Muscleblind-like protein 1 nuclear sequestration is a molecular pathology marker of DM1 and DM2

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Myotonic dystrophies (DM) are repeat expansion diseases in which expanded CTG (DM1) and CCTG (DM2) repeats cause the disease. Mutant transcripts containing CUG/CCUG repeats are retained in muscle nuclei producing ribonuclear inclusions, which can bind specific RNA-binding proteins, leading to a reduction in their activity. The sequestration of muscleblind-like proteins (MBNLs), a family of alternative splicing factors, appears to be involved in splicing defects characteristic of DM pathologies. To determine whether MBNL1 nuclear sequestration is a feature of DM pathologies, we have examined the *in vivo* distribution of MBNL1 in muscle sections from genetically confirmed DM1 (n=7) and DM2 (n=9) patients, patients with other myotonic disorders (n=11) and from patients with disorders caused by repeat expansions, but not DM1/DM2 (n=3). The results of our immunofluorescence study indicate that, among patients examined, MBNL1 nuclear sequestration in protein foci is a molecular pathology marker of DM1 and DM2 patients where ribonuclear inclusions of transcripts with expanded CUG/CCUG repeats are also present. These findings indicate that MBNLs might be important targets for therapeutic interventions to correct some of the specific features of DM pathology.

Key words: myotonic dystrophies; MBNL1, muscle biopsy, immunofluorescence.

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Myotonic dystrophies (DM) are dominantly inherited multisystemic disorders with similar clinical, histological and genetic features. They belong to the category of diseases now commonly know as unstable repeat expansion disorders since they are caused by two different microsatellite expansions. Myotonic dystrophy type 1 (DM1) is caused by a CTG trinucleotide expansion in the 3' untraslated region of the dystrophya myotonica-protein kinase (DMPK) gene on chromosome 19 (Brook *et al.,* 1992, Fu *et al.,* 1992; Mahadevan *et al.,* 1992), whereas myotonic dystrophy type 2 (DM2) is caused by a CCTG tetranucleotide expansion in intron 1 of the zinc finger protein 9 (*ZNF9*) gene on chromosome 3 (Ranum *et al.,* 1998; Liquori *et al.,* 2001). A characteristic disease feature is that transcripts from the mutant allele cannot be exported to the cytoplasm but are retained in muscle nuclei producing ribonuclear inclusions that can be evidenced by in situ hybridization (Taneja *et al.,* 1995; Davis *et al.,* 1997; Liquori *et al.,* 2001; Mankodi *et al.,* 2001, 2003; Cardani *et al.,* 2004).

One proposed mechanism to explain the molecular basis of DM pathogenesis is that nuclear accumulation of mutant RNAs that contain expanded CUG or CCUG repeats adversely affects nuclear functions. An RNA gain of function mechanism via sequestration or aberrant activation of RNA-binding proteins, is one of the mechanism proposed to explain how ribonuclear inclusions might play a role in DM pathogenesis. Several proteins have been suggested to interact with CUG/CCUG repeats in *in vitro* or *in vivo* experiments, including CELF proteins (CUG-binding protein 1 (CUG-BP1) and elav-like RNA-binding protein 3 (ETR3)), protein kinase R (PKR) and muscleblind-like proteins (MBNLs) (Timchenko *et al.,* 1996; Tian *et al.,* 2000; Miller *et al.,* 2000).

MBNLs were identified as a family of RNA-binding proteins by Miller *et al.,* (2000). These proteins are homologus of muscleblind in *Drosophila*

melanogaster where it is involved in the terminal differentiation of muscle (Artero *et al.,* 1998) and of photoreceptor cells in the eye (Begemann *et al.,* 1997). Three mammalian muscleblind genes have been recognized although their function is not well understood: MBNL1, MBNL2 and MBNL3 (Miller *et al.,* 2000; Fardaei *et al.,* 2002). It has been demonstrated that MBNLs interact with CUG/CCUG repeats and with other repetitive sequences in a yeast three-hybrid system and in *in vitro* binding experiments (Kino *et al.,* 2003, 2004). By using fluorescence in situ hybridization (FISH), it has been reported that MBNLs, in contrast to CUG-BP and PKR, showed a clear colocalization with DM1 or DM2 ribonuclear inclusions both in muscle tissue and cell cultures (Fardaei *et al.,* 2001, 2002; Mankodi *et al.,* 2001, 2003). These observations seem indicate that muscleblind proteins are sequestered in CUG/CCUG containing RNA foci and they cannot perform their normal functions. An indication that sequestration of MBNLs plays a central role in DM pathogenesis comes from recent studies of Kanadia *et al.,* (2003). These authors developed a muscleblind knockout model of myotonic dystrophy in which disruption of the mouse Mbnl1 gene leads to muscle, eye and RNA-splicing abnormalities that are characteristic of DM disease. More recently Ho *et al.,* (2004) demonstrated that these proteins are splicing factors and compete with CUG-BP1 to regulate alternative splicing of specific pre-mRNA that are misregulated in DM pathogenesis. At least 5 of the 13 misregulated splicings observed in DM pathologies are consistent with the loss of MBNL1 and/or gain of CUG-BP1 activity. Splicing alterations that lead to aberrant expression of several proteins, including chloride channel, insulin receptor and cardiac troponin c, underlie the complex multisystemic phenotype of DM disorders like myotonia, insulin resistance and cardiac defects (Gatchel *et al.,* 2005; Day and Ranum, 2005).

In our study we have examined if MBNL1 depletion, due to nuclear sequestration, is a characteristic feature of DM pathologies where ribonuclear inclusions are also present. Thus, we have compared the *in vivo* MBNL1 distribution observed in muscle biopsies from DM patients with that observed in muscle biopsies from patients with other myotonic disorders and in patients with other types of disorders caused by repeat expansions similar to those of DM pathologies. Spinobulbar muscular atrophy (SBMA) and Huntington's disease (HD) are neurological diseases caused by the expansions of CAG repeat that lead to proteins with expanded polyglutamine stretches. However, evidences suggest that, as for DM pathologies, RNA with expanded repeat are present in the nucleus and form RNA-protein complexes with RNA-binding proteins that lead to neuronal and muscle degeneration (Broude and Cantor, 2003).

Materials and Methods

Tissue samples and patients

Human muscle biopsies from biceps brachii muscle were taken under sterile conditions and prepared by snap-freezing in cooled isopentane. The biopsies were used for this study after informed consent from patients.

The clinical diagnosis of DM1 (n=7) and DM2 patients (n=9) was based upon the criteria set by the International Consortium for Myotonic Dystrophies (Moxley *et al.,* 2002). The histological diagnosis was performed on serial sections (10 μm) of muscle biopsies from all of these patients processed for routine histological or histochemical stainings. A standard myofibrillar ATPase staining protocol was used after preincubation at pH 4.3, pH 4.6, and pH 9.4 or 10.4 (Dubowitz, 1985). Antibodies against different myosin heavy chain (MHC) isotypes were used for immunohistochemical identification of very small fast or slow fibers according to Vihola *et al.,* (2003).

The genetic confirmation of DM2 was performed by repeat assay described by Bachinski *et al.,* (2003). DM1 patients were genetically confirmed by PCR or Southern blot on DNA lymphocytes. Fluorescence in situ hybridization was performed on frozen sections from DM1 or DM2 patients using a (CAG)$_6$CA or (CAGG)5 probe respectively, as reported by Cardani *et al.,* (2004).

A total of 8 patients with non-dystrophic myotonic disorders (6 chloride channelopathies, 2 sodium channelopathies), 3 non-DM1/DM2 patients, 1 patient affected by spinal and bulbar muscular atrophy (SBMA) and 2 affected by Huntington's disease (HD) were also examined. Control muscle biopsies were taken from healthy subjects (n=5).

MBNL1 immunofluorescence

Transverse muscle cryostat sections (5-6 μm) were dried for 1 hour at room temperature and then

fixed in 2% paraformaldehyde for 30 min. at 4°C. Sections were washed five times in phosphate-buffered saline (PBS) for 3 min. at room temperature and then preincubated with normal goat serum (DAKO) at a dilution 1:20 in PBS containing 2% bovine serum albumin (BSA) for 20 min. at room temperature. The primary antibody, a polyclonal rabbit anti-muscleblind1 (MBNL1; gift of Prof. C.A. Thornton, University of Rochester, New York, USA) was applied at a dilution of 1:1000 in PBS+2% BSA overnight at 4°C. After washing in PBS 3 times for 5 min., sections were incubated with secondary antibody (Alexa488-labeled goat anti rabbit; Molecular Probes) diluted 5 μg/μL in PBS+2% BSA for 1 hour at room temperature. Following incubation, sections were washed 3 times with PBS and nuclei were stained with 165 nM 4,6 diamidino-2-phenylindole (DAPI). Sections were then mounted with Gel Mount mounting medium (Biomedia corp) and examined using a fluorescence microscope.

FISH and immunofluorescence

FISH procedure, using RNA (CAG)6CA or (CAGG)5 Texas red labelled probes (IDT, Coralville, IA) on DM1 or DM2 muscle sections respectively, was carried out as previously reported by Cardani *et al.*, (2004). Following the 1XSSC post-hybridization wash without DAPI, the immunofluorescence protocol was performed as described above starting from the incubation with normal goat serum. The interaction between ribonuclear inclusions and MBNL1 nuclear accumulation was examined by analysis of images obtained at confocal microscopy.

Results

We have used an anti-MBNL1 polyclonal antibody to examine the distribution of MBNL1 in sections of skeletal muscle from DM1 or DM2 patients, in patients with other myotonic disorders and in patients with other types of repeat expansions disorders. In skeletal muscle from healthy patients, MBNL1 is localised primarily in the nucleus where is uniformly distributed. The same distribution is observed in nuclei of skeletal muscles from the 7 patients with chloride or sodium channelopathies, from the 3 non-DM1/DM2 patients and from the patients affected by SBMA or HD (Figure 1A-C). In these patients, the intensity of immunofluorescence nuclear staining appears to be equal to that observed in muscle nuclei of healthy controls. In DM1 and DM2 patients, myonuclei show intensely fluorescent MBNL1 foci that represent high concentration of the protein (Figure 1D, E). Although MBNL1 nuclear sequestration, MBNL1 is still expressed in the nucleoplasm both in DM1 and DM2 myonuclei, but on the whole, the immunofluorescence intensity of the nucleoplasm seems to be lower in DM nuclei than in the nuclei of all the other muscle sections examined. The analysis of the number of MBNL1 foci per nucleus reveals that the majority of DM1 myonuclei show 1 focus per nucleus and more rarely 2 foci per nucleus, whereas 1 to 3 foci per nucleus are found in DM2 muscle sections. However, nuclei without protein foci are also present both in DM1 and DM2 muscle sections. Moreover, it should be noted that MBNL1 foci appear to be larger in DM2 than in DM1 nuclei. The nuclear MBNL1 accumulation observed in DM1 and DM2 muscle nuclei is completely absent from the myonuclei of all the control muscle sections examined.

FISH analysis, using (CAG)6CA or (CAGG)5 probe on DM1 and DM2 muscle sections respectively, has confirmed that DM patients used in this study showed nuclear accumulations of mutant transcripts, with no foci observed in nuclei of muscle sections from healthy patients. By using FISH in combination with immunofluorescence, it appears that focal accumulation of MBNL1 in DM myonuclei co-localize precisely with the foci of transcripts containing the CUG/CCUG expansions (Figure 1F-H).

Discussion

Evidences suggest that in DM disease, ribonuclear inclusions are directly involved in pathogenesis through a gain-of-function mechanism that involves protein sequestration. Among the different proteins suggested to interact with CUG/CCUG repeats, only muscleblind proteins show a clear colocalization with RNA foci both in muscle tissue and cell cultures (Fardaei *et al.,* 2001, 2002; Mankodi *et al.,* 2001, 2003).

Our examination of MBNL1 expression in skeletal muscle sections show that nuclear sequestration of this protein is a feature of DM pathology where ribonuclear inclusions of transcripts with expanded CUG/CCUG repeats are also present. So, the pres-
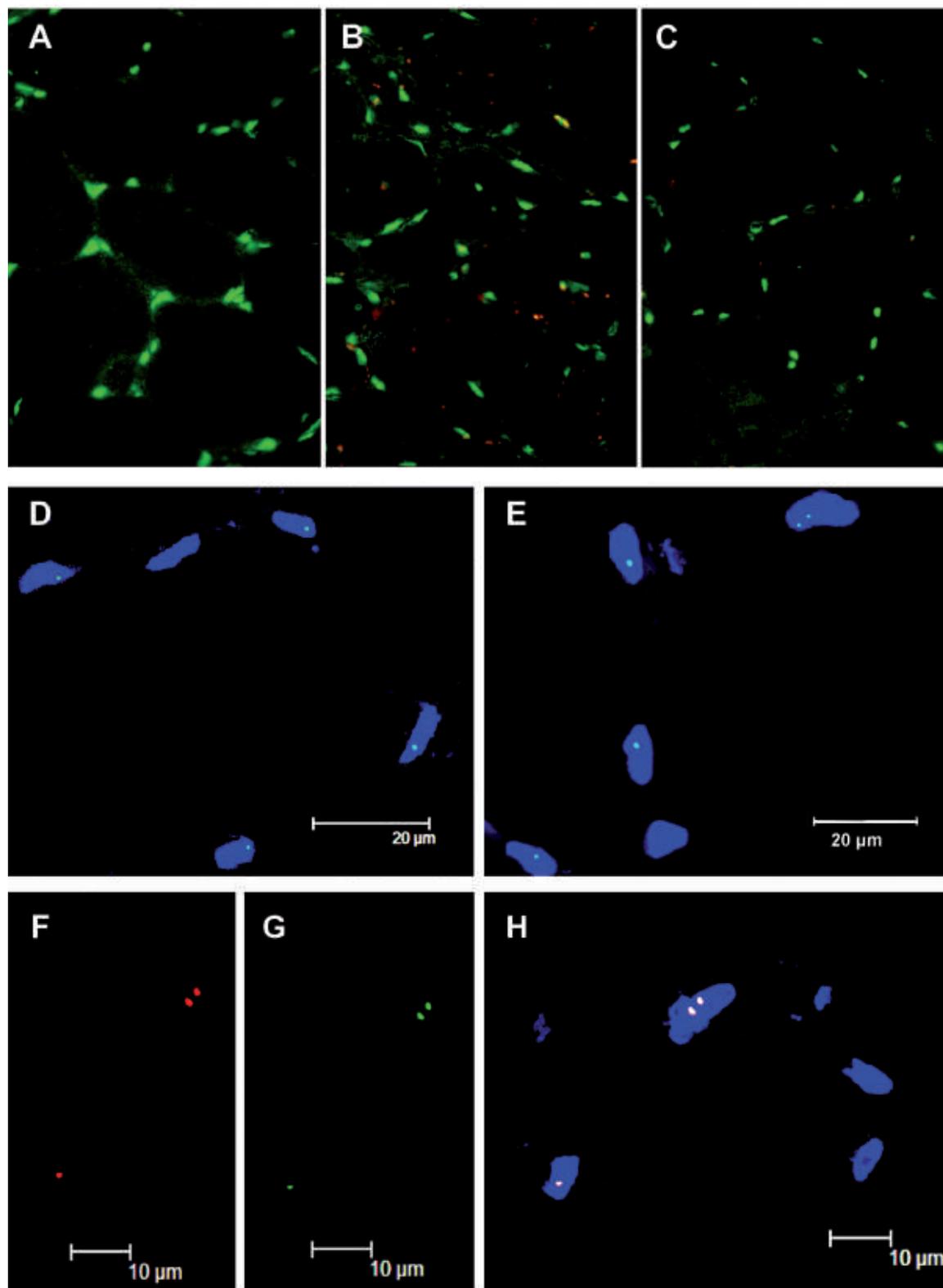
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Figure 1. A-C. Immunofluorescence analysis of MBNL1 distribution in human skeletal muscle tissue at fluorescence microscopy. MBNL1 is localized primarily in the nucleus of muscle sections from patient with chloride channelopathy (A), from a non-DM1/DM2 patient (B) and from a patient affected by Huntington's disease (C). Brown material present in (B) is autofluorescent lipofuscin. Original magnification x600. D-H. Analysis of MBNL1 distribution at confocal microscopy in DM muscle sections. Foci of MBNL1 are present in muscle nuclei of a DM1 patient (D) and of a DM2 patient (E). Ribonuclear inclusions (red) (F) and foci of MBNL1 (green) (G) in DM2 muscle evidenced on the same muscle section using FISH in combination with immunofluorescence and acquired at two different channels at confocal microscopy. Merged images show that MBNL1 co-localize with ribonuclear inclusions within DM2 muscle nuclei (blue) (H).

ence of nuclear foci of MBNL1 in DM muscle sections might be considered a molecular pathology marker. Indeed no nuclear foci of the protein are detectable either in non-DM patients with myotonic disorders or in patients with neurological disorders that are caused by unstable repeats similar to those observed in DM patients. The size and number of MBNL1 foci appear to be greater in DM2 nuclei than in DM1, as expected since ZFN9 mRNA is more abundant than DMPK mRNA and the size of expanded repeats is greater in DM2 than in DM1 (Mankodi and Thornton, 2002) This could indicate that MBNL1 is sequestred in ribonuclear inclusions more in DM2 than in DM1, even if this suggestion does not seem to be associated to a reduction of protein expression in DM2 myonuclei more than in DM1 myonuclei. The observation at confocal microscope of muscle sections stained by FISH in combination with immunofluorescence, show a clear colocalization of ribonuclear inclusions with MBNL1 foci. Moreover a reduction of nucleoplasm immunofluorescence staining in the most of DM myonuclei as compared to non-DM muscle nuclei is also appreciable. Taken together these observations indicate that MBNL1 is strongly recruited to ribonuclear inclusions leading to a protein depletion in the nucleoplasm. Similar results are reported by Jiang *et al.,* (2004) whose quantitative analysis of MBNL1 fluorescence intensity showed a lower mean immunofluorescence intensity for MBNL1 in the nucleoplasm in DM1 neurons than in controls.

These results support the hypotesis that depletion of MBNL1 due to sequestration of the protein in ribonuclear inclusions, is involved in several aspects of DM. Indeed, it has been shown that loss of MBNL1 and/or gain of CUGBP1 activity lead to at least 5 misregulated splicing events in DM pathologies that result in aberrant proteins expression. Myotonia, cardiac defects and insulin resistance could be caused by defective regulation of alternative splicing for chloride channel 1, cardiac troponin T and insulin receptor respectively (Savkur *et al.,* 2001, 2004; Philips *et al.,* 1998, Charlet *et al.,* 2002; Mankodi *et al.,* 2002;). Alterations in the splicing of myotubularin-related 1 gene have been also described in muscle tissue or cells from DM1 patients (Buj-Bello *et al.,* 2002). Moreover, splicing alterations of the RNA encoding the microtubule-associated Tau protein could underlie the several CNS alterations observed in

DM patients (Sergeant *et al.,* 2001).

Nevertheless the whole range of aberrantly spliced genes that are involved in DM pathology need to be clarified as well as the protein content of the ribonuclear inclusions besides MBNLs. On the other hand, it is well known that several human disorders are associated with splicing defects and several researches are in progress to test molecular therapies targeting alternative splicing. As alternative splicing factors, MBNLs might be important targets for therapeutic interventions to correct some of the specific features of DM disease.

Acknowledgements

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