Neurogenetics (2004) 5:27–34
DOI 10.1007/s10048-003-0165-9

ORIGINAL ARTICLE

Nacim Louhichi · Chahnez Triki · Susana Quijano-Roy · Pascale Richard · Samira Makri · Mériem Méziou · Brigitte Estournet · Slah Mrad · Norma B. Romero · Hammadi Ayadi · Pascale Guicheney · Faiza Fakhfakh

New *FKRP* mutations causing congenital muscular dystrophy associated with mental retardation and central nervous system abnormalities. Identification of a founder mutation in Tunisian families

Received: 31 July 2003 / Accepted: 13 October 2003 / Published online: 2 December 2003

Abstract The congenital muscular dystrophies (CMD) constitute a clinically and genetically heterogeneous group of autosomal recessive myopathies. Patients show congenital hypotonia, muscle weakness, and dystrophic changes on muscle biopsy. Mutations in four genes (*FKT1, POMGnT1, POMT1, FKRP*) encoding putative glycosyltransferases have been identified in a subset of patients characterized by a deficient glycosylation of α-dystroglycan on muscle biopsy. *FKRP* mutations account for a broad spectrum of patients with muscular dystrophy, from a severe congenital form with or without mental retardation (MDC1C) to a much milder limb-girdle muscular dystrophy (LGMD2I). We identified two novel homozygous missense *FKRP* mutations, one, A455D, in six unrelated Tunisian patients and the other, V405L, in an Algerian boy. The patients, between the ages of 3 and 12 years, presented with a severe form of MDC1C with calf hypertrophy and high serum creatine kinase levels. None had ever walked. Two had cardiac dysfunction and one strabismus. They all had mental retardation, microcephaly, cerebellar cysts, and hypoplasia of the vermis. White matter abnormalities were found in five, mostly when cranial magnetic resonance imaging was performed at a young age. These abnormalities were shown to regress in one patient, as has been observed in patients with Fukuyama CMD. Identification of a new microsatellite close to the *FKRP* gene allowed us to confirm the founder origin of the Tunisian mutation. These results strongly suggest that particular *FKRP* mutations in the homozygous state induce structural and clinical neurological lesions in addition to muscular dystrophy. They also relate MDC1C to other CMD with abnormal protein glycosylation and disordered brain function.

Keywords Congenital muscular dystrophy · *FKRP* gene · Founder haplotype · Mental retardation · Cerebellar cysts

N. Louhichi · H. Ayadi · F. Fakhfakh (✉)
Laboratoire de Génétique Moléculaire Humaine,
Faculté de Médecine de Sfax,
Avenue Majida Boulila, 3029 Sfax, Tunisia
e-mail: faiza.fakhfakh@gnet.tn
Tel.: +216-74-246946
Fax: +216-74-246946

C. Triki · M. Méziou
Service de Neurologie,
CHU Habib Bourguiba,
3029 Sfax, Tunisia

S. Quijano-Roy · B. Estournet · S. Mrad
Service de Pédiatrie,
Rééducation et Réanimation Neurorespiratoire,
Hôpital Raymond-Poincaré,
92380 Garches, France

P. Richard
UF Cardio-MyoGénétique,
Groupe Hospitalier Pitié-Salpêtrière,
Paris, France

S. Makri
Etablissement Hospitalier Spécialisé Ali Ait Idir,
16000 Hahad, Alger, Algeria

N. B. Romero · P. Guicheney
INSERM U582 Institut de Myologie,
Groupe Hospitalier Pitié-Salpêtrière,
Paris, France

Introduction

Congenital muscular dystrophies (CMD) constitute a heterogeneous family of autosomal recessively inherited diseases, presenting at birth or within the first few weeks of life with hypotonia, delayed motor development, and dystrophic changes on skeletal muscle biopsy [1]. Clinical heterogeneity is evidenced by different patterns of motor involvement, variable serum creatine kinase (CK) levels, and the presence or not of mental retardation and structural defects of the

central nervous system (CNS). At least one-third of patients in western countries have a primary deficiency in the laminin $\alpha 2$ chain (merosin) resulting from mutations in the *LAMA2* gene [2, 3, 4]. This type of CMD, usually referred to as merosin-deficient CMD, has now been classified as MDC1A and is always associated with diffuse white matter abnormalities, and occasionally cerebellar hypoplasia or abnormal lesions of the cerebral gyri [5, 6, 7]. Epileptic seizures have been reported in a subset of these patients. A secondary deficiency of laminin $\alpha 2$ and of α-dystroglycan is found in some CMD forms, including muscle-eye-brain disease (MEB), Walker-Warburg syndrome (WWS), Fukuyama CMD (FCMD), and MDC1C, mapped to chromosomes 1p32, 9q34, 9q31, and 19q13, respectively [8, 9, 10, 11]. These CMD are caused by defects in genes encoding putative glycosyltransferases: the *POMGnT1* gene coding for an *O*-mannose glycosyltransferase (MEB) [8], the *POMT1* gene coding for *O*-mannosyltransferase 1 (WWS) [9], the *FKT1* gene encoding a protein of unknown function called fukutin (FCMD) [10], and *FKRP*, a homologue of the *FKT1* gene (MDC1C) [11]. Clinically, the first three types show severe symptoms and structural CNS involvement. MDC1C is characterized by marked elevation of serum CK, early onset hypotonia, delayed or arrested motor development, muscle hypertrophy, and variable cardiomyopathy. However, no major CNS abnormalities have been described in initial reports of even the most-severe forms [11, 12]. Very recently, two homozygous *FKRP* mutations associated with mental retardation and cerebellar cysts have been identified in two Turkish patients [13]. They were isolated cases and, therefore, it was not possible to conclude if particular *FKRP* gene mutations may constitute a subset of patients with a particular phenotype characterized by severe neurological involvement.

To address this issue, we report two new *FKRP* mutations associated with CMD and mental retardation, one in a series of six unrelated Tunisian families and another in an Algerian patient. All patients had mental retardation and neuroimaging showed a variable combination of CNS abnormalities, including cerebellar cysts or atrophy with or without white matter lesions. These results suggest strongly that certain *FKRP* mutations induce structural and clinical neurological abnormalities in a subset of patients with MDC1C. They may contribute to the better understanding of this entity and relate this type of CMD to others with abnormal protein glycosylation and structural CNS abnormalities. In addition, identification of a new microsatellite close to the *FKRP* gene was used to investigate the founder origin of the Tunisian mutation.

Patients and methods

Patients

Seven unrelated patients belonging to six consanguineous Tunisian CMD families from southern Tunisia (patients 1–6) and one consanguineous Algerian CMD family (patient 7) were studied. The diagnosis of CMD was made on the basis of hypotonia and weakness at birth or within the first months of life, and dystrophic changes on muscle biopsy. In addition, all patients had a marked increase in serum CK. Muscle biopsies were obtained from the deltoid muscle in five patients and from the tibialis anterior muscle in patients 6 and 7. Brain magnetic resonance imaging (MRI) was available for patients 1, 2, 3, 4, 6, and 7 and patient 5 had a cerebral computed tomographic (CT) scan. Blood samples were collected from the members of these families after informed consent.

Immunohistochemistry

Unfixed frozen 8-μm sections were incubated with monoclonal antibodies binding specifically to the human laminin $\alpha 2$ chain 80-kilodalton (kDa) fragment towards the C-terminal region (MAB 1922 Chemicon) and the human merosin 300-kDa fragment towards the amino-terminal region (NLC-merosin Novo-Castra) in all patients. In addition, muscle sections of patients 6 and 7 were immunolabelled with α-dystroglycan antibodies (V1A4-1, 05298, Euromedex). All primary antibodies were applied for 1 h and developed using an appropriate secondary antibody (fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins, Dako).

Linkage and haplotype analysis

Genetic analysis was performed on genomic DNA extracted from blood leukocytes using a standard phenol-chloroform procedure [14]. Microsatellites spanning the *FKRP* locus (D19S606, D19S412, D19S219, and fkrp 52) were studied. PCR amplification was carried out in 50 μl with 60 ng of genomic DNA, 1 μM of each primer, 125 μM dNTPs, 1.5 mM $MgCl_2$, 5 mM KCl, 10 mM TRIS-HCl, pH 8.8, and 1 U of Taq DNA polymerase. PCR products were analyzed on 6% denaturing polyacrylamide gels, transferred to a N^+ Hybond membrane (Amersham Pharmacia Biotech), and hybridized with a poly AC probe labeled with α-^{32}P. The following primers were designed to amplify the new microsatellite, fkrp 52 (ID=ss12568442 in the NCBI SNP database): 5′-TCTCCAAAAAACAACAACAAC-3′ and 5′-CTAGTGTTCTGGGACCTTT-3′. The sequence of the amplified fragment is available in the Third Party Annotation Section of the DDBJ/EMBL/Genbank databases under the accession number TPA: BK001438.

FKRP mutational analysis

A 1.8-kb fragment containing the *FKRP* coding sequence of exon 4 and its bordering intronic sequences was amplified using five primer sets (Table 1). The overlapping PCR products were purified (Qiagen) and used for direct sequencing on both strands. Sequencing reactions were carried out using an ABI Prism Big Dye

Table 1 PCR primers used to amplify the coding sequence of the *FKRP* gene located within exon 4

Name	Position on mRNA (GI:15866719)	Forward primer	Reverse primer	Product
FKRP 4-1	−170ATG/517	5′CAAAGCTGAAACCAAATAGGGA3′	5′GCTGGGCTGGGTCTTGCTG3′	401 bp
FKRP 4-2	422/830	5′GCCCCCGTGTCACCGTCCT3′	5′TAGCGGGCGGTCCACTCTC3′	408 bp
FKRP 4-3	660/1170	5′TGTGGCCCTAGTACCTGATG3′	5′GCGCGTGGTCTCCTTGTTG3′	510 bp
FKRP 4-4	1112/1558	5′TAGTGAGCTGGGAAGGCGG3′	5′CATTGCGGGGGTAGAAGGG3′	446 bp
FKRP 4-5	1500/1921	5′GCAGTACAGCGAAAGCAACC3′	5′GCCTTCTCTCATGCTCTCCT3′	421 bp

Terminator Cycle Sequencing Kit (Applied Biosystems). The 1364C>A mutation induces the loss of a *Nae*I restriction site. The *Nae*I restriction pattern of the FKRP 4-5 fragment (421 bp) was used to confirm the presence of the mutation in patients' relatives and to screen 100 unrelated healthy Tunisian individuals. Digestion of PCR products was performed according to the manufacturer's instructions (New England Biolabs), followed by separation on 2% Nusieve gels. The 1213G>T mutation identified in the FKRP 4-5 fragment was investigated by direct sequencing in 50 Algerian and 100 French healthy control individuals.

Results

Clinical and complementary findings

The main clinical features of the Tunisian (patients 1–6) and Algerian patients are summarized in Table 2.

Patients 1, 2, and 3

These patients have previously been described [15]; they presented a severe form of CMD with mental retardation and cerebellar cysts. The condition of these patients worsened progressively. For patient 1, brain MRI performed at ages 3 and 6 years showed a significant reduction of the white matter abnormalities with age (Fig. 1). In contrast, the hypoplasia of the pons and cerebellar cysts remained unchanged.

Patient 4

This patient was a 5-year-old girl. She had a severe form of CMD with mental retardation. Electromyography (EMG) was myopathic and muscle biopsy showed severe dystrophic features. Cranial MRI at 5 years of age revealed only cerebellar cysts without any white matter or other cerebellar abnormality (Fig. 2). Echocardiography showed left ventricular hypertrophy.

Patient 5

This patient was a 6-year-old girl. She presented at birth with a severe form of CMD with axial and facial weakness and had mental retardation. The ophthalmological examination was normal. Brain CT scan had been performed at the age of 3 years and showed white matter abnormalities (not shown).

Patient 6

Patient 6 was a 12-year-old mentally retarded girl who had an older brother with CMD associated with encephalopathy who had died at the age of 3 years due to a pneumopathy. She was the oldest child of this series and was the most severely affected patient. She never walked.

Table 2 Summary of the clinical features of the seven patients (*CK* creatine kinase, *ND* no data)

Patient	1	2	3	4	5	6	7
Sex	F	M	F	F	F	F	M
Age of onset	Birth	Birth	Birth	4 months	2 months	Birth	Birth
Current age (years)	7	6	6	5	6	12	3
Serum CK	4,400 IU/I	5,500 IU/I	7,330 IU/I	2,978 IU/I	2,600 IU/I	4,997 IU/I	1,370 IU/I
Maximum motor milestone	Sat unsupported	Sat unsupported	Sat unsupported	Sat unsupported	Sat unsupported	Sat unsupported	Sat unsupported
Contractures	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Hypertrophy	Calf	Calf	No	Calf	No	Tongue	Calf
Mental retardation	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Head circumference	−2 SD	−2 SD	−2 SD	−2 SD	−2 SD	−2 SD	−2 SD
White matter changes	Yes (3 years)	Yes (3 years)	No (6 years)	No (5 years)	Yes (3 years)	Yes(1 year)/±(4 years)/−(12 years)	Yes (2.5 years)
Cerebellar cysts	Yes	Yes	Yes	Yes	ND	Yes	Yes
Hypoplasia of vermis	Yes	Yes	Yes	No	ND	Yes	Yes
Microcephaly	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Eye involvement	No	Strabismus	No	No	No	Ophthalmoplegia	ND
Cardiac function	Normal	Normal	ND	Left ventricular hypertrophy	ND	Left ventricular dilatation	Normal
Respiratory function	Normal	Normal	Normal	Normal	Normal	Mechanical ventilation	Normal
Scoliosis	Yes	Yes	No	Yes	No	Vertebral arthrodesis	No

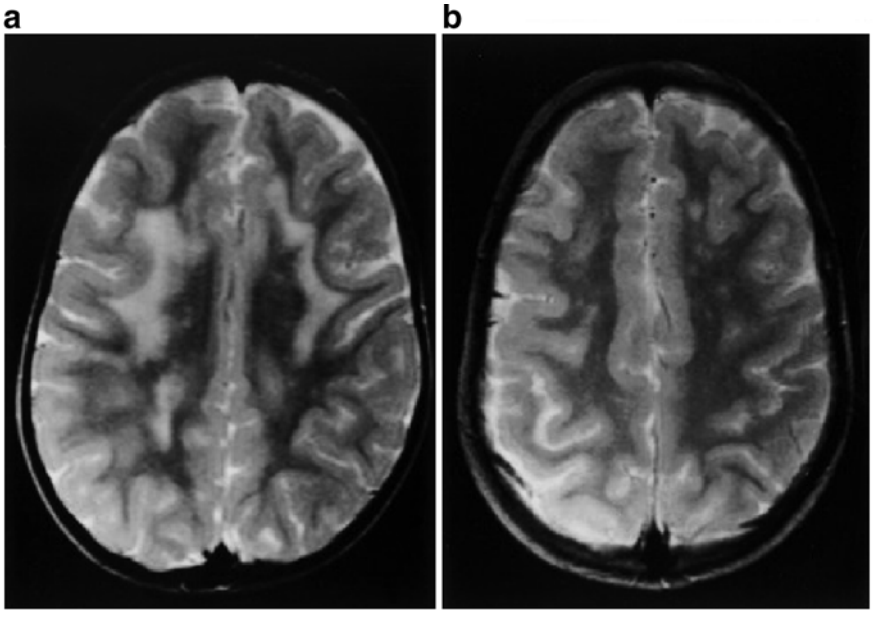

Fig. 1 T2-weighted axial magnetic resonance imaging (MRI) of the brain of patient 1 shows abnormal high white matter signal intensities at age of 3 years (**a**) and regression of these abnormalities on follow-up examination at the age of 6 years (**b**)

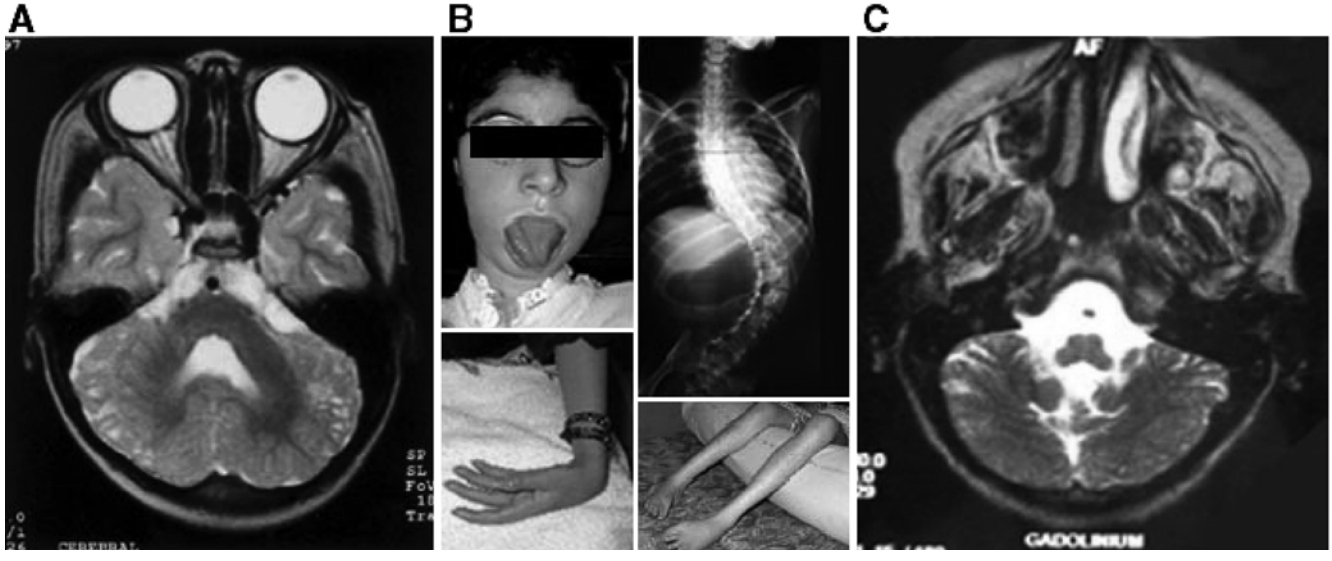

Fig. 2 **A** Transverse T2-weighted MRI of patient 4 showing cerebellar cysts. **B** Clinical features of patient 6: severe facial weakness and tongue enlargement, wrist and flexor finger contractures without elbow contractures, severe distal amyotrophy, and spinal deformity. **C** Transverse T2-weighted MRI of patient 6 showing hypoplasia of cerebellar vermis and cerebellar cysts

She showed a severe facial weakness, progressive weakness, macroglossia, limited ocular excursion, and scoliosis that was treated surgically. She was mechanically ventilated by 10 years of age because of severe restrictive respiratory insufficiency. At 12 years she was only able to perform minimal movements of the fingers, which were contracted in flexion (Fig. 2) and a mild cardiac involvement was detected, with a reduced shortening fraction and a normal ejection fraction. She did not speak before 5 years of age. At 12 years, she was able to say only single simple words of two or three syllables (verbal IQ 50) and her brain MRI showed cerebral atrophy, large white matter abnormalities, and hypoplasia of cerebellar vermis and cerebellar cysts (Fig. 2). The abnormal white matter signal was very diffuse in early life and regressed progressively.

Patient 7

This patient was a 3-year-old mentally retarded boy. He presented at birth with a severe form of CMD. EMG showed myopathic features and the muscle biopsy of the tibialis muscle showed severe dystrophic features. Electrocardiography, electroencephalography, and ophthalmological examination were normal. Brain MRI revealed

cerebellar cysts, megacisterna with hypoplasia of the vermis, and an abnormally high periventricular intensity on T2-weighed images. At the age of 3 years, he was microcephalic and could say only a few words.

Immunocytochemistry

All the patients showed histological changes characteristic of muscular dystrophy, and reduced immunohistochemical staining of laminin $\alpha 2$ chain using antibodies against the 80- and the 300-kDa fragments. α-Dystroglycan expression was markedly reduced in the only two samples available (data not shown).

Mutation screening of the *FKRP* gene

After exclusion of the CMD loci FCMD, MEB, MDC1A, and MDC1B in families 1–7 (data not shown), all patients were found to be homozygous for markers surrounding the *FKRP* locus (D19S219, D19S412, D19S606) on chromosome 19q13.3, suggesting homozygous mutations in *FKRP*. Sequencing of the *FKRP* coding region revealed the same homozygous transversion, 1364C>A, in the six Tunisian patients inducing the change of alanine 455 to a negatively charged aspartic acid (A455D) (Fig. 3). We took advantage of the loss of a *Nae*I restriction site induced by this mutation to exclude it as a non-pathogenic polymorphism. This variant was not found in 200 control chromosomes. Using *Nae*I digestion and direct sequencing, the mutation was found to segregate with the disease in an autosomal recessive fashion in the six consanguineous families (Figs. 4 and 5). A second homozygous transversion 1213G>T was identified in the Algerian patient changing valine 405 to leucine (V405L). This change was not found by direct sequencing in 50 Algerian and 100 French healthy control individuals.

Since the six families originated from Tunisia, we searched for a founder origin of the mutation. The known markers flanking *FKRP*, D19S219, D19S412, and D19S606, were physically mapped according to the Human Genome Browser and Ensembl Genome Server maps (http://genome.cse.ucsc.edu/goldenPath/hgTracks.html and http://www.ensembl.org). They are located at 1,255 kb and 238 kb proximal and 722 kb distal to *FKRP*, respectively. Among the families tested, families 1–4 from the same region in the south of Tunisia shared the same alleles (Fig. 5), while the two others did not. We searched for new (CA)n microsatellite markers closer to *FKRP*. One was identified, fkrp 52, with alleles varying from 92 to 122 bp, 42 kb proximal to *FKRP*. A rare120-bp allele containing 26 CA repeats was shared by the six families, suggesting that the mutation has been transmitted by a common ancestor (Fig. 5).

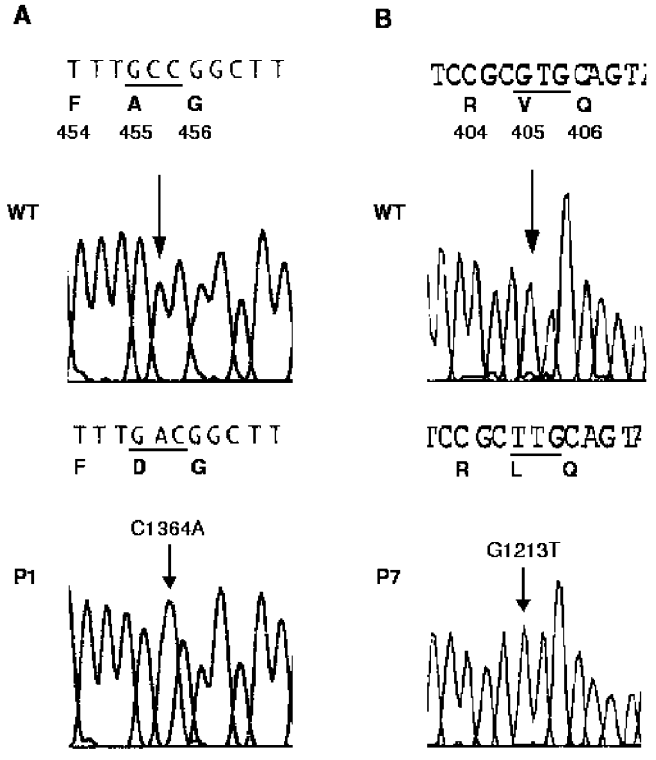

Fig. 3A, B Sequence chromatograms from normal individual (wild type, *WT*) and affected individuals (*P*) are shown together with the expected amino acid changes. Nucleotide variations are indicated by an *arrow*. **A** 1364C>A inducing the A455D mutation in patient 1 (*P1*), **B** 1213G>T inducing the V405L mutation in patient 7 (*P7*)

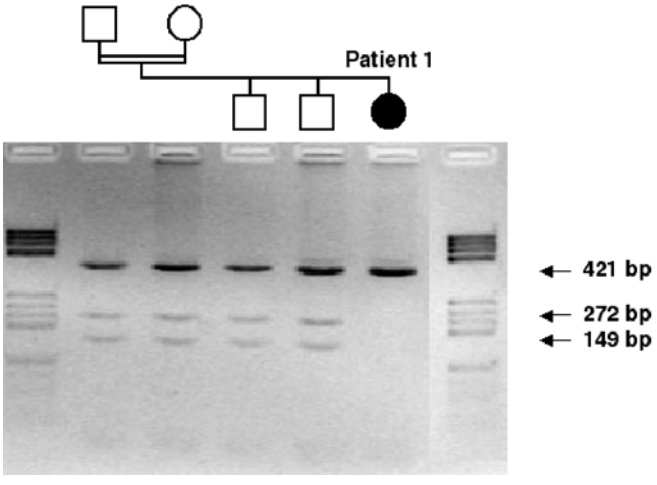

Fig. 4 Restriction enzyme analysis showing the inheritance of the 1364C>A mutation in family 4. A 421-bp PCR fragment was digested with *Nae*I. Normal control DNA is cut into two fragments of 272 bp and 149 bp, whereas products containing the mutation remained uncut at 421 bp in patient 1. Only one of the two alleles was cut for parents and the two brothers who are heterozygous carriers of the A455D mutation

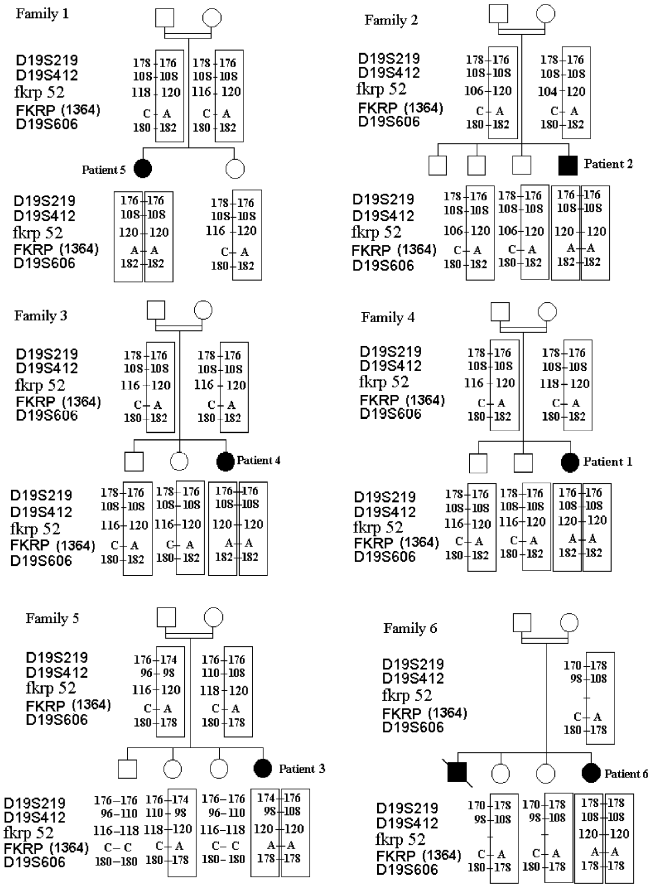

Fig. 5 The pedigrees of the six Tunisian families showing the inheritance of the 1364C>A mutation. The haplotypes containing the mutation are *boxed*. We note the same founder haplotype for families 1, 2, 3, and 4. Families 5 and 6 had the same mutation but not the same haplotype. With the fkrp 52 all families shared the same founder allele (120 bp) transmitted with the Tunisian mutation

Discussion

In this study, we report two new homozygous missense *FKRP* mutations in seven patients with MDC1C associated with mental retardation and CNS abnormalities. Six from Tunisia share the same mutation, which strongly suggests that this particular *FKRP* mutation in the homozygous state invariably lead to the CNS involvement.

Mutations in the *FKRP* gene previously reported account for a broad spectrum of patients with muscular dystrophy, from a severe congenital form (MDC1C) [11] to a much milder limb-girdle muscular dystrophy (LGMD2I) [16]. There are intermediate phenotypes that look like Duchenne muscular dystrophy. The intermediate and congenital forms may share an identical end-stage picture characterized by a paralytic atrophic-hypertrophic phenotype, including extreme diffuse weakness, tongue hypertrophy, and the need for continuous mechanical ventilation [11, 12]. Genotype-phenotype correlation has been shown by the identification of "mild" and "severe" mutations in cases with LGMD2I and MDC1C respectively, and which lead to an intermediate phenotype when combined [17, 18, 19]. In spite of differences in the degree of severity, a common phenotypic pattern, including some features such as high CK levels, muscle hypertrophy, a progressive course, and reduced expression of laminin $\alpha 2$ chain and α-dystroglycan in muscle, characterizes this entity.

The patients of our series presented with a typical MDC1C phenotype, but they also had severe psychomotor retardation and developed a microcephaly that was not observed at birth. They showed late and unsteady head control, inability to stand or walk, and marked mental retardation. Very high CK levels were common to all, and calf hypertrophy was noted in four patients. The oldest patient developed the typical paralytic atrophic-hypertrophic FKRP phenotype at the end of the first decade. This was not observed in the other six patients, probably because they were too young to manifest this progression, which is not usually observed in the first years of life [12]. All patients in our series showed white matter changes and/or cerebellar structural abnormalities on neuroimaging. We have previously reported minor signs of central involvement, such as speech delay, brain atrophy, and mild white matter lesions in some MDC1C patients. However, such findings were thought to be non-specific or secondary to hypoxic complications related to the respiratory insufficiency in advanced stages of the disease [12]. Therefore, MDC1C was initially classified as a CMD without neurological involvement, as distinct from the CMD syndromes with structural brain abnormalities, such as MEB, FCMD, and WWS. Brain abnormalities such as agyria, pachygyria or polymicrogyria, hypomyelination of the white matter, cerebellar cysts, or pontocerebellar hypoplasia are frequent findings in these three conditions [8, 9, 10]. In our series, brain MRI was available for six of the seven patients and revealed cerebellar cysts in all and hypoplasia of the vermis in five. MRI has not been performed in patient 5 and, therefore, we cannot exclude the presence of posterior fossa abnormalities in this patient. Our patients did not show any gyral malformation in contrast to what may be observed in MEB, FCMD, or WWS. White matter abnormalities were observed in five of our patients, with significant regression in one detected by serial MRI at 3 and 6 years of age. Such transient infra-myelination has also been described in MEB and FCMD [19, 20], while in primary laminin $\alpha 2$ chain-deficient CMD, brain white matter abnormalities do not regress with time. It is also noteworthy that one patient presented with strabismus and another with ophthalmoplegia in the 2nd decade of life.

Very recently, different *FKRP* homozygote mutations were identified in three Turkish patients, two with mental retardation, white matter changes, and cerebellar cysts [13], and another with a typical WWS (Beltran-Valero de Bernabé et al, personal communication). This finding raised the question of the role of specific homozygous *FKRP* mutations in the development of neurological disturbances. Our study revealing two new homozygous

missense *FKRP* mutations, A455D and V405L, in seven additional patients of similar phenotype clearly supports this hypothesis. Fukutin-related protein has been localized in the Golgi apparatus [21]. It is involved in the glycosylation processing of α-dystroglycan, a heavily glycosylated membrane protein that forms a link between the actin-associated cytoskeleton and the extracellular matrix via the laminin α2 chain [22]. Hypoglycosylation of α-dystroglycan abolishes binding not only to laminin, but also to neurexin and agrin, and such abnormal interactions underlie the pathogenic mechanism of muscular dystrophy with brain abnormalities [23, 24]. The mutations identified in our patients, V405L and A455D, are localized in the C-terminal domain (catalytic domain) of the fukutin-related protein, where other severe MDC1C mutations (P448L, Y465S) without CNS involvement have also been identified. These different mutations may affect in various degrees the conformation or the catalytic activity of the protein and/or recognition sites for α-dystroglycan and other potential substrates [16]. In contrast, the two mutations reported by Topaloglu et al. [13], P315T and S221A, which cause a similar phenotype with CNS involvement are located outside the catalytic domain in the stem region. This region is believed to be involved in protein-protein interaction.

In summary, the report describes two new missense mutations in the *FKRP* gene in seven children with MDC1C, mental retardation, and CNS abnormalities. While brain white matter changes may regress with time, structural cerebellar abnormalities were present early in life and did not seem to progress. Our results support the existence of particular homozygous *FKRP* mutations associated with severe neurological involvement, and relate MDC1C to other CMD with abnormal protein glycosylation affecting brain function.

Acknowledgements We thank the patients and their families for their participation. This work was supported by funds from the Secrétariat d'Etat à la Recherche Scientifique et à la Technologie (Tunisia), the Institut National de la Santé et de la Recherche Médicale (INSERM), Association Française contre les Myopathies (AFM), and the European Commission (contract no. QLG1-CT1999-00870).

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