Novel mutations and genotype–phenotype relationships in 107 families with Fukuyama-type congenital muscular dystrophy (FCMD)

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Fukuyama-type congenital muscular dystrophy (FCMD), one of the most common autosomal recessive disorders in the Japanese population, is characterized by congenital muscular dystrophy in combination with cortical dysgenesis (micropolygyria). Recently, we identified, on chromosome 9q31, the gene responsible for FCMD, which encodes a novel 461 amino acid protein which we have termed fukutin. Most FCMD-bearing chromosomes examined to date (87%) have been derived from a single ancestral founder, whose mutation consisted of a 3 kb retrotransposal insertion in the 3′ non-coding region of the fukutin gene. FCMD is the first human disease known to be caused primarily by an ancient retrotranspositional integration. We undertook a systematic analysis of the FCMD gene in 107 unrelated patients, and identified four novel non-founder mutations in five of them: one missense, one nonsense, one L1 insertion and a 1 bp insertion. The frequency of severe phenotypes, including Walker–Walberg syndrome-like manifestations such as hydrocephalus and microphthalmia, was significantly higher among probands who were compound heterozygotes carrying a point mutation on one allele and the founder mutation on the other, than it was among probands who were homozygous for the 3 kb retrotransposon. Remarkably, we detected no FCMD patients with non-founder (point) mutations on both alleles of the gene, and suggest that such cases might be embryonic-lethal. This could explain why few FCMD cases are reported in non-Japanese populations. Our results provided strong evidence that loss of function of fukutin is the major cause of FCMD, and appeared to shed some light on the mechanism responsible for the broad clinical spectrum seen in this disease.

INTRODUCTION

Fukuyama-type congenital muscular dystrophy (FCMD; MIM 253800), the second most common muscular dystrophy in Japan, is an autosomal-recessive disorder characterized by brain malformation, principally cerebral and cerebellar cortical dysplasia, and by primary dystrophic changes in skeletal muscle (1–4). It is recognized almost exclusively in Japanese infants. Clinically, a patient’s peak motor function usually consists only of unassisted sitting or sliding on the buttocks. Intellectual, cognitive and communicative functions are, without exception, moderately delayed; the most common brain anomalies are micropolygyria, pachygyria and agyria. Ophthalmological findings such as peripheral abnormalities of the retina or abnormal eye movements are often observed in FCMD patients (3).

Recently, we reported the identification of the gene responsible for FCMD, located on chromosome 9q31 (5). After localization of the FCMD gene to a region of <100 kb that contained D9S2107 (6–8), we demonstrated that a founder haplotype (138–192–147–183, in terms of sizes of PCR products of markers lying in an ~200 kb critical region of chromosome 9q31; i.e. D9S2105–D9S2170–D9S2171–D9S2107) accounted for >80% of FCMD chromosomes. This haplotype no longer occurred in normal chromosomes, indicating that most FCMD-bearing chromosomes were derived from a single ancestor (9). A 3 kb retrotranspositional insertion of novel tandemly-repeated sequences was present within the candidate interval in all FCMD chromosomes that carried the founder haplotype. This insertion, located in the 3′-untranslated region of a novel gene, caused a significant reduction of the corresponding mRNA. Two independent point mutations found among FCMD patients in our original panel of 81 families (nonsense and frameshift in exons 3 and 4, respectively) confirmed that this gene is responsible for FCMD (5). The cDNA possesses an open reading frame of 1383 bp encoding a predicted 461 amino acid protein, ‘fukutin’, which bears no

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significant similarity to previously described proteins. The gene spans >100 kb of genomic DNA and is composed of 10 exons. Identification of this gene and its protein product has permitted direct genetic diagnosis of FCMD. FCMD is the first human disease known to be caused by an ancient retrotransposon integration.

Walker–Warburg syndrome (WWS) and muscle–eye–brain disease (MEB) are well-known congenital muscular dystrophies (CMDs) that also involve central nervous system abnormalities and ocular malformations (10–13). MEB is found mainly in Finland, and WWS is prevalent in other Caucasian populations. Both syndromes are characterized by brain malformations (type II lissencephaly with progressive hydrocephalus and agyria) and ocular abnormalities (severe myopia, corneal opacities, retinal dysplasia and microphthalmia) that are generally more severe than in FCMD. The gene underlying MEB has been mapped to chromosome 1p32–34 (14), but the chromosomal location of the WWS gene is still unknown; WWS is likely to be a heterogeneous disorder. Although the clinical features of FCMD are well characterized, recent analyses have demonstrated a wide clinical spectrum, from mild to severely affected cases: a few FCMD patients are able to walk at some point, while some never achieve the capacity to hold their heads completely upright; sometimes it is nearly impossible to differentiate severe FCMD cases from WWS on clinical grounds alone (15,16; K. Saito, M. Osawa, Z.P. Wang, K. Ikeya, Y. Fukuyama, E. Kondo-Iida, T. Toda, H. Ohashi, K. Kurosawa, S. Wakai and K. Kaneko, in preparation).

Here we describe the identification of several new mutations in the FCMD gene, which include a de novo alteration confirmed in this disease for the first time. We also report correlation of genotype with phenotype, in a panel of 107 unrelated patients. The results could help explain the broad clinical spectrum of FCMD.

**RESULTS**

**Screening for the 3 kb retrotransposon insertion**

The 81 FCMD families that provided DNA for previous studies (5–9) were re-analyzed, and an additional 26 families permitted analysis of their DNA samples. All available members of the 26 newly ascertained families were genotyped with polymorphic microsatellite markers D9S2105–D9S2170–D9S2171–D9S2107. When these results were combined with previous data, the 3 kb insertion was found to be present on 85.6% of FCMD disease chromosomes examined. Eighty probands (75%) were homozygous for the 3 kb insertion, 25 (23%) were heterozygous, and two did not show the 3 kb insertion on either allele (Table 1).

**Table 1.** Genotype–phenotype correlation in FCMD patients

<table>
<thead>
<tr>
<th>Haplotypes for the founder haplotype</th>
<th>Location</th>
<th>Nucleotide changes</th>
<th>Mutation effect</th>
<th>No. of patients</th>
<th>Phenotypea</th>
</tr>
</thead>
<tbody>
<tr>
<td>(138–192–147–183)</td>
<td>3′ untranslated region</td>
<td>3 kb retrotransposon insertion</td>
<td>Instability of mRNA</td>
<td>80</td>
<td>Mild/typical/severe/un-informed</td>
</tr>
<tr>
<td>Heterozygous for the founder haplotypeb</td>
<td>exon 3</td>
<td>C250T/Arg47Stop</td>
<td>Nonsense</td>
<td>7</td>
<td>Severe (severe hydrocephalus:1)</td>
</tr>
<tr>
<td>130–201–157–183</td>
<td>exon 4</td>
<td>298–299 del 2 bp/Met63Val/75Stop</td>
<td>Frameshift</td>
<td>1</td>
<td>Severe</td>
</tr>
<tr>
<td>138–195–143–191</td>
<td>exon 6</td>
<td>T859G/Cys250Gly</td>
<td>Missense</td>
<td>1</td>
<td>Severe</td>
</tr>
<tr>
<td>158–201–151–197</td>
<td>exon 7</td>
<td>T1017A/Cys302Stop</td>
<td>Nonsense</td>
<td>1</td>
<td>Typical</td>
</tr>
<tr>
<td>126–200–149–183</td>
<td>intron 7</td>
<td>1.2 kb L1 insertion/Aberrant splicing</td>
<td>Exon skipping</td>
<td>2</td>
<td>Severe</td>
</tr>
<tr>
<td>150–196–147–193</td>
<td>exon 9</td>
<td>1279insA/Phe390Ile/403Stop</td>
<td>Frameshift</td>
<td>1</td>
<td>Severe (microphthalmia)</td>
</tr>
<tr>
<td>None of the founder haplotype</td>
<td>142–196–147–193</td>
<td></td>
<td></td>
<td>1</td>
<td>Severe</td>
</tr>
<tr>
<td>144–203–157–183</td>
<td>144–201–149–183</td>
<td></td>
<td></td>
<td>1</td>
<td>Typical</td>
</tr>
<tr>
<td>144–201–153–183</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each allele is indicated by the size of its PCR product. Haplotypes were for D9S2105–D9S2170–D9S2171–D9S2107.

aP < 0.0001 (homozygous versus heterozygous).
bHaplotypes other than the founder’s.
cReported previously by Kobayashi et al. (5).
Table 2. PCR primers used to amplify exons of the FCMD gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(the first half) ex1F: 4: GCTGCTTCACGTTCATCTTC</td>
<td>ex1R: 4- CGGAAAAAGGCCGCGTCAAGT</td>
<td>282</td>
</tr>
<tr>
<td></td>
<td>(the latter half) ex1F: 1F: AGATATTAAAAGGAGGAATGCG</td>
<td>ex1R2: 2- TACCTGCTGAAGGCGCC</td>
<td>227</td>
</tr>
<tr>
<td>2</td>
<td>(the first half) ex2F: 2F: CTATGGTGGTATACAGTAC</td>
<td>0.1R: CTGTCAGCTTTAAAGGCC</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>(the latter half) ex2F: 1F: TGAACAGACTGAGATCTTTT</td>
<td>ex2R2: GCAAAGAAGGTGTGACGAGA</td>
<td>232</td>
</tr>
<tr>
<td>3</td>
<td>(the first half) ex3F: 3F: GTGGACTGGACATTTGAA</td>
<td>ex3R: CATAGAGCATTTGGAAGGCC</td>
<td>278</td>
</tr>
<tr>
<td></td>
<td>(the latter half) ex3F: 1.1F: CTTCTCAGTGGCTCTCTACAC</td>
<td>ex3R2: TTATACACTGCTAATACACGGA</td>
<td>214</td>
</tr>
<tr>
<td>4</td>
<td>(the first half) ex4F: 4F: GACCTGTTGGTGCTCTCTCTCTCTG</td>
<td>1R: TGAAGACATCTCCAAGGAC</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td>(the latter half) 2.01F: CTTCTCAGTGGCTCTCTACAC</td>
<td>ex5R: GACTGAACACACTCTACTTCAC</td>
<td>246</td>
</tr>
<tr>
<td>5</td>
<td>(the first half) ex5F: 6F: TGTTCTTCCACTGTTGAAGCC</td>
<td>ex6R: CCCTCACTTACCACCCCTC</td>
<td>232</td>
</tr>
<tr>
<td></td>
<td>(the latter half) ex5F: 7F: TCAGATTTAAATCTCAGTCCACA</td>
<td>ex7R: TCCCTAAGGCAGAGCC</td>
<td>271</td>
</tr>
<tr>
<td>6</td>
<td>(the first half) ex6F: 8F1: GTGCACTTAGATACTTTGCC</td>
<td>ex8R1: TTTCTGAGTGATGCTGGCTGC</td>
<td>293</td>
</tr>
<tr>
<td></td>
<td>(the latter half) ex6F: 8F2: TTTTAGGATGGTAACCAAATGC</td>
<td>ex8R2: ATTCTTTACATCTCAGTACG</td>
<td>226</td>
</tr>
<tr>
<td>7</td>
<td>(the first half) ex7F: 9 (the first half) ex7F: ex7F: GTGCACTTAGATACTTTGCC</td>
<td>ex9R1: TTTCTGAGTGATGCTGGCTGC</td>
<td>293</td>
</tr>
<tr>
<td></td>
<td>(the latter half) ex7F: ex7F: TTTTAGGATGGTAACCAAATGC</td>
<td>ex9R2: ATTCTTTACATCTCAGTACG</td>
<td>226</td>
</tr>
<tr>
<td>8</td>
<td>(the first half) ex8F1: GTGCACTTAGATAAGAATACCTT</td>
<td>2.02R: TTTCTGAGTGATGCTGGCTGC</td>
<td>263</td>
</tr>
<tr>
<td></td>
<td>(the latter half) ex8F1: GTGCACTTAGATAAGAATACCTT</td>
<td>ex9R: AACTTTATTTCTACGCTT</td>
<td>187</td>
</tr>
<tr>
<td>9</td>
<td>(the first half) ex9F2: TTTAGGATGGTAACCAAATGC</td>
<td>ex10R: TTTTCAAGGAGAAATCTTACCC</td>
<td>295</td>
</tr>
<tr>
<td></td>
<td>(the latter half) ex9F2: TTTAGGATGGTAACCAAATGC</td>
<td>ex10R: TTTTCAAGGAGAAATCTTACCC</td>
<td>295</td>
</tr>
</tbody>
</table>

10 exons of the FCMD gene and their flanking intronic sequences were amplified (Table 2); SSCP analysis was performed; and any shifted bands were sequenced. When no aberrant bands could be detected by SSCP, all exons were sequenced. By this strategy we identified four novel mutations including an L1 insertion, a missense mutation, a nonsense mutation and a 1 bp insertion.

Southern analysis detected the 1.2 kb L1 insertion in DNA from patients A.F. and A.M., each of whom carried the founder insertion on one allele and a 128–196–159–183 haplotype for D9S2105–D9S2170–D9S2171–D9S2107 on the other (Fig. 1). When the 1.6 kb EcoRI–XbaI fragment of cosmid cH1 was used as a probe, we found that PstI-digested DNAs from patient A.F. and her mother showed a specific new band. Sequence analysis revealed that the 3′ region of an L1 repetitive element had been inserted 24 bp before the intron 7–exon 8 boundary. The patient’s RNA was tested for the effects of the insertion by means of reverse transcriptase–polymerase chain reaction (RT–PCR) analysis, using primers 2.01F and 3R that amplify exons 5–10. Products of various sizes were obtained, and sequencing revealed that the main band lacked exons 7 and 8; another band lacked only exon 7; another lacked exons 7, 8 and 9. These results were suggestive of exon skipping.

SSCP and direct sequencing detected two point mutations (Fig. 2). A missense mutation (T859G, Cys250Gly) in exon 6 was identified in patient H.H., who carried the founder insertion from her father and the 158–201–151–197 haplotype from her mother. Other members of the family were tested by SSCP to confirm that this mutation co-segregated with FCMD; in addition, 50 normal individuals were tested as controls to exclude the possibility of polymorphism (data not shown). On the other hand, a nonsense mutation in patient S.H. (exon 7; T1017A, Cys302Stop) was identified only by sequencing all exons. S.H. carried the founder insertion from his mother and the 126–200–149–183 haplotype from his father. Sequencing of DNA from other family members verified that the non-founder mutant allele was derived from the father (data not shown).

In patient N.S. we identified a 1 bp insertion (1279insA) in exon 9 that caused a frame-shift and a premature stop at codon 403. This patient carried the founder insertion from her mother and the 150–196–147–193 haplotype from her father (Fig. 3), and she manifested microphthalmia. However, the 1 bp insertion could not be detected in the father by SSCP (data not shown) or by direct sequencing, and we concluded that a de novo mutation had occurred in this patient.

Altogether, mutations other than the 3 kb insertion were identified in 13 of the 27 probands examined (Table 1). Of the other 14, nine showed one unique haplotype (139–201–155–183) on the non-founder allele. However, we were unable to detect mutations in even one allele in either of the two patients without the 3 kb insertion on either chromosome.

Genotype–phenotype correlation
We investigated the phenotypes of probands whose specific mutations had been identified on both chromosomes (Table 1), after classifying the clinical phenotypes into the following three groups according to the patients’ maximum motor abilities: (i) ‘typical’ phenotype was assigned to patients who were able to sit unassisted or to slide on the buttocks (levels 2–4); (ii) patients assigned the ‘mild’ phenotype were able to stand or walk with or without support (levels 5–8); and (iii) patients with ‘severe’ phenotype could sit only with support or often had no head control (levels 0–1).

Among patients homozygous for the founder haplotype, 22 (27.5%) and 51 (64%) were mild and typical cases, respectively, two (2.5%) cases were severe, and clinical details were unknown in five cases (6%). On the other hand, among patients heterozygous for the founder haplotype, 12 (92%) showed severe phenotypes, while only one showed a typical phenotype. That is to say, clinical severity clearly tended to be greater among compound heterozygotes than among patients who were homozygous for the founder mutation (P < 0.0001). We note here that two of the compound-heterozygous patients showed WWS-like phenotypes such as hydrocephalus and microphthalmia. The two patients without the founder haplo-
type on either chromosome 9q showed typical and severe phenotypes, respectively, but we were unable to detect any mutations of the FCMD gene in either case.

RNA expression analysis
To compare the level of RNA expression with the phenotypes, we performed RT–PCR analysis of RNA isolated from lymphoblasts and/or skeletal muscle of several patients whose samples were available (Fig. 4). The level of amplified products was very low in patients who carried the founder insertion homozygously, and lower than normal in patients heterozygous for the insertion and another mutation. The patient who carried the founder insertion on neither chromosome (Table 1, the lowest line) showed the normal level of the product.

DISCUSSION
We analyzed a total of 107 unrelated FCMD patients. All but two of them carried at least one chromosome 9q bearing the FCMD founder haplotype, represented as 138–192–147–183 (i.e. sizes in bp of PCR products from markers D9S2105–D9S2170–D9S2171–D9S2107). This haplotype was present in 173 of the 202 (85.6%) FCMD chromosomes, and all of those chromosomes also had the founder’s 3 kb retrotransposon insertion in the FCMD gene. Ten haplotypes other than the founder’s were found in our panel of patients; two of those were found to be associated with point mutations described previously (a nonsense mutation and a 2 bp deletion). In the present study, our mutational analyses resulted in characterization of four novel mutations (L1 insertion, missense, nonsense and 1 bp insertion). All six of the non-founder mutations identified to date are likely to cause serious structural changes in the predicted protein product, fukutin. Together, the results provide strong evidence that loss of function of fukutin is the major cause of FCMD. The mutations associated with the remaining four haplotypes are still unknown.

The clinical severity of FCMD ranges from relatively mild to severe (15,16; K. Saito et al., in preparation). To investigate a correlation between genotype and phenotype, we set criteria for subdividing FCMD patients according to motor ability level (18). In patients who were homozygous for the 3 kb founder insertion, 91.5% showed mild or typical phenotypes.
However, 92% of heterozygous patients showed a severe phenotype. What is the explanation for this effect? We speculated that because the 3′-untranslated region of a gene affects the stability of its mRNA, the 3 kb sequence inserted in that portion of the FCMD gene may alter the secondary structure of FCMD mRNA and render it unstable (5). This notion is supported by our RT–PCR analysis that revealed low levels of the expected amplification product occurred in patients who were homozygous for the founder’s mutation (Fig. 4). In other words, chromosomes carrying the 3 kb insertion may merely produce a lower level of mature fukutin than normal and generate a relatively mild FCMD phenotype, as opposed to mutations that cause serious structural changes in fukutin. The antibodies against fukutin might well resolve this issue, though our attempts to obtain them have failed so far, despite repeated trials.

Although brain and ophthalmological involvement in FCMD is generally milder than in WWS and MEB (10–13), two compound heterozygous patients in the present study showed WWS-like phenotypes; one of them had progressive hydrocephalus that required a shunt operation and the other had microphthalmia, which is regarded as a hallmark of WWS or MEBD. These data suggest that the spectrum of clinical variability of FCMD is wider than recognized previously.

It is remarkable that we were unable to detect any mutations (even in one allele) in DNA from either of the two patients who did not carry the 3 kb insertion on either allele of the FCMD gene. These patients showed typical and severe FCMD phenotypes, respectively. We have three conjectures regarding this puzzle: (i) mutations elsewhere on both alleles; (ii) genetic heterogeneity in FCMD; or (iii) a diagnosis other than FCMD. RT–PCR analysis that revealed a normal expression in one of these two patients would rather support (ii) or (iii). We determined an allele frequency of 85.6% for the 3 kb insertion in our panel of Japanese patients. A Hardy–Weinberg distribution would indicate that the proportion of FCMD patients carrying mutations other than the 3 kb insertion on both chromosomes would be (14.4%)² ≈ 2.07%. It is difficult to judge whether we simply have not come across such patients yet. Nevertheless, considering the fact that point mutations have been seen to render the FCMD phenotype rather severe, inactivation of both alleles by point mutations might be embryonic-lethal, and that could explain why few FCMD cases are reported in non-Japanese populations.

As far as we know, the frameshift mutation (1279insA) reported here is the first instance of a de novo mutation being confirmed genetically in FCMD. If more de novo mutations are discovered in the future, we will need to be more careful in the matter of prenatal diagnosis.

Although FCMD, WWS and MEB are nosologically separate disease entities (19), all three entities are characterized by combinations of muscular, central nervous and ocular abnormalities. Why do the phenotypes of these diseases resemble each other or overlap? The MEB gene was recently localized to chromosome 1p32–34 (14), but the genomic location of the WWS gene is unknown. Moreover, according to our preliminary data no mutations of the FCMD gene have been detected in WWS patients. The clinical features may result from disturbances in basement-membrane components that are common to muscle, eye and brain, since several investigators have observed aberrations in extracellular matrix proteins in these diseases. For example, decreased immunostaining of the laminin α2 chain has been reported in muscle tissue from FCMD and MEB patients (20,21), whereas it is consistently preserved in WWS muscle (22). Laminin β2 chain protein is severely deficient in WWS (23), but mildly deficient in FCMD (20) and normal in MEB (21). A study of fukutin will open new avenues for understanding the pathophysiological mechanisms underlying these complex disorders consisting of cortical dysgenesis, congenital muscular dystrophy and eye malformation.
MATERIALS AND METHODS

Patients

The 81 FCMD families that provided DNA for previous studies (5–9) were re-analyzed, and an additional 26 families permitted analysis of their DNA samples. Fifteen affected individuals in 12 families were the offspring of consanguineous marriages, and in all cases FCMD had been diagnosed on the basis of standard clinical criteria (3). The probands from all 107 families were examined for specific mutations.

Haplotype analysis

All available members were genotyped with polymorphic microsatellite markers D9S2105–(FCMD)–D9S2170–D9S2171–D9S2107 as described previously (9). For all cases from consanguineous marriages, only a single FCMD chromosome was counted.

Analysis for the 3 kb retrotranspositional insertion and other genomic rearrangements

Genomic Southern blot analysis was used to detect the 3 kb insertion in DNA from patients who carried the founder haplotype. Genomic DNA was digested with PvuII. The blots were prehybridized overnight at 65°C in 10% SDS and 7% PEG containing 100 µg/ml sonicated salmon sperm DNA. Then the probe (fEco8-1, a 1.4 kb EcoRI fragment of cE6) (5), radiolabeled with [α-32P]dCTP using the Megaprime labeling kit (Amersham Pharmacia Biotech, Little Chalfont, UK), was hybridized with the membranes. The final washing stringency was 0.1× SSC, 0.1% SDS at 63–65°C.

To screen other genomic rearrangements, we hybridized each cosmid clone defining the FCMD contig on 9q31 (17) to Southern blots of genomic DNA from FCMD patients carrying non-founder haplotypes. Membranes were prehybridized using sonicated human placental DNA (200 µg/ml) in place of salmon sperm DNA. Cosmids were digested with NotI to separate DNA inserts. Each probe was prehybridized in the same solution at 65°C for 1 h (24).

SSCP analysis and sequencing

Primers to amplify each exon and surrounding intronic sequences (Table 2) were designed from genomic sequence of the FCMD gene (unpublished data). Each PCR was performed in a final volume of 50 µl containing 100 ng of genomic DNA, 50 pmol of each primer, 0.2 mM each dNTP and 1.0 U of EX Taq polymerase (Takara). Samples were incubated in a DNA thermocycler (GeneAmp 9600; Perkin Elmer, Rockville, MD) under the following conditions: 94°C for 30 s, 58 or 60°C for 1 min, followed by 30 cycles of 94°C for 30 s, 58 or 60°C for 1 min, and 72°C for 1 min. SSCP analysis was performed essentially as described elsewhere (25), using a 10% polyacrylamide gel with or without 5% glycerol. Each sample was electrophoresed for ~60 min in a ResolMax apparatus (Atto, Tokyo, Japan). The single-stranded DNA fragments separated in the gel were visualized by silver staining. PCR products exhibiting band shifts were directly sequenced using the PRISM ReadyReaction DyeDeoxy Terminator Cycle-sequencing kit with AmpliTaq FS (Perkin Elmer). Data were acquired on an ABI Model 377 DNA sequencer (Perkin Elmer).

RNA extraction and analysis

Total RNA was isolated from lymphoblasts and biopsied skeletal muscle by TRIzol Reagent (Gibco BRL, Rockville, MD). RNA was reverse-transcribed with oligo(dT) and SuperscriptII reverse transcriptase (Gibco BRL). Primers in the latter half of exon 5 (2.01F; Table 2) and exon 10 (3R: 5′-TGGTTC-CCACTTATGTTTGCAG-3′) were used to amplify the cDNA from patient A.F. Primers in the end of exon 9 (LAex9F; 5′-GAAGAAACTGTACATGTTGGAAATGGAGGC-3′) and exon 10 (GP1; TGAGTACTGGATCAGCGAGCAGTCTTGG-3′) and those for GAPDH (5′-CAAACATGTTTAC-GTTC-3′ and 5′-GCCAGTGGGACTCCACTCCAGAC-3′) were used for comparative expression analysis.

Statistical analysis

The statistical significance of phenotypical differences between genotype groups was calculated using Fisher’s exact test.

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