MUTATION IN BRIEF

Vacuolating Megalencephalic Leukoencephalopathy With Subcortical Cysts: Functional Studies of Novel Variants in *MLC1*

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Nine new unrelated patients presenting vacuolating myelinopathy with subcortical cysts were identified and analyzed for variations in the *MLC1* gene. We detected 12 mutations (p.Leu37fs, p.Met80Val, p.Leu83Phe, p.Pro92Ser, p.Ser93Leu, p.Ile108fs, p.Gly130Arg, p.Cys171fs, p.Glu202Lys, p.Ser269Tyr, p.Ala275Asn, and p.Leu310_311insLeu) of which nine were novel. In one patient we did not detect mutations. Using a heterologous system, three new missense variants (p.Glu202Lys, p.Ser269Tyr, and p.Ala275Asn) and a single leucine insertion (p.Leu310insLeu) — lying in a stretch of seven leucines — were functionally assayed by determining total protein levels and mutant protein expression at the plasma membrane. No correlation was observed between mutation, clinical features, and plasma membrane expression of mutant protein. © 2006 Wiley-Liss, Inc.

KEY WORDS: mutation detection; *MLC1*; leukodystrophy

INTRODUCTION

The syndrome of megalencephalic leukoencephalopathy with subcortical cysts (MLC, MIM# 604004) is a rare vacuolating myelinopathy characterized by early-onset progressive ataxia, and pyramidal tract involvement initially confined to lower limbs with subsequent diffusion to upper limbs (van der Knaap et al., 1995). Late occurrence of motor dysfunction provoking severe walking disability, onset of seizures, and progressive cognitive impairment contribute significantly to the overall disability. Brain MRI is diagnostic in MLC (van der Knaap et al., 1995; De

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Stefano et al., 2001). The distinctive features are megalencephaly – present at birth or from the first year of life – and diffuse white matter abnormalities with subcortical cysts in the tips of the temporal lobes and in frontoparietal subcortical areas (van der Knaap et al., 1995). Unlike other leukoencephalopathies, MLC has a relatively mild clinical course.

Genetically, MLC is a heterogeneous condition. A first locus was mapped on chromosome 22q13.33 in Turkish families (Topcu et al., 2000). Mutations in *MLC1* were later found to be the molecular basis for this disorder in families of different ethnicities (Leegwater et al., 2001; Ben-Zeev et al., 2002; Leegwater et al., 2002; Blattner et al., 2003; Saijo et al., 2003; Gorospe et al., 2004), including our own series from the Mediterranean area (Patrono et al., 2003). Additionally, families unlinked to the MLC1 locus have been reported (Leegwater et al., 2001) and the existence of at least another locus is hypothesized (Blattner et al., 2003; Patrono et al., 2003).

MLC1 encodes a 377-amino acid protein which is believed to be a putative membrane protein with eight predicted transmembrane domains. MLC1 is expressed in neurons — preferentially in certain axonal tracts —, in astrocyte-astrocyte membrane contact regions (Boor et al., 2005), and in other neuronal barriers, such as the pia mater and the ependyma (Teijido et al., 2004). On the basis of its weak structural homologies, the gene product is believed to be similar to an ion channel (Lehmann-Horn and Jurkat-Rott, 1999) but, as yet, there is no strong experimental evidence that it is the case.

There is a wide array of mutations in *MLC1*, without a major common mutation. In some specific areas, ancestral mutations have been found consistent with a founder effect (Meyer et al., 2001; Ben-Zeev et al., 2002; Leegwater et al., 2002; Gorospe et al., 2004). Almost nothing is known about the pathogenic mechanism of these mutations, but recent heterologous expression studies proposed that gene mutations impair protein folding (Teijido et al., 2004).

In the wake of molecular confirmation of new cases of MLC, we studied nine patients, identified *MLC1* mutations in eight of them, expressed heterologously and studied biochemically some of the new mutant alleles; we also attempted to correlate residual protein presentation at the plasma membrane with clinical features.

MATERIALS AND METHODS

Patients

This study was performed according to a protocol reviewed and approved by the Ethics Committees of our various institutions. A total of nine probands from unrelated families of different ethnicities diagnosed with MLC in different neuropediatric centers were analyzed. All the patients were evaluated by at least two expert neurologists and one neuroradiologist who have a wide knowledge of genetic disorders of the white matter. All the patients underwent a careful recording of their cognitive and motor milestones and were neurophysiologically assessed as in our previous work (Patrono et al., 2003). The patients then underwent molecular testing at a single center (EB and FMS) after fulfilment of institutional guidelines for genetic analyses. Autosomal recessive (AR) inheritance was present in two families with multiple affected siblings. The remaining cases were apparently sporadic.

Genetic analyses

Total genomic DNA was purified from peripheral leukocytes or lymphoblastoid cell lines using a standard salting-out procedure. No prior linkage data were available. Oligonucleotide primers to amplify all *MLC1* exons were as previously described (Patrono et al., 2003). Identical amplification conditions were used in a total volume of 50ul containing 250nM dNTPs, 150ng of template DNA, 0.1mM of each of the primers, 1.0U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA), in 1X reaction buffer (10mM Tris HCl pH 8.3, 50mM KCl, 2.5mM $MgCl_2$). The specificity of the amplified products was verified by 1% agarose gel electrophoresis before further analysis. After amplification, fragments were gel-purified with MinElute Spin Columns (Quiagen, Hidden, Germany) and submitted to direct sequencing on an ABI377 sequencer (Applied Biosystems, Foster City, CA). Changes were confirmed by a repeated PCR and by sequencing both strands of the resulting product. When a new *MLC1* mutation was identified in a proband, a PCR-restriction fragment length polymorphism (PCR-RFLP) method was employed to confirm the change within the family, whenever possible. A panel of 300 ethnically matched control chromosomes was also screened to substantiate novel variants and rule out their presence in normal chromosomes.

Molecular biology and expression in *Xenopus* oocytes

Mutant constructs were made with recombinant PCR, cloned into the vector pTLN-MLC1 containing two HA epitope tags (Teijido et al., 2004), and sequenced completely. Capped complementary RNA of the different constructs was obtained using SP6 polymerase and the mMESSAGE mMachine transcription kit (Ambion, Austin, TX), and expressed in Xenopus oocytes as described (Teijido et al., 2004).

Oocyte extraction and Western blot analysis

Oocytes were homogenized in lysis buffer containing 150mM NaCl, 20mM Tris (pH 7.6), 1% Triton X-100, and a protease inhibitor mixture (Complete; Roche Molecular Biochemicals, Germany). Insoluble material was separated by centrifugation, and the clear supernatant was processed for Western blot analysis on a 10% SDS-polyacrylamide gel. We used a rat monoclonal antibody (3F10; 200ng/ml; Roche Molecular Biochemicals, Germany) directed against the HA epitope we had added to MLC1 constructs. Primary and secondary (horseradish peroxidase-conjugated donkey anti-rat IgG, 1:10,000) antibodies were diluted in PBS containing 5% nonfat milk powder and 0.1% Triton X-100. Antigen-antibody complexes were detected by enhanced chemioluminescence (ECL, Amersham, UK) and exposed on X-ray films. Quantification of bands was performed using the ImageJ program (http://rsb.info.nih.gov/ij/).

Measurement of surface expression in *Xenopus* oocytes by luminescence

HA antibodies and chemiluminescence were used to measure surface expression in Xenopus, as previously described (Teijido et al., 2004). Oocytes were placed in ND96 with 1% BSA for 30 min at 4°C, then incubated with 1 g/ml rat monoclonal anti-HA antibody (3F10, Roche Molecular Biochemicals, Germany) in 1% BSA/ND96 for 60 min at 4°C, washed at 4°C, and incubated with horseradish peroxidase-coupled secondary antibody (donkey anti-rat IgG, Jackson ImmunoResearch Europe, Soham, UK), in 1% BSA for 30-60 min at 4°C). Oocytes were washed thoroughly (1% BSA, 60 min, 4°C) and transferred to ND96 without BSA. Individual oocytes were placed in 50 l of Power Signal Elisa solution (Pierce, USA). Chemiluminescence was quantified in a Turner TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA, USA).

RESULTS

A total of nine unrelated MLC patients were studied. Table 1 summarizes the clinical and molecular findings in our patients.

Table 1. Summary of major clinical and molecular findings in MLC patients

Pt.	Sex	Age	Macrocephaly (months)	Independent walking (months)	Loss of walking (years)	Mental retardation	Seizures	*MLC1* mutation at the cDNA levels (protein effect)
1	F	6 y	6	24	-	no	yes	c.109delC **(p.Leu37fs)**
2	M	25 y	5	30	23	yes	yes	c.237A>G **(p.Met80Val)**
3	M	15 y	4	17	4	yes	yes	c.387G>A **(p.Gly130Arg)**
4	F	21 y	12	13	20	yes	yes	c.603G>A/c.805C>A **(p.Glu202Lys / p.Ser269Tyr)**
5	M	6 y	6	20	-	yes	yes	c.277C>T/c.823C>A (p.Ser93Leu / **p.Ala275Asn)**
6	M	26 y	birth	30	-	yes	yes (24 y)	c.249G>T (p.Leu83Phe)
7	M	21 mo	6	19	-	no	no	c.323delT/c.929insGCT**(p.Ile108 fs / p.Leu310_311insLeu)**
8	F	24 mo	1	24	-	yes	yes	c.274C>T/c.514_515delAA (p.Pro92Ser /**p.Cys171fs)**
9	M	15 y	1	14	-	no	no	None detected

New mutations are in bold. Mutation nomenclature follows the format indicated in http://www.hgvs.org/mutnomen and it considers as +1 the A of the first methionine in the reference cDNA sequence (NM_015166.2).

A total of twelve different mutations were identified, of which nine were novel. A sample of the novel mutations identified in this study is shown in Figure 1. Nomenclature of mutations follows the format indicated in http://www.hgvs.org/mutnomen and refers to the cDNA sequence (Genbank mRNA no. NM_015166.2) in which we designated the A of the first methionine as nucleotide +1. The corresponding protein changes are also shown.

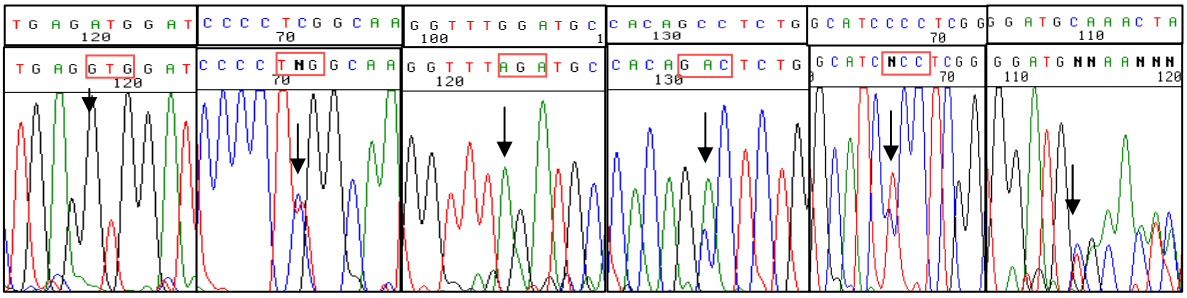

Figure 1. Identification of novel variants in *MLC1*. A sampling of electropherogram obtained from MLC1 patients harboring novel gene variants in this study. The novel mutations are indicated (codons are boxed). The control sequence is superimposed.

Three frameshift mutations were identified. The c.109delC (p.Leu37fs), the c.323delT (p.Ile108fs), and the c.514_515delAA (p.Cys171fs) changes were clearly pathogenic in that they led to premature translation termination with the predicted protein shortened by 85, 68, 52%, respectively. A heterozygous single leucine insertion (c.929insGCT, p.Leu310_311insLeu) was found in patient #7. The presence of an extra leucine might perturb the protein structure and probably impair its function. The remaining mutations (p.Met80Val, p.Gly130Arg, p.Glu202Lys, p.Ser269Tyr, and p.Ala275Asn) were all missense, occurred in either homozygosity or heterozygosity (see Table 1), affected amino acids conserved between rat, mouse and human proteins (Fig. 2), and were not detected in 300 control chromosomes. On the basis of these indirect criteria, the aforementioned changes were deemed pathogenic. Three additional missense changes (c.249G>T, p.Leu83Phe, c.274C>T, p.Pro92Ser, and c.277C>T, p.Ser93Leu) have been already associated with MLC elsewhere (Leegwater et al., 2002; Patrono et al., 2003; Saijo et al., 2003). Interestingly, p.Ser93Leu is considered a “common” Japanese *MLC1* variant (Saijo et al., 2003).

We did not detect *MLC1* mutations in one child born to consanguineous parents. Another child, related to the family, is reportedly affected. Although our PCR-based mutation strategy cannot formally rule out the presence of heterozygous large deletions and mutations in intronic regulatory elements, or even in the as yet unknown promoter region, it is possible that mutations in a different gene are responsible in these two cases. Preliminary results of analyses of polymorphic microsatellites located in and around the MLC1 locus on chromosome 22qter, and used for genotyping the patients, are consistent with this hypothesis (data not shown).

Of 14 relatives investigated, we detected the specific mutation in 13. They were all clinically and neuroradiologically healthy.

In this study we also identified two polymorphisms (p.Cys171Phe and p.Ans344Arg) already reported in patients bearing *MLC1* mutations (Leegwater et al., 2002; McQuillin et al., 2002). However, we did not test their frequency in control chromosomes and cannot rule out a possible role as gene modifiers.

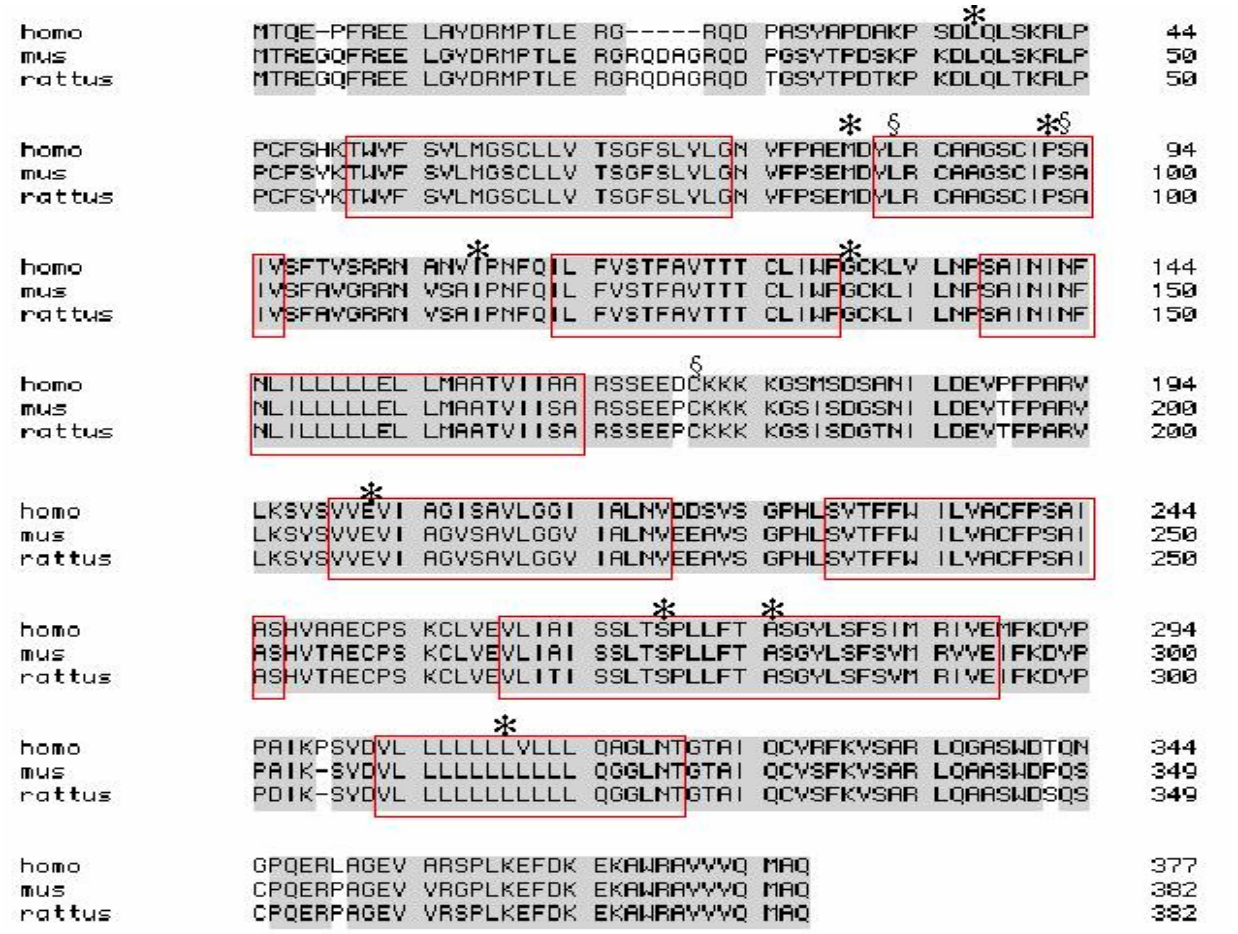

Figure 2. Protein alignment of MLC1 and new variants identified in this study. Novel mutations identified in this study are marked with a star. Variants described elsewhere are marked with a § symbol. SwissProt accession numbers of cited proteins are as follows: *Homo sapiens* MLC1 (homo) Q15049; *Mus musculus* MLC1 (mus) Q8VHK5; *Rattus norvegicus* MLC1 (rattus) XP_235558 (predicted).

To gather more information on the effect of *MLC1* variants, three new missense mutations (p.Glu202Lys, p.Ser269Tyr, p.Ala275Asn), as well as the single leucine insertion identified in this study, were functionally expressed in *Xenopus* oocytes (Teijido et al., 2004). The criteria for selecting mutations were either first detection in our cases or interesting position in the protein, or both. Mutations were introduced by PCR in human MLC1 protein containing two *HA* epitope tags. The insertion of a leucine at residue 311 is present in a conserved poly-leucine stretch. We injected cRNA of wild-type MLC1 and each mutant into oocytes and measured MLC1 expression by Western blot analyses (Fig. 3A) and plasma membrane expression by a luminescence method (Fig. 3B).

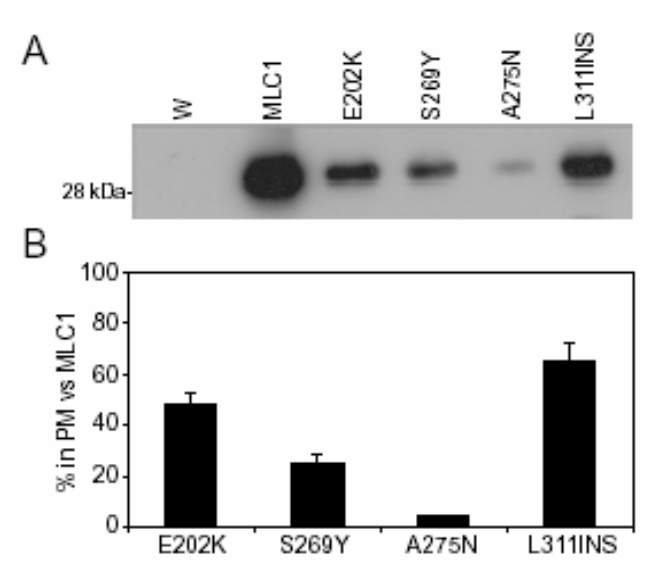

Figure 3. Biochemical studies of new *MLC1* mutations in Xenopus oocytes. Oocytes were injected with 10 ng of each cRNA construct. Plasma membrane levels (**B**) were measured as described in the methods section, and the same oocytes were processed for Western blotting (**A**). The signal of uninjected oocytes was always 5% below the signal of MLC1 wild-type injected oocytes. Surface expression of the mutant proteins was normalized with the expression levels of MLC1 wild-type. Lanes E202K, S269Y, A275N, and L311ins correspond to mutations p.Glu202Lys,p. Ser269Tyr, pAla275Asn, p.L310_311insLeu, respectively. The figure summarizes the results of three experiments involving the measurement of n > 30 oocytes. One typical Western blotting experiment is shown, and two other independent experiments gave similar results.

All *MLC1* mutations produced a decrease in total MLC1 protein expression. In two independent experiments, the expression of p.Glu202Lys was reduced to about 40%, p.Ser269Tyr to about 20%, p.Ala275Asn to about 11% and p.Leu310_311insLeu to about 55% of wild-type values. MLC1 plasma membrane expression was also decreased in parallel. In comparison to MLC1 expression at the plasma membrane (100%), the expression of p.Glu202Lys was reduced to 48% (n = 47), p.Ser269Tyr to 25% (n = 35), p.Ala275Asn to 4 % (n = 31) and p.Leu310_311insLeu insertion to 65% (n = 54).

DISCUSSION

First described in 1995 (van der Knaap et al., 1995), and confirmed the following year in a different population (Singhal et al., 1996), the syndrome of megalencephalic leukoencephalopathy with subcortical cysts (or simply MLC) is a relatively rare myelinopathy associated with mutations in a gene (*MLC1*) of still unknown function. MLC usually occurs in infancy and is easily differentiated from other conditions associated with megalencephaly ("big head circumference") presenting in infancy. In the differential diagnosis, one should consider Alexander disease, which shows white matter degeneration of the frontal lobe and is associated with mutations in the *GFAP1* gene, Canavan disease, which is accompanied by blindness and spasticity but has a more severe course, and glutaric aciduria type I, which is more easily identified through complete determination of urinary organic profiles (reviewed in Schiffmann and van der Knaap, 2004). Genetic testing appears to assist in clinical diagnosis.

Although MLC patients have a relatively mild clinical course compared to cases presenting with similar white matter disorders, the impairment of motor functions, the occurrence of seizures — which are well controlled by therapy with drugs such as carbamazepine and valproic acid — and most notably the presence of a clear cognitive decline, prevent MLC patients from leading a normal life.

MLC has a wide heterogeneity both within and between families and we speculated that this might be related to specific genetic determinants (Patrono et al., 2003). In an attempt to understand the heterogeneity of the phenotype between different mutations in *MLC1*, we wanted to compare the clinical phenotype of the patients with the biochemical phenotype of the corresponding mutations (Teijido et al., 2004). Interestingly, although all the studied mutations reduced total and plasma membrane expression in comparison with wild-type MLC1, not all the mutations produced the same effect, being the insertion of a single leucine in the poly-leucine stretch of the

putative last transmembrane domain the least severe. In other membrane proteins, poly-leucine stretches have been related to oligomerization (Torres et al, 2003). It remains to be determined whether this process also occurs in MLC1.

We did not detect straightforward correlation between mutation, severity of amino acid change, predicted length of the residual protein (in cases of frame-shift variants), and clinical manifestations. In particular, no major difference was observed between patients who presented early in infancy (pts# 3, 4) and the patient who manifested his symptoms in late adolescence (pt # 7). Moreover, the extent of the mental retardation did not appear to be influenced by the genetic defect (Table 1). Nonetheless, the patient carrying the mutation with the mildest biochemical phenotype (pt # 7, Leu310_311insLeu) shows neither mental retardation nor seizures. Although the cognitive deterioration needs to be more carefully defined, it is tempting to speculate that additional genetic factors or epigenetic determinants somehow influence clinical presentation and progression in this condition.

The patient without mutations in *MLC1* did not significantly differ from those harboring variants in the gene. This agrees with our early report (Patrono et al., 2003). It is highly possible that an unknown protein that acts in partnership with MLC1 accounts for a smaller subset of MLC patients.

Summarizing, we reported new variants in MLC patients, expressed some of them functionally studying their effects in a heterologous system, and did not find clear evidence of straightforward genotype-phenotype correlations. More functional approaches must be tried in the attempt to uncover the correlations that may exist between gene mutation and clinical manifestations.

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