SHORT REPORT

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6q23

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J Med Genet 2004;**41**:273–277. doi: 10.1136/jmg.2003.014787

Background: Joubert syndrome (JS) is a recessively inherited disorder characterised by hypotonia at birth and developmental delay, followed by truncal ataxia and cognitive impairment, characteristic neuroimaging findings (cerebellar vermis hypoplasia, ''molar tooth sign'') and suggestive facial features. JS is clinically heterogeneous with some patients presenting with breathing abnormalities in the neonatal period, oculomotor apraxia, retinal dystrophy, retinal coloboma, ptosis, hexadactyly, and nephronophtisis or cystic dysplastic kidneys. JS is also genetically heterogeneous, with two known loci, on 9q34 (JBTS1) and 11p11-q12 (CORS2), representing only a fraction of cases.

Methods: A large consanguineous Joubert family (five affected) was analysed for linkage with a marker set covering the entire genome and 16 smaller families were subsequently tested for candidate loci.

Results: We report here the identification of a third locus in 6q23 (JBTS3) from the study of two consanguineous families. LOD score calculation, including the consanguinity loops, gave a maximum value of 4.1 and 2.3 at q=0 for the two families, respectively.

Conclusions: Linkage between the disease and the D6S1620–D6S1699 haplotype spanning a 13.1 cM interval is demonstrated. Genotype-phenotype studies indicate that, unlike CORS2, JBTS3 appears not to be associated with renal dysfunction.

Joubert syndrome (JS) (also known as Joubert-Boltshauser syndrome) is a recessively inherited disorder first described by Joubert *et al*[1] in a family of Canadian origin and further characterised by Boltshauser and Isler[2] in families of Swiss and German origin. Since then, many cases have been reported with various geographic origins. Consistent features of this condition are developmental delay, hypotonia, cerebellar ataxia, and suggestive facial features. Abnormalities on axial MRI are the neuroimaging hallmarks of JS, and include cerebellar vermis hypoplasia and abnormalities at the pontomesencephalic junction that lead to a characteristic ''molar tooth'' appearance.[3] This neuroradiological pattern results from an abnormally deep interpeduncular fossa and thick superior cerebellar peduncles orientated perpendicular to the brainstem. A wide clinical variability within the sibships and between families is observed with a marked variation in severity and the inconsistent presence of the following features: episodic apnea-hyperpnea which disappears with increasing age, abnormal eye movements (jerky eye movements, nystagmus, delay in saccadic initiation), rhythmic protrusion of the tongue, occipital meningoencephalocele, polydactyly, nephronophthisis or cystic dysplasia of the kidney, chorioretinal coloboma, and retinal dysplasia. There is a significant clinical overlap between JS and other cerebello-oculo-renal syndromes (CORS) such as Arima, Senior-Loken, and COACH syndromes, and furthermolecularinvestigationsshouldhelptoachieveafinal classification of these conditions.[4] This wide clinical heterogeneity hampers the gathering together of families into genetically homogeneous groups needed for linkage studies. Indeed, the first linkage studies failed to identify a specific chromosomal locus for JS providing evidence that JS and related syndromes are genetically heterogeneous.[5 6] In addition, candidate gene approaches have failed so far to detect mutations in the *WNT1*, *EN1*, *EN2*, and *FGF8* genes of patients with JS.[6 7] Homozygosity mapping can be used as an alternative method to bypass the problem of genetic heterogeneity by studying large consanguineous families with autosomal recessive disorder.[8] Using this strategy, Saar *et al* identified a JS locus on chromosome 9q34.3 (JBTS1) in two families of Omani origin.[9] However most of the families studied by Blair *et al*[6] and by us (unpublished results) are excluded for linkage to this locus. Recently, Valente *et al*[10] and Keeler *et al*[11] identified a second JS locus associated with nephronophtisis on chromosome 11p11-q12 (cerebello-oculo-renal syndrome 2, CORS2). We report here the identification of a third locus on 6q23 (JBTS3) from the study of two consanguineous families, including the second reported JS family.[2] On the other hand, linkage to 6q23 was excluded for nine out of 15 additional families diagnosed with JS, the remaining families being too small to allow conclusions to be drawn.

METHODS

Subjects

In family 1, of Turkish origin, five children born from consanguineous parents have Joubert syndrome (fig 1, table 1). All affected individuals have marked cognitive impairment, requiring special education. Expressive speech was markedly affected, and two children remained without verbal communication. Motor milestones were extremely slow and the ability to walk unaided was never reached before the age of 7 years, reflecting hypotonia and truncal ataxia. The patients had significantly reduced vision with retinal dystrophy at later ages, but no nystagmus and no retinal coloboma. Electroretinography was not performed. There was no evidence of renal dysfunction and all patients had normal serum creatinin levels at their present ages, ranging from 17 to 28 years. Neuroimaging confirmed cerebellar vermis hypoplasia and molar tooth sign. All patients, the parents, and three out of five healthy siblings were available for study.

Family 2, of Swiss origin, is one of the original families described by Boltshauser and Isler.[2] The parents are third

Abbreviations: CORS, cerebello-oculo-renal syndrome; JS, Joubert syndrome

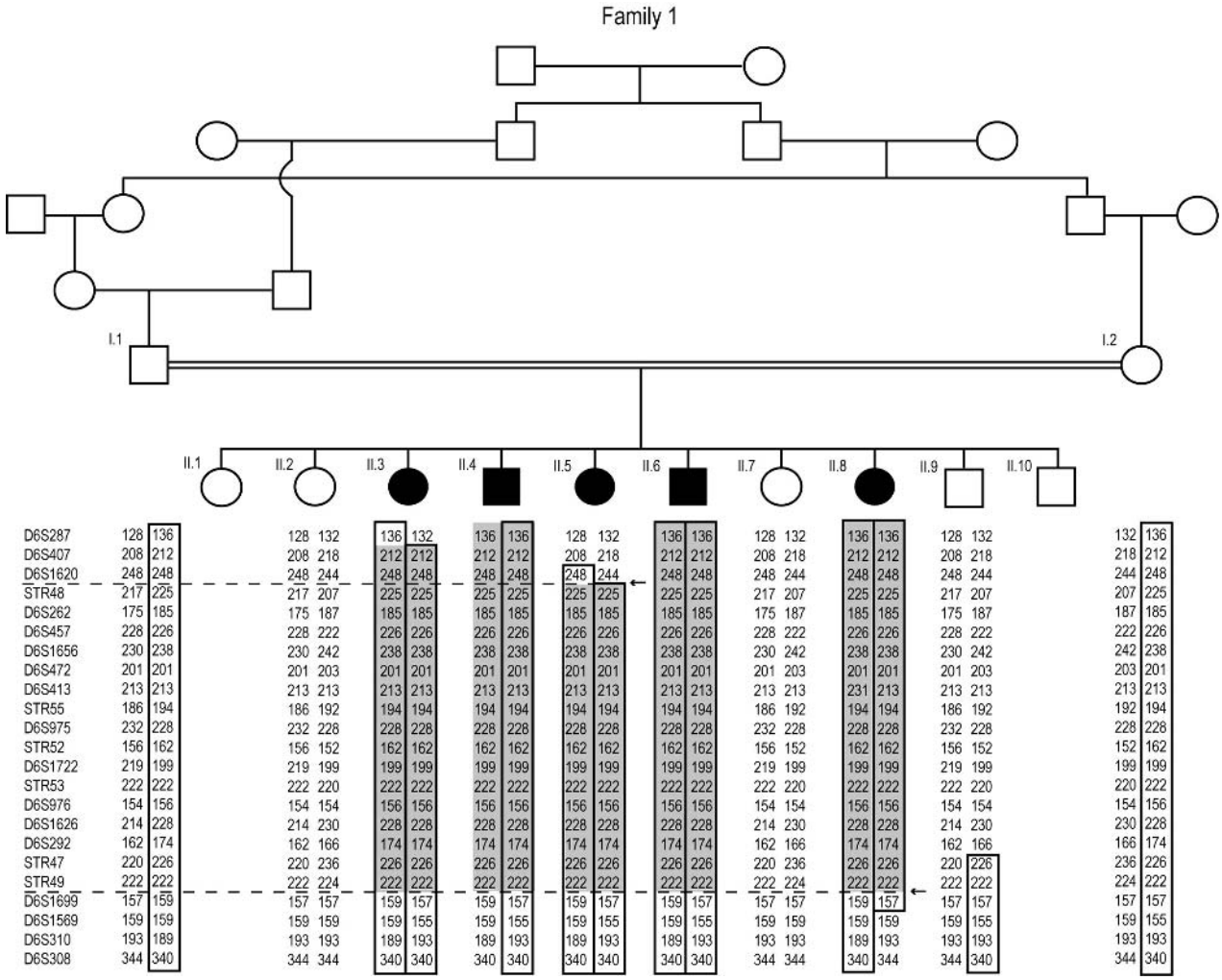

Figure 1 Genotyping results of family 1 for the chromosome 6q23 region. DNA samples of healthy siblings II.1 and II.2 were not available for study. Markers are indicated on the left and are organised from top to bottom in centromeric to telomeric order. The marker order is based on the UCSC Assembly of Human Genome browser (April 2003 update, http://genome.ucsc.edu). Parental haplotypes linked with the disease are boxed. The region of homozygosity by descent is highlighted in grey. Dotted lines indicate the centromeric and telomeric boundaries of the JBTS3 locus defined, respectively, by a maternal recombination in patient II.5 and heterozygosity in patients due to an ancestral recombination (arrows).

Table 1 Clinical features of the JBTS3 patients

Clinical features	Family 1					Family 2	
Origin	Turkey					Switzerland	
Patient	1	2	3	4	5*	1	2
Sex	F	M	F	M	F	F	F
Present age (years)	28	27	23	21	17	†	23
Early hypotonia	+	+	+	+	+	+	+
Age at independent walking (years)	10	10	9	WCB	WCB	†	7
Cognitive impairment	++	++	++	++++	++++	NA	+
Neonatal breathing problems	NA	NA	NA	NA	NA	+	+
Cerebellar ataxia	±	−	+	−	−	+	+
Nystagmus	−	−	+	−	−	+	+
Optic atrophy	−	−	−	−	NA	−	−
Retinal dystrophy	−	+	−	−	NA	NA	+
Reduced vision	+	+	+	+	+	NA	+
Kyphoscoliosis	+	+	−	−	−	−	−
Retarded skeletal growth	NA	NA	+	+	+	−	−
Renal dysfunction	−	−	−	−	−	−‡	−
MTS	+	+	+	+	+	+§	+
Cerebellar vermis hypoplasia	+	+	+	+	+	+	+

F, female; M, male; NA, not available; MTS, molar tooth sign; WCB, wheelchair bound.
*Also presented with spasticity, microcephaly and seizures; †deceased at 23 months; ‡normal kidney histology at autopsy; §abnormal ponto-mesencephalic junction and upper cerebellar pedoncules at autopsy.

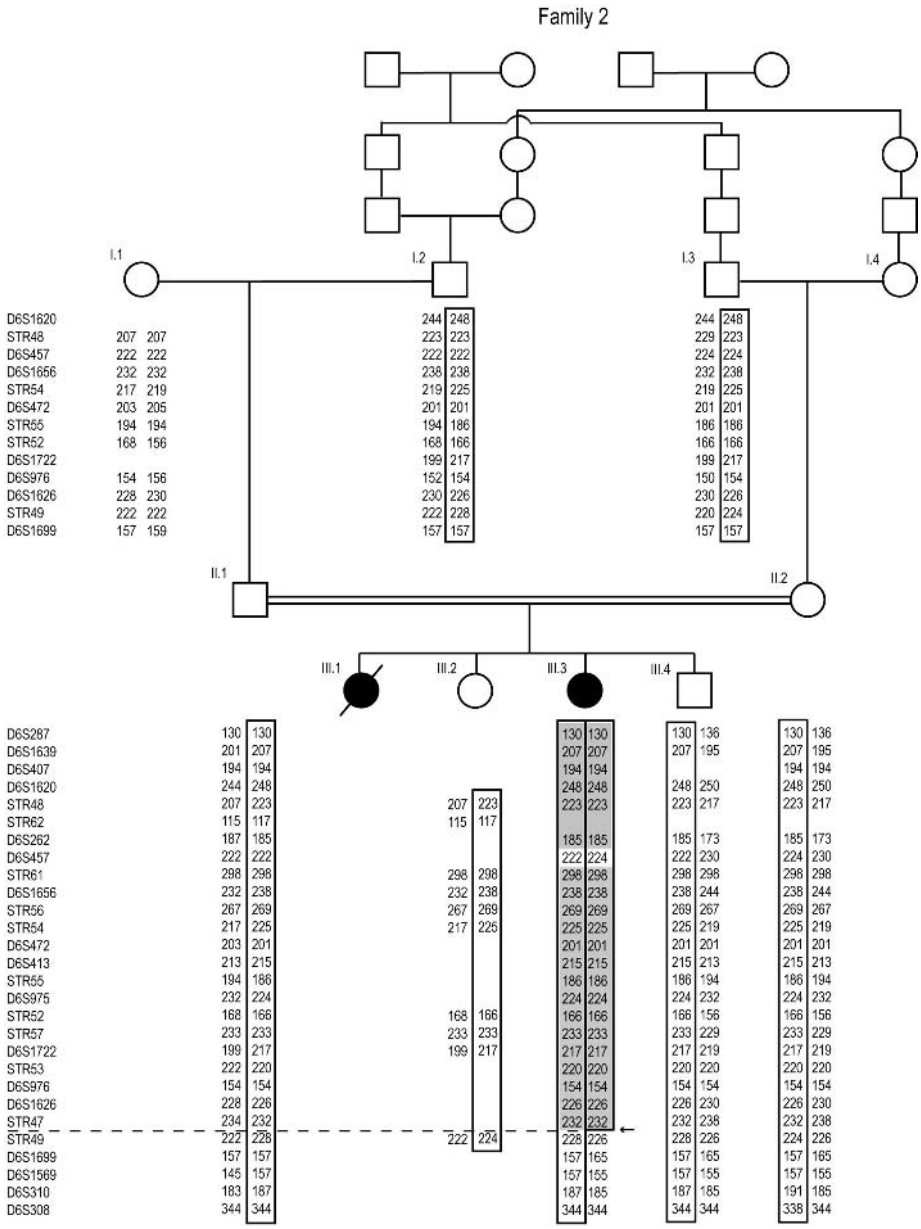

Figure 2 Genotyping results of family 2 for the chromosome 6q23 region. Markers and haplotypes are indicated as in figure 1. The patient is homozygous for 23 consecutive markers, with the exception of D6S457 which is assumed to represent an ancient allelic mutation, since both parents and transmitting grandparents carry different alleles at this locus. The patient is heterozygous for marker STR49 (arrow) and further telomeric markers, allowing reduction of the critical interval. The dotted line defines the telomeric boundary of this interval.

degree cousins and have two healthy children (fig 2). A second affected daughter was born subsequently to the initial report.[12 13] The index patient had poor developmental progress and died at 23 months of age, before DNA sampling was available. Her autopsy report[14] was the first detailed pathological description of JS. The surviving patient is now 23 years old and has a favourable, but clearly subnormal, cognitive development, with independence in her daily activities and fair reading and writing skills. She has normal ultrasonography of kidney and liver, normal serum creatinin levels, and no evidence of kidney involvement. She has pigmentary retinopathy, which has been non-progressive until now, with flat electroretinogram since the age of 10 (table 1).

Fifteen additional families (22 patients in total) were also included in the study. The selected families had at least one healthy child available for genetic study or had documented consanguinity. Fourteen patients have been previously described.[13 15 16] All patients conform to the following diagnostic criteria: hypotonia and developmental delay, followed by truncal ataxia and cognitive impairment, presence on MRI of the molar tooth sign and cerebellar vermis hypoplasia, and suggestive facial features (high-rounded eyebrows, ptosis, broad nasal bridge with mild epicanthus, anteverted nostrils, triangular shaped open mouth, and low-set ears as illustrated in Maria *et al*[17]). In addition, some patients presented with breathing abnormalities in the neonatal period, abnormal eye movements, or retinal dystrophy. Two patients, from two families, had renal failure before 12 years of age followed by renal transplantation, and an affected brother had reduced cortico-medullary differentiation on renal ultrasounds and high serum creatinin levels since the age of 10.

Genotyping

Blood samples were obtained with informed consent. Genomic DNA was extracted from the peripheral blood leukocytes by a standard phenol/chloroform method.

A whole-genome screen was initiated with family 1 using a microsatellite marker set developed and commercialised by PE Biosystems (ABI Linkage Mapping Set version 2, medium density set 10, MD-10). This set comprises 400 fluorescently labelled microsatellite markers selected from the Généthon human linkage map,[18] with an average spacing of 10 cM and an average heterozygosity of 75%. PCR-multiplex protocol and fragment analysis were performed as described.[19]

Additional CA/TG microsatellite markers from the Généthon human linkage map[18] and new polymorphic markers were amplified with a universal fluoresceinated primer as described.[19] To identify new polymorphic markers, we searched for $(CA)_n$ repeats in the corresponding sequence of the human genome and designed flanking PCR primers for repeats with more than 12 motif units. The primer sequences of the new polymorphic markers, derived from the BAC clones (http://genome.ucsc.edu), are given in table 2. All annealing temperatures for PCR amplification were set at 60°C. PCR products were resolved on ABI 377 or ABI 3100 DNA sequencers (PE Applied Biosystems) and analysed using ABI PRISM GeneScan Analysis Software.

Linkage analysis

Part of the linkage power of families 1 and 2 is due to the consanguinity loop(s) and linkage is supported when the patients are homozygous for a rare haplotype.[8] This information was included in a two-point LOD score calculation by considering the non-recombinant haplotype as a single locus.[8] The frequency of the homozygous haplotype was calculated as the product of the frequency of the individual alleles estimated from a reference white population. In order to eliminate biases due to possible linkage disequilibrium, only one marker was taken into account when two were less than 500 kb apart on the human genome sequence. Two-point LOD scores with consanguinity loops were calculated by using the MLINK program of the FASTLINK package.[20] We assumed a fully penetrant autosomal recessive mode of inheritance, and a gene frequency of 0.001 that certainly represents an upper limit for this rare condition.

RESULTS

A total of 390 markers of the ABI PRISM Linkage Mapping Set were tested with family 1. Given the close consanguinity of the parents and the close spacing of the markers, we selected the regions for which at least one marker was homozygous in all five affected individuals and heterozygous in the three healthy siblings. We identified only one such region, on chromosome 6q23, in which the consecutive markers D6S262 and D6S292 were homozygous in all patients. The study of a dense set of microsatellite markers from this region confirmed linkage to the 6q23 locus, since the five patients were homozygous for at least 16 consecutive markers and the healthy siblings were heterozygous for 14 of these markers (fig 1). LOD score calculation, including the consanguinity loop,[8 21] gave a value of 4.1 at a recombination fraction θ of 0, demonstrating linkage between the disease and the 6q23 haplotype. On the centromeric side, a maternal recombination in patient II.5 excluded marker D6S1620 from linkage. On the other side, marker D6S1699 and further telomeric markers were heterozygous in all patients, indicating the occurrence of an ancestral recombination. The recombinant markers define a 13.1 cM interval containing the JS gene.

The set of 6q23 microsatellite markers was tested in 16 smaller families, of which six had documented consanguinity. Linkage to 6q23 was excluded in nine families either because patients and a healthy sibling shared the same haplotypes or because homozygosity by descent was not present in consanguineous families. Five of these families were compatible with linkage to CORS2, and three to JBTS1, including two compatible with both (LOD scores in favour of linkage ranging from 0.25 to 1.1, not shown). Six families were compatible with linkage to 6q23, albeit this is most likely due to small family size in most cases (LOD scores in favour of linkage ranging from 0.125 to 0.725), since five of these families were equally linked to the CORS2 locus, and two to the JBTS1 locus (not shown). In all cases, sufficient markers were tested in order to have fully informative families. Finally, linkage to 6q23 was demonstrated for family 2. The affected patient was homozygous for 22 consecutive markers (with the exception of a probable allelic mutation at D6S457) and the two healthy siblings did not share the same haplotypes (fig 2). LOD score calculation, including the consanguinity loops, gave a value of 2.3, at a recombination fraction θ of 0, demonstrating linkage between the disease and the 6q23 locus in this family.[22] Marker STR49, which is 1.3 Mb centromeric to D6S1699, as well as more distal markers were heterozygous in the patient, allowing us to reduce the critical interval and to exclude seven genes located between STR49 and D6S1699 from being candidate JS genes. The final interval is 8.2 Mb in size and contains 45 known genes. None of them seemed to be a good candidate gene for Joubert syndrome.

Table 2 Primer sequences for PCR amplification of the new microsatellite markers

Marker	Primer sequence	BAC clones	Size (bp)
STR48	5′-aagtcggtctgctgtttcca-3′ 5′-tgttcctaagtcttctctccatcc-3′	AL358943	~210
STR55	5′-atcaccactgatcccacaga-3′ 5′-gattcaacttcttcccggttt-3′	AL513524	~190
STR52	5′-ccaaaaggccaagagaaggt-3′ 5′-ggttggggagagatcacagg-3′	AC068005	~160
STR53	5′-acttctagtctggttccctagagttt-3′ 5′-tggctaaagtgtgttggcttc-3′	AC023293	~220
STR47	5′-agttttctggccccctctat-3′ 5′-caccttcatgggtgtccttt-3′	AL023284	~230
STR49	5′-tgctacgattgacccaactct-3′ 5′-caaccagtgaaaagcaacaca-3′	AL357060	~220

Despite early descriptions of JS in 1969 and 1977,[1 2] and numerous subsequent reports since then, the search for the defective genes has remained elusive so far, mostly due to tremendous heterogeneity both at the clinical and genetic levels. Identification of a first JS locus on chromosome 9q34 (JBTS1),[9] confirmed this heterogeneity, since most JS families are not linked to this locus.[6 9] We report here the identification of another locus, on 6q23, based on homozygosity of patients in two consanguineous families. While the LOD score in favour of linkage in family 1 (4.1) is sufficient to demonstrate the existence of this locus, the LOD score for family 2 (2.3) is sufficient to confirm that it is linked to the same locus as family 1. Here again, the JS locus represents only a fraction of the JS families. Indeed, it is anticipated that in most of the six small families for which the disease segregates with 6q23 it does so by chance and only a few subjects will have mutations in the 6q23 locus.

Another locus has recently been identified on chromosome 11p11-q12, based on the study of a large Sicilian consanguineous family[10] and of three smaller consanguineous families.[11] In the Sicilian family, two patients also developed

renal failure at age 17 and 15, respectively, while the two youngest patients, aged 12 and 8 years, had only altered urinary concentration test and no polyuria/polydipsia. Three patients had increased kidney echogenicity but no renal cysts. None had retinal abnormalities. Patients from the three smaller families were clinically heterogeneous and presented with either kidney cysts, suggestive nephrocalcinosis, posterior encephalocele, hydrocephalus, corpus callosum and occipital lobe dysplasia, coloboma of the retina, or retinal dystrophy.[11] The age at last examination of patients without renal problems was not indicated. The 11p11-q12 locus was named CORS2 for cerebello-oculo-renal syndrome, with JBTS1 corresponding to CORS1. CORS2 patients contrast with the six patients of families 1 and 2, who have not developed renal failure past the age of 17. Moreover, reduced vision with retinal dystrophy was found in both families, while retinal presentation of CORS2 patients was more heterogeneous. The 6q23 locus was therefore named JBTS3, which may correspond to CORS3 if future linked families reveal renal heterogeneity. The knowledge of three JS loci opens up the possibility of investigating genotype/phenotype correlations that should help clarify nosologic delineation among CORS and facilitate the search for defective genes from homogeneous patient groups.

ACKNOWLEDGEMENTS

We wish to thank Drs D Chaigne, J Messer, and B Rousselle for referring JS samples and clinical information, Dr E M Valente for sharing data prior to publication, and M Moreira and J-L Mandel for helpful discussion and support. We also wish to thank L Reutenauer, E Troesch, F Ruffenach, I Colas, and S Vicaire for excellent technical help.

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Genetic studies were supported by funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Hôpitaux Universitaires de Strasbourg (PHRC régional), and the GIS-Maladies Rares. CL-T was supported by the Fondation pour la Recherche Médicale and by the Association Française contre les Myopathies.

Conflict of interest: none declared.

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Revised version received 1 January 2004
Accepted for publication 6 January 2004

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