった。その中には、血管新生促進因子LI-8といった既報告の遺伝子に加え[2]、未報告の遺伝子の発現も確認された。

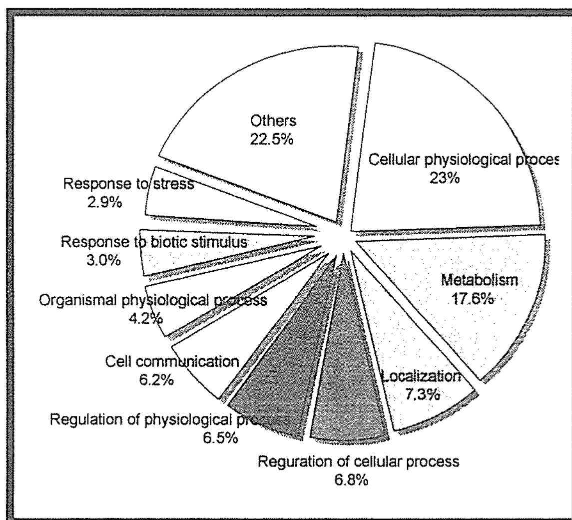

図1　FatiGOによる抽出遺伝子の機能分類

D．考察

既報告の遺伝子に加え、未報告の遺伝子の関与が示唆された。今後、抽出遺伝子群の構造と機能および増殖組織での局在を把握することで、新規の分子マーカーの同定による、エビデンスに基づいた新しい診断や治療につながる可能性があると考えた。

E．結論

糖尿病網膜症に伴う増殖組織のEST解析を行い、発現遺伝子は、細胞生理反応や代謝などに機能分類できた。PVR、iERMとの遺伝子発現の比較により増殖組織生成に関するこれまでの既知の遺伝子に加えて未知の遺伝子発現を同定した。

F．健康危険情報　　なし

G．研究発表

1．論文発表　　なし

2．学会発表　　なし

H．知的財産権の出願・登録状況

1．特許取得　　なし

2．実用新案登録　　なし

3．その他　　なし

I．参考文献

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Rapid detection of *SAG* 926delA mutation using real-time polymerase chain reaction

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Purpose: Mutation 926delA of the arrestin/S-antigen *SAG* gene is the main cause of Oguchi disease in the Japanese. The purpose of this study was to develop a rapid diagnostic assay to detect mutations in the *SAG* gene.

Methods: Two sequence-specific primers and fluorophore-labeled probes for exon 11 of the *SAG* gene were designed, and the region spanning the mutations was amplified by polymerase chain reaction (PCR) using the LightCycler detection system (Roche Diagnostics, Mannheim, Germany). The mutations were then identified by melting curve analyses of the hybrid formed between the PCR product and a specific fluorescent probe.

Results: We clearly distinguished each *SAG* genotype (homozygous and heterozygous 926delA and wild type) by the distinct melting peaks at different temperatures. One thermal cycling required approximately 54 min to process, and the results were 100% in concordance with the genotypes determined by DNA sequencing.

Conclusions: We have succeeded in developing a rapid method to detect the most frequent mutation in the *SAG* gene. This method will help in identifying gene mutations associated with Oguchi disease with a rapid and reliable identification or the exclusion of the frequent mutations in the *SAG* gene.

Oguchi disease is a rare, autosomal recessive form of congenital stationary night blindness [1]. Patients with Oguchi disease, usually have normal visual acuity, visual fields, and color vision. A diagnostic feature of the disorder is a golden discoloration of the fundus, which disappears in the fully dark-adapted state and reappears shortly after the onset of light (the Mizuo-Nakamura phenomenon) [2]. The course of dark-adaptation of the rod photoreceptors is extremely slow in patients with Oguchi disease while that of the cones appears to proceed normally [3]. Mutations of the arrestin/S-antigen (*SAG*) gene [4] and the G protein-coupled receptor kinase 1 (*GRK1*) gene [5] have been identified as the causes of Oguchi disease. Both genes encode an intrinsic rod photoreceptor protein that participates in the recovery phase of the light transduction cascade.

A homozygous deletion of adenine at nucleotide 926 (926delA: Asn309 (1-bp del)) of the *SAG* gene is the main cause of Oguchi disease in the Japanese [4,6-9]. The 926delA mutation was formerly referred to as 1147delA, but it has been renamed in accordance with the recommended nomenclature system for human mutations [10,11]. The initial report on the SAG gene mutation identified a homozygous 926delA in five of six unrelated Japanese patients [4]. Thereafter, the causative mutations in Oguchi's disease in six additional Japanese families were described; all had the same homozygous 926delA mutation in the arrestin gene [6-9].

Although it has been suggested that there is generally a clear genotype/phenotype correlation associated with mutations of the *SAG* gene, the clinical manifestation of the same mutation can vary, probably modified by the stage of the disease, aging, and/or gene modifiers. In addition, it has been shown that the 926delA mutation is also responsible for autosomal recessive retinitis pigmentosa [12]. Therefore, it would be useful to develop a rapid diagnostic method to identify the 926delA mutation.

Among the several techniques for molecular genetic mutation screening, the current standard for experimental detection of mutations is the direct sequencing of DNA samples. However, gel electrophoresis makes the isolation of the mutations time-consuming. The LightCycler detection system (Roche Diagnostics) is a combined microliter volume thermal cycler with an integrated fluorometer [13-15]. This system offers a high-throughput, semiautomatic method that permits fast genotyping of mutation sites. By use of real-time polymerase chain detection followed by melting curve analysis with hybridization probes, the system can be adapted to become a highly sensitive, rapid, and an efficient alternative approach to detect mutations. Using this system, we have recently succeeded in developing a method to detect mutations in the *transforming growth factor β-induced* (*TGFBI*) gene rapidly, and found that the detection system was reliable and accurate [16].

In this study, we considered whether if this system was also able to detect a common mutation in the *SAG* gene.

METHODS

Clinical examinations: Full medical histories were taken from the patient and his parents, who also received ophthalmologic examinations, including best-corrected visual acuity, slit-lamp biomicroscopy, kinetic visual field examination, fundus ex-

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