Allelic Variation of the FRMD7 Gene in Congenital Idiopathic Nystagmus

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Objective: To perform a genotype-phenotype correlation study in an X-linked congenital idiopathic nystagmus pedigree (pedigree 1) and to assess the allelic variation of the FRMD7 gene in congenital idiopathic nystagmus.

Methods: Subjects from pedigree 1 underwent detailed clinical examination including nystagmography. Screening of FRMD7 was undertaken in pedigree 1 and in 37 other congenital idiopathic nystagmus probands and controls. Direct sequencing confirmed sequence changes. X-inactivation studies were performed in pedigree 1.

Results: The nystagmus phenotype was extremely variable in pedigree 1. We identified 2 FRMD7 mutations. However, 80% of X-linked families and 96% of simplex cases showed no mutations. X-inactivation studies demonstrated no clear causal link between skewing and variable penetrance.

Conclusions: We confirm profound phenotypic variation in X-linked congenital idiopathic nystagmus pedigrees. We demonstrate that other congenital nystagmus genes exist besides FRMD7. We show that the role of X inactivation in variable penetrance is unclear in congenital idiopathic nystagmus.

Clinical Relevance: We demonstrate that phenotypic variation of nystagmus occurs in families with FRMD7 mutations. While FRMD7 mutations may be found in some cases of X-linked congenital idiopathic nystagmus, the diagnostic yield is low. X-inactivation assays are unhelpful as a test for carrier status for this disease.

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Nystagmus is a disorder of oculomotor control and can occur as an isolated inherited trait, a congenital idiopathic nystagmus, or as secondary to other visual (sensory deficit nystagmus) or neurological (neurological nystagmus) diseases.1 In all cases, the underlying pathophysiology is poorly understood. Furthermore, it is unclear which characteristics of the nystagmus phenotype, if any, are reliable as a diagnostic tool to help identify underlying etiology.

Congenital idiopathic nystagmus is genetically heterogeneous and has been described as an autosomal dominant,2 autosomal recessive,3 X-linked dominant,3 or X-linked recessive4 trait. X-linked loci have been identified at Xp11.4-p11.35 and Xq26-q27.35 In November 2006, Tarpey et al8 identified 22 nystagmus-causing mutations in the FERM domain-containing 7 (FRMD7) gene, which resides within the Xq26-q27 interval. Sixteen X-linked families underwent linkage analysis and 15 of these pedigrees had causative FRMD7 mutations. An additional 14 small pedigrees with congenital idiopathic nystagmus and an inheritance pattern consistent with X linkage were screened and FRMD7 mutations were found in 8 of the pedigrees (57%). Forty-two simplex cases were screened for mutations in this gene and yielded 3 mutations (7%). Therefore, the overall contribution of FRMD7 mutations to the cause of both X-linked and singleton cases remains relatively unexplored.

In X-linked congenital idiopathic nystagmus pedigrees, penetration among female obligate carriers has been variable,3-5,7 ranging from 30% to 100%. Possible mechanisms for this variability include skewed X inactivation, genetic modifiers (such as polymorphisms within interacting proteins), and other nongenetic influences (such as environment) on oculomotor development. These factors may also explain why X-linked dominant and recessive pedigrees, with nystagmus or other ocular diseases, can both show linkage to the same region.5,8,9
This article describes high-resolution phenotyping using infrared limbal tracking, electrophysiology, and clinical examination in affected and unaffected members of a single large congenital nystagmus pedigree (pedigree 1) (Figure 1). The role of FRMD7 mutations in 9 other X-linked families and 28 simplex cases is investigated and, in addition, studies of X inactivation are performed to investigate the hypothesis that skewing of X inactivation may be a major contributor to the variable penetrance seen in X-linked nystagmus pedigrees.

The study had the approval of the local and regional ethics committees and conform to the tenets of the Declaration of Helsinki. Twenty-nine individuals (7 affected males, 2 affected females, 11 obligate female carriers, and 9 unaffected males) in a single congenital idiopathic nystagmus pedigree underwent detailed clinical examination (Figure 1), including tests for logarithm of the minimum angle of resolution visual acuity, refraction, color vision, intraocular pressure; anterior and posterior segment slitlamp examination, including iris transillumination testing in a darkened room; and orthoptic assessment. Twenty-four of these patients had detailed recordings of their nystagmus waveform performed using Skalar IRIS IR Light Eye Tracker equipment (Cambridge Research Systems Ltd, Rochester, England). Twenty-four eye movement recordings were completed for each patient. Binocular and unidirectional saccades were recorded to calibrate amplitude measurements at ±10° and ±20° from fixation in the horizontal plain using a 1° red square target moving at 500-millisecond intervals. Binocular and unidirectional optokinetic nystagmus (OKN) measurements were performed using infrared limbal tracking, electrophysiological examination but without detailed eye movement recordings.

International Society for Clinical Electrophysiology of Vision–standardized electrophysiology (http://www.iscev.org/) and visual evoked potential tests were completed in 2 affected males and 2 obligate female carriers. Monocular stimulation and a 3-channel transoccipital electrode montage were employed for visual evoked potential recordings to optimize detection of neuronal misrouting suggestive of ocular albinism. Informed consent was obtained from all subjects for genetic studies, and genomic DNA was isolated from either blood or Oragene saliva sample kits (DNA Genotek Inc, Ottawa, Ontario, Canada).

**GENOTYPING AND LINKAGE ANALYSIS**

We had previously shown by conventional linkage mapping techniques that the nystagmus gene in pedigree 1 links to an 8-cM region at Xq24-q26.3. This contains the FRMD7 gene, which is now known to be a cause of X-linked nystagmus and was thus investigated in this family. DNA was amplified using standard polymerase chain reaction (PCR) protocols. Primers for FRMD7 (complementary DNA sequence NM_194277) were designed to include all 12 exons, splice sites, and both the 5’ and 3’ untranslated regions in amplimers with fewer than 250 base pairs (primers available on request). Pedigrees 2 through 10 were not large enough to provide linkage information individually, and to avoid errors in light of known X-linked congenital idiopathic nystagmus locus heterogeneity, these families were not combined. Therefore, linkage information was not ascertained for these families and definitive proof of X linkage was not possible.

**SINGLE-STRAND CONFORMATIONAL POLYMORPHISM ANALYSIS**

Single-strand conformational polymorphism (SSCP) was performed using standard techniques. Specifically, polyacrylamide gels with glycerol, 6%, were run for 3.5 hours at 25 W per gel at room temperature. We screened for sequence variations in 96 female controls (182 control X chromosomes), followed by 2 affected subjects from the first pedigree, 9 pro-
bands from other X-linked nystagmus pedigrees, and 28 nystagmus simplex cases.

SEQUENCING

Sequencing was performed for samples with either shifts or failed PCR seen on SSCP gels. Following standard PCR, each fragment was sequenced using the Big Dye Terminator Cycle Sequencing kit, version 1.1, and an ABI 3100 Automated Capillary DNA Sequencer (Applied Biosystems, Foster City, California) following the manufacturer’s protocol.

RESTRICTION ENZYME DIGEST

Cosegregation of an identified mutation with the nystagmus phenotype in pedigree 1 was tested using a restriction enzyme digest following standard PCR. The PstI enzyme was used according to the manufacturer’s protocol (Promega, Madison, Wisconsin) and chosen to cut wild type (CTGCAG) but not mutant sequence (CTGCAAG) mutations in this pedigree.

X-INACTIVATION ANALYSIS

Two assays comprising amplification of a short fragment of X chromosome (amplimer) were used to determine the activation status of each X chromosome (ie, the maternal or paternal X chromosome) in females. Each amplimer contains a polymorphic marker adjacent to the promoter of a gene, which is unmethylated on the active X chromosome and methylated on the inactive chromosome. The 2 genes investigated were the androgen receptor Humar and ZNF261. Both genes are in Xq13. Prior to amplification, both assays employ a methylation-sensitive restriction enzyme, which cuts at the promoter site on the active (unmethylated) but not on the inactive (methylated) X chromosome. Therefore, by examining the size and peak heights of the amplification products and by tracking the inheritance of alleles from parents to offspring, we ascertained the percentage of cells in which the maternal or paternal X chromosome had been inactivated. To calculate the X-inactivation ratio, each sample was set up in duplicate: 1 digest and 1 mock digest without enzyme. Both were then amplified by PCR. For heterozygotes, the ratio of the 2 peaks were compared between the undigested and digested samples. Male samples were used for haplotype reconstruction.

RESULTS

CLINICAL PHENOTYPING STUDIES

Flash and pattern electroretinograms and occipital pattern visual evoked potential recordings in 2 affected males and 2 obligate female carriers were normal, thus excluding masquerading eye conditions. Computerized tomographic brain scans were performed on 2 affected individuals at diagnosis with no abnormal findings. Ophthalmic examination results were also normal except for nystagmus in affected patients. The prevalence of strabismus was 44% in affected subjects (4 of 9) and 15.8% in unaffected subjects (3 of 19). Refractive errors ranged from unilocular logarithm of the minimum angle of resolution acuities of 0.1 to 0.5 in affected subjects. We have published the detailed results previously and they are in agreement with previous findings in congen-
Eye movement recording results are presented in Table 1 for all patients with findings positive for nystagmus. Examples of nystagmus waveforms seen in this pedigree are presented in Figure 3. Eighteen unaffected subjects, including 6 obligate female carriers, had no abnormalities on any of the 24 recordings and so were not included in Table 1. Also included in Table 1 is a female carrier with nystagmus, possibly secondary to a cochlear implant (III:16), and a male subject (III:8) who is affected but was excluded owing to a history of congenital cataract. The results also exclude an affected subject (IV:15) for whom eye movement recordings were not collected.

SSCP AND SEQUENCING RESULTS

Thirteen gel shifts were found in 9 amplimers in both the control and affected probands and these were checked by sequencing and corresponded to known single nucleotide polymorphisms. A shift was found for both samples from pedigree 1 in an amplimer containing part of exon 9. Sequencing revealed this was caused by a single base insertion (“A”) after base 880 (complementary DNA sequence NM_194277) designated 880insA, 293fs mutation. This frameshift mutation is predicted to cause a premature stop codon after 9 amino acids, thereby truncating the FRMD7 protein (Lasergene, version 6.1.3; DNASTAR Inc, Madison) or leading to nonsense-mediated decay of the messenger RNA.

In an affected male from X-linked pedigree 2 and an unrelated singleton female, a shift was seen in an amplimer containing exon 4. Sequencing identified a point mutation in the first intronic base after exon 4 in both subjects, designated IVS4 + 1G→A. This sequence had been previously described by Tarpey et al and the change is predicted to cause a splice recognition site mutation leading to continued transcription into intron 4-5 resulting in a premature stop codon after 9 amino acids, thereby truncating the FRMD7 protein or leading to nonsense-mediated decay of the messenger RNA. These mutations were not seen in controls and are summarized in Table 2.

RESTRICTION ENZYME DIGEST RESULTS FOR PEDIGREE 1

The results of Psrl restriction enzyme digest of the exon 9 PCR amplimer for individuals in pedigree 1 are illustrated in Figure 4, along with a condensed pedigree to aid subject identification. Mutation 880insA, 293fs cosegregates with all affected and female carriers but not with unaffected patients. Notably, affected individual III:8, who was excluded from initial work because of a history of congenital cataract, is also hemizygous for the 880insA, 293fs mutation.

X-INACTIVATION STUDIES

Results for the X-inactivation assays are in Table 3. Ten of the 16 females who were tested demonstrated at
least moderate skewing, including both affected females, and 3 cases showed skewing of more than 95%. However, as illustrated, it is not always possible to tell which X chromosome is inactivated. Mutation status of each X chromosome was known for 1 affected female and the X carrying the FRMD7 mutation was active in 73% of cells. For informative unaffected carriers, the X chromosome carrying the FRMD7 mutation was active in 6% to 80% of cells. Therefore, there was no clear-cut difference in the pattern of X inactivation between af-

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**Table 2. Mutations Identified in FRMD7**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pedigree</th>
<th>Class</th>
<th>Mutation</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>XN3P</td>
<td>X-linked (pedigree 1)</td>
<td>Truncating</td>
<td>880insA, 293fs</td>
<td>England</td>
</tr>
<tr>
<td>NRD2</td>
<td>Singleton</td>
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<td>IVS4 + 1G→A</td>
<td>England</td>
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<tr>
<td>XNTW1</td>
<td>X-linked (pedigree 2)</td>
<td>Truncating</td>
<td>IVS4 + 1G→A</td>
<td>England</td>
</tr>
</tbody>
</table>

*The reference complementary DNA sequence NM_194277 is used as a basis for numbering the nucleotide of the mutation. All mutations are located relative to the A of the first coding ATG at position 179. The reference protein sequence NP_919253 is used as the basis for numbering the amino acid variation starting from the first methionine at position 1.*
We have shown that congenital idiopathic nystagmus patients in a single family with this novel 880insA, 293fs-truncating FRMD7 mutation have extremely variable nystagmus phenotypes. Classically, the hallmarks of the congenital idiopathic nystagmus phenotype are accelerating slow phases, loss of OKN, and no changes in phenotype on monocular viewing. In pedigree 1, however, 7 of 9 patients with congenital idiopathic nystagmus had 3 or more waveforms, including prolonged periods of decelerating or linear slow phases, which are more common seen in patients with nystagmus of neurologic origin. Similarly, OKN was preserved to some degree in 2 patients, which is considered to be rare in congenital idiopathic nystagmus. Interestingly, in subject III:16 (female carrier but also known to have a cochlear implant), OKN was preserved, the nystagmus amplitude was very low, and no accelerating slow phases were seen (also rare in congenital idiopathic nystagmus). These findings might suggest that her nystagmus is secondary to the cochlear implant and not congenital idiopathic nystagmus. Interestingly, in subject III:16 (female carrier but also known to have a cochlear implant), OKN was preserved, the nystagmus amplitude was very low, and no accelerating slow phases were seen (also rare in congenital idiopathic nystagmus). These findings might suggest that her nystagmus is secondary to the cochlear implant and not congenital idiopathic nystagmus. Subject III:8, who has nystagmus but was excluded owing to a history of congenital cataract, has nystagmus waveforms that are very similar to the affected subjects, including disrupted OKN and accelerating slow phases. These findings would be more suggestive of congenital idiopathic nystagmus than sensory deficit nystagmus due to congenital cataract. This subject was subsequently found to be hemizygous for the causative mutation in this family.

The clinical phenotyping demonstrated that in some cases waveform characteristics were indicative of underlying etiology in this family; but conversely, many of the waveform characteristics seen in individuals of this pedigree would suggest wholly differing etiologies. These results support the opinion that waveforms alone should be interpreted with caution when employed as a diagnostic tool in congenital idiopathic nystagmus. However, detailed eye movement recordings may be necessary to assign affection status in congenital idiopathic nystagmus pedigrees and the families of congenital idiopathic nystagmus singleton cases. An example is individual V:9 from pedigree 1 who had been previously diagnosed as unaffected by an experienced ophthalmologist but was found to have subtle nystagmus only after examination with infrared oculography (and was subsequently found to be hemizygous for the causative mutation in this family). This has significant implications for diagnosis and genetic counseling for this individual and other currently undiagnosed individuals from congenital idiopathic nystagmus pedigrees.

We confirm that FRMD7 mutations are a cause of X-linked congenital idiopathic nystagmus and describe a novel protein-truncating mutation. By screening all exons and splice sites of the FRMD7 gene, we identified mutations in 20% (2 of 10) of apparent X-linked pedigrees. This percentage is significantly smaller than that found by Tarpey et al, who identified mutations in 8 of 14 (57%) apparent X-linked pedigrees and 15 of 16 (94%) proven X-linked pedigrees. This may be because we used SSCP as our initial method of mutation detection. In our experience, SSCP has a sensitivity of 89% (95% confidence interval, 79%-96%) compared with direct DNA sequencing. Therefore, our detection rate is likely to be lower than that found by direct DNA sequencing. However, additionally, the cohort used by Tarpey et al had been previously used for linkage analysis to identify the causative gene inevitably leading to an ascertainment bias. We also identified FRMD7 mutations in 1 of 28 (3.6%) singleton cases, which is similar to the 3 of 42 (7%) singleton cases found by Tarpey et al. Therefore, according to our results, most (80% in this study) unselected X-linked families and 96.4% of single-

**Figure 4.** Restriction enzyme digest of DNA samples from an X-linked congenital idiopathic nystagmus pedigree (pedigree 1) using the enzyme PstI. The mutant (uncut) allele generates a 267–base pair (bp) fragment, while the wild-type allele (cut) produces fragments of 133 bp and 134 bp. Control lanes are included. Asterisk indicates unaffected male III:17 with no PstI enzyme; and triangle, unaffected male III:17 with PstI enzyme.
ton congenital idiopathic nystagmus cases do not have FRMD7 mutations as a cause. This suggests the existence of other prevalent nystagmus genes on the X chromosome. Alternatively, mutations in as yet uncharacterized regulatory elements for FRMD7, such as promoters, silencers, or enhancers, may also exist but were not detected by our assays.

FRMD7 mutations found by Tarpey et al are clustered around the B41 and FERM-C domains. The FARP2 gene on chromosome 2 shares significant homology with a large portion of FRMD7, including these domains. It is also known that FARP2 alters neurite length and degree of sprouting in rat embryonic cortical neurons. This has led to the hypothesis that mutations in FRMD7 may cause nystagmus by altering the neurite length and degree of branching of neurons as they develop in the midbrain, cerebellum, and retina. The mutations identified in our study are also clustered around these domains and thus our results support the hypothesis that mutations in this region of FRMD7 are particularly important for nystagmus. FARP2 and other homologues may also be potential candidates for nystagmus genes.

We had previously proposed that the likeliest explanation for the variable phenotype in female carriers in pedigree 1 was variability in the pattern of X inactivation. Skewed X inactivation (significant deviation away from the expected 50:50 contribution of each X chromosome) was observed in some female carriers, suggesting that the expression of FRMD7 is modulated by the pattern of X inactivation.

### Table 3. Humar and ZNF261 X-Inactivation Assays for Pedigree 1

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<tr>
<th>Sample</th>
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<th>Status</th>
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<th>Allele 2</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Degree of Skewing</th>
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<td></td>
<td>DNA</td>
<td></td>
<td></td>
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<td>Activity, %</td>
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<td>Activity, %</td>
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<td>233 47</td>
<td>270 55</td>
<td>272 45</td>
<td>Randomb</td>
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</tbody>
</table>

*a* Two activity values are shown for each assay, as all experiments were performed twice. Empty cells show that there is no second allele. X denotes that a percentage calculation was not possible, as the patient was homozygous for this marker.

*b* Mean result of skewing from 50% to 70%.

*c* Mean result of skewing greater than 90%.

*d* Mean result of skewing between 70% and 90%.
mosome) has been described in other ocular diseases and may explain why other X-linked nystagmus pedigrees linked to the locus containing FRMD7 have shown either dominant or recessive inheritance patterns. The vast majority of genes on the long arm of the X chromosome are subject to X inactivation, including those immediately flanking FRMD7, like MST4, MBNL3, and RAP2C. Because genes that are subject to or escape from X inactivation tend to be clustered into domains, it is very likely that FRMD7 is inactivated. Only a very small proportion of healthy females show significant levels of skewed inactivation. However, our results suggest an excess of skewing in pedigree 1 (10 of 16 females including 3 with >95% skewing). Interestingly, of the 10 females with skewing, 7 are younger than 40 years; for 3 of these patients, the assays were performed on saliva samples. This suggests that the observed skewing in pedigree 1 is not because of age-related secondary skewing or specific to blood.

There are 3 possible interpretations of the X-inactivation results from blood or saliva. First, the effect of X inactivation on penetrance may be subtle, rather than all or nothing, as neither unaffected nor affected female carriers have complete skewing. It is possible that the proportion of cells in which the mutation-carrying X is active may be higher in the crucial tissues in the affected cases than in carrier females. Many X-linked disorders do not fit classic dominant or recessive modes of inheritance and female carriers display variable penetrance.

For example, mutations in the dystonia-deafness peptide gene cause incomplete penetrance in females with variable X inactivation ratios in blood from 50:50 to more than 95:5. Thus, mutations in X-linked genes can cause partial cell selection and incompletely skewed X inactivation. This could explain variable clinical expression within the same family.

Second, the results obtained from lymphocytes and saliva samples may not necessarily reflect the patterns of X inactivation in the tissue whose pathophysiology causes nystagmus (as yet unknown). In healthy females, X-inactivation ratios are normally similar between tissues. However, for certain X-linked conditions, complete skewing may be restricted to specific cell lineages, and different patterns of X inactivation are seen in all other tissues. Selection against cells that have inactivated the normal X may be more pronounced in tissues where most FRMD7 expression occurs, possibly in parts of the developing brain and retina.

Third, variable penetrance may be at least in part because of other genetic factors. The results of the X-inactivation studies give a clear overlap in the patterns of X inactivation between unaffected and affected female carriers. Individual IV:18, who is negative for the FRMD7 mutation, is one of the females with severe skewing. It is possible that the higher frequency of skewed X inactivation seen in this family may be unrelated to nystagmus, as we know that skewed X inactivation per se may run in families.

Importantly, by showing that there is no definitive pattern of X inactivation in carrier or manifesting females, we have shown that determining the X-inactivation pattern in blood or saliva cannot be used to identify carrier status for nystagmus caused by FRMD7 mutations. This is important from a diagnostic viewpoint, as X inactivation is used as a surrogate test for carrier status in other X-linked conditions.

In summary, we have demonstrated the variability of the congenital idiopathic nystagmus phenotype in patients with FRMD7 mutations. We report a novel FRMD7 mutation and confirm that mutations in this gene may cause nystagmus. This work suggests that other nystagmus genes exist, that FRMD7 is rarely mutated in simplex nystagmus cases, and that routine FRMD7 mutation screening would not have a sufficiently high detection rate to warrant routine use in congenital idiopathic nystagmus cases or suspected carriers.

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REFERENCES

Retained foreign bodies were found in 731 of 3,882 eyes of soldiers studied at the Army Institute of Pathology during World War II. All globes with penetrating wounds were examined roentgenologically and were searched for foreign bodies. In many instances the particles were so small or so deeply embedded in organizing hemorrhage or inflammatory membrane that they were not recovered from the gross specimens and became visible only on microscopic examination. These could not be subjected to the magnet test, but sections containing them were stained with prussian blue.