A Novel *CACNA1F* Gene Mutation Causes Åland Island Eye Disease

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Purpose. Åland Island eye disease (AIED), also known as Forsius-Eriksson syndrome, is an X-linked recessive retinal disease characterized by a combination of fundus hypopigmentation, decreased visual acuity, nystagmus, astigmatism, protan color vision defect, progressive myopia, and defective dark adaptation. Electroretinography reveals abnormalities in both photopic and scotopic functions. The gene locus for AIED has been mapped to the pericentromeric region of the X-chromosome, but the causative gene is unknown. The purpose of this study was to identify the mutated gene underlying the disease phenotype in the original AIED-affected family.

Methods. All exons of the *CACNA1F* gene were studied by DNA sequencing. *CACNA1F* mRNA from cultured lymphoblasts was analyzed by RT-PCR and cDNA sequencing.

Results. A novel deletion covering exon 30 and portions of flanking introns of the *CACNA1F* gene was identified in patients with AIED. Results from expression studies were consistent with the DNA studies and showed that mRNA lacked exon 30. The identified in-frame deletion mutation is predicted to cause a deletion of a transmembrane segment and an extracellular loop within repeat domain IV, and consequently an altered membrane topology of the encoded α_1-subunit of the $Ca_v1.4$ calcium channel.

Conclusions. Mutations in *CACNA1F* are known to cause the incomplete form of X-linked congenital stationary night blindness (CSNB2). Since the clinical picture of AIED is quite similar to CSNB2, it has long been discussed whether these disorders are allelic or form a single entity. The present study clearly indicates that AIED is also caused by a novel *CACNA1F* gene mutation. (*Invest Ophthalmol Vis Sci.* 2007;48:2498–2502) DOI:10.1167/iovs.06-1103

Åland Island eye disease (AIED), also known as Forsius-Eriksson syndrome (MIM 300600; Mendelian Inheritance in Man; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), was originally reported in 1964 in a farmer's family on the Åland Islands in the Baltic Sea.[1] Affected males show a combination of fundus hypopigmentation, decreased visual acuity due to foveal hypoplasia, nystagmus, astigmatism, protan color vision defect, myopia, and defective dark adaptation.[1–3] Except for progression of axial myopia, the disease can be considered to be a stationary condition. Electroretinography (ERG) is abnormal, showing defects in both scotopic and photopic functions.[2,4] AIED was initially thought to be a variant of ocular albinism[1]; however, latent nystagmus of extraocular origin[5] and absence of macromelanosomes in skin biopsy specimens[6] differentiates AIED from Nettleship-Falls type ocular albinism (MIM 300500). Furthermore, optic fiber misrouting, which is present in all persons with albinism, has been shown to be absent in a patient with AIED.[7] Female carriers do not show any features of the disease, except for slight latent nystagmus in some cases.[5] In addition to the original AIED-affected family, a few families with a similar, AIED-like phenotype have been reported.[2,8–10]

The AIED gene locus has been localized to the pericentromeric region of the X-chromosome, between the markers MAOA and DXS559 (Ref. 11 and Alitalo T, unpublished linkage data, 1999). Another X-linked retinal disease, incomplete congenital stationary night blindness (CSNB2), maps to Xp11.23 within the AIED minimal region. Besides overlapping genetic intervals, these two diseases share many clinical similarities. X-linked congenital stationary night blindness (CSNBX) is a nonprogressive retinal disease characterized by a negative ERG (i.e., the amplitude of the a-wave is larger than that of the b-wave[12]). Typical clinical features of CSNBX are defective night vision, myopia, nystagmus, strabismus, and reduced visual acuity, despite corrected refraction.[13–15] However, the expression of the disease is variable, and one or more of the typical symptoms may be absent, as documented in patients with a CSNB2 founder mutation.[15] Based on ERG findings, CSNBX can be divided clinically into two subtypes. Patients with the complete type of CSNBX (type 1, CSNB1) lack a detectable scotopic rod-derived b-wave, whereas in the incomplete type (type 2, CSNB2) the rod b-wave is diminished but recordable.[16,17] Also, the photopic cone function is more impaired in the incomplete type. The genetic background of CSNBX has been resolved by positional cloning efforts. CSNB1 (MIM 310500) is caused by mutations in the *NYX* gene (Xp11.4, MIM 300278),[18,19] whereas CSNB2 (MIM 300071) results from mutations in the calcium channel α_1-subunit gene, *CACNA1F* (Xp11.23; MIM 300110).[20,21]

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It has long been discussed whether the two X-linked retinal disorders AIED and CSNB2 can be separated clinically as well as genetically from each other. *CACNA1F* mutations have been identified in patients with an AIED-like phenotype, but a previous effort failed to reveal any *CACNA1F* mutations in patients of the original AIED family.[22] Because analysis of the coding regions does not necessarily reveal intronic mutations, which may affect exon splicing, in this study, we screened the *CACNA1F* gene by using both genomic DNA and lymphoblastoid RNA of a patient belonging to the original AIED-affected family.

Materials and Methods

Subjects

Members of the original AIED family participated in the study. The research adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants in accordance with the requirements of the University of Helsinki, Department of Medical Genetics, Ethics Committee. Blood samples had been collected previously, and the Epstein-Barr virus–transformed lymphoblastoid cell cultures were established earlier as well.[11] A total of 29 samples, of which 6 were from affected males, were included in the study (Fig. 1). Clinical studies of the family members have been published elsewhere.[1,3–5,7] DNAs from 121 healthy, unrelated, Finnish male blood donors[11] and RNAs from nine lymphoblast cell lines from unrelated and unaffected males and females were used as control samples.

Molecular Studies

All 48 exons and flanking intronic regions of the *CACNA1F* gene were PCR-amplified from the genomic DNA of an affected male individual, VII-5, by using published primer sequences.[23] Because we failed to amplify exon 30 robustly with these primers, new primers flanking a larger region were used (forward primer 5′-GATGGCCCTGTTCACTGTCT-3′ in exon 27 and reverse primer 5′-AAGAGCGTCAAACGTGTTCC-3 in exon 31). For PCR amplification, 50 ng DNA was used in a 25-μL volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 250 μM dNTPs, 5 picomoles of each primer and 0.75 U DNA polymerase (AmpliTaq Gold; Applied Biosystems, Inc. [ABI], Foster City, CA). Reaction conditions were the following: initial denaturation at 95°C for 10 minutes, 35 cycles of 95°C for 1 minute, 59°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. PCR fragments were gel purified and sequenced.

RNA was extracted from Epstein-Barr virus–transformed lymphoblastoid cells of the affected male VII-5 and the nine control samples (RNeasy Mini Kit; Qiagen GmbH, Hilden, Germany). cDNA was synthesized by using the M-MLV (H-) RT enzyme and random 6-mer primers (Promega, Madison, WI), according to the manufacturer's instructions. Exons surrounding the mutation were amplified from *CACNA1F* cDNA with exon 27 forward primer and exon 31 reverse primer. Amplified fragments were purified (QIAquick PCR Purification Kit; Qiagen) and sequenced (Prism BigDye Terminator ver. 3.1 Cycle Sequencing Kit and 310 Genetic Analyzer; ABI). Complementary DNA from a cDNA library (Human Retina QUICK-Clone cDNA library; BD-Clontech, Palo Alto, CA) and lymphoblast cDNAs from the patient with AIED and nine control samples were amplified, to study the expression of previously identified *CACNA1F* gene splice variants of exons 31 and 32.[24] PCR was performed as previously described, with the exon 28 forward primer 5′-GCGAGCAGGAGTACCAAAAC-3′ and exon 34 reverse primer 5′-GAAGAGCCACATAGGGCAAG-3′.

Results

Mutation analysis of the *CACNA1F* gene in genomic DNA in a patient from the original AIED family[1] revealed a novel deletion of a single exon and portions of adjacent introns. The deletion covered 425 bp, including 133 bp of intron 29, 111 bp of exon 30, and 181 bp of intron 30 (Fig. 2A). Sequencing of the lymphoblast cDNA of a patient with AIED confirmed that exon 30 was absent from the *CACNA1F* mRNA (Fig. 2B). This deletion mutation cosegregated with the disease phenotype—that is, it was observed in all the affected patients ($n = 6$) and carrier females ($n = 8$). We did not find the mutation in samples from 121 Finnish male control subjects.

To verify that the skipping of exon 30 is not just a normal splice variant in lymphoblasts, we also studied the splicing of *CACNA1F* mRNA in an unrelated control sample with the exon 27 and 31 primer pairs and identified only a transcript containing exon 30 (data not shown).

The identified mutation is in-frame and predicted to lead to the deletion of the transmembrane domain IVS2 and the preceding extracellular loop of the $Ca_v1.4$ α_1-subunit (α_{1F}; Fig. 3).

We had identified normal *CACNA1F* mRNA splice variants in control lymphoblast cells lacking either exon 32 or exons 31 and 32.[24] The variant, which lacks both exons 31 and 32, leads to the deletion of the IVS3 transmembrane segment and part of the IVS3-S4 linker region. Because this variant is predicted to cause an in-frame deletion of the $Ca_v1.4$ α_1-subunit, similar to the AIED mutation, we studied the expression of the splice variant in several lymphoblast cell lines and in the retina to see whether the change is a polymorphism that could modify the disease phenotype or a lymphoblast-specific variant that does

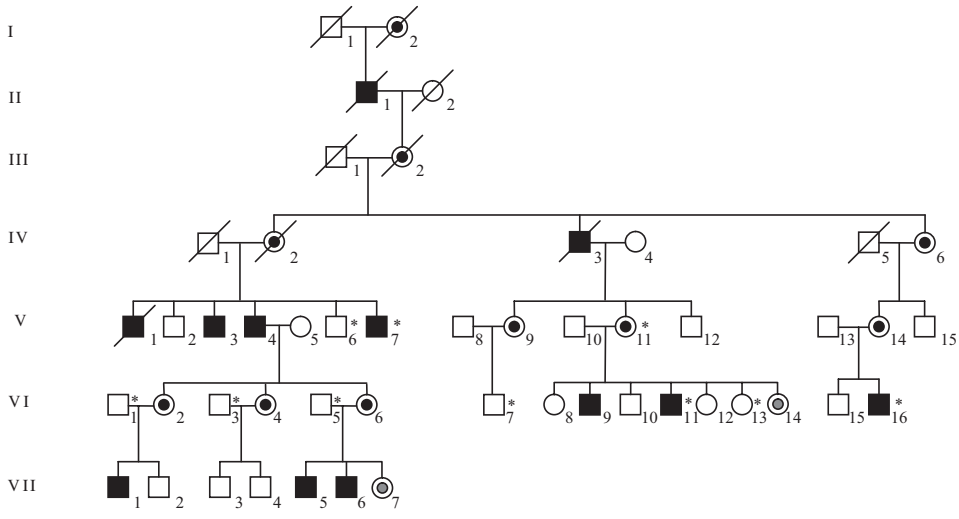

Figure 1. Pedigree of the AIED family. *Blood/DNA sample not available.

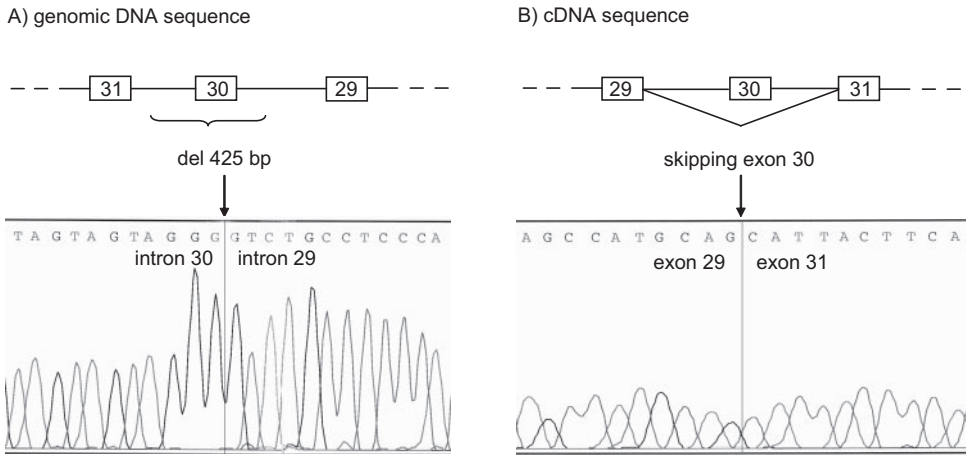

FIGURE 2. The novel *CACNA1F* mutation segregating in the AIED family. (**A**) Electropherograms of the antisense strand of an amplified genomic DNA fragment of *CACNA1F* from a patient with AIED, showing the deleted region. (**B**) Electropherogram of the sense strand of an amplified cDNA fragment from a patient with AIED, showing the exclusion of *CACNA1F* exon 30.

not exist in the retina. cDNAs of lymphoblast cell lines of a patient with AIED, nine control cDNAs, and a human retinal cDNA library were analyzed with exon 28 and 34 primer pairs. All 10 lymphoblast cell lines expressed the splice variant lacking exons 31 and 32, as well as the variant lacking exon 32. In contrast, only the wild-type cDNA containing all exons and the variant lacking exon 32 were seen in the retinal cDNA library (data not shown).

DISCUSSION

We have identified a novel 425-bp deletion mutation encompassing exon 30 and portions of adjacent introns of *CACNA1F* in patients of the original AIED-affected family. In a previous study by Wutz et al.[22] two patients in the same family (V-4 and VI-9) were studied, but no mutation was found. In this study, we used the same primer pairs as Wutz et al.[22] used, but failed to amplify exon 30 robustly with these primers. Amplification with a new primer pair, located in nearby exons, however, revealed the 425-bp deletion. Sequence analysis indicated that the original primers for exon 30 were located in the deleted region. The newly identified mutation is predicted to cause a deletion of the transmembrane domain IVS2 and the preceding extracellular loop and consequently an altered membrane topology for the C-terminal part of the α_{1F} protein (Fig. 3). Such an important alteration of the protein structure suggests a total absence or significantly altered function of the channel.

In a previous study, we identified a similar kind of deletion in control lymphoblast cells, involving exons 31 and 32 of the *CACNA1F* mRNA, which is a normal splice variant due to alternative splicing.[24] In the present study, we found that the same variant was expressed in all lymphoblast cell lines studied, but was absent from the human retinal cDNA library, suggesting that this splice variant has a specific role in lymphoblasts. The variant, which lacks exons 31 and 32, is predicted to lead to the deletion of the IVS3 transmembrane segment and part of the IVS3-S4 linker region and to an altered membrane topology for the C-terminal part of the protein. The same kind of splice variant has also been reported for another L-type calcium channel gene, *CACNA1D,* in rat neuroendocrine GH_3 cells.[26] This kind of splicing is likely to cause marked changes in the channel function, but the actual functional significance of these variants still must be clarified.

Several splice variants observed within voltage-dependent calcium channels have already been studied functionally (see review by Jurkat-Rott and Lehmann-Horn[27]). For example, an N-type calcium channel gene (*CACNA1B*) splice variant, which generates an insertion of two amino acids to a loop between transmembrane domains IVS3 and S4 of $Ca_v2.2$, has been shown to have an impact on the activation kinetics and voltage

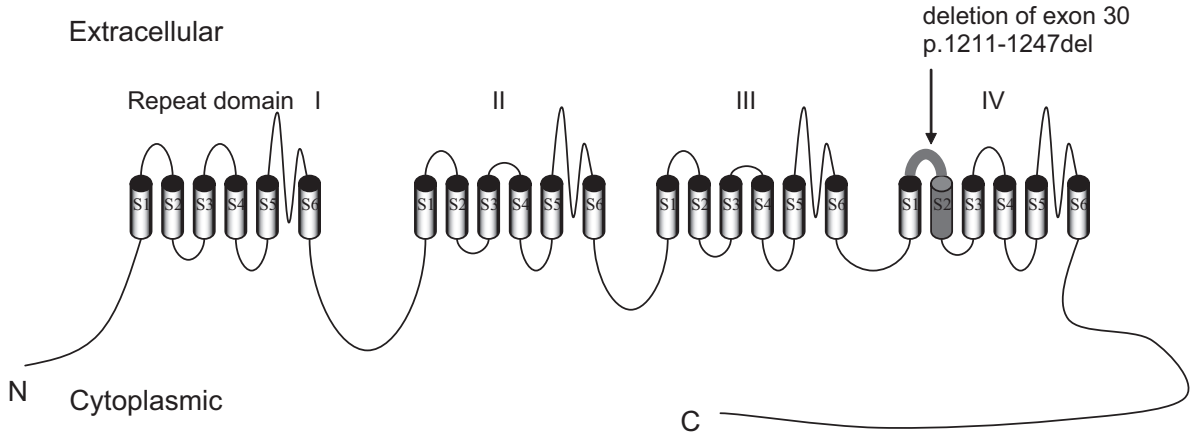

FIGURE 3. The predicted consequence of the identified AIED mutation is shown in the putative membrane topology of the human L-type calcium channel $Ca_v1.4$ α_{1F}-subunit encoded by *CACNA1F.* The α_1-subunit is the pore-forming part of the voltage-dependent calcium channels (VDCCs).[25] It has a tetrameric motif, composed of four homologous domains (I-IV), each containing six transmembrane α helices (S1–S6) and a membrane-associated loop between the S5 and S6 segments. In patients with AIED, the S2 transmembrane segment and the preceding extracellular loop of the repeat domain IV are deleted.

dependence of gating.[28] More dramatic changes are caused by variants that generate truncated proteins. For example, a mutant calcium channel $Ca_v2.2$ consisting of only one or two of the four repeat domains is not functional when expressed alone. Instead, it suppresses the expression of the full-length channel.[29] As yet, the functional consequence of the novel deletion of exon 30 identified in the AIED family has not been established. Though, it is possible that this mutation leads to an altered channel function, possibly in the same manner as splice variants that are predicted to lead to membrane topology changes.

A total of 56 *CACNA1F* gene mutations have been described to date. The mutation spectrum is wide, including missense, nonsense, splice site, deletion, and insertion mutations. Mutations of *CACNA1F* were originally found to be the underlying cause of CSNB2.[20,21] Subsequent studies have shown that *CACNA1F* mutations can also lead to distinct but partially overlapping phenotypes, such as an AIED-like phenotype[2,22]; retinal and optic disc atrophy and progressive decline of visual function[30]; severe CSNB2-like disease, with associated intellectual disability and female carrier symptoms[31]; and X-linked progressive cone-rod dystrophy, CORDX3.[24]

Reevaluation of the clinical features of the patients with AIED-like disease has led to the conclusion that AIED-like disease and CSNB2 are identical disorders.[22] Is AIED then the same disease as CSNB2? It was suggested in a 1977 study[32] that AIED should be classified as a form of CSNB. In 1989, Weleber et al.[33] reported ocular findings in a boy who had signs of both AIED and CSNB and stated that the two diseases appeared to be identical. The linkage studies of the original AIED-affected family indicated that in addition to the clinical features, the genetic intervals of AIED and CSNB2 were overlapping.[11] Our current finding of a novel mutation in *CACNA1F* now establishes that AIED and CSNB2 are allelic diseases. Moreover, the clinical features of these two diseases seem to be nearly identical considering that clinical variability is not uncommon among patients with CSNB2, even among patients with the same *CACNA1F* mutation.[15] However, there are a few distinct differences between the symptoms of the patients in the original AIED family and those described as having CSNB2. AIED has progressive myopic refraction, foveal dysplasia with no foveal reflex, and a protan defect in color vision,[1,8] whereas CSNB2 is apparently stationary with a normal fovea and mostly normal color vision, with tritan or mixed defects in some cases.[14,34,35] These differences may be attributable to differences in genetic background (i.e., other genes, possibly genetic modifiers).

The clinical picture of progressive retinal and optic disc atrophy[30] differs from both CSNB2 and AIED. Of interest, the same *CACNA1F* gene mutation that is causative of retinal and optic disc atrophy has also been identified in patients with a typical CSNB2 phenotype.[30,36] The severe retinal disease described in a Maori family[31] shows similarities to CSNB2 and AIED, but distinctive features also exist, such as abnormal intelligence and manifestations in female carriers.

In comparison to CORDX3, AIED has several features that are different. It is considered to be a congenital disease, whereas CORDX3 can start in childhood or adulthood. Except for the refraction, AIED is stationary, whereas CORDX3 shows progression in refraction, visual acuity, color vision, and visual fields. AIED shows nystagmus and astigmatism >1.5 D, but CORDX3 has neither of these. AIED has foveal dysplasia with no foveal reflex, whereas the fovea in CORDX3 is normal. In AIED, visual fields are normal, and dark adaptation shows a biphasic curve, but in CORDX3, visual fields have central scotomas and the dark-adaptation curve can lack the cone threshold.[1,8,37]

Two recent studies of *Cacna1f* mutant mice provide clues to the pathophysiology of diseases caused by *CACNA1F* mutations by indicating the essential role of the $Ca_v1.4$ calcium channel in the development and/or maintenance of ribbon synapses between photoreceptors and second-order neurons.[38,39] Of note, there are some differences between the phenotypes of the two *Cacna1f* mutant mouse strains, even if both carry a loss-of-function mutation in the *Cacna1f* gene. According to anatomic and functional characterizations of the retina, the phenotype of naturally occurring *Cacna1f* null-mutant mouse, nob2 (no b-wave 2), resembles the phenotype in CSNB2 patients.[39] In contrast, the *Cacna1f*-knockout mouse constructed by Marsergh et al.[38] is phenotypically more like a cone–rod dystrophy. These phenotypic differences observed in the *Cacna1f* mutant mice and the fact that in humans the clinical variability of patients does not fully correlate with the *CACNA1F* genotype[15] further support the contribution of other genetic and/or environmental factors to the phenotypic expression of the *CACNA1F* mutations.

In summary, the variability of clinical features among patients with *CACNA1F* mutations seems to be wide. Different mutations of *CACNA1F* can lead to several phenotypes having a few or more symptoms in common, suggesting the need for a thorough clinical examination with visual function tests together with mutation analysis, to reach a correct diagnosis. In addition to a wide phenotypic spectrum associated with different *CACNA1F* mutations, phenotypic differences can be found even among patients who share the same *CACNA1F* mutation. Our findings on AIED further expand our knowledge concerning the clinical spectrum caused by mutations in *CACNA1F*.

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