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Neonatal, Lethal Noncompaction of the Left Ventricular Myocardium Is Allelic with Barth Syndrome

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Summary

Loss-of-function mutations in the *G4.5* gene have been shown to cause Barth syndrome (BTHS), an X-linked disorder characterized by cardiac and skeletal myopathy, short stature, and neutropenia. We recently reported a family with a severe X-linked cardiomyopathy described as isolated noncompaction of the left ventricular myocardium (INVM). Other findings associated with BTHS (skeletal myopathy, neutropenia, growth retardation, elevated urinary organic acids, and mitochondrial abnormalities) were either absent or inconsistent. A linkage study of the X chromosome localized INVM to the Xq28 region near the BTHS locus, suggesting that these disorders are allelic. We screened the *G4.5* gene for mutations in this family with SSCP and direct sequencing and found a novel glycine-to-arginine substitution at position 197. This position is conserved in a homologous *Caenorhabditis elegans* protein. We conclude that INVM is a severe allelic variant of BTHS with a specific effect on the heart. This finding provides further structure-function information about the *G4.5* gene product and has implications for unexplained cases of severe infantile hypertrophic cardiomyopathy in males.

Introduction

We recently described the clinical and pathological findings of a four generation Utah family (fig. 1) in which six males were affected with severe X-linked cardiomyopathy (Bleyl et al. 1997). Consistent findings in affected males included neonatal onset of ventricular dysfunction frequently complicated by arrhythmias and cardiac failure within the first year. Echocardiographic findings were diagnostic of isolated noncompaction of the left ventricular myocardium (INVM) (Chin et al. 1990). Gross cardiac pathology showed dilation and hypertrophy with endocardial fibroelastosis (EFE) and numerous prominent trabeculations and deep intertrabecular recesses within the left ventricle, characteristic of INVM. Histologically, the myocardium showed loosely arranged fascicles of myocytes, especially in the subepicardial regions and more prominent in the left ventricle. One patient survived after cardiac transplantation and is now age 7 years. The most recent case, now age 14 mo, has survived with aggressive medical management.

Originally described in a Dutch family by Barth et al. (1983), Barth syndrome (BTHS) is an X-linked recessive disorder associated with dilated cardiomyopathy, skeletal myopathy, neutropenia, and short stature. Additional findings with variable expression include early onset with death in infancy or early childhood, EFE, borderline plasma carnitine deficiency, low muscle carnitine, elevated urinary organic acids, and myopathic facies. The early and severe presentation of cardiomyopathy in the Utah kindred described in this article, as well as the lack of consistent BTHS-associated findings, suggested a distinct disorder of which INVM is the most consistent and diagnostic finding. Linkage colocalization of INVM and BTHS to Xq28 and some phenotypic overlap between the disorders suggested that they might be allelic.

Subjects and Methods

Patients

A complete clinical description of this family appears elsewhere (Bleyl et al., 1997). In brief, all six affected relatives presented postnatally (birth to 7 mo) with left ventricular failure and arrhythmias and with the pathognomonic echocardiographic findings of INVM. Four of the affected individuals died during infancy, one is in cardiac failure at age 14 mo, and one is alive following cardiac transplant at age 9 mo. The usual findings of BTHS were either absent or inconsistent findings. Neutropenia was seen in two patients (III-2 and III-3). Muscle weakness was seen in one patient (III-5). Neither of two patients tested (III-5 and IV-3) had abnormal plasma free carnatine or urinary organic acids. Abnormal mitochondria were seen in the explanted myocardium of one patient (III-5), but this was an inconsistent finding. All hearts examined on explant or autopsy showed the gross and histological findings of INVM.

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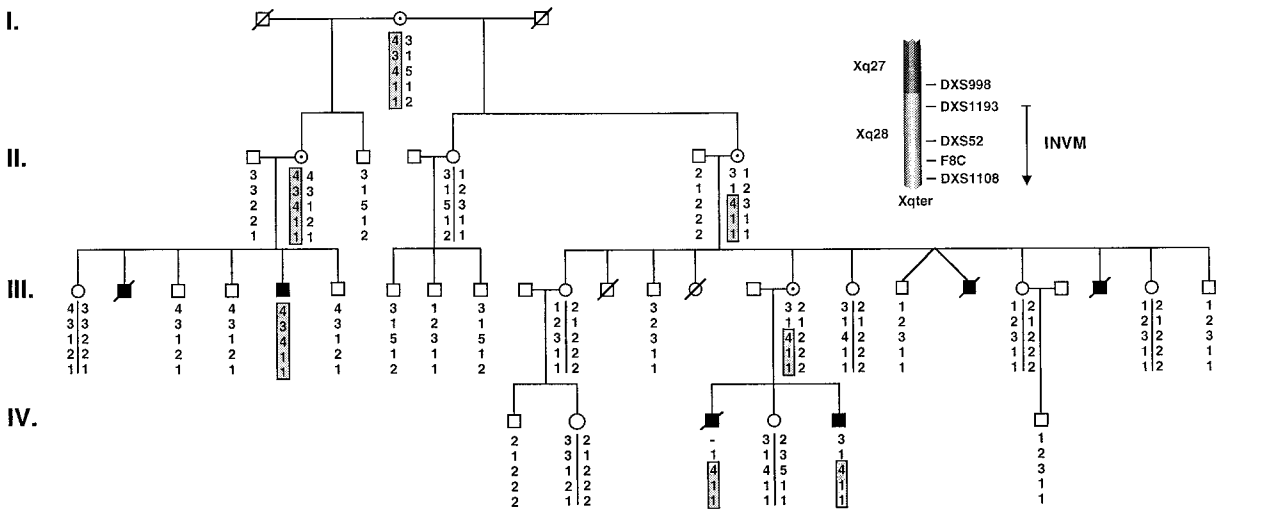

Figure 1 X-linked INVM pedigree. Haplotypes for STR markers shown in upper-right schematic are listed in order below each individual. The INVM-linked haplotype is boxed.

Linkage Analysis

Blood samples were acquired after patients granted informed consent, and DNA was extracted by standard methods (Sambrook et al. 1989). DNA extraction from paraffin-embedded tissues was performed using the Ex-Wax DNA extraction kit (Oncor). Multiplex amplification, gel electrophoresis, and genotyping of X-chromosome short-tandem-repeat (STR) markers was performed as described by Bleyl et al. (1995). Two-point LOD scores were calculated using MLINK program of the LINKAGE package, version 5.1 (Lathrop et al. 1985), on the assumption of an X-linked recessive model, a gene frequency of .001, and a penetrance of 0.95.

Amplification of G4.5 Exons

Primers were synthesized using the *G4.5* gene sequence in the European Molecular Biology Laboratory database (X92763 and X92764) to amplify each of the 11 exons including ⩾10 bp of flanking intron (not shown). Exon 8 was amplified using primers Ex 8A (5′-GCTCA GGGCC CAGCT TATGC TAACA T-3′) and Ex 8B (5′-GGCAG ACAGC CGAAG GTCAG CAGTC-3′), resulting in a 152-bp product. PCR reactions contained 150 ng of genomic DNA, 10 pg of each oligo, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris pH 8.4, 40 mM $NaCl_2$, 0.25 mM spermidine (Sigma), and .625 U *Taq* DNA polymerase (Perkin-Elmer) in a final volume of 25 μl. "Hot" reactions also contained 0.002 mCi α^{32}P-dCTP (DuPont NEN). Reactions were performed in the GeneAmp PCR System 9600 (Perkin-Elmer) with the following cycling conditions for exon 8: 5 min at 95°C; 5 cycles of 10 s at 95°C, 10 s at 65°C and 20 s at 72°C; and 30 cycles at 10 s at 95°C, 10 s at 60°C, and 20 s at 72°C.

SSCP Analysis

SSCP analysis was performed as follows. Radiolabeled PCR products were diluted 1:3 in formamide loading buffer, denatured for 5 min at 95°C, and electrophoresed in 0.6× tris-borate EDTA (TBE) buffer at 4 W overnight in either 1.0× Mutation Detection Enhancement (MDE) polyacrylamide gel (FMC BioProducts) with 5% glycerol at room temperature or 1.0× MDE gel at 4°C. Gels were dried and exposed to film without intensification for 12–36 h. Mutant and control bands from exon 8 were excised, and the DNA was eluted in water for 1 h at 37°C for reamplification and sequencing. The exon 8 variant was confirmed using nonradiolabeled PCR amplification and "cold SSCP" separation on the Thermoflow system (Novex). PCR products were electrophoresed in a 20% polyacrylamide TBE gel at 15°C for 3.0 h in 1.25× TBE. The gel was stained with ethidium bromide for 30 min, and images were captured using the ImageStore 7500 gel documentation system (Ultraviolet Products).

Sequence Analysis

Excised SSCP bands (two mutant and two control) were reamplified and size fractionated on a 2% Seakem agarose (FMC Bioproducts) 1× tris-acetate EDTA gel and purified using the GeneClean spin system (BIO 101). Purified products were sequenced using the ABI PRISM dye terminator cycle sequencing protocol and were analyzed on an ABI 373 automated sequencer (Perkin-Elmer). Sequence data was aligned using the Sequencher 3.0 software (Gene Codes). BLAST analysis (Altschul et al. 1990) was performed using the NCBI Entrez Browser (http://www3.ncbi.nlm.nih. gov/Entrez/). Protein alignment was performed using the ALIGN portion of the FASTA 2.0 program package (Pearson 1990) through

the BCM search launcher (http://kiwi.imgen.bcm.tmc.edu:8088/).

Mutagenically Separated (MS) PCR

MS-PCR (Rust et al. 1993) genotyping was performed using the following primers: Ex 8Mut (5′-GCCCA GCTTA TGCTA ACATT AGTAC CTCCC CCCTG GGCAG GATTC A-3′); Ex 8WT (5′-TCTAC CTCCC CCCTG GGCAG GAAAC G-3′); and Ex 8B (5′-GGCAG ACAGC CGAAG GTCAG CAGTC-3′). Engineered mismatches are underlined. A 20-bp difference in the lengths of primers Ex 8Mut and Ex 8WT results in MS-PCR products of 145 bp and 125 bp for mutant and wild-type genotypes, respectively. Reactions contained 12.8 pg each of oligos Ex 8WT and Ex 8B and 6.4 pg of oligo Ex 8Mut. Conditions were otherwise identical to those described above for exon amplification. MS-PCR products were run in 5% Nuseive 3:1 agarose for 2 h and were imaged as described.

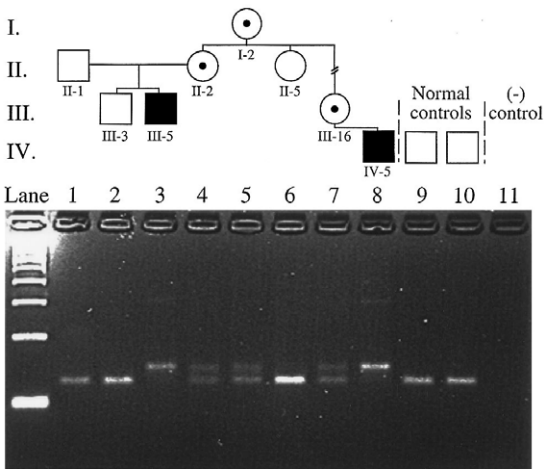

Figure 2 MS-PCR genotyping of the G4.5 mutation in the INVM family. A 100-bp ladder is shown at the left. The long primer (Ex 8Mut) anneals to the mutant allele and results in a 145-bp product (*lanes 3-5* and *7-8*). The short primer (Ex 8WT) anneals to the wild-type allele and results in a 125-bp product (lanes *1, 2, 4–7, 9,* and *10*).

Results

Linkage Analysis

The apparent X-linked recessive inheritance of INVM in this family was verified by linkage analysis. Initial haplotyping for 12 STR markers spread across the X chromosome at 15–20-cM intervals indicated cosegregation of the INVM phenotype with the Xq28 region (data not shown). The family was then genotyped for five STR markers mapping to the Xq28 region (*DXS998, DXS1193, DXS52, F8C,* and *DXS1108*) (Willard et al. 1994; Palmieri et al. 1994). Haplotype analysis for these five markers (fig. 1) showed complete segregation of INVM with the three most distal markers (*DXS52, F8C,* and *DXS1108*). Recombinational events were seen between INVM and markers *DXS998* and *DXS1193,* indicating that the defect in INVM lies distal to *DXS1193* in a region corresponding to ~6.8 Mb of DNA (Palmieri et al. 1994). The maximum LOD score was 3.64 at $\theta = 0$ from *DXS52*.

Mutation Analysis

Linkage localization to the Xq28 region and phenotypic overlap with BTHS suggested that INVM in this family might be caused by mutations in the *G4.5* gene. PCR primers were designed to amplify all 11 exons of *G4.5* from genomic DNA of INVM patients and controls and were used to screen for mutations using SSCP analysis. Amplification products from exon 8 demonstrated a mobility shift in INVM patients versus controls. Sequence analysis revealed a G→A transition mutation at nt 6 of exon 8 that results in a glycine-to-arginine change at residue 197 of the *G4.5* gene product. MS-PCR was used to verify that the mutation segregated with INVM through the family (fig. 2) and that it is not present in 300 unrelated females (not shown).

Blast Search and Alignment

While the function of the *G4.5* gene products, known as *tafazzins,* is unknown, a homologous *C. elegans* protein called zk809.2 (Wilson et al. 1994)) was found in a BLAST search (Altschul et al. 1990) using the full-length tafazzin protein. Bione et al (1996) noted two regions of the *G4.5* gene product that might be functionally significant. Alignment of tafazzin and zk809.2 (fig. 3) shows that the first region, a 30-residue hydrophobic N terminus, is mostly missing. The second region is a 71-residue hydrophilic domain coded for by exons 5–7. Residues corresponding precisely to exon 5, which is frequently spliced out in humans, are missing from zk809.2. Strong conservation is seen (65% identity) across the remaining hydrophilic domain, coded for by exons 6 and 7 and the first part of exon 8. Of this conserved central domain, only the portion coded for in the first part of exon 8 is common to all tafazzins. It is in this invariable region where the Gly 197 Arg mutation occurs (fig. 3), further supporting the hypothesis that it is responsible for INVM in this family.

Discussion

Two transcription start sites and alternative splicing of the *G4.5* mRNA results in at least five different transcripts, some with tissue-specific patterns of expression (Bione et al. 1996). All BTHS mutations reported so far have resulted in inactivation of several but not all tafazzin proteins (Bione et al. 1996), either by introducing

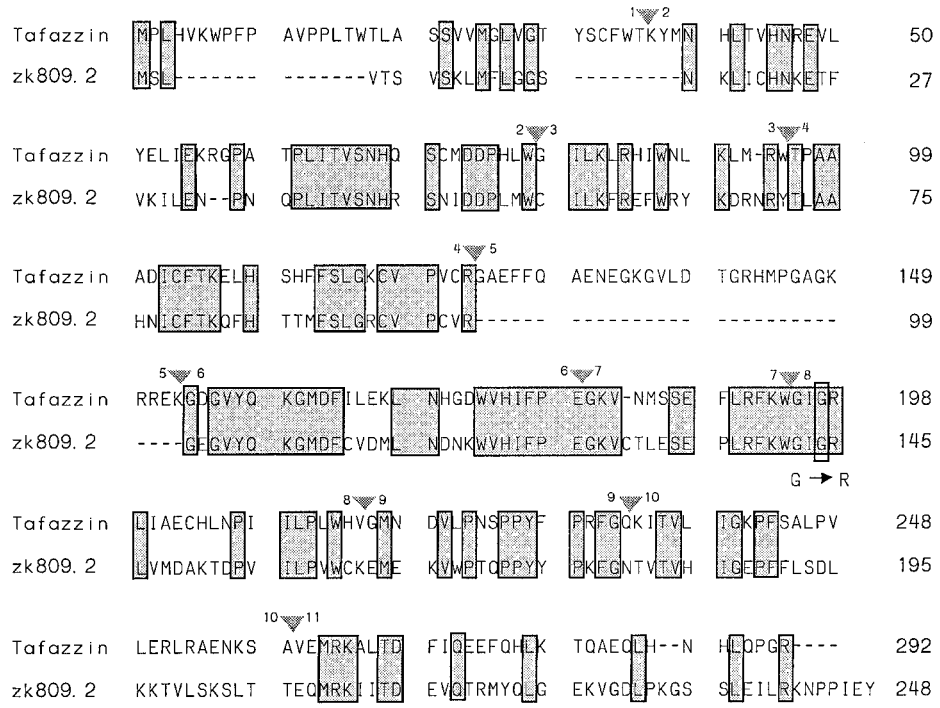

Figure 3 Alignment of the predicted full-length tafazzin protein and the *C. elegans* protein zk809.2. Identical residues are shaded. Arrowheads and numbers indicate *G4.5* exon boundaries. The exon 8, Gly 197→Arg 197, mutation is labeled and boxed.

premature stop codons or by abolishing splice sites. We report a nonconservative change in a domain common to all tafazzin splice forms that results in a disorder with severe and somewhat specific effects on the heart.

As yet, no function has been established for tafazzins. Bione et al. (1996) noted that a central hydrophilic domain of the G4.5 gene product might be functionally significant, and we have found this domain to be highly conserved in a putative *C. elegans* homologue. Thus, the discovery of a nonconservative missense mutation in this region, in an invariant part of the putative protein, could be important in light of the severe cardiac phenotype it produces in this family. One potential mechanism for this cardiac-specific effect could be expression of heart-specific *G4.5* splice forms that are particularly sensitive to this mutation.

In conclusion, we report a novel missense mutation in the *G4.5* gene in a family with severe infantile X-linked INVM without the usual findings of BTHS. Considerable variability is seen in the clinical and pathological findings in our report and in families with BTHS. Gedeon et al. (1995) reported a family with a fatal infantile cardiomyopathy without the usual characteristics of BTHS that map to Xq28. The consistent finding of INVM in association with this *G4.5* mutation underscores its possible utility in the diagnosis of BTHS and perhaps other allelic X-linked cardiomyopathies. Finally, the Gly 197 Arg mutation gives further structure-function information about tafazzin proteins. While this mutation has a consistent and severe effect on the neonatal myocardium, effects on skeletal muscle and leukocytes seem attenuated, even in the surviving transplant recipient. This suggests that the mutation effects a domain that is particularly important for cardiac muscle function in the context of tissue specific expression of various *G4.5* splice forms. It is hoped that future studies of tafazzin expression and function will explain this specific effect.

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