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Mutations in the *MTM1* gene implicated in X-linked myotubular myopathy

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X-linked recessive myotubular myopathy (XLMTM) is characterized by severe hypotonia and generalized muscle weakness, with impaired maturation of muscle fibres. The gene responsible, *MTM1*, was identified recently by positional cloning, and encodes a protein (myotubularin) with a tyrosine phosphatase domain (PTP). Myotubularin is highly conserved through evolution and defines a new family of putative tyrosine phosphatases in man. We report the identification of *MTM1* mutations in 55 of 85 independent patients screened by single-strand conformation polymorphism for all the coding sequence. Large deletions were observed in only three patients. Five point mutations were found in multiple unrelated patients, accounting for 27% of the observed mutations. The possibility of detecting mutations and determining carrier status in a disease with a high proportion of sporadic cases is of importance for genetic counselling. More than half of XLMTM mutations are expected to inactivate the putative enzymatic activity of myotubularin, either by truncation or by missense mutations affecting the predicted PTP domain. Additional mutations are missenses clustered in two regions of the protein. Most of these affect amino acids conserved in the homologous yeast and *Caenorhabditis elegans* proteins, thus indicating the presence of other functional domains.

INTRODUCTION

X-linked recessive myotubular myopathy (XLMTM; OMIM31040) is a congenital muscle disorder characterized by severe hypotonia and generalized muscle weakness. Spontaneous movements are weak or absent. Prenatal onset can often be indicated by polyhydramnios and decreased fetal movements. Miscarriages and stillbirths are frequently observed among obligate carriers. No significant clinical symptoms or signs of myopathy occur in female carriers. The characteristic muscle histopathology consists of small rounded muscle cells with centrally located nuclei surrounded by a halo devoid of contractile elements. These resemble fetal myotubes, and it has been suggested that the disorder results from an arrest in the normal development of muscle fibres (1). XLMTM is usually lethal in males because of respiratory failure. Most patients die within the first months of life. A few males have survived several years with a spontaneous improvement of the respiratory function after birth. The reason for this clinical improvement is unknown. Since the first report of myotubular myopathy (2), ~100 cases have been documented (3–5). Autosomal centronuclear/myotubular myopathies (CNM) are related disorders with similar histopathological findings, but the age of onset is later with a milder clinical course (4).

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Table 1. SSCP primers and PCR conditions

Exon	Exon limits	Proximal primer 5′→3′	Distal primer 5′→3′	PCR fragments size (nt)	Annealing (°C)	Final mM $MgCl_2$	Glycerol 10%
1	1–44	GGCGCCCAGTCCAACTTC	CCAAGGAGTCCCAACTTC	455	52	1.5	–
2	45–117	AGAACCTGTAAAGTAGTACC	TAGGCCAGCCCAAAATGAC	272	52	1.5	–
3	118–190	AACAGTGTGTAAATGTAACGTC	AGACTTCTCCTCAAGTTATGC	172	55	1.5	+
4	191–285	AGTGCCATTTGTTGTGTATC	TGACCCACAGTCAATCTTGC	255	55	1.5	+
5	286–396	TAATTATACTGACAGAAATACTG	TCCACATTAATTGTCTACTATC	234	55	2	–
6	397–498	TTGAAGACTGAACTGTCATAC	AACCTTCCCACGCTGAGG	175	55	1.5	–
7	499–582	AGATGTACTATAATAGTAGAC	AGGTTCATCACATACCAGAC	188	52	1.5	–
8	583–732	TCCAGAGATGAGGTCAAGC	GGTGCTCTTCAAGAGAAACG	239	55	1.5	+
9	733–921	TTGATAGCTTAAACTTTCTGAC	TCCAGCACATCATTAAGTCC	247	55	1.5	–
10	922–1107	CTGATTGTTTGTATTTCATG	TATATACACAAATATTTTCAC	251	52	2	+
11	1108–1314	AACTCCCTACTGACTCACG	AATCCTGAATGGTAGTGATCT	292	55	1.5	+
12	1315–1407	ATGCTTTCTCAGTTTTGTACC	TAAACAATGAGTTGAATGTA	195	52	2	+
13	1408–1521	TTATAAAGTTTCAGTCCCAG	TTTGGCAAGCCTAATGTATC	211	52	2	+
14	1522–1698	TTTTACTTAGGCTCTCCAC	CTATCTTTTAACAGTGCTAC	248	52	2	+
15	1699–3411	AGTGTAACTCAAGTCTCTGG	AAATGAAAGTCAGCTATCGC	256	52	1.5	+

Exons a, b, c, d and e from Laporte *et al*. (13) are numbered respectively exon 8, 9, 11, 14 and 15. The stop codon is 1863–1865.

The locus responsible for the disease (*MTM1*) was mapped to Xq28 by linkage analysis (6,7). By combining linkage analysis with the study of patients carrying deletions, we restricted the candidate region to 280 kb (8–12), and the *MTM1* gene was isolated recently by a positional cloning strategy (13). The *MTM1* gene is ubiquitously expressed and shows a muscle-specific alternative transcript due to the use of a different polyadenylation signal. It encodes a protein which contains the consensus sequence for the active site of tyrosine phosphatases, a wide class of proteins involved in signal transduction, control of cell growth and differentiation. The *MTM1* gene product (myotubularin) is highly conserved in *Saccharomyces cerevisiae* and *Caenorhabditis elegans*, which is surprising for a gene implicated in a muscle-specific disorder. Three new homologous human genes were found, and, together with *MTM1*, they define a new family of putative tyrosine phosphatases in man (13).

The cloning of the *MTM1* gene has greatly improved the prospects for diagnosis and genetic counselling of the disease. Moreover, identification of the mutations responsible for XLMTM should give some insights into its molecular and physiological basis. We have determined the exon–intron structure of the *MTM1* gene (Laporte *et al*., in preparation), and performed mutation screening in 85 independent patients. Mutations were found in 55 of them, and this allows accurate counselling in the relevant families. The identification of 19 missense mutations and small insertions or deletions of amino acids should be useful for functional studies of myotubularin.

RESULTS

The *MTM1* gene, located in the proximal part of Xq28, consists of 15 exons, for which flanking intronic sequences have been determined (Laporte *et al*., in preparation). In the original description of the cDNA sequence, the location of the translation initiation site had not been determined. More recent data using antibodies directed against myotubularin indicate that the protein is translated starting from the methionine codon at position 19 in the putative amino acid sequence previously reported [(13), GenBank U46024]. The endogenous protein immunoprecipitated from myoblast extracts has the same size as that synthesized in COS cells transfected with the full-length cDNA, where the first ATG codon is at position 19 (unpublished results). We have thus renumbered the amino acid positions.

The *MTM1* gene was screened for mutations using single-strand conformation polymorphism (SSCP) followed by sequencing of abnormal bands, based on the knowledge of intronic sequences flanking all of the 15 exons (see primers and conditions in Table 1). Among 85 patients screened for mutations for all the coding sequence, we found 52 point mutations (Table 2). Three patients had large deletions. Seven of the point mutations and two large deletions were reported previously (11,13). The 52 point mutations consist of 33 base changes and 19 small insertions/deletions. Sixteen of the base substitutions affect CpG dinucleotides. Twenty seven mutations are frameshift or nonsense mutations. Alteration of donor or acceptor splice sites account for six of the mutations. Including the large deletions, 65% of the mutations would result in the absence or truncation of myotubularin.

An A(1314–10)G change in intron 11 was found in four unrelated patients and creates a new functional acceptor site that leads to the insertion of three amino acids in a conserved region of the protein as indicated by RT-PCR experiments (14,28).

Missense mutations were found concentrated in three areas: exons 4 and 5, exons 8 and 9 and exons 11 and 12 (Fig. 1). At least 12 of the 16 different missenses or single amino acid deletions affect residues that are completely conserved in the homologous proteins predicted from yeast and *C.elegans* sequences (13), as indicated in Table 2. The N-terminal domain of myotubularin (from exons 2 to 7) is only weakly homologous to its counterparts in yeast and *C.elegans*, making the alignment less reliable. The four other missense mutations are in any case non-conservative (del K47, R69C, L70F and S229P). We tested all exons on at least 40 normal chromosomes under the same SSCP conditions, and did not find these mutations. This further supports the conclusion that these alterations are indeed disease causing.

Table 2. Mutations in the *MTM1* gene

Patient[a]	Exon intron	Nucleotide change[b]	Predicted protein or splicing alteration[c]	Yeast or *C.elegans* aa[d]	Age at death[e]	Mutation origin[f]	Referring physician	Ref.
N1-44	ex2	G103T	E17X			mc	P.G. Barth/P. Bolhuis	
4399	ex3	C124T*	R24X				E. Flory	
G92-628	ex3	C163T*	R37X			mc	W. Kress	
NL16	ex3	C163T*	R37X			maternal	P.G. Barth/ P. Bolhuis	
1-3228	in3	(191–4)delATAG	destroy acceptor splice site		7 months		C. Francannet	
CNM25	ex4	193delAAA	K47del	A/E(?)	11 months	mc	E. Kattner	
CNM10	ex4	195delAGAA	frameshift 48		6 months	mc	W. Kress/T. Grimm	14
CNM17	ex4	195delAGAA	frameshift 48		1 month	mc	E. Bertini	
CNM21	ex4	195delAGAA	frameshift 48		8 and 10 days	mc	M. Huggins/ T. Costa	
4715	ex4	C259T*	R69C	H/R (?)	still alive at 3 years		N. Dahl	
NL20	ex4	C262T	L70F	T/Q (?)			G. van Wijngaarden/ P. Barth	
DA1	ex4	266delATTT	Y71X			grand paternal	S. Manouvrier-Hanu	16
G93-652	ex5	T314C	L87P	L/L (?)	still alive at 3 years		W. Kress	
DF34	ex5	351insCG	frameshift 100			mc	Boute/Pevissen	
CU15	in5	A(397–2)C	destroy acceptor splice site				J. Boue	
4711	ex6	451delAT	frameshift 133		neonatal/alive at 7 years		Kubota	
CNM1	ex7	515del18bp and 515insAAA	L154X		3 days	mc	S. Braga	
CNM22	ex7	G(582+1)A	destroy donor splice site				S. Liechti-Gallati	
3096	ex8	A620G	N189S	N/N	3 months	maternal	N. Dahl	
CNM32	ex8	643delACTT	frameshift 198		3 weeks		D. Lev	13
GM-06813	ex8	646delT and C648A	frameshift 198		10 months	mc	NHGMCR[g]	13
CNM23/G90-520	ex8	C668T*	P205L	P/P	2 months	maternal	W. Kress/R. Kraschl	13
CNM2	ex8	703insG	frameshift 217		4 and 5 days	mc	S. Braga	
CNM28	ex8	C718T*	R222X		still alive at 14 years		B. Wittwer/ B. Lemcke	
CNM20	ex8	C724T*	R224X			maternal	S. Liechti-Gallati	
CU32	ex8	C724T*	R224X				M.P. Cordier-Alex	
G93-401	ex8	C724T*	R224X		6 months	maternal	W. Kress	
G93-258	in8	(732+1)delG	destroy donor splice site		6 months		W. Kress	
CNM9	in8	G(733–1)A	destroy acceptor splice site		22 days	grand maternal	G. Wolff	14
DY89	ex9	T739C	S229P	T/I	still alive at 1 year		W. Kress	
3097	ex9	763delAC	frameshift 237				N. Dahl	13
CNM19	ex9	763insA	frameshift 237			mc	M.C. Zahller	
NL15	ex9	C775T*	R241C	R/R	still alive at 4 years	mc	P.G. Barth/P. Bolhuis	
CNM12	ex9	819delA	frameshift 256		1 month	grand paternal	A. Schinzel/ E. Boltshauser	
CNM8	ex9	890delC	frameshift 279		still alive at 8 years	mc	H. Jacobi	
MA3	ex11	1147insT	frameshift 365				N. Dahl	13
CF88	ex11	G1181A	S376N	S/S	7 months	grand parental	Chery/Vigneron	13
IT	ex11	G1186A*	G378R	G/G	2 weeks		N. Dahl	13
CNM14	ex11	1233insGTTG	frameshift 394		2 months	mc	W. Kress/E. Passarge	
MT65	ex11	A1244G	Y397C	Y/Y	29 days	maternal	F. Samson	15
G95–127	ex11	G1259C	G402A	G/G			W. Kress	
CNM16	in11	G(1314+1)C	destroy donor splice site			mc	A. Steen	
CNM4	in11	A(1315–10)G: new splice site	420insFIQ		6 months		G. Wolff/ D. Emmerich	
CNM15	in11	A(1315–10)G: new splice site	420insFIQ		3 months		H. Schroeder/ J. Sonntag	14
MTMFIN2	in11	A(1315–10)G: new splice site	420insFIQ		11 months		L. Pääkkönen/ L. Paljärvi	

Table 2. *Continued*

EA5	in11	A(1315–10)G: new splice site	420insFIQ			maternal	K. Tezcan	
G92-120	ex12	G1316A*	R421Q	R/R	3 months		W. Kress	
GM-10680	ex12	G1345A* and G1351A	D431N and D433N	D/D and D/D	6 weeks	mc	NHGMCR[g]	
NL21	ex13	A1460C	H469P	H/H			P.G. Barth/P. Bolhuis	
CNM5	ex13	C1474T*	R474X			mc	E. Boltshauser	
DM92	ex13	C1474T*	R474X		2 weeks		N.Piquion	
1-3884	ex13	C1474T*	R474X		3 weeks		Jarreau	
CM73	del	from ex2 to ex 8 included					J. Boue/ J.A. Urtizberea	
G89-441	del	from ex1 to ex13 included			11 months		W. Kress	11
G89-474	del	all gene deleted			3 months		W. Kress	11

[a]Each patient represents one family. For DA1 and CNM5, CNM10, CNM14 and CNM19, maternal DNA was analysed, as no DNA was available from the affected boys.
[b]Sequence changes are presented according to the nomenclature proposed by the Ad Hoc Committee on Mutation Nomenclature (27). Nucleotide numbers refer to their positions in the sequence of the *MTM1* transcript (GenBank U46024). Starred nucleotide changes affect a CpG dinucleotide.
[c]The amino acid numbering starts at the first methionine of the open reading frame [codon 19 in (13)]. The numbering has therefore been modified, for the mutations previously reported (13).
[d]In regions where the homology between myotubularin and yeast or *C.elegans* homologues is low (N-terminal domain of the protein), a question mark indicates that the alignment is ambiguous.
[e]In a few cases, two brothers were affected, and their age at death is indicated.
[f]In the mutation origin column, mc means that the mother carries the mutation, other mentions (maternal, grand paternal etc.) indicate the origin of a new mutation.
[g]NHGMCR stands for Nigms Human Genetic Mutant Cell Repository.

Table 3. Polymorphisms and rare variants

Exon/ intron	Nucleotide change	Frequency
in2	(118–50)delAG	1/128
in2	(118–50)A/T	1/128
in2	(118–14)T/C	1/128
ex8	615T/C wobble base	1/150
ex9	738G/C wobble base	1/150
ex11	1308T/C wobble base	2/150
in11	(1315–19)T/G	2/150
in11	(1314+3)A/G consensus donor site	23/47
in11	(1314+17)A/G	3/150
ex15[a]	1755C/T wobble base	1/150

Rare variants were found in either normal individuals or in patients carrying a characterized mutation, except for 1755C/T, found in a patient for whom no mutation has been detected yet. The first base indicated corresponds to the common allele and the frequency is calculated for the rare allele.
[a]See Laporte *et al*. (13).

Although no point mutations were found in exons 1, 10, 14 and 15, our results are consistent with a widespread occurrence of mutations along the gene. The C-terminal end of the protein may be of lesser functional importance as it is not well conserved in yeast and *C.elegans*, and as one would expect ~10 mutations in exons 14 + 15, based on the length of their coding sequences. Exons 4, 8, 9, 11 and 12 showed a higher tendency to mutate in this panel of patient samples.

Polymorphisms

Ten polymorphisms or rare variants have been characterized to date (Table 3). The T→C transition at position 615 affects a wobble base and was found in only one patient (CU32) who carries a deleterious mutation (R224X). The (1314+17)A/G was found in G93-401, who already has the same R224X mutation, and in two normal chromosomes (15). The frequent (1314+3)A/G polymorphism is located in the donor splice site of intron 11. It is unlikely that it affects the splicing efficiency in the normal population as G or A are equally accepted at the +3 position of the consensus donor site. The 1755C/T variant that does not affect the amino acid sequence was found in a patient for whom no other mutation has yet been observed, but this variant is unlikely to be pathological.

Large deletions

Three large deletions encompassing part or all of the *MTM1* gene were found, including two previously reported ones (11). Patients G89-441 and G89-474 carried the largest deletions and were also found to have an abnormal genital development (hypospadias). Patient CM73, who shows an intragenic deletion encompassing exons 2–8, had normal genitalia and no additional phenotypes. As proposed previously (11), these findings suggest that a gene centromeric to *MTM1* is important for male genital development.

Recurrent and new mutations

The nonsense mutations R37X, R224X and R474X, corresponding to C→T transitions at CpG sites, were found in two, three and three independent families, respectively. A 4 bp frameshift deletion in exon 4 was found in three patients. This recurrent deletion does not correspond to a replication slippage affecting a tandem repeat, a frequently observed cause of small deletions. The splice mutation in intron 11 (A 1314–10G) which

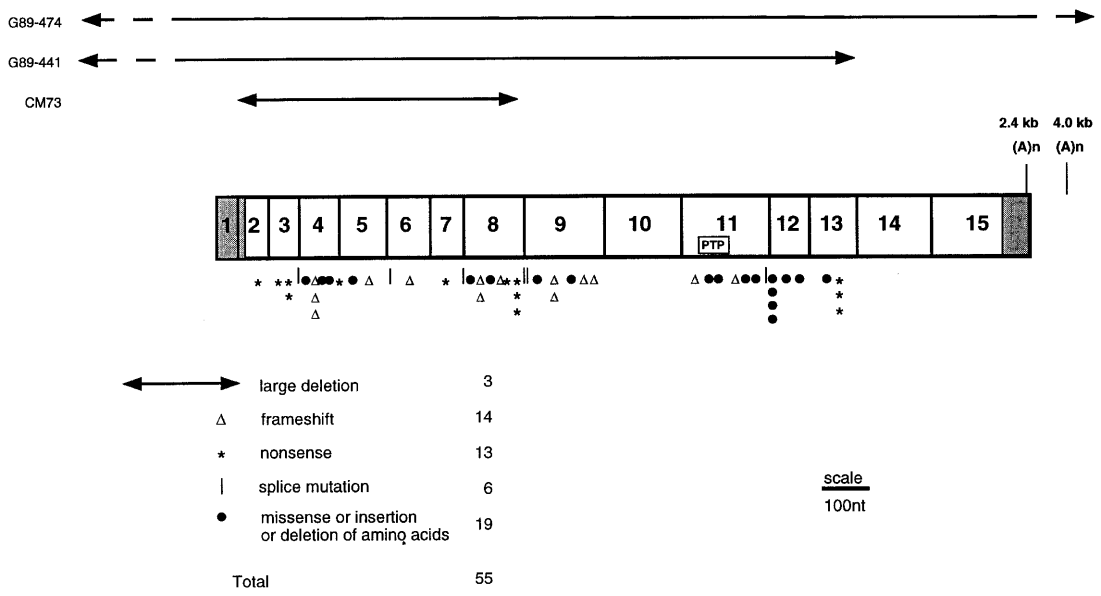

Figure 1. Distribution of mutations along the open reading frame of the *MTM1* transcript. Exons are drawn to scale, numbered 1–15, and their boundaries are indicated. The 5′ and 3′ untranslated sequences (UTRs) are in grey. The 2.4 kb muscle- and testis-specific transcript and the 4.0 kb ubiquitous transcript differ only by the length of the 3′UTR. The putative tyrosine phosphatase active site is indicated in exon 11. Recurrent mutations in unrelated patients are vertically aligned. Large deletions occurring in patients G89-441, G89-474 and CM73 are represented by the upper lines.

was found four times, shows no obvious feature that could account for its high incidence.

Patients showing the same mutation were analysed with microsatellites in the *MTM1* region [*DXS8377* and *7423* (17)] which confirmed that they were from different families (not shown), except for patients CNM15 and EA5 who carry the splice mutation in intron 11 on the same haplotype. The mutation in EA5 is a maternal new mutation, indicating that the mutations in EA5 and CNM15 are independent.

Although in many cases we did not have DNA from the mother or other family members to trace the inheritance of the mutation, in the cases where such analyses could be performed, we found a high proportion of maternal or grandparental new mutations. In particular, seven of 28 mothers tested did not carry their son's mutation, which is close to the expected one-third (Table 2).

DISCUSSION

The *MTM1* gene, which encodes a 603 amino acid protein, was screened for mutations in 85 patients by SSCP analysis, over all the coding sequence. Fifty five mutations and 10 polymorphisms and rare variants were identified. As expected for a lethal X-linked disease, we found a high heterogeneity of mutations, widespread through the coding sequence, of which a high proportion were new mutations. Truncated proteins are predicted to result from ~65% of the mutations.

Five recurrent mutations account for nearly one-third of all mutations, and single nucleotide changes affect preferentially CpG dinucleotides. The only exception is the splice mutation in intron 11 resulting in the insertion of three amino acids (FIQ) at position 420 and observed in four independent patients. There are no obvious sequence features that may account for the high incidence of this mutation (also observed by de Gouyon *et al.*, 28), and for the recurring 4 bp deletion in exon 4 (195delAGAA). The parental origin of these mutations is under investigation.

The number of mutations found is lower than expected, considering that we screened all the coding sequence. This might be accounted for by preferential localization of mutations in regions not yet analysed (regulatory sequences, introns) or by the fact that we did not detect all mutations by SSCP analysis. As sporadic cases account for a high proportion of the patients, some patients may not have XLMTM. We cannot exclude the possibility, at least in a small proportion of patients, of another gene defect causing a similar phenotype. We reported recently the finding of an *MTM1* mutation in a family previously thought to indicate genetic heterogeneity in XLMTM (18). There is thus no more evidence of genetic heterogeneity in proven X-linked cases (15).

Diagnostic applications

The proportion of sporadic cases in an X-linked lethal disease like XLMTM is >50%. Genetic counselling is especially difficult in such cases, as there is no reliable way of assessing carrier status in females (4,15). Direct mutation detection thus becomes the most reliable method for prenatal diagnosis and carrier detection (13,14). Exons 4, 8, 9, 11 and 12 showed a higher tendency to mutate and may be tested first. Together, these five exons accounted for 73% of all the mutations found. Three of the recurrent mutations are included in these five exons. RT-PCR can be an alternative method in order to avoid analysis of individual exons, but this requires adequately stored cells.

In the future, the assessment of the presence of the protein in muscle biopsies or in leukocytes by Western blot or immunohistochemistry (19) may become an alternative to mutation screening, if the level of expression is sufficient. Sixty five percent of the mutations are predicted to truncate the protein, thus probably leading to its degradation. Furthermore, some missense

mutations may also lead to selective degradation of myotubularin by affecting its stability or its appropriate targeting to subcellular compartments.

Functional implications

The *MTM1* gene is probably involved in a signal transduction pathway necessary for late myogenesis, although its ubiquitous expression suggests a wider function. Almost all changes, insertion or deletion of amino acids, observed to date in patients affect regions and residues that are conserved in the homologous yeast and *C.elegans* proteins, indicating their functional importance. A tyrosine phosphatase active site is predicted in myotubularin. Although phosphatase activity has not yet been demonstrated, two of the disease-causing mutations are missenses within the PTP signature (amino acids 373–385) and eight involve neighbouring amino acids (encoded in exons 11 and 12) that may belong to the broader PTP domain which usually encompasses ~250 amino acids around the active site (20). The A(1314–10)G intronic mutation causes an insertion of three amino acids (420insFIQ) in a very conserved region close to the PTP site, where we also found the R421Q missense.

We observed two other preferential regions for missenses. Four missense mutations were found in exons 8 and 9, corresponding to a region conserved throughout evolution (13). The other region corresponds to exons 4 and 5, but here the conservation in the yeast or *C.elegans* homologous genes is low. These two domains of the protein may be involved in substrate recognition, appropriate targeting to cellular compartments or interaction with other proteins. The missense mutations we observed will be useful when studying putative functions or protein interactions of myotubularin. Although no missense mutations were found in exons 14 and 15, the recurring R474X nonsense mutation truncates this part of the protein, indicating that the C-terminal domain is important for the structure/stability of myotubularin.

Milder autosomal forms of CNM have been described (4). Candidate genes for such forms are the MTM-related genes, and genes coding for putative proteins that interact with myotubularin or belong to the same signal transduction pathway. Identification of genes implicated in autosomal CNM may thus shed light on the function of myotubularin.

Genotype–phenotype correlations

Given the ubiquitous expression of the gene and the existence of a well-conserved gene in yeast, it is surprising that no tissues other than muscle have been clearly found to be affected in XLMTM patients (21). Other associated phenotypes might have been missed, as most patients die very early. Alternatively, tissue-specific pathology could result from interaction with muscle-specific proteins, or from a different balance of proteins with overlapping functions encoded by the MTM-related genes.

Additional clinical features were only described for patients with large deletions, and suggest a contiguous gene syndrome (11). A gene involved in male sexual development must be centromeric to *MTM1* in the region deleted in G89-441 and G89-474, but present in CM73. The *F18* gene and *XAP80–XAP87* transcripts represent candidates for such function (22,23).

From our data, there is no obvious correlation between the type of mutation and the severity of the disease. Some patients with nonsense or frameshift mutations have survived 7–14 years (mutations 451delAT, C718T and 890delC), while other patients with missense mutations died in the neonatal period. Moreover, patient 4711 (451delAT) is still alive at 7 years but a brother died neonatally. The missense R241C led to a mild phenotype in two cases [patient N2-9 in this study, and (24)], while a R241L mutation caused a severe phenotype (28). Because of the existence of such milder phenotypes, it may be important to check for mutations in cases diagnosed as autosomal recessive centronuclear/myotubular myopathy if the autosomal inheritance is not well established.

It is possible that in most cases survival depends more on clinical treatment and other parameters (prematurity, putative modifying genes) than on the nature of the mutation. It has indeed been suggested that, once a patient survives beyond a certain point, he may have improvement in his condition (4).

We conclude that the *MTM1* gene is indeed the principal and probably the only gene implicated in XLMTM. Mutation detection has already proven to be very useful for genetic counselling, carrier detection and prenatal diagnosis in families.

MATERIALS AND METHODS

Identification of mutations

Genomic DNA was prepared from cultured fibroblasts, cultured lymphoblastoid cell lines or venous EDTA blood (25). Primer sets were designed from intronic and exonic sequences flanking the 15 exons of the *MTM1* gene. Primer sequences and PCR conditions are summarized in Table 1. Briefly, 100 ng of patient DNA were amplified in 10 mM Tris–HCl pH 8.3, 50 mM KCl, 200 μM dNTP, the defined $MgCl_2$ concentrations, with 10 pmol of each primers, 1 nmol (3μCi) of [^{32}P]dCTP and 1 U of *Taq* polymerase in a total volume of 25 μl. Thermocycling was carried out in the PTC-100 Programmable Thermal Controller (MJ Research, Inc.) at 94°C for 5 min followed by 30 cycles at 94°C for 10 s, annealing temperature for 10 s, 72°C for 10 s, and a final elongation step at 72°C for 5 min. For exon 1, the PCR was performed with 3 min elongation at each cycle and with 10% dimethylsulfoxide (DMSO) and 3.75 nmol of C7deaza guanine; then the product was digested by *Sal*I into two fragments (176 and 279 nucleotides).

After PCR, the products were denaturated at 94°C for 10 min with 6 μl of SSCP loading buffer (0.2 M NaOH, 0.25% w/v each bromophenol blue and xylene cyanol in formamide) and separated on a 6% native polyacrylamide gel. For each primer set, gels were run at 4°C under two conditions: overnight at 6 W and 5 h at 30 W. Shifted bands were excised from the gel and reamplified in 200 μl, then purified using the Geneclean Kit (Bio 101, Inc.) and sequenced on an Applied Biosystems (ABI) automated sequencer with fluorescent dideoxynucleotides using the same primers as for PCR.

For patients identified as CNMx, the same PCR amplification was carried out in a Thermal Cycler 2400 (Perkin Elmer). Initial denaturation was at 95°C for 2 min, followed by 28 cycles of 95°C for 15 s, annealing for 15 s (at 52°C: exons 5, 10, 12, 13, 14; at 56°C: exons 3, 4, 6, 8, 9, 11, 15), and 72°C for 45 s. Final extension was at 72°C for 7 min. PCR products were analysed after denaturation on 12% acrylamide gels containing 7.25% glycerol using a two-buffer system (Liechti-Gallati *et al*., submitted). DNA was visualized by silver staining (26). Sequence changes were detected either by double or single strand band shifts or by the generation of heteroduplex on the same gel.

SSCP variants were purified directly using QIAquick PCR purification columns (QIAGEN) according to the supplier and sequenced.

Large deletions were confirmed by PCR amplification of the different exons using the primers in Table 1, and by Southern blot from patient genomic DNA (11) digested with *Bst*YI or *Msp*I and hybridized with exon-specific PCR products.

Microsatellite genotyping

In order to confirm that patients with recurrent mutations were from different families, they were all genotyped using polymorphic markers *DXS8377* and *DXS7423* closely flanking the *MTM1* gene (17).

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REFERENCES

1. Sarnat, H.B. (1990) Myotubular myopathy: arrest of morphogenesis of myofibres associated with persistence of fetal vimentin and desmin. Four cases compared with fetal and neonatal muscle. *Can. J. Neurol. Sci.*, **17**, 109–123.
2. Spiro, A.J., Shy, G.M. and Gonatas, N.K. (1966) Myotubular myopathy, persistence of fetal muscle in an adolescent boy. *Arch. Neurol.*, **14**, 1–14.
3. Wallgren-Pettersson, C. and Thomas, N.S. (1994) Report on the 20th ENMC sponsored international workshop: myotubular/centronuclear myopathy. *Neuromuscul. Disord.*, **4**, 71–74.
4. Wallgren-Pettersson, C., Clarke, A., Samson, F., Fardeau, M., Dubowitz, V., Moser, H., Grimm, T., Barohn, R.J. and Barth, P.G. (1995) The myotubular myopathies: differential diagnosis of the X linked recessive, autosomal dominant, and autosomal recessive forms and present state of DNA studies. *J. Med. Genet.*, **32**, 673–679.
5. Thomas, N. and Wallgren-Pettersson, C. (1996) X-linked myotubular myopathy. 33rd ENMC International Workshop Soest. The Netherlands, 9–11 June 1995. *Neuromuscul. Disord.*, **6**, 129–132.
6. Thomas, N.S., Williams, H., Cole, G., Roberts, K., Clarke, A., Liechti-Gallati, S., Braga, S., Gerber, A., Meier, C., Moser, H. *et al*. (1990) X linked neonatal centronuclear/myotubular myopathy: evidence for linkage to Xq28 DNA marker loci. *J. Med. Genet.*, **27**, 284–287.
7. Liechti-Gallati, S., Muller, B., Grimm, T., Kress, W., Muller, C., Boltshauser, E., Moser, H. and Braga, S. (1991) X-linked centronuclear myopathy: mapping the gene to Xq28. *Neuromuscul. Disord.*, **1**, 239–245.
8. Dahl, N., Samson, F., Thomas, N.S., Hu, L.J., Gong, W., Herman, G., Laporte, J., Kioschis, P., Poustka, A. and Mandel, J.L. (1994) X linked myotubular myopathy (MTM1) maps between DXS304 and DXS305, closely linked to the DXS455 VNTR and a new, highly informative microsatellite marker (DXS1684). *J. Med. Genet.*, **31**, 922–924.
9. Dahl, N., Hu, L.J., Chery, M., Fardeau, M., Gilgenkrantz, S., Nivelon-Chevallier, A., Sidaner-Noisette, I., Mugneret, F., Gouyon, J.B., Gal, A. *et al*. (1995) Myotubular myopathy in a girl with a deletion at Xq27–q28 and unbalanced X inactivation assigns the MTM1 gene to a 600-kb region. *Am. J. Hum. Genet.*, **56**, 1108–1115.
10. Hu, L.J., Laporte, J., Kioschis, P., Heyberger, S., Kretz, C., Poustka, A., Mandel, J.L. and Dahl, N. (1996) X-linked myotubular myopathy: refinement of the gene to a 280-kb region with new and highly informative microsatellite markers. *Hum. Genet.*, **98**, 178–181.
11. Hu, L.J., Laporte, J., Kress, W., Kioschis, P., Siebenhaar, R., Poustka, A., Fardeau, M., Metzenberg, A., Janssen, E.A., Thomas, N., Mandel, J.L. and Dahl, N. (1996) Deletions in Xq28 in two boys with myotubular myopathy and abnormal genital development define a new contiguous gene syndrome in a 430 kb region. *Hum. Mol. Genet.*, **5**, 139–143.
12. Smolenicka, Z., Laporte, J., Hu, L.J., Dahl, N., Fitzpatrick, J., Kress, W. and Liechti-Gallati, S. (1996) X-linked myotubular myopathy: refinement of the critical gene region. *Neuromuscul. Disord.*, **6**, 275–281.
13. Laporte, J., Hu, L.J., Kretz, C., Mandel, J.L., Kioschis, P., Coy, J.F., Klauck, S.M., Poustka, A. and Dahl, N. (1996) A gene mutated in X-linked myotubular myopathy defines a new putative tyrosine phosphatase family conserved in yeast. *Nature Genet.*, **13**, 175–182.
14. Tanner, S.M., Laporte, J., Guiraud-Chaumeil, C. and Liechti-Gallati, S. (1997) Confirmation of prenatal diagnosis results of X-linked recessive myotubular myopathy by mutational screening, and description of three new mutations in the MTM1 gene. *Hum. Mutat.*, in press.
15. Guiraud-Chaumeil, C., Vincent, M.C., Laporte, J., Fardeau, M., Samson, F. and Mandel, J.L. (1997) A mutation in the MTM1 gene invalidates a previous suggestion of non allelic heterogeneity in X-linked myotubular myopathy. *Am. J. Hum. Genet.*, in press.
16. Vincent, M.-C., Guiraud-Chaumeil, C., Laporte, J., Manouvrier-Hanu, S. and Mandel, J.-L. (1997) Extensive germinal mosaicism in a family with X-linked myotubular myopathy simulates genetic heterogeneity. *J. Med. Genet.*, in press.
17. Hu, L.J., Laporte, J., Kress, W. and Dahl, N. (1996) Prenatal diagnosis of X-linked myotubular myopathy: strategies using new and tightly linked DNA markers. *Prenat. Diagn.*, **16**, 231–237.
18. Samson, F., Mesnard, L., Heimburger, M., Hanauer, A., Chevallay, M., Mercadier, J.J., Pelissier, J.F., Feingold, N., Junien, C., Mandel, J.L. *et al*. (1995) Genetic linkage heterogeneity in myotubular myopathy. *Am. J. Hum. Genet.*, **57**, 120–126.
19. Willemsen, R., Mohkamsing, S., De Vries, B., Devys, D., van den Ouweland, A., Mandel, J.L., Galjaard, H. and Oostra, B. (1997) Rapid antibody test for fragile X syndrome. *Lancet*, **345**, 1147–1148.
20. Fauman, E. and Saper, M.A. (1996) Structure and function of the protein tyrosine phosphatases. *Trends Biochem. Sci.*, **21**, 413–417.
21. Joseph, M., Pai, G.S., Holden, K.R. and Herman, G. (1995) X-linked myotubular myopathy: clinical observations in ten additional cases. *Am. J. Med. Genet.*, **59**, 168–173.
22. Kioschis, P., Rogner, U.C., Pick, E., Klauck, S.M., Heiss, N., Siebenhaar, R., Korn, B., Coy, J.F., Laporte, J., Liechti-Gallati, S. and Poustka, A. (1996) A 900-kb cosmid contig and 10 new transcripts within the candidate region for myotubular myopathy (MTM1). *Genomics*, **33**, 365–373.
23. Laporte, J., Kioschis, P., Hu, L.J., Kretz, C., Carlsson, B., Poustka, A., Mandel, J.L. and Dahl, N. (1997) Cloning and characterization of an alternative spliced gene in proximal Xq28 deleted in two patients with intersexual genitalia and myotubular myopathy. *Genomics*, **41**, 458–462.
24. Donelly, A., Haan, E., Manson, J. and Mulley, J. (1997) A novel mutation in exon b (R259C) of the MTM1 gene is associated with myotubular myopathy. *Hum. Mutat.*, in press.
25. Miller, S., Dykes, D. and Polesky, H. (1988) A simple salting out method for extracting DNA from human nucleated cells. *Nucleic Acids Res.*, **16**, 1215.
26. Budowle, B., Chakraborty, R., Giusti, A.M., Eisenberg, A.J. and Allen, R.C. (1991) Analysis of the VNTR locus D1S80 by the PCR followed by high-resolution PAGE. *Am. J. Hum. Genet.*, **48**, 137–144.
27. Ad Hoc Committee on Mutation Nomenclature (1996) Update on nomenclature for human gene mutations. *Hum. Mutat.*, **8**, 197–202.
28. de Gouyon, B.M., Zhao, W., Laporte, J., Mandel, J.-L., Metzenberg, A. and Herman, G.E. (1997) Characterization of mutations in the recently identified myotubularin gene in 26 patients with X-linked myotubular myopathy. *Hum. Mol. Genet.* **6**, 1499–1504.