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Address for correspondence and reprints: Dr. David H. Ledbetter, Center for Medical Genetics, The University of Chicago, 924 East 57th Street, Room R110, Chicago, IL 60637-1470. E-mail: dhl@genetics.uchicago.edu

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## DNA Studies of Limb-Girdle Muscular Dystrophy Type 2A in the Amish Exclude a Modifying Mitochondrial Gene and Show No Evidence for a Modifying Nuclear Gene

*To the Editor:*

Limb-girdle muscular dystrophy type 2A (LGMD2A) is characterized by slowly progressive muscle weakness, usually first evident in the pelvic girdle and then spreading to the upper limbs *while sparing facial muscles*. Onset of symptoms is variable (mean age 9 years), and creatine kinase (CK) levels are elevated from early infancy and remain elevated until the individual is well past this age (Jackson and Strehler 1968). Affected individuals are often wheelchair-bound 20–30 years after the onset of symptoms. There is variability in the age of death, and most individuals die in middle age.

The gene for LGMD2A was first linked to chromosome 15 by Beckmann et al. (1991). Allamand et al. (1995) narrowed the region to 15q15.1-q15.3, using large kindreds from the Isle of La Réunion and the northern Indiana Amish. The muscle-specific calcium-activated neutral protease 3 or calpain 3 (*CANP3*) gene, a possible candidate gene in the 15q15.1-q15.3 region, was examined by Richard et al. (1995). Fifteen different mutations, including missense, splice-site, frameshift, and nonsense mutations, were identified in LGMD2A patients, and many others have subsequently been identified. Since the affected patients in La Réunion belong to a genetic isolate presumed to derive from a single ancestor who immigrated to the island during the late 1670s, it was expected that all affected patients from La Réunion would have the same LGMD2A mutation. Paradoxically, six different mutations were identified. This paradox led the investigators to propose digenic inheritance, in which the founder effect is due to an as-yet-unidentified modulating gene (either nuclear or mitochondrial) that permits mutations in *CANP3* to express LGMD2A. This hypothesis does not require the presence of multiple mutations, since the genetic principles of digenic inheritance should apply to all populations with LGMD caused by calpain-3 mutations.

In the Amish of northern Indiana, Richard et al. (1995) identified a single mutation in *CANP3* (CGG→CAG, R769Q) in a homozygous state in affected patients. The authors speculated that the complete penetrance of this disease in the Amish and in the other LGMD2A pedigrees might also be under the control of a second locus. One expectation of the digenic hypothesis would be that some individuals homozygous for the mutation would be clinically unaffected (i.e., CK is normal and there are no physical findings suggestive of LGMD). Because of the possible implications in genetic testing and counseling, we analyzed 580 DNA samples from Amish individuals in one northern Indiana county for the presence of the R769Q mutation, looking for evidence of phenotypically normal R769Q homozygotes.

We initiated the countywide screen by first identifying carrier couples. Appropriate informed consent was obtained from all individuals. In order to identify R769Q carriers in this population, we specifically approached members of 16 previously studied nuclear LGMD2A families from this county. We obtained blood samples

from spouses of already tested siblings of affected individuals and from siblings of known carriers and their siblings' spouses, because within this group there was an increased likelihood of finding couples in which both members were carriers. When carriers were identified outside this group, their siblings and their siblings' spouses were genotyped. For couples in which both individuals were heterozygous for R769Q, blood samples were requested from their children, for DNA studies (fig. 1) and CK analysis. Previous studies (C. E. Jackson, unpublished data) have shown that nonpaternity is rare in the Amish. All children of couples in which both parents were homozygous wild type were assumed to be homozygous wild type. The results are summarized in table 1. An exact gene frequency in this particular Amish isolate is not known at this time.

In addition, we searched for evidence of possible modifying mitochondrial effects by studying maternal lineages and mtDNA haplotypes. Genealogical analyses of LGMD patients with the R769Q mutation embedded in the same haplotype revealed 11 maternal lineages for the 23 northern Indiana and the three Pennsylvania Amish sibships (traceable to ancestors born 150–275 years ago). This suggests that the mutation came from the same founder. Samples from 5 of these 11 lineages were screened for mtDNA restriction-site polymorphisms characteristic of previously identified Caucasian groups of related haplotypes (haplogroups). The restriction analyses were conducted on mtDNA PCR products, as described by Torroni et al. (1994). In two of the Pennsylvania lineages and three of the northern Indiana lineages covering 15 (58%) of 26 sibships, the mtDNA analyses revealed four separate previously known northern European haplogroups. These four haplogroups are widely dispersed over the Caucasian phylogenetic tree and are differentiated from each other. Hence, any mtDNA mutation of relevance to the expression of the phenotype would have to have arisen independently in each of the relevant maternal lineages, an unlikely event. These results strongly suggest that the mitochondrial genetic background does not play a major role in the type of LGMD in this population.

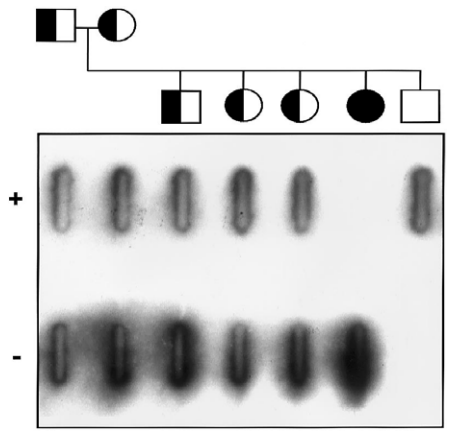


**Figure 1** Representative family of five offspring from two carrier parents in which R769Q-mutation analysis by allele-specific oligonucleotide assay was performed. The first three children are heterozygous for R769Q. The fourth child is homozygous for R769Q, and the fifth child is homozygous wild type. Genomic amplification of exon 22 of *CANP3* was performed as described by Richard et al. (1995). The allele-specific oligonucleotide primers for R769 and Q769 are 5′-TTACCATGCGGTACGCAGA-3′ and 5′-TTACCATGCAGTACGCAGA-3′, respectively (the variant nucleotide is underlined).

**Table 1**

**Results of R769Q-Mutation Screening in Northern Indiana Amish Couples**

| Matings[a] | No. of Couples |
|---|---|
| +/+ and +/+ | 73[b] |
| +/+ and spouse not tested | 133[c] |
| +/+ and +/− | 118 |
| +/− and spouse not tested (11 deceased) | 12 |
| +/− and +/− | 9 |
| Obligate +/− and +/− | 10[d] |
| +/+ and −/− | 1 |

[a] A plus sign (+) denotes wild type; and a minus sign (−) denotes R769Q mutation.

[b] In 6 couples the carrier status of both partners was presumed to be +/+, on the basis of the +/+ status of both parents of each individual.

[c] In 107 individuals the carrier status of one of the partners was presumed to be +/+, on the basis of the +/+ status of both parents of that individual.

[d] Original 10 couples with known affected children.

The failure to identify any R769Q-homozygous individual who was phenotypically normal (i.e., in whom CK is normal) is evidence against a segregating modifying gene in this population. Since the ratio of affected to unaffected siblings is as expected within nuclear families, the presence of a modifying mitochondrial gene was considered. Since all individuals within a sibship would inherit the same mtDNA, the presence of a shared "permissive" mitochondrial gene would cause that disorder to appear as a simple recessive disorder. Our data do not exclude a fixed second nuclear locus in the northern Indiana Amish population, nor do they exclude the possibility of a second locus for digenic inheritance in the Réunion population. A widespread permissive gene could be present in the Amish population but not in the Réunion population. Similar studies in the Réunion population could help to resolve the question of digenic inheritance.

Since digenic inheritance is unlikely in the Amish population, the Réunion paradox still needs to be resolved. Zlotogora et al. (1996) observed several different muta-

tions in the genes for metachromatic leukodystrophy and Hurler disease in a small geographic location, Lower Galilee. They suggested that a high mutation rate and selective advantage of the carriers were responsible for the multiple mutations, and they proposed that a similar event may have occurred in the Réunion population. Perhaps there are multiple founders despite the evidence of a single common ancestor in the Réunion families, or, less likely, perhaps there is in the *CANP3* gene a mutation-rate increase due to exogenous (differential exposure to mutagens) or endogenous (unequal distribution of mutator genes) factors. Finally, perhaps there are unknown environmental or genetic factors that influence the manifestations of mutations in the gene in the Réunion population. The search for possible modifying genetic or environmental factors should continue, since it could disclose both the mechanism of action of mutated *CANP3* in LGMD2A and, possibly, factors of therapeutic importance (Beckmann 1996). Currently, we are offering this Amish population carrier testing and genetic counseling, on the basis of both the R769Q mutation analysis and monogenic autosomal recessive inheritance for LGMD2A.

V. M. Pratt,[1,*] C. E. Jackson,[1] D. C. Wallace,[2] D. S. Gurley,[2] A. Feit,[1] and G. L. Feldman[1]
[1]*Henry Ford Hospital, Detroit; and* [2]*Emory University School of Medicine, Atlanta*

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Address for correspondence and reprints: Dr. Gerald L. Feldman, Medical Genetics and Birth Defects Center, 2799 West Grand Boulevard-CFP4, Detroit, MI 48202. E-mail: glfeldman@pol.net

*Present affiliation: Department of Pediatrics, Meharry Medical College, Nashville.


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## A C2055T Transition in Exon 8 of the ATP7A Gene Is Associated with Exon Skipping in an Occipital Horn Syndrome Family

*To the Editor:*

Mutations associated with abnormal splicing account for 10%–20% of all gene mutations (Krawczak et al. 1992). Mutations leading to abnormal splicing usually are located within the donor or acceptor splice sites. However, exonic consensus sequences that are implied in the splicing process have been described outside the splice sites (Ligtenberg et al. 1990; Steingrimsdottir et al. 1992). Direct study of the genomic DNA may not easily reveal the splicing mutations. However, reverse transcription (RT) followed by PCR analysis is likely to detect such mutations.

In the ATP7A gene, which encodes a copper-transporting P-type ATPase (Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993), splicing mutations are frequent in patients with Menkes disease (Das et al. 1994; Kaler et al. 1994, 1995; Tümer et al. 1997) and also are described in patients with occipital horn syndrome (OHS) (Kaler et al. 1994; Das et al. 1995). OHS, previously known as "X-linked cutis laxa" (MIM 304150), is a connective-tissue disorder characterized by skin laxity, hyperextensible joints, skeletal anomalies, including occipital exostoses, and inconstant mild mental retardation (Lazoff et al. 1975; Tsukahara et al. 1994). Here, we report an unusual splice mutation, which was detected by RT-PCR, in the ATP7A gene of an OHS family.