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Mutant (CCTG)n Expansion Causes Abnormal Expression of *Zinc Finger Protein 9 (ZNF9)* in Myotonic Dystrophy Type 2

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The mutation that underlies myotonic dystrophy type 2 (DM2) is a (CCTG)n expansion in intron 1 of *zinc finger protein 9* (*ZNF9*). It has been suggested that *ZNF9* is of no consequence for disease pathogenesis. We determined the expression levels of ZNF9 during muscle cell differentiation and in DM2 muscle by microarray profiling, real-time RT-PCR, splice variant analysis, immunofluorescence, and Western blotting. Our results show that in differentiating myoblasts, ZNF9 protein was localized primarily to the nucleus, whereas in mature muscle fibers, it was cytoplasmic and organized in sarcomeric striations at the Z-disk. In patients with DM2, ZNF9 was abnormally expressed. First, there was an overall reduction in both the mRNA and protein levels. Second, the subcellular localization of the ZNF9 protein was somewhat less cytoplasmic and more membrane-bound. Third, our splice variant analysis revealed retention of intron 3 in an aberrant isoform, and fourth quantitative allele-specific expression analysis showed the persistence of intron 1 sequences from the abnormal allele, further suggesting that the mutant allele is incompletely spliced. Thus, the decrease in total expression appears to be due to impaired splicing of the mutant transcript. Our data indicate that ZNF9 expression in DM2 patients is altered at multiple levels. Although toxic RNA effects likely explain overlapping phenotypic manifestations between DM1 and DM2, abnormal ZNF9 levels in DM2 may account for the differences in DM1. *(Am J Pathol 2010, 177:3025–3036; DOI: 10.2353/ajpath.2010.100179)*

Myotonic dystrophy (*Dystrophia myotonica*; DM) is the most common inherited muscular dystrophy in adults. Both myotonic dystrophy type 1 (DM1; Steinert's disease [Online Mendelian Inheritance in Man (OMIM) number 160900]) and type 2 (DM2; Proximal Myotonic Myopathy (PROMM) [OMIM number 602668]) are dominantly inherited disorders with an estimated prevalence of 1/8000 in DM1.[1] The prevalence of DM2 is not established, but estimated to be similar to DM1 in European populations.[2] DM1 is caused by a $(CTG)_n$ trinucleotide repeat expansion mutation in the 3′ untranslated region (UTR) of the *dystrophia myotonica protein kinase* gene in chromosome 19q13.3.[3–5] The mutation underlying DM2 is a tetranucleotide $(CCTG)_n$ repeat expansion in intron 1 of the *zinc finger protein 9* (*ZNF9*) gene, also known as *cellular nucleic acid-binding protein*, in chromosome 3q21.[6,7] The pathogenic repeat size in patients with DM2 ranges from approximately 50 to 11,000.[6,8] In contrast to DM1 there

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appears to be no clear genotype/phenotype correlation between expansion size and disease severity in DM2.[2]

The major symptoms of DM2 include late-onset proximal muscle weakness, myalgic muscle pain and/or stiffness, cataracts, myotonia, tremors, cardiac conduction defects, and endocrinological abnormalities.[2] However, compared with adult-onset DM1 clinical symptoms are more inconsistent and extremely diverse in DM2.[2,9] In any given DM2 patient, any of these symptoms may be absent, and myotonia may be variable over time in the same individual. A number of less consistent findings are occasionally associated with this disorder, making the clinical diagnosis a challenge.[2,10] Histopathological features in DM1 and DM2 are also different. Ring fibers and sarcoplasmic masses are characteristic features only in DM1, whereas nuclear clump fibers are prominent in DM2 and are present even before clinical muscle weakness.[1,11,12] In DM1, type 1 fiber atrophy may be present, whereas in DM2 a subpopulation of type 2 fibers, including the nuclear clump fibers, most of which are not detected by conventional ATPase staining, are extremely atrophic.[11]

The prevailing paradigm is that DM1 and DM2 are toxic RNA diseases: transcription of the repeats into mutant $(CUG)_{DM1}$/$(CCUG)_{DM2}$-containing RNAs is both necessary and sufficient to cause disease by formation of ribonuclear foci and interference of the splicing of downstream "effector" genes through *trans*-acting splicing factors, particularly CUG binding protein 1[13] and muscleblind 1.[14,15] For DM1 it has also been suggested that the pathogenic mechanism might be a dominant-negative mutation manifested at RNA level.[16] Two studies—one using four immortalized lymphoblastoid cell lines,[17] the other primary myoblast cultures from a heterozygous and a homozygous patient[18]—showed no effect of the $(CCTG)_{DM2}$ expansion on ZNF9 mRNA and protein levels.[17,18] However, heterozygous $Znf9^{+/-}$ knockout mice developed a multiorgan phenotype resembling DM,[19] and recently a growing number of studies showed that ZNF9 may not be normally expressed in DM2 muscle.[20–22] Thus, the DM2 phenotype may not depend solely on the expression of toxic mutant $(CCUG)_{DM2}$ transcripts, but may also be due to alterations in ZNF9 expression. Using a large series of patient skeletal muscle biopsies and myoblast cultures, we show here that ZNF9 expression is altered at multiple levels in DM2.

Materials and Methods

Patient Samples

Enrollment of patients was approved by the respective local institutional review boards. After obtaining informed consent from the patients, according to the Declaration of Helsinki, muscle biopsies were obtained. The patients and their biopsies used for the different analyses are summarized in Table 1. All DM1 and DM2 diagnoses were based on DNA mutation testing.[7,23] Muscle biopsies of 11 patients with DM2 were used for immunofluorescence (IF) and Western blotting (WB) studies. In addition to five normal control muscle tissue samples, skeletal muscle biopsies from seven patients with DM1 were used as disease controls. RNA extracted from five DM1 and five DM2 muscle biopsies was used for RT-PCR analysis. Another separate 10 DM1, 20 DM2, and six normal controls were used for microarray expression profiling.[24] We also performed ZNF9 IF studies on primary myoblast cultures at different time points during differentiation to myotubes.

Microarray Expression Profiling

Preparation and Labeling of RNA

Microarray gene expression data are available (NCBI, *http://www.ncbi.nlm.nih.gov/geo/*, GEO series numbers GSE7014, last accessed on January 28, 2010).[24] Skeletal muscle biopsies were homogenized by using a shark-tooth pulveriser with TRIzol (Invitrogen, Carlsbad, CA), and total cellular RNA was extracted according to the manufacturer's suggestions. RNA was further purified by using the RNeasy kit (Qiagen, Valencia, CA). The quality and integrity of the RNA was analyzed on an Agilent BioAnalyzer by using the RNA 6000 Nano LabChip (Agilent, Santa Clara, CA); samples with a RIN (RNA integrity number) >7 were used. For RNA expression profiling on the U133Plus2 GeneChip (Affymetrix, Santa Clara, CA), a total of 5 μg of total cellular RNA from each sample was used for cDNA synthesis according to the manufacturer's protocol. Briefly, a mixture of *in vitro* transcribed cRNAs of cloned bacterial genes for *lysA*, *pheB*, *thrB*, and *dap* (American Type Culture Collection, Manassas, VA) was added as external controls to monitor the efficiency of cRNA synthesis. First-strand cDNA synthesis was performed at 42°C for 1 hour with the Superscript II system (GIBCO Invitrogen, Carlsbad, CA) at a final concentration of 1× first-strand synthesis buffer, 10 mmol/L dithiothreitol, 500 μmol/L deoxynucleotide triphosphates, 100 pmol of T7-$(T)_{24}$ primer, and 200 units of reverse transcriptase. Second-strand cDNA synthesis was performed at 16°C for 2 hours at a final concentration of 1× second-strand buffer, 250 μmol/L dNTP, 65 U/ml DNA ligase, 250 units/ml DNA polymerase I, and 13 U/ml *RNase* H. Second-strand synthesis reaction mixtures were cleaned with an Affymetrix cDNA purification column. *In vitro* transcription labeling with biotinylated UTP and CTP was performed according to the manufacturer's recommendations (Enzo Diagnostics, Plymouth Meeting, PA) for 16 hours at 37°C. Amplified cRNA was purified on a cRNA purification column (RNeasy, Qiagen), and the quality of the amplification was verified by analysis on an Agilent BioAnalyzer. Labeled cRNAs were fragmented for 35 minutes at 94°C in 40 mmol/L Tris-acetate, pH 8.1/100 mmol/L KOAc/30 mmol/L $Mg(OAc)_2$. The hybridization cocktail consisted of 10 μg fragmented cRNA in 200 μl, containing 50 pM control oligonucleotide B2, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated bovine serum albumin (BSA), 100 mmol/L Mes, 20 mmol/L EDTA, 0.01% Tween 20 (total Na^+ = 1 M), and bacterial sense cRNA controls for *bioB*, *bioC*, *bioD*, and *cre* at 1.5, 5.0, 25, and 100 pM, respectively. Fragmented cRNAs were then hybridized to Af-