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-
amination, fluorescein angiography, and electroretinography (ERG) [17]. The color vision was tested 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 Committees of the International Society for Clinical Electrophysiology of Vision (ISCEV). After pupillary dilatation was achieved with 0.5% tropicamide and 0.5% phenylephrine hydrochloride and 30 min of dark adaptation, the scotopic ERGs were elicited by a white stimulus at a luminance of 80 x 0.12 cd/sec µm². The rod-cone single-flash ERGs were elicited by a white stimulus at an intensity of 20 cd/sec µm². The photopic single flash ERG and the 30-Hz flicker ERG were elicited by a white stimulus at a luminance of 1.2 and 0.6 cd/sec µm², respectively, on a white background of 25 cd/m².

**DNA sequence analysis:** DNA was extracted from the blood of the patient and his parents with the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), and the DNA was used to screen for genetic mutations following protocols described in references [18-21]. The genomic DNAs of exon 11 of the SAG gene were amplified using appropriate forward primer (SAG11F, 5’-GGT CCA TGG CAG CTT TGA TG-3’) and reverse primer (SAG11R, 5’-CTT A TT CCC TGA GCC TCG AG-3’). The conditions for polymerase chain reaction (PCR) were as follows: 10 min at 94 °C, followed by 35 cycles of 30 s at 72 °C, 94 °C for 30 s, and 60 °C for 30 s with a final extension step at 72 °C for 5 min. The PCR products were purified and sequenced using the Big Dye Terminator sequencing kit (Perkin Elmer Applied Biosystems, Foster City, CA). The products were resolved on an ABI Prism 3100 sequencer (Perkin Elmer Applied Biosystems). The mutated nucleotides and substituted amino acids were numbered according to the Genbank accession number NM_000541.

**LightCycler polymerase chain reaction:** PCR and melting curve analyses were performed on the LightCycler detection system (Roche Diagnostics) using protocols in references [16,22-25]. Schematic diagrams of the LightCycler hybridization analyses used in our assay are shown in Figure 1. The hybridization probes for the SAG gene were designed with the LightCycler Probe Design Software (Roche Diagnostics). The probe sequences were as follows: the anchor probe SAG11Flu, AGG CAT TGC CCT GGA TGG GAA AA T CAA G-Fluorescein, and the sensor probe SAG11LCRed, LC Red 640-ACG AGG ACA CAA ACC TTG.

When the probes hybridized to the same DNA strand internal to the PCR primers, the probes came in close proximity and produced a fluorescence resonance energy transfer (FRET). During the FRET, a donor fluorophore, which is excited by the LED light source, transfers its energy to an acceptor fluorophore only when it is positioned in close vicinity of the former. After a complete PCR run, it is possible to perform a melting point analysis, during which the temperature is lowered below the annealing temperature of the probes and then slowly increased. The fluorescence signal decreases when the detection probe melts off its target.

The reaction mixtures were prepared in glass capillaries containing 0.5 µl of purified genomic DNA, 0.2 µM of each primer, 0.2 µM of each probe, 10 µ LightCycler Fast Start DNA Master Hybridization Probes mix, and processed according to the manufacturer’s instructions (Roche Diagnostics).

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**Figure 1.** Schematic illustration of polymerase chain reaction primers, anchors, and detection probes for the SAG gene. The probe for SAG11Flu is labeled with fluorescein at the 3’ end and serves as an anchor probe for SAG11LCRed. The sensor probes, SAG11LCRed, is labeled with LCRed640 at the 5’ end and spans the DNA region having the mutations. The region of the mutations of the SAG gene is shown below the illustration, and the mutation sites corresponding to 926delA are shown in bold letters and are indicated by arrows. Circle represents fluorescein; double circle represents LC-Red 640.
Real-time PCR was performed by an initial denaturation at 95 °C for 10 s, followed by 40 cycles of 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 9 s. Following the amplification phase, a melting curve analysis was performed at 40 °C for 20 s followed by a slow heating at 0.2 °C/s to 85 °C to determine the melting points by monitoring the reporter dye fluorescence emission in channel F2/F1 (640 nm).

Melting curves were converted into melting peaks by plotting the negative derivative of the fluorescence signal against the temperature. The sudden drop in the fluorescence signal is then transformed to a peak, allowing the easy identification of the melting temperature (Tm) mismatches between the hybridization probe and the target.

RESULTS
Case report: Our case was a 14-year-old man who first visited us three years ago for evaluation of night blindness. He had a slight myopia with a correction of -2.5 diopters in both eyes, and his best-corrected visual acuity was 1.5 in each eye. There was no consanguinity in his family, but his paternal grandmother had a history of night blindness. He had normal color vision according to the results of the Farnsworth Panel D-15. The Goldmann kinetic visual fields showed no abnormalities. Fundus examination revealed a golden-yellow glinting color throughout the retinas of both eyes, but after 4 h of dark adaptation, this fundus appearance became normal, confirming the presence of a Mizuo-Nakamura phenomenon (Figure 2).

The full-field rod ERGs after 30 min of dark adaptation could not be recorded in both eyes. The photopic ERGs and the 30-Hz flicker ERGs were normal. The bright-flash, mixed cone-rod ERGs demonstrated a negative-type ERG with the amplitude of the b-wave smaller than that of the a-wave. In addition, the amplitude of the a-wave was reduced (Figure 3).

LightCycler analyses: Direct sequencing of all coding regions of the SAG gene in the patient revealed a homozygous 926delA (Asn309 (1-bp del)) of the SAG gene (Figure 4), which meant there would be a shift in the reading frame and a pre-
mature termination of translation. Both parents were heterozygous for the same deletion mutation.

To develop a reliable and rapid method of genotyping based on the hybridization probe assays, we designed two sequence-specific primers and fluorophore-labeled probes for exons 11 of the SAG gene were (Figure 1). The LightCycler detection system was standardized by analyzing the sequence-verified DNA from the patient, his parents, and normal controls. (For normal controls, three normal volunteers were recruited. With appropriate correction they had a full visual acuity, and there was no history of eye diseases and no serious general diseases. Their ages were 25, 28, and 29 years.) With this technique, we were able to distinguish the disease genotypes by the distinct melting peaks (Figure 5). Homozygous wild-type (926AA) showed a single peak at 62.0 °C. Heterozygous 926delA decreased the Tm with a heterozygous pattern of two peaks at 62.0 °C and 54.0 °C. The single peak at 54.0 °C indicated a homozygous 926delA mutation (Figure 5).

It required approximately 54 min for one thermal cycle to complete. DNA samples were examined under the same PCR conditions in triplicate, and all results were 100% in concordance with the genotypes determined by DNA sequencing. We observed scant intra assay variation, and neither shifts of the hybridization probe melting temperatures nor inaccurate melting curves of individual samples were observed (data not shown).

**DISCUSSION**

We have developed a rapid PCR-based fluorescein assay for the detection of the 926delA mutation, a hotspot in patients with Oguchi disease, in the SAG gene. Because the 926delA mutation accounts for most Japanese patients with Oguchi disease, this probe set can detect the most common mutation in the SAG gene, and is thus particularly useful for a rapid initial screening in the clinic. The precision of the assays was 100% demonstrating a complete reliability of our new high-speed genotyping assay [16].

One conventional method used to detect mutations in patients with Oguchi disease is direct genomic sequencing. Even...
though the method provides more data, it is more labor-intensive and expensive. Another method, the PCR-restriction fragment length polymorphism (RFLP) assay for detecting the 926delA genotypes, requires a PCR amplification followed by digestion with a mutation-specific restriction enzyme of the amplicons and then gel electrophoresis. Both methods require several hours and more manual interventions, such as gel electrophoresis, resulting in a higher risk of contamination by displaced amplicons and exposure of the investigators to potentially toxic reagents such as the ethidium bromide stain. In contrast, our rapid PCR-based fluorescein assay is a closed-tube system that does not require any post-PCR sample manipulation, which enables reliable genotyping in less than one hour and is suitable for routine clinical use. In addition, our new assay can also be used for screening autosomal recessive retinitis pigmentosa caused by the 926delA mutation of the SAG gene.

It was suggested that mutation 926delA of the SAG gene is the mutational hot spot in Oguchi disease, however there have been reports of increasing numbers of rare gene mutations, such as a homozygous 577C>T (Arg193stop) [26], a compound heterozygous 523C>T (Arg175stop) and 926delA [27], a homozygous 874C>T (Arg292stop) of SAG gene, [27] and compound heterozygous 1139T>A (Val380Asp) and 1607del4bp [5], and a homozygous deletion of Exon 5 of GRK1 gene [5] have been recently reported. More recently, a variant form of Oguchi disease was reported with a c.827+623_883del mutation of the SAG gene [28]. These findings indicate that there is likely to be a broader spectrum of genetic heterogeneity associated with Oguchi disease than previously believed. Our method should help identify the gene defects associated with SAG or GRK1 mutations in Oguchi disease with a rapid and reliable identification and the exclusion of the frequent mutations in the SAG gene. This should accelerate our ability to gain insight into Oguchi disease enable us to and understand more precisely the mechanisms underlying night blindness associated with clinically diagnosed Oguchi disease or SAG-associated retinitis pigmentosa.

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