Biomolecular identification of (CCTG)n mutation in myotonic dystrophy type 2 (DM2) by FISH on muscle biopsy

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Myotonic dystrophy type 2 (DM2) is a dominantly inherited disorder with multisystemic clinical features, caused by a CCTG repeat expansion in intron 1 of the zinc finger protein 9 (ZNF9) gene. The mutant transcripts are retained in the nucleus forming multiple discrete foci also called ribonuclear inclusions. The size and the somatic instability of DM2 expansion complicate the molecular diagnosis of DM2. In our study fluorescence-labeled CAGG-repeat oligonucleotides were hybridized to muscle biopsies to investigate if fluorescence in situ hybridization (FISH), a relatively quick and simple procedure, could be used as a method to diagnose DM2. When FISH was performed with $(CAGG)_5$ probe, nuclear foci of mutant RNA were present in all genetically confirmed DM2 patients (n = 17) and absent in all patients with myotonic dystrophy type 1 (DM1; n = 5) or with other muscular disease (n = 17) used as controls. In contrast, foci were observed both in DM1 and DM2 myonuclei when muscle tissue were hybridized with $(CAG)_6CA$ probe indicating that this probe is not specific for DM2 identification. The consistent detection of ribonuclear inclusions in DM2 muscles and their absence in DM1, in agreement with the clinical diagnosis and with leukocyte (CCTG)n expansion, suggests that fluorescence in situ hybridization using $(CAGG)_5$ probes, may be a specific method to distinguish between DM1 and DM2. Moreover, the procedure is simple, and readily applicable in any pathology laboratory.

Key words: myotonic dystrophy type 2; diagnostic method; fluorescence in situ hybridization; FISH; CAGG probe; ribonuclear inclusions.

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Myotonic dystrophy type 2 (DM2) is an autosomal dominant, multisystemic disease. It is characterized by muscle weakness, myotonia and by non-muscle manifestations including cataracts, cardiac arrhytmias and hypogonadism (Meola *et al.*, 1999; Moxley *et al.*, 1998, 2002; Meola, 2000; Day *et al.*, 2003; Udd *et al.*, 2003). Muscle biopsies are characterized by fibers with increased number of central nuclei, angulated atrophic fibers, occasional ring fibers, type I fiber predominance and a preferential atrophy of type II fibres. (Day *et al.*, 2003; Schoser *et al.*, 2004; Vihola *et al.*, 2003). Nuclear clump type II fiber atrophy is considered typical of DM2, although, in our experience, 20% of patients with clinical and genetic confirmation of DM2 mutation have normal histograms and no nuclear clump type 2 fiber atrophy (Meola *et al.*, 2004).

The DM2 locus is mapped to a 10 c-M region of chromosome 3q (Ranum *et al.*, 1998) and recently Liquori *et al.* (2001) have reported that DM2 is caused by a large expansion of a CCTG repeat located in intron 1 of the zinc finger protein 9 (ZNF9) gene. ZNF9 contains seven zinc finger domains and is thought to be an RNA binding protein that is expressed in many different tissues. In DM2, expanded allele sizes range from 75 to 11000 CCTG repeats, with a mean of approximately 5000 repeats (Liquori *et al.*, 2001). The mutant ZFN9 gene is transcribed into RNA that contains expanded CCUG repeats and these transcripts are retained in the muscle nucleus, producing foci of mutant RNA also called ribonuclear inclusions (Liquori *et al.*, 2001; Mankodi *et al.*, 2001, 2003). A large body of evidence indicates that these ribonuclear inclusions are responsible for the clinical manifestations of DM2. It has been suggested that CCUG expansion expressed at RNA level could themselves be pathogenic and cause the multisystemic features of DM2 (Liquori *et al.*, 2001). Moreover recent studies have demonstrated that intranuclear RNA foci bind specific

Table 1. F = female; M = male; LLW = lower limb weakness; MRC = Medical Research Council Scale (Medical Research Council. Aids to the examination of the peripheral nervous system. Memorandum 45. 1976, Pendragon House, London.); CK = creatine kinase; AV = atrioventricular; OGTT = oral glucose tolerance test; γ-GT = γ-glutamyl transferase; SVEB = supraventricular ectopic beats.

Patient	*Age/ sex*	*Disease duration*	*Age at onset*	*Symptoms at onset*	*MegaMRC*	*MRC biceps*	*multisystem involvement*
BC	59/F	14	45	proximal LLW	118	4,5	hypoacusia, hyperthyroidism, CK x4
CI	47/F	10	37	distal LLW	134	4,5	diabetes, hyperthyroidism, cataracts, memory loss
CS	49/F	10	39	proximal LLW	118	4	cataracts
FE	60/M	6	54	lower limb stiffness	150	5	visual-spatial deficits
GL	55/F	10	45	proximal LLW	118	4	cataracts, visual spatial deficits
GE	36/M	21	15	myotonia	150	5	neurosensory hypoacusia, 2nd degree AV block, CK x4
GM	43/F	25	18	myalgia	138	5	neurosensory hypoacusia, sacral cysts, uterine fibroma, high glucose levels after OGTT, CK x2, triglycerides x2, visual-spatial deficits, WMHL on brain MRI
NG	62/M	12	40	proximal LLW	124	4	neurosensory hypoacusia, paresthesias of the hands, coronary heart disease, cataracts, cerebellar vermis and corpus callosum atrophy, cholesterol x2, triglycerids x3, CX x2
PV	47/M	17	30	myotonia	132	4,5	testicular motility, baldness, CK x5, γ-GT x2, cholesterol x2,
PL	65/M	15	50	proximal LLW	105	4,5	cataracts, baldness, diabetes, high blood pressure, triglycerides x4, mild mitral prolapse, choreic movements, oro-mandibular dyskinesia
RG	63/M	27	36	proximal LLW	127	4,5	cataracts, feet paresthesias, truncal lipomas, erectile dysfunction, visual-spatial deficits, bradycardia, neurosensory hypoacusia, lower limb venous incontinence, restless leg syndrome, CK x3, γ-globulin < 10%, low back pain
RL	32/F	6	26	myotonia	150	5	none
RO	40/F	15	35	proximal LLW	146	4,5	cataracts, visual spatial deficits, low back pain, hand paresthesias, neurosensory hypoacusia, infertility, tachycardia, CK x2, γ-globulin <10%
RR	44/F	28	16	proximal LLW	77	2,5	visual spatial-deficits, low-back pain, cervical herniated discs, diffuse trunk and limb lipomas, initial baldness, fine distal tremor, SVEB, CK x2
RS	43/M	21	22	proximal LLW	146	4,5	visual spatial-deficits, low-back pain, lumbar herniated discs, initial baldness, SVEB, CK x2
RA	20/F	2	18	myotonia	150	5	none
RAm	71/F	46	25	proximal LLW	112	3	extrapyramidal signs, cataracts, 1st AV block, chronic obstructive broncopulmonary disease

RNA binding proteins and muscleblind that are sequestered in DM2 nuclei, leading to depletion of transcripts that require these proteins for normal gene expression (Mankodi *et al.*, 2001, 2003; Fardaei *et al.*, 2002; Kanadia *et al.*, 2003).

The discovery of DM2 mutation raised the possibility to use molecular testing for a more accurate diagnosis of patients with suspected DM2 since the clinical and histological features of DM2 are similar even if not identical to those of myotonic dystrophy type 1 (DM1) and it is not always easy to distinguish by routine histological and histochemical technique on muscle biopsy these two diseases (Meola, 2000; Moxley *et al.*, 2002). This is especially true when family trees are uninformative and core clinical findings, like clinical EMG are extremely fluctuating and maybe absent when the patient is first seen. Similarly, cataracts, one of the core clinical diagnostic features for DM2, may be absent because evaluation occurs early in the disease. However, molecular testing is not applicable as yet on a routine basis as is CTG expansion PCR or Southern Blot for DM1. The size and somatic instability of the DM2 repeat in fact, complicate the interpretation of data obtained by PCR and Southern analysis that sometimes fail to detect the DM2 expansions (Liquori *et al.*, 2001; Bachinski *et al.*, 2003; Day *et al.*, 2003). To determine the presence of DM2 mutation, Bachinski *et al.* (2003) developed a repeat-primed PCR (RP-PCR), which used in combination with a Field Inversion Electrophoresis (FIGE) Southern blot also determines the size of the expansion. Furthermore, Day *et al.* (2003) describe a repeat assay (RA) based on the amplification of the genomic region with the repeat expansion followed by the Southern analysis of the PCR products. Although these tests increase the detection rate of the DM2 expansion, still they are expensive, not available as routine diagnostic test and much time consuming methods for DM2 diagnosis. Only recently nuclear clump type 2 fiber atrophy (Vihola *et al.*, 2003) and type 2 fiber atro-

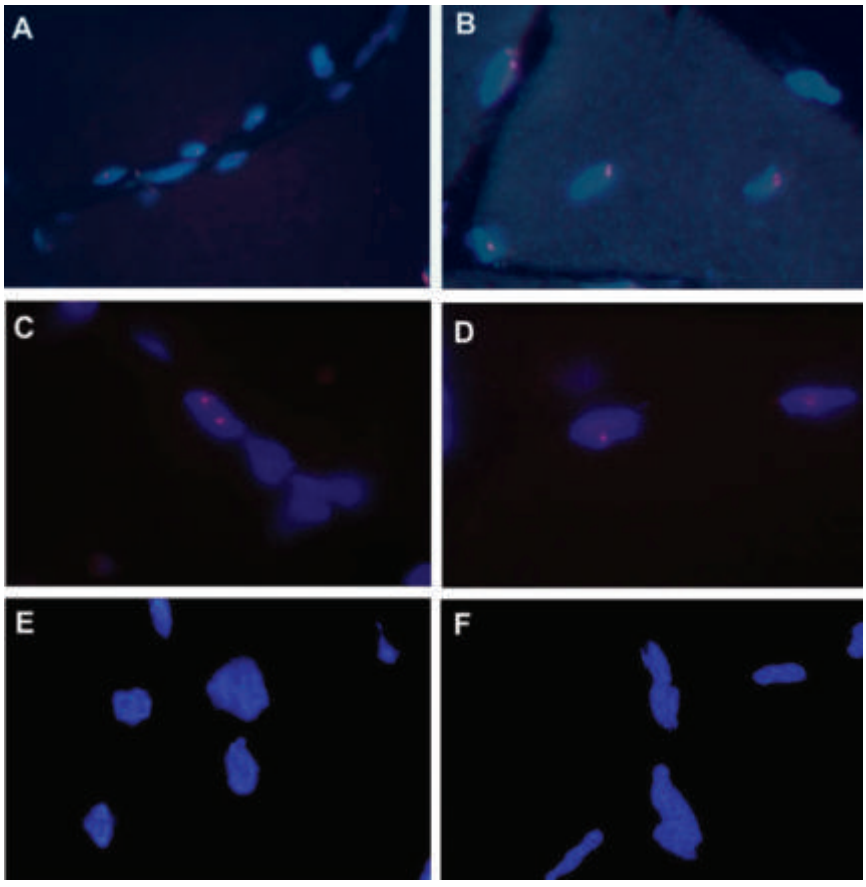

Figure 1. Fluorescence in situ hybridization (FISH) with $(CAGG)_5$ probe on muscle tissue. A-D. Ribonuclear inclusions (red) are present in myonuclei (blue) of muscle tissue from DM2 patients (A: original magnification 400×; D original magnification 1000×). B. Mutant RNA foci (red) are also present in central nuclei (blue) of muscle tissue: note the rod shape of foci suggesting the confluence of 2 or more spot signals (original magnification 630×). C. Multiple ribonuclear inclusion in muscle nucleus in a DM2 patient (original magnification 1000×). E-F. Ribonuclear inclusions are absent in myonuclei in DM1 patient (E: original magnification 1000×) and in patient with other muscular disease (F: original magnification 1000×)

phy (Schoser *et al.*, 2004) have been found typical of DM2 and could be considered an useful tool to target subsequent biomolecular analysis.

Fluorescence in situ hybridization (FISH) is a powerful technique used to localize nucleic acid sequences within the cell. In the present study, fluorescent labelled antisense oligonucleotide probes to the CCUG repeat were hybridized to muscle biopsy tissue from genetically confirmed DM2 patients for the direct visualization of mutant RNA in myonuclei. Our aim was to asses if FISH could be used as a method to diagnose DM2, since the procedures for the application of this technique on tissues are quick, not very complex and share many similarities to those for immunohistochemistry, which is widely used in both basic research and diagnostic laboratories.

Materials and Methods

Patients and tissue samples

The diagnosis of DM2 was based upon the clinical diagnostic criteria set by the International Consortium for Myotonic Dystrophies (Moxley *et al.*, 2002). A description of the clinical features of the DM2 patients used in this study are reported in Table I. Biceps brachii muscle biopsies from genetically confirmed DM2 patients (n=17) were prepared by snap-freezing in cooled isopentane.

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 fast and slow MHC fibers according to Vihola *et al.* (2003). The genetic confirmation of DM2 was performed by repeat assay described by Bachinski *et al.* (2003).

Muscle biopsies from patients with myotonic dystrophy type 1 (DM1; n=5) or with other muscular diseases (n=17) were used as controls. The biopsies were used for this study after informed consent from patients.

Fluorescence In Situ Hybridization (FISH)

Transverse muscle cryostat sections (6 μm) were

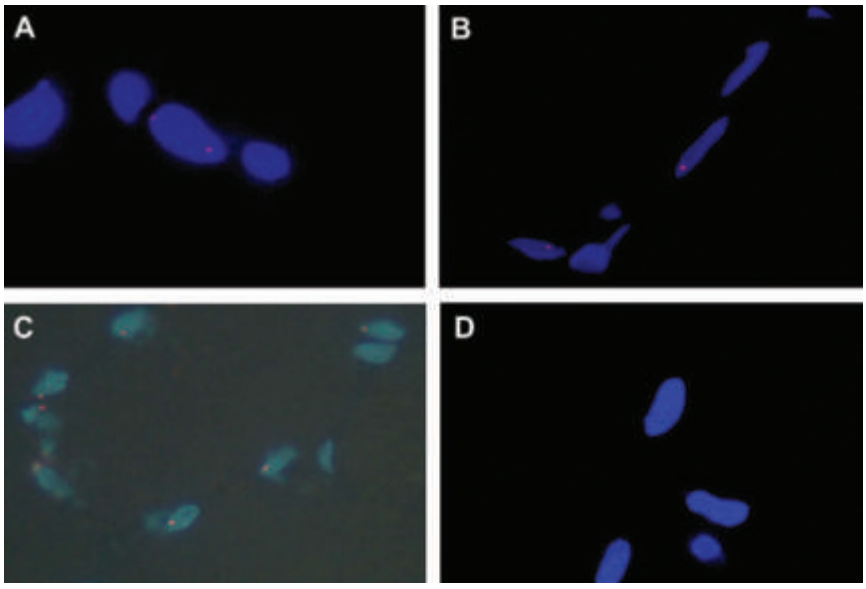

Figure 2. Fluorescence in situ hybridization (FISH) with $(CAG)_6CA$ probe on muscle tissue. A,B. Ribonuclear inclusions (red) are present in nuclei (blue) of muscle tissue from DM1 patients (A: original magnification 1000×; B original magnification 1000×). C. ribonuclear inclusion (red) are also present in nuclei (blue) of muscle tissue from DM2 patient (original magnification 400×).D. No foci are present in myonuclei in patient with other muscular diseases (F: original magnification 1000×)

dried for 30 min at room temperature and fixed in 2% paraformaldehyde for 30 min at 4°C. After fixation, sections were washed five times in phosphate-buffered saline (PBS) for 3 min at room temperature, and permeabilized in 2% acetone PBS (pre-chilled at –20°C) for 5 min. After washing in PBS, sections were incubated in 30% formamide and 2xSSC for 10 min at room temperature and then hybridized with probe (1 ng/μL for 2 h at 37°C in 30% formamide, 2xSSC, 0.02% BSA, 67 ng/μL yeast tRNA, 2 mM vanadyl ribonucleoside complex. Sections were washed first in 30% formamide and 2xSSC at 45°C for 30 min, then in 1xSSC and 165 nM 4,6 diamidino-2-phenylindole (DAPI) at room temperature for 30 min. Sections were then mounted with Gel Mount mounting medium (Biomedia Corp). Probes were HPLC-purified 2-O-methhyl RNA CAGG or CAG repeat 20-mers and 5'Texas red labelled (IDT, Coralville, IA). Muscle sections were examined using a fluorescence microscope or sometimes using a confocal microscope. In order to get the same treatment, one section from both DM2 and control patient muscle tissue were placed on the same slide.

Results

Fluorescently-labelled antisense oligonucleotide $(CAGG)_5$ probe was used for in situ hybridization to detect the ribonuclear inclusions containing the CCUG expansion in muscles from genetically confirmed DM2 patients. Intense nuclear foci of hybridization were observed in muscle tissue from each of 17 DM2 individuals considered (Figures 1A-D). The number of foci was 1 to 3 per nucleus with no foci detected in the cytoplasm (Figures 1A, B). In contrast, no nuclear foci were detected when $(CAGG)_5$ probe was hybridized to muscle tissue from DM1 patients or from subjects with other muscle diseases used as controls (Figures 1E, F). $(CAGG)_5$ probe generated a high background in the nucleoplasm both in DM2, DM1 and control muscles, but foci of hybridization were easily distinguished since they resulted more intensely fluorescent than the background hybridization. When $(CAG)_6CA$ probe was hybridized to DM2 muscle tissue, mutant RNA was also identified and RNA foci appeared to be smaller and less fluorescent than those identified by $(CAGG)_5$ (Figure 2C). As expected, $(CAG)_6CA$ probe also evidenced ribonuclear inclusions myonuclei of DM1 muscle sections (Figure 2A, B). Indeed DM1 patients, unlike DM2 patients, have an abnormally high number of CTG repeats in the gene for myotonic dystrophy (DM protein kinase gene) (Brook *et al.*, 1992; Mahadevan *et al.*, 1992). As for DM2, mutant transcripts containing the expanded repeats are retained in myonuclei producing inclusions similar to those of DM2 (Taneja *et al.*, 1995). Nuclear foci were not observed in control muscle sections hybridized with $(CAG)_6CA$ probe (Figure 2D). No nuclear background was observed in any muscle sections when hybridized with $(CAG)_6CA$ probe. In summary our results show that $(CAGG)_5$ probe

identifies ribonuclear inclusions in DM2 patients only, whereas $(CAG)_6CA$ probe can detect foci of mutant RNA both in DM1 and DM2 patients.

Discussion

Conventional genetic tests used for myotonic dystrophy type 1 diagnosis, such as Southern blot analysis or PCR, are not reliable for DM2 identification (Liquori *et al.*, 2001; Bachinski *et al.*, 2003; Day *et al.*, 2003). Molecular diagnostic protocols, recently described by Bachinski *et al.* (2003) and by Day *et al.* (2003), can be used for detecting the DM2 mutation, but are still too long and complicated to be part of routine laboratory diagnostics. In agreement with previous data (Liquori *et al.*, 2001; Mankodi *et al.*, 2003; Sallinen *et al.*, 2004), the present study clearly demonstrates that the DM2 mutation can be identified by FISH using CAGG probe to visualize nuclear foci in muscle biopsies. Moreover, our results show a consistent detection of ribonuclear inclusions by $(CAGG)_5$ in DM2 muscles and their absence in DM1 and in other control muscles, suggesting that the use of this probe allows a specific method for DM2 diagnosis. Performing FISH with CAG oligonucleotides instead, reveals ribonuclear inclusions both in DM1 and DM2 muscles as previously reported by other Authors (Mankodi *et al.*, 2001, 2003). This aspecific finding indicates that $(CAG)_6CA$ probe cannot be used for a differential diagnosis between DM2 and DM1.

It is worth emphasizing that ribonuclear inclusions by FISH, were present irrespective of degree of muscle involvement or of severity of clinical involvement. Three of 17 patients had normal histograms, with no preferential nuclear clump type II fiber atrophy as is usually found in DM2. Yet FISH demonstrated nuclear foci in these patients.

The nuclear accumulation of mutant RNA in DM1 and DM2 muscle tissue, is a molecular feature shared by these two disorders. This may indicate that some characteristic they share, like myotonia, cardiac arrhythmias and insulin resistance, as well as other aspects of the multisystemic involvement of these disorders, result from a common mechanism such as altered RNA splicing of several genes. Alteration of the chloride channel splicing leads to the loss of chloride channel protein that results in myotonia (Charlet *et al.*, 2002; Mankodi *et al.*, 2002); changes in cardiac troponin T splicing (Philips *et al.*, 1998) may be implicated in the cardiac disfunction; insulin receptor splicing alterations in DM2 may be implicated in insulin resistance which characterizes the disease (Savkur *et al.*, 2001, 2004). The role of ribonuclear inclusions in disease pathogenesis remains unclear. Nevertheless ribonuclear inclusions and splicing changes are present before any histological abnormality manifestations (Mankodi *et al.*, 2001; Savkur *et al.*, 2004). This could be important for an early diagnosis before the spectrum of clinical signs of muscle disease appear.

In conclusion, FISH with $(CAGG)_5$ is a method that allows the direct visualization of the mutant RNA on muscle biopsy. Therefore it may be a simple approach for DM2 diagnosis, which can be performed in a rapid and sensitive manner in any pathology laboratory. Moreover, it permits a differential diagnosis between DM2 and DM1. We suggest that FISH with $(CAGG)_5$ should be considered as a routine laboratory procedure to confirm or refute the clinical suspicion of DM2. It should also be applied routinely to screen patients with myotonic disorders. This procedure may contribute for example to differentiate chloride channelopathies from DM2 and better target subsequent genetic screening. It may also allow to recognize preclinical or sporadic presentations of DM2 in patients with unknown neuromuscular disorders or asymptomatic high CK levels, in whom a biopsy is performed for diagnostic purposes.

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