Novel CACNA1F Mutations in Japanese Patients with Incomplete Congenital Stationary Night Blindness

Makoto Nakamura, Sei Ito, Hiroko Terasaki, and Yozo Miyake

PURPOSE. Although it was reported that congenital stationary night blindness (CSNB) could be divided into complete and incomplete CSNB clinically in 1986, it was not until 1998 that the two types were found to be distinct clinical diseases by molecular genetic analysis. The purpose of this article is to report mutations in the retina-specific calcium channel a1-subunit gene (CACNA1F) in Japanese patients with incomplete CSNB and to describe the clinical features of these patients.

METHODS. Seven patients from five separate Japanese families with incomplete CSNB were examined. Genomic DNA was extracted from leukocytes of the peripheral blood, and all 48 exons of the CACNA1F were amplified by polymerase chain reaction and directly sequenced. A complete ophthalmic examination was performed, including best corrected visual acuity, slit lamp and fundus examinations, fundus photography, and electroretinography.

RESULTS. A mutation in the CACNA1F was identified in all the patients. The identified mutations were a missense mutation (Gly609Asp); a nonsense mutation (Arg913stop); a splice donor site mutation of G to C at nucleotide 2571+1; a G insertion at nucleotide 709, resulting in a frame shift with a predicted stop codon at codon 247; and a 4-bp deletion at nucleotides 271 to 274, with a replacement by an abnormal 34-bp sequence. Clinically, each patient had essentially normal fundi, mildly reduced corrected visual acuity, and slight myopia or hyperopia with astigmatism. Electrophysiologically, the mixed rod-cone ERG showed a negative configuration with recordable oscillatory potentials. The rod ERG was recordable but subnormal, and the cone and 30-Hz flicker ERGs were markedly depressed.

CONCLUSIONS. Five novel mutations were identified in the CACNA1F in five Japanese families with incomplete CSNB, leading to the conclusion that in most Japanese patients, incomplete CSNB is caused by a CACNA1F mutation. (Invest Ophthalmol Vis Sci. 2001;42:1610–1616)

The Schubert-Bornschein type of congenital stationary night blindness (CSNB) is characterized by night blindness and the so-called negative-type electroretinogram (ERG) in which the amplitude of the b-wave is smaller than that of the a-wave. Nystagmus and amblyopia sometimes accompany the other signs. The fundus is essentially normal, except for high myopic changes and temporal pallor of the optic discs. The symptoms appear from early childhood and are stationary. The hereditary pattern is X-linked recessive or autosomal recessive.

The negative-type ERG is also recorded in other diseases including retinoschisis, Oguchi disease, fundus albipunctatus, Batten disease, and ischemic retinal diseases such as central retinal artery occlusion. However, these retinal diseases differ from CSNB in that they involve fundus abnormalities, and patients with negative-type ERG without fundus abnormalities most likely have CSNB.

In 1986, we first reported that the Schubert-Bornschein type of CSNB could be divided clinically into two subtypes: the complete type of CSNB (complete CSNB), in which rod function is completely absent, and the incomplete type of CSNB (incomplete CSNB) in which rod function is present but decreased. Since then, we have reported other differences in the two types of CSNB, by using various kinds of functional examinations.

In 1998, the a1-subunit of the L-type calcium channel gene (CACNA1F) located on the X chromosome was identified as the mutated gene in incomplete CSNB by two research groups. Mutations of CACNA1F were found in 10 of 13 families and in all 20 families with incomplete CSNB. Since then, there has been only one study of the genotype-phenotype correlation with a known common mutation; studies on additional mutations of this gene have not been published.

There has not been a study of the genotype-phenotype correlation in nonwhite patients. The purpose of this study was to examine the CACNA1F in seven patients from five Japanese families in whom incomplete CSNB was clinically diagnosed. We have identified five novel mutations in the gene in all the patients. These results established clearly that incomplete CSNB is caused by CACNA1F mutations in Japanese patients, some of whom were the founders of this clinical entity.

METHODS

This study conformed to the tenets of the Declaration of Helsinki, and informed consent was obtained from the subjects after an explanation of the purpose of the study.

Seven patients with incomplete CSNB from five Japanese families (referred to by the letters A through E) were analyzed. To the best of our knowledge, the families were not related. All individuals examined had been under observation in the Department of Ophthalmology of Nagoya University, Japan. The ophthalmic examination included best corrected visual acuity, refraction, biomicroscopy, ophthalmoscopy, fundus photography, and ERG. The diagnosis in these patients was based on the following clinical characteristics of incomplete CSNB: essentially normal fundus, mildly depressed visual acuity, slightly myopic or hyperopic refraction, and ERG abnormalities. Electrophysiologically, the mixed rod-cone ERG showed a negative configuration with recordable oscillatory potentials. The rod ERG was recordable but subnormal, and the cone and 30-Hz flicker ERGs were markedly deteriorated.

Genomic DNA was extracted from leukocytes of the peripheral blood. Exons 1 through 48 of the CACNA1F were amplified by poly-
merase chain reaction (PCR) using a thermal cycler (DNA Thermal Cycler 9700; Perkin Elmer Applied Biosystems, Foster City, CA). Primers were purchased from Life Technologies Oriental, Inc. (Tokyo, Japan) using the previously published sequences.10 For all exons, 200 ng genomic DNA was amplified in a 50-μl reaction with 0.5 μM of each primer, 0.2 mM of each dNTP, and DNA polymerase (AmpliTaq Gold; Perkin Elmer Applied Biosystems). The PCR conditions were as follows: 5 minutes at 94°C; 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds; and a final extension step at 72°C for 7 minutes. The PCR products were purified (High Pure PCR Purification Kit; Boehringer Mannheim, GmbH, Mannheim, Germany) and then directly sequenced using a DNA sequencing kit (Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin Elmer Applied Biosystems). An automated DNA sequencer (Model 373; Applied Biosystems) was used. Primers for the sequence reaction were the same as those for the PCR reaction. To search for polymorphisms, exons 4, 7, 14, 15, 22, 24, 28, and 48 of the CACNA1F from 100 alleles (34 men and 33 women) from unrelated normal Japanese individuals were directly sequenced.

Standardized ERGs were elicited by Ganzfeld stimuli after 30 minutes of dark adaptation. The scotopic (rod) ERGs were elicited by a blue light at an intensity of 5.2 × 10^{-2} candelas (cd)/m^2 per second. The mixed rod–cone single-flash ERGs were elicited by a white stimulus at an intensity of 44.2 cd/m^2 per second. The cone ERG and the 30-Hz flicker ERG were elicited by a white stimulus at an intensity of 4 cd/m^2 per second and 0.9 cd/m^2 per second, respectively.

### RESULTS

Families A, B, and E showed an X-linked recessive heredity pattern. No family history was obtained from families C and D (Fig. 1). Molecular genetic examination revealed mutations of CACNA1F in all the patients. A missense mutation of G to A at nucleotide 1826 in exon 15 predicting a Gly609Asp amino acid substitution was found in family A. In family B, a G insertion at nucleotide 709 in exon 7 resulted in a frame shift with a predicted stop codon at codon 247. A nonsense mutation of C to T at nucleotide 709 in exon 7 resulted in a frame shift with a predicted stop codon at codon 247. A nonsense mutation of C to T at nucleotide 2737 in exon 24 resulting in the substitution of arginine to a stop at codon 913 was detected in family C. A splice donor site mutation of G to C at nucleotide 2571 in intron 22 was detected in family D. A 4-bp deletion at nucleotides 271 to 274 was found in family E, with insertion of an abnormal 34-bp sequence in exon 4 causing amino acid substitution of Ser91 and Ala92 with 12 unusual residues consisting of ValGlyValLeuHisProValGlyValLeuHisPro (Fig. 2, Table 1). In this study, we numbered the mutated nucleotides and substituted amino acids according to the sequence reported by Bech-Hansen et al.,11 which is slightly different in exons 1, 2, and 9 from that reported by Strom et al.,10 probably because of splice variants.

The mutated sequence in family E was very interesting, because the first 5-bp sequence of the insertion, 5'-GTAGG-3', was the same as the normal antisense strand just downstream.

### Table 1. Mutations and Sequence Variants in CACNA1F Identified in This Study

<table>
<thead>
<tr>
<th>Nucleotide Variants</th>
<th>Exon</th>
<th>Predicted Change</th>
<th>Family</th>
<th>Control Alleles (n = 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>271–274 del/ins 34 bp</td>
<td>4</td>
<td>Unusual 12 residues</td>
<td>E</td>
<td>0</td>
</tr>
<tr>
<td>709insG</td>
<td>7</td>
<td>Frame shift</td>
<td>B</td>
<td>0</td>
</tr>
<tr>
<td>G1826A</td>
<td>15</td>
<td>Gly609Asp</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>2571+1G→C</td>
<td>22 intron</td>
<td>Splice variant</td>
<td>D</td>
<td>0</td>
</tr>
<tr>
<td>C2737T</td>
<td>24</td>
<td>Arg913stop</td>
<td>C</td>
<td>0</td>
</tr>
</tbody>
</table>

Sequence variants:
- C1647T (rod) | 14 | Gly549Gly | A, B, C, E | 60 |
- T3114C | 28 | His1048His | A, B, C, E | 63 |
- G5594A | 48 | Arg1865His | D | 21 |
of the deletion. This was followed by an insertion of a 13-bp sequence, 5'-GGTGCTCCACCCC-3', which is the same sequence as the normal sense strand just upstream of the deletion. These two kinds of abnormal sequences were repeated sequentially twice. Furthermore, the 6-bp sequence, 5'-GGGGTG-3', which included the joint of the two kinds of abnormal sequences, was the same as the normal antisense strand just upstream of the deletion (Fig. 3).

All five of the mutations were novel. The segregation in family A was confirmed, because the Gly609Asp mutation was detected in affected patients (III:5, IV:6, and IV:7), as well as heterozygously in a female carrier (III:6), and an unaffected member of the family (III:8) showed a normal sequence (Fig. 4). In families B and D, the mothers of the patients were heterozygotes and the fathers were normal. None of the five mutations was found in 100 normal alleles.

In four families (A, B, C, and E), two nucleotide sequences were found that differed from those in the GenBank (accession number AJ006216; National Center for Biotechnology Information, Bethesda, MD; available in the public domain at http://www.ncbi.nlm.nih.gov)—namely, nucleotide 1647 C to T (Gly549Gly) in exon 14 and nucleotide 3114 T to C (His1038His) in exon 28. These are probably polymorphisms, because amino acid residue changes were not found. In family D, both nucleotides were the same as those in the GenBank sequence. An examination of exons 14 and 28 of 100 normal alleles revealed that nucleotide 1647 C is 40% and T in 60%, and nucleotide 3114 C is 63% and T in 37% in Japanese subjects. In family D, a missense sequence change of G to A at nucleotide 5594 in exon 48 predicting an Arg1865His amino acid substitution was found in the proband (III:4) and heterozygously in his mother (II:6). However, the proband's unaffected father (II:1) also showed this sequence change, and the examination of exon 48 in 100 normal alleles revealed that nucleotide 5594 was G in 79% and A in 21%. G in 25 and A in 9 of the males and homozygously G in 22 of the control females, heterozygously G and A in 10, and homozygously A in 1. We thus concluded that this mutation is not the disease-causing mutation but a polymorphism in Japanese subjects. Because these polymorphisms have not been reported previously, this infor-

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933588/)

**Figure 2.** Nucleotide sequences of the CACNA1F using sense primers in the patients. Bars: positions of the mutations. (A) A missense mutation of G to A at nucleotide 1826 (Gly609Asp) in exon 15, patient A-IV:7; (B) a G insertion at nucleotide 709, resulting in a frame-shift stop at codon 247 in exon 7, patient B-III:3; (C) a nonsense mutation of C to T at nucleotide 2737 (Arg913stop) in exon 24, patient C-III:2; (D) a splice donor site mutation of G to C at nucleotide 2571+1 in intron 22, patient D-III:4; and (E) a 4-bp deletion at nucleotides 271 to 274 with a replacement by an abnormal 34-bp sequence, causing amino acid substitution of Ser91 and Ala92 into 12 unusual residues in exon 4, patient E-III:1.

![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933588/)

**Figure 3.** Changes of the nucleotide sequence associated with nucleotides 271 to 274 del/ins 34 bp in exon 4 of the CACNA1F identified in family E. The first 5-bp sequence of the abnormal insertion is the same as the normal antisense strand just to the 5' side of the deletion (lines above the nucleotide sequence), which was assumed to be an early Okazaki fragment. The next 13-bp sequence in the insertion is the same as the normal strand just to the 5' side of the deletion (underline). These two abnormal sequences were repeated twice. The 6-bp sequence of the normal antisense strand just to the 5' side of the deletion is seen at the joint of the abnormal sequences (dotted lines above the sequence).
The idea that the Schubert-Bornschein type of CSNB included two different clinical entities was first proposed by our laboratory from the results of electrophysiological examinations (Fig. 5). The idea that the Schubert-Bornschein type of CSNB included two different clinical entities was first proposed by our laboratory from the results of electrophysiological examinations (Fig. 5). After that, the question was raised as to whether complete and incomplete CSNB were different stages of the same clinical disease or were indeed separate diseases. With the development of molecular biology, the causative gene for the two types of CSNB were mapped to different loci of the X chromosome: complete CSNB on Xp.11.4 and incomplete CSNB on Xp.11.23. In addition, leucine-rich proteoglycan nylctalin was recently found to be the causative gene of complete CSNB, and the difference seemed to be resolved. However, there has not been another molecular genetic study in patients with incomplete CSNB after the first two studies, and there has not been a report involving nonwhite patients.

In this study, we conducted a molecular genetic analysis of Japanese patients with incomplete CSNB, some of whom had been involved in the identification of this disease. Mutations of the CACNA1F gene were detected in all our patients, confirming the earlier observation that incomplete CSNB is caused by CACNA1F defects. Because we have identified CACNA1F mutations in all the typical patients with incomplete CSNB, we believe that in most affected Japanese persons, incomplete CSNB is caused by CACNA1F defects. No founder effect was observed in the five families examined; a different mutation was detected in each family.

Of the five novel mutations in CACNA1F and the point protein structure, four seemed to be severe. One is a nonsense mutation (Arg913stop) with a predicted protein missing 52.3% of the C terminus; the second is a frame-shift mutation (nucleotide 709 ins G) that causes premature truncation and in which 87.7% of the C terminus of the protein would be lost with a replacement by 10 foreign amino acid residues; the third is a splice-site mutation (nucleotide 2571 +1 G to C) that may cause a premature truncation that is missing 55.2% of the C terminus; and the fourth (nucleotide 271-274 del/ins 34 bp) led to in-frame insertion of 12 abnormal amino acids, which would probably change the conformation of the protein. These mutations are likely to result in the inability of cells to form calcium channels. Because the clinical features in family A with Gly609Asp mutation were similar to those with the other four mutations, the Gly609Asp mutation was also presumed to be critical for the function of the protein. A summary diagram of the CACNA1F protein showing all reported mutations is presented (Fig. 7). We assume the other amino acid substitution, Arg1865His, does not cause CSNB because the position is near the C terminus of the protein, and both arginine and histidine are hydrophilic and basic.

Based on our clinical and electrophysiological findings, there were no significant phenotypic differences correlated with the position of the mutation in CACNA1F. However, additional data from the long-term follow-up of the patients is needed for an exact genotype-phenotype correlation and to determine whether this disease is associated with retinal atrophy or progressive degeneration. Actually, although one of the

**FIGURE 4.** CACNA1F mutation in family A. DNA sequences in exon 15 demonstrated a missense mutation of G to A in the three affected members (III:5, IV:6, and IV:7). The unaffected member (III:8) showed a normal sequence and the carrier female (III:6) was heterozygous for the mutation.

**FIGURE 5.** Fundus photographs of patients with mutations of the CACNA1F. (A) Left eye, patient A-III:5; (B) right eye, patient A-IV:7. The patient number and the age (in years) are indicated in each photograph. There was no abnormality except for myopic changes and tilted discs.
characteristics of CSNB is normal fundi, we recently examined some patients with retinal atrophy or abnormality in whom the CACNA1F was mutated. The details of these cases will be presented (manuscript in preparation).

The mutation in family E with a 4-bp deletion and 34-bp insertion seemed to be very unusual. Because the 5-bp sequence on the 5' end of this insertion is the same as that of the normal antisense strand just downstream of the deletion, the cause of this mutation was assumed to be an early Okazaki fragment.21 This mutation may have resulted during DNA replication. For eye diseases, there is only one mutation reported in which a possible early Okazaki fragment was found, in a family with X-linked retinoschisis.21

The CACNA1F is expressed only in the retina.10,11 It is expressed strongly in the outer and inner nuclear layers as well as weakly in the ganglion cells.10 Calcium channel α1-subunits form the calcium channel, together with α2-, β1-, β2-, γ- and δ-subunits, and the α1-subunits are considered to function as pore and voltage sensors.22,23 It is assumed that abnormal molecules of the α1-subunits can result in the impairment of the influx of Ca2+ that is required for the release of glutamate as a neurotransmitter from the photoreceptor cells. This would result in the reduction of signal transfer to the ON-bipolar cells.10,11 Thus, the ON-bipolar cells would be continuously depolarized, and secondarily, the Müller cells would be depolarized. Because the ERG b-wave originates from the Müller

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**TABLE 2. Clinical Characteristics and CACNA1F Mutations in Patients with Incomplete CSNB**

<table>
<thead>
<tr>
<th>Family</th>
<th>Patient</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Corrected Visual Acuity</th>
<th>Refraction</th>
<th>Fundus Appearance</th>
<th>Visual Field</th>
<th>Night-Blindness Symptoms</th>
<th>Nystagmus</th>
<th>Strabismus</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>III:5</td>
<td>49</td>
<td>M</td>
<td>0.6 OD</td>
<td>OD -2.50–1.50 × 180</td>
<td>Myopic; tilted disc</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>Orthophoria</td>
<td>Gly609Asp</td>
</tr>
<tr>
<td>A</td>
<td>IV:6</td>
<td>14</td>
<td>M</td>
<td>0.7 OD</td>
<td>OD -1.00–1.75 × 180</td>
<td>N</td>
<td>N</td>
<td>–</td>
<td>–</td>
<td>Orthophoria</td>
<td>Gly609Asp</td>
</tr>
<tr>
<td>A</td>
<td>IV:7</td>
<td>9</td>
<td>M</td>
<td>0.3 OD</td>
<td>OD -1.00–1.75 × 170</td>
<td>Myopic; tilted disc; goldish metallic reflex</td>
<td>N</td>
<td>–</td>
<td>+</td>
<td>Orthophoria</td>
<td>Gly609Asp</td>
</tr>
<tr>
<td>B</td>
<td>III:3</td>
<td>8</td>
<td>M</td>
<td>0.5 OD</td>
<td>OD plano–2.00 × 5</td>
<td>Tilted disc</td>
<td>ND</td>
<td>–</td>
<td>+</td>
<td>Exotropia</td>
<td>709 ins G</td>
</tr>
<tr>
<td>C</td>
<td>III:1</td>
<td>27</td>
<td>M</td>
<td>0.6 OD</td>
<td>OD -7.50–3.50 × 180</td>
<td>Myopic; tilted disc</td>
<td>N</td>
<td>–</td>
<td>–</td>
<td>Orthophoria</td>
<td>Arg107stop</td>
</tr>
<tr>
<td>D</td>
<td>III:4</td>
<td>12</td>
<td>M</td>
<td>0.8 OD</td>
<td>OD -0.50–2.00 × 180</td>
<td>Goldish metallic reflex</td>
<td>N</td>
<td>–</td>
<td>+</td>
<td>Orthophoria</td>
<td>2571 + 1 G to C</td>
</tr>
<tr>
<td>E</td>
<td>III:1</td>
<td>35</td>
<td>M</td>
<td>0.6 OD</td>
<td>OD +2.50–5.00 × 180</td>
<td>N</td>
<td>N</td>
<td>–</td>
<td>+</td>
<td>Exotropia</td>
<td>271–274 del/ ins 34 bp</td>
</tr>
</tbody>
</table>

N, normal; ND, not determined.

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![Figure 6](https://iovs.arvojournals.org/pdfaccess.aspx?url=/data/journals/iovs/933588/)

**Figure 6.** Full-field ERGs recorded after 30 minutes of dark adaptation in a normal subject and six affected individuals. The rod ERG amplitudes were mildly reduced, and the cone ERG amplitudes were significantly reduced or were not recordable. The bright-flash cone-rod mixed ERG amplitudes were the negative type in all. Arrows: stimulus onset. The number of each patient is noted at left.
cells and the depolarizing ON-bipolar cells, this mechanism is suggested to be the reason for the decreased b-wave amplitude as well as the night blindness in incomplete CSNB.\(^{10,11}\)

However, the exact pathogenesis of incomplete CSNB is still not known. We have evidence that the off pathway is abnormal in incomplete CSNB, because the off responses were selectively diminished when the on- and off-responses of photopic ERG were recorded, with rectangular light stimuli in patients with incomplete CSNB.\(^{4,24}\) This observation is supported by the similarity in the shape of the responses elicited by rectangular light stimuli when \(\text{cis}-2,3\)-piperidine dicarboxylic acid (PDA) or kynurenic acid (KYN) were injected into the vitreous of experimental animals. These two agents selectively block signal transmission between the photoreceptors and OFF-bipolar cells.\(^{25}\) Furthermore, the blue cone ERG, which is made up of only the ON-pathway, are recordable in incomplete CSNB,\(^{26}\) whereas it is not recordable in complete CSNB.\(^{26,27}\)

These observations have led us to suggest that the pathogenesis of incomplete CSNB is mainly a blockage of signal transfer from photoreceptors to OFF-bipolar cells. If this hypothesis is correct, the calcium channel abnormality may cause the reduction of the release of glutamate from the cone photoreceptors, leading to a failure to hyperpolarize the OFF-bipolar cells. In such cases, the reason for the night blindness in incomplete CSNB may be the dysfunction of signaling from rods proximal to the bipolar cells. These abnormalities are thought to be generated around the inner plexiform layer or between the inner plexiform layer and ganglion cells and is thought to reflect the function of rod signaling proximal to the bipolar cells.\(^{28-30}\)

It is not clear whether the pathogenesis of incomplete CSNB is due to the continuous depolarization of ON-bipolar cells, or to the obstruction of the off pathway, or to both. Evidence to support one of these explanations should be obtained with additional studies on the localization and the determination of the function of the \(\text{CACNA1F}\).\

**Acknowledgments**

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