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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.

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

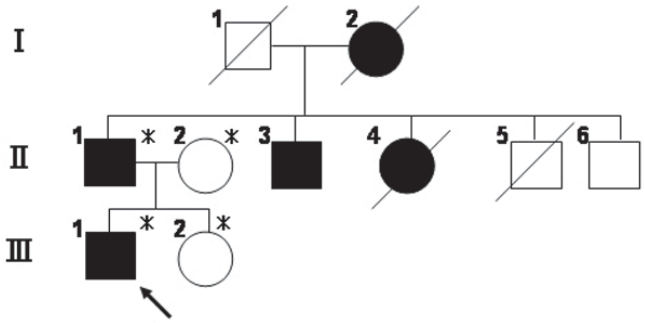

Figure 1. Pedigree of the family. Solid black symbols represent affected members who have autosomal dominant cone-rod dystrophy; white symbols represent unaffected members. Circles and squares indicate women and men, respectively. Those who underwent DNA testing are indicated by asterisk, and the is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

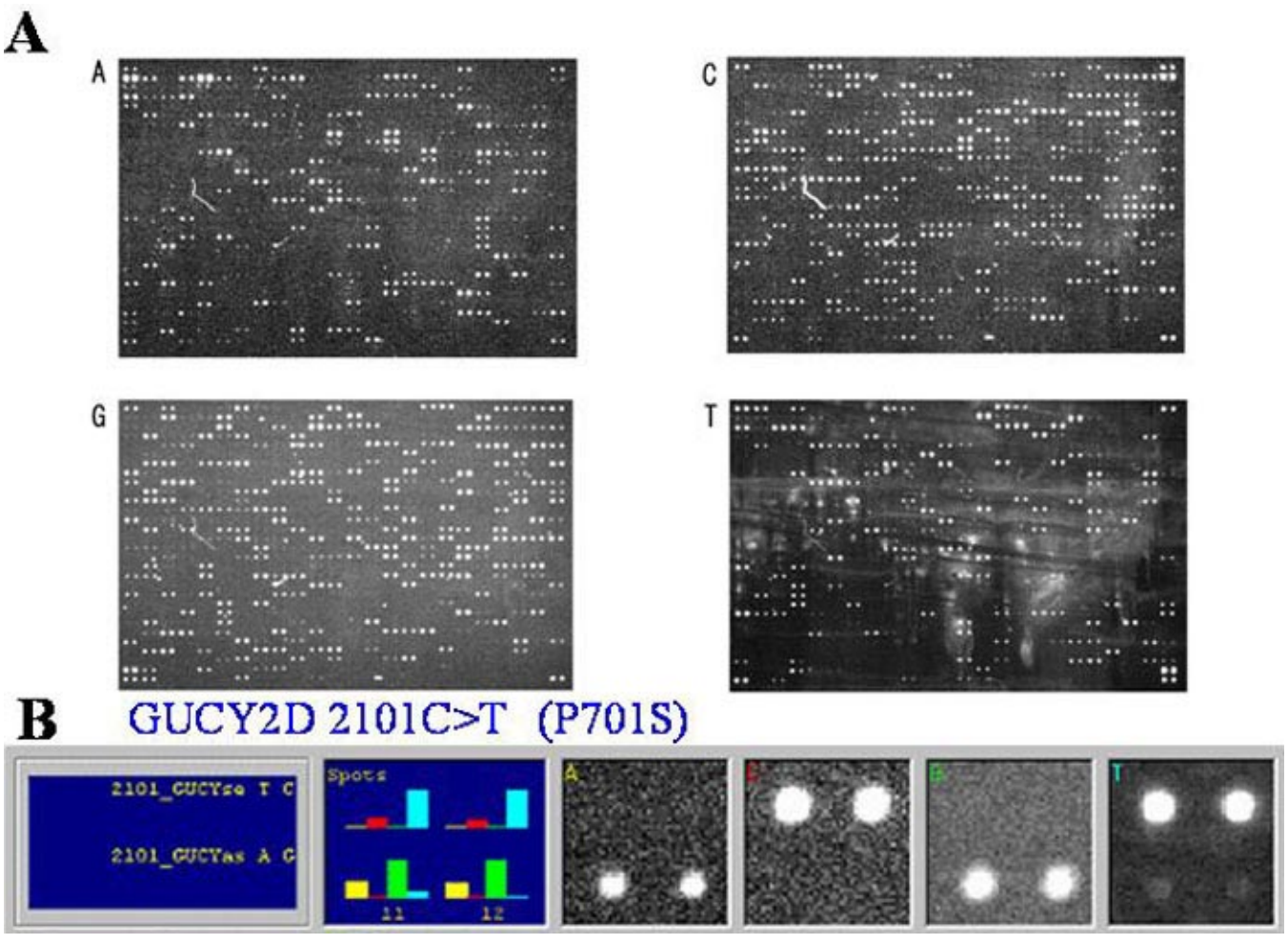

Figure 2. Leber congenital amarousis arrayed primer extension-based microarray assay hybridized to probes generated from proband's genomic DNA. **A**: Grayscale images for each fluorescent dideoxy nucleotide are used for the sequence analysis. **B**: Sequence alteration in the third base of codon 701 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-

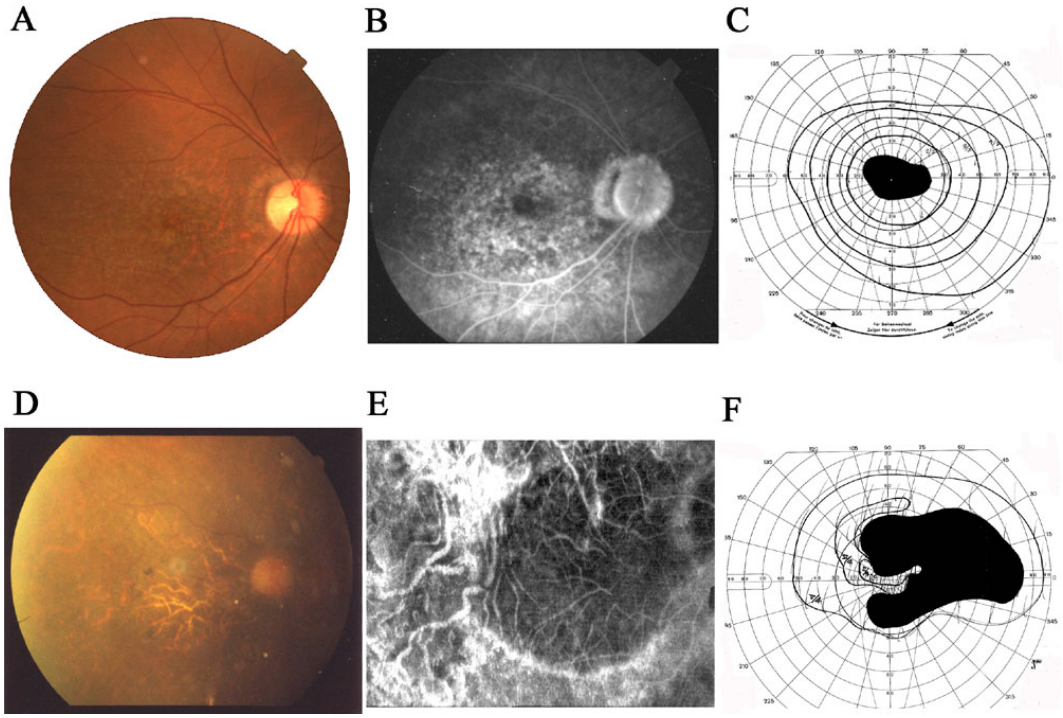

Figure 3. Ophthalmologic and functional analyses. Fundus photographs (**A**, **D**), fluorescein fundus angiograms (**B**, **E**), and Goldmann kinetic perimetric fields (**C**, **F**) of patients with mutations of the *GUCY2D* gene. (**A**, **B**, **C**) Right eye, proband; (**D**, **E**, **F**) Right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

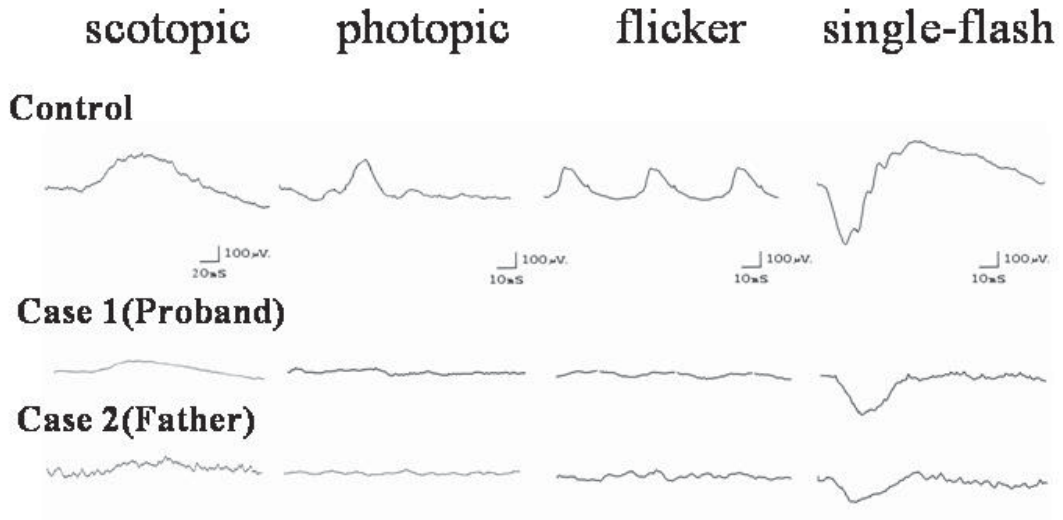

Figure 4. Electrophysiological recordings from the affected individuals and a normal subject. The scotopic rod electroretinogram (ERG), photopic cone ERG, 30-Hz flicker ERG, and bright flash rod cone mixed ERG amplitudes are markedly reduced in the proband. Those of the proband's father exhibited a similar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

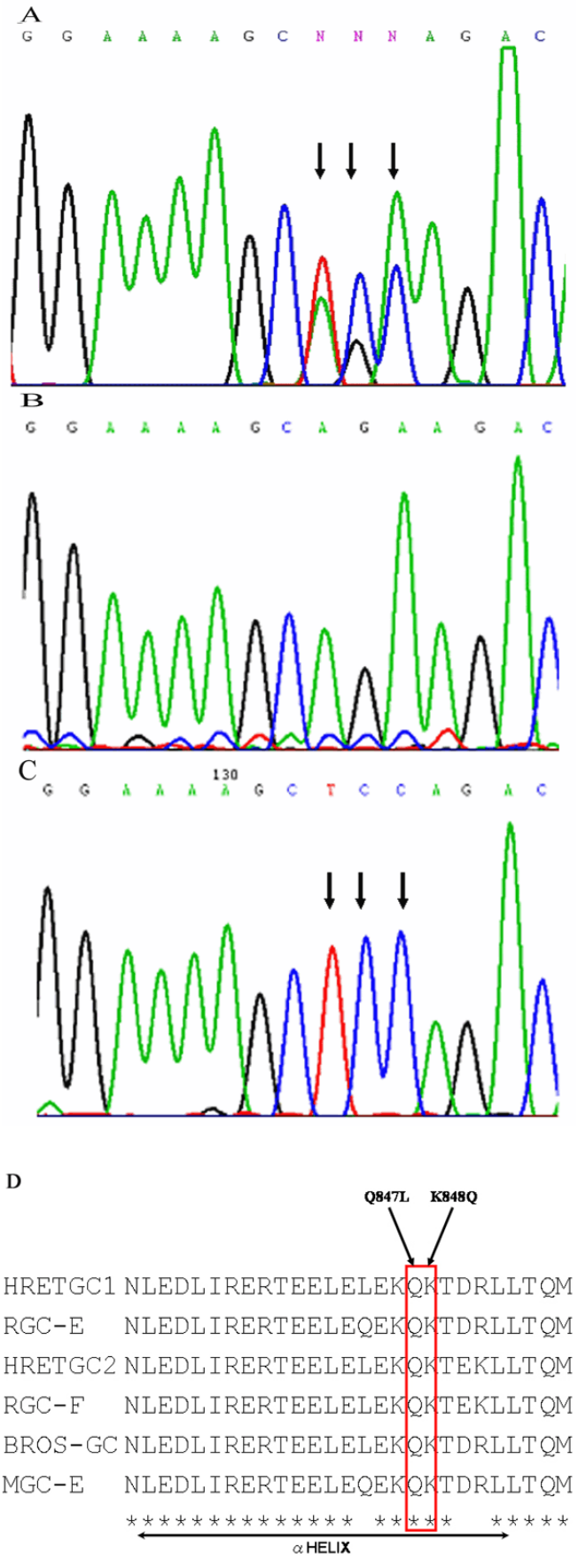

Figure 5. Nucleotide and amino acid sequence of exon 13 of the GUCY2D. **A**: Electropherogram of the sense strand of genomic DNA from the affected proband (III-1) and his father (II-1), showing a novel heterozygous multiple missense mutation of c.2540_2542delinsTCC in exon 13 (p.Gln847_Lys848delinsLeuGln). 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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