

# Genotype–phenotype correlation in myotonic dystrophy

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Myotonic dystrophy (DM) is caused by a mutation in the length of a trinucleotide (CTG) repeat in the 3' untranslated region of the myotonin protein kinase gene located on chromosome 19q13.3. The normal gene has between 5 and 36 CTG trinucleotide repeats, whereas minimally affected individuals have 50 copies and severely affected DM-patients have several thousands of such repeats. Since no information on a genotype–phenotype correlation in Austrian DM-patients is available, we examined a small group of these patients for the unstable trinucleotide repeat. Molecular analysis was used to clarify equivocal clinical diagnoses and confirm clinical findings. We studied eight DM-families, a total of 57 individuals, of whom 18 were diagnosed with a trinucleotide repeat expansion. Twenty-six unrelated individuals served as a control. Clinical assessment was based on the muscular disability rating scale (MDRS) and a sum of symptoms score (SSS). There was a significant correlation between the clinical scores (MDRS: Spearman  $r = 0.51$ ;  $p = 0.029$ ; SSS: Spearman  $r = 0.538$ ;  $p = 0.0259$ ) used and the size of the amplification of the trinucleotide repeat. The largest expansion found in our group of patients was 6 kb. Furthermore, we observed both expansion and contraction of the enlarged fragment during transmission from one generation to the next.

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Myotonic dystrophy (DM) or Curschmann–Steinert's disease is the most frequent autosomal dominant myopathy in adults with an incidence of 1 in 8000 among Caucasians (1). Common clinical findings are progressive muscle weakness, myotonia, cardiac conduction disturbances, cataracts, smooth muscle involvement, endocrine changes and brain alterations. DM is exceptionally variable both within and between families (2), and shows anticipation (3, 4) with earlier age at onset and more severe clinical features in successive generations.

The genetic defect responsible for DM is an unstable expansion of a trinucleotide CTG repeat (5–9) in the 3'-untranslated region of a gene located on chromosome 19q13.3 (10–12), encoding a putative serine/threonine kinase. Because the DM-gene product displays kinase activity (13), it is presumed to be part of a signal transduction pathway. It has been suggested that ion channels are candidates for DM-proteinkinase substrates (14).

So far eight human neurodegenerative diseases have been identified as expanded trinucleotide repeat disorders. This mutation has been found to

contain two types of sequences: CAG (with complement CTG) and CGG (with complement CCG) (15). Recently, however, there was a report of a third type of sequence, namely the association of Friedreich's ataxia with an intronic GAA triplet repeat expansion (16). The trinucleotide repeat regions in these genes are highly polymorphic, not only in affected individuals but also in the normal population (6, 17, 18). The DM-locus shows considerable variability: over 75% of normal individuals are heterozygous. In the normal population up to 36 CTG repeats occur (19), while in DM-patients extreme expansions up to 2000 repeats and more (2, 6, 7, 9, 20) can be observed. A general correlation has been demonstrated between the degree of repeat expansion and clinical symptoms (21–23) and an inverse correlation was found between age at onset of the disease and repeat length (24–26).

The aim of our study was to analyze the genotype–phenotype relationship in a group of Austrian DM-patients using clinical phenotype as defined by clinical scores and molecular assessment

of the (CTG)<sub>n</sub> repeat number in the DM-gene. Furthermore, we wanted to see whether there are changes in genotype–phenotype status upon transmission in affected patients.

### Materials and methods

#### Patients and controls

Eighteen DM-patients, aged 11–88 years, from eight unrelated families were diagnosed at the Department of Neurology, Rosenhügel, Vienna, Austria. The diagnosis of DM was based on clinical, electromyographic (high frequency repetitive discharges), ophthalmologic, endocrine and cardiac investigations (27). Additionally, 39 family members who consented to undergo a clinical, neurologic, electromyographic and molecular investigation were included in the study. Twenty-six unrelated individuals served as a control.

Among the 18 DM-patients analyzed, one had only electromyographic alterations but no other clinical symptoms and another one had as a sole sign cataract without pathologic electromyography (EMG). In these two patients the definite diagnosis of DM was not established before DNA testing was performed.

In DM-patients, the degree of muscle impairment was assessed by using the muscular disability grading (Muscular Disability Rating Scale, MDRS). The MDRS is based on a five-point scale as described by Mathieu et al. (28). Grade 1, no clinical muscular impairment (diagnosis made by EMG, slit-lamp examination or DNA analysis); grade 2, minimal signs (myotonia, jaw and temporal wasting, facial weakness, sternocleidomastoid wasting/weakness, ptosis, nasal speech, no distal weakness except isolated flexor weakness); grade 3, distal weakness (no proximal weakness except isolated triceps brachii weakness); grade 4, mild or moderate proximal weakness; grade 5, severe proximal weakness (confined to wheelchair for short or long distances).

In addition to the MDRS, we assessed cognitive impairment, cataract, cardiac involvement, diabetes, hypogonadism and motor impairment. To further define the DM-phenotype, we devised a second score (sum of symptoms score, SSS) with a grading from 1 to 6 based on the cumulative number of clinical symptoms other than muscle impairment being present in a patient.

#### DNA analysis

All patients, their relatives and control subjects were studied by polymerase chain reaction (PCR) and Southern blot analyses. DNA was extracted

from peripheral blood leukocytes (PBL) using the QIAamp Blood Kit, according to the manufacturer's protocol (QIAGEN, Hilden, Germany). To determine the size of the expanded and normal CTG repeat, PCR amplification with primers 101 (5'-CTTCCCAGGCCTGCAGTTTGCCCATC-3') and 102 (5'-GAACGGGGCTCGAAGGGTC-CTTGTAGC-3') (6) was performed. One primer, primer 102, was fluorescently labeled with a 6-FAM dye phosphoramidite. PCR amplification was performed in a final reaction volume of 25 µl containing 200 ng of genomic DNA, 5 pmol of each primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM MgCl<sub>2</sub>, 200 µM each dNTP and 1.25 U of *Taq* polymerase (AmpliTaq™ DNA Polymerase, PE Applied Biosystems, Foster City, CA). PCR reactions were carried out using a Perkin Elmer Thermal Cycler apparatus (GeneAmp PCR-System 2400). Samples were denatured for 2 min at 94°C, followed by 35 cycles at 94°C for 30 s, 70°C for 30 s, and 72°C for 1 min, with a final extension for 5 min at 72°C. Fifteen µl of each PCR product was electrophoresed on a 3.5% MetaPhor™ agarose gel and visualized by ethidium bromide staining with UV transillumination. For accurate sizing the GeneScan™ Analysis program on an automated sequencer (310 Genetic Analyzer, PE Applied Biosystems) was used. Twelve microliter of formamide and 1 µl of marker (Prism™ Genescan-500 Tamra, PE Applied Biosystems) were added to 1 µl of the PCR-reaction, denatured at 95°C for 2 min before capillary electrophoresis on a POP-4 polymer™ (PE Applied Biosystems).

All samples found to have only one allele were further analyzed using Southern blotting. Ten µg of lymphocyte genomic DNA was digested to completion with *SacI* according to the manufacturer's instruction (Boehringer Mannheim, Germany). A second Southern analysis with the restriction enzyme (*BglII*) was used to confirm the first analysis. Digestion products were run on 1% agarose gels and transferred onto nylon membranes (Duralon-UV membrane, Stratagene, La Jolla, CA). Southern Blots were hybridized with cDNA probe p5B1.4 (29), a 1.4 kb *BamHI* fragment from cDNA25 (7) containing a (CTG)<sub>5</sub> repeat (6). The probe was digoxigenin-UTP labeled using a DIG RNA Labeling Kit (Boehringer Mannheim, Germany) according to the manufacturer's instruction. Prehybridization and hybridization were performed at 50°C in DIG Easy Hyb™ solution (Boehringer Mannheim, Germany). Blots were washed twice for 5 min in 2 × sodium citrate (SSC)/0.1% sodium dodecyl sulphate (SDS) at room temperature and twice for 15 min in 0.1 × SSC/0.1% SDS at 68°C. After washing the mem-

brane was treated according to the protocol for DIG Nucleic Acid detection (Boehringer Mannheim) and then visualized by overnight exposure using Kodak X-OMAT AR film. Expanded fragments were sized by measuring the midpoint of the band, as larger bands tend to be diffuse owing to somatic heterogeneity.

Statistical analysis

The length of the CTG trinucleotide expansion in DM-patients was correlated with the MDRS and SSS using a Spearman's rank coefficient. Correlation coefficients were considered significant at  $p < 0.05$ .

Results

The distribution of CTG repeat lengths in controls and DM-patients is shown in Figs. 1 and 2, respectively. In the healthy controls studied, a total of 13 different alleles were found, the most common allele had five repeats (36.5% of chromosomes), followed by 13 repeats (15.4%), 14 repeats (11.6%), and 12 repeats (9.6%). The highest repeat number observed in controls was 30 repeats. Trinucleotide repeat expansions were found in each DM-patient from eight unrelated families (Table 1). Two of these 18 affected individuals had a clinically equivocal DM diagnosis (patient F1-I and patient F5-III) which we were able to clarify by means of DNA-analysis. One case with the mildest form seen in old age (patient F1-I, Table 1) was diagnosed with cataract as a sole clinical sign. DNA-analysis revealed an expanded allele harboring approximately 100 CTG-repeats. The son of this patient, patient F1-II, clearly showed the phe-

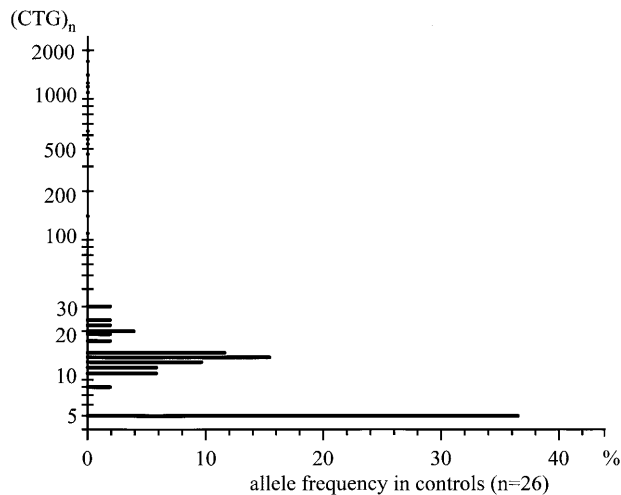


Fig. 1. Histogram showing the frequency of alleles (in %) of control subjects ( $n = 26$ ).

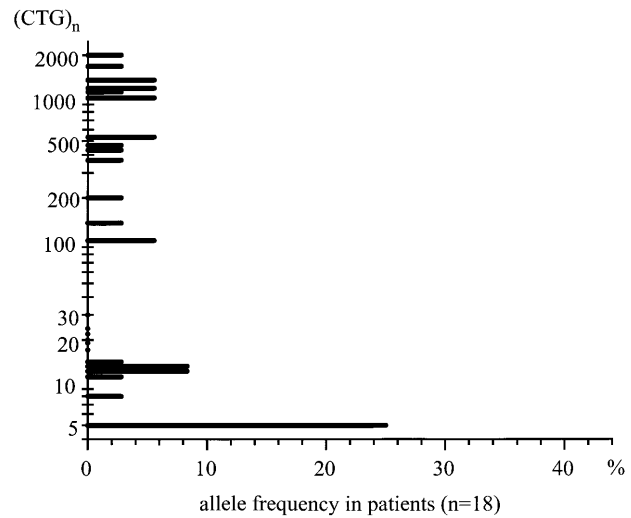


Fig. 2. Histogram showing the frequency of alleles (in %) of DM-patients ( $n = 18$ ).

nomenon of anticipation with earlier onset of the disease accompanied by increasing severity (Table 1). In the third generation of this family, there was no transmission of the affected DM-allele. Both offspring's were heterozygous normal for a 5 and 13 repeat allele, respectively (data not shown). Patient F5-III, the daughter of patient F5-II, was clinically normal despite a pathologic EMG. On the DNA-level, patient F5-III, had an obvious retraction from a 3.3 kb expanded DM-allele seen in her father F5-II to a 1.6 kb CTG trinucleotide repeat containing allele (see Table 1). All patients, except patient F1-I, belonged to the classical form with juvenile/adult onset DM. As mentioned above, there were no cases belonging to the congenital type of this myopathy. We observed a rather wide range of expansion, ranging from 0.3 to 6.0 kb (Table 1).

In four of the DM-families studied, the size of the expanded fragment increased upon transmission while in two families a decrease was seen (Table 1). In the remaining two families the parents were not available for analysis. Patient F4b-I (Table 1), who is clinically affected, has two healthy children. The genotype of the 28-year-old daughter showed heterozygosity for the (CTG)<sub>11</sub> and (CTG)<sub>12</sub> alleles and the 26-year-old son is heterozygous for the (CTG)<sub>5</sub> and (CTG)<sub>12</sub> alleles, respectively (data not shown).

In all 18 DM-patients the clinical picture reflected the known variability in phenotype of this autosomal muscular dystrophy. EMG revealed myotonic discharges in muscles of all patients studied except for patient F1-I who was only mildly affected. Endocrine dysfunction such as hypogonadism was seen in 7 patients (Table 1). All of

Table 1. Clinical and molecular findings in 18 DM-patients.

Patient	Sex	Age	Cogn. imp.	Cataract	Cardiac involv.	Diabetes	Hypogonadism	Motor imp.	EMG	MDRS	Normal allele (CTG) <sub>n</sub>	Expanded allele (kb)
F1-I	m	88	–	+	–	–	–	–	–	1	8	~0.3
F1-II	m	49	–	+	+	–	–	+	+	4	13	~1.6
F2-I	f	48	–	+	+	+	+	+	+	3	13	~3.0
F2-II	m	29	+	+	+	–	+	+	+	3	11	~5.0
F3-I	f	57	+	+	+	–	–	+	+	4	5	~6.0
F3-II	f	29	+	–	+	–	+	+	+	3	13	~3.5
F4a-I	m	52	–	–	+	+	+	+	+	5	5	~3.5
F4b-I	m	49	–	–	+	–	–	+	+	4	12	~3.0
F5-II	m	47	+	–	+	–	–	+	+	4	5	~3.3
F5-III	f	11	–	–	–	–	–	–	+	1	5	~1.6
F6a-II	m	31	+	–	+	–	+	+	+	3	5	~1.1
F6b-II	f	43	–	+	+	–	–	+	+	3	5	~0.4
F6-III*	m	13	–	–	–	–	–	+	+	2	12	~1.3
F6c-II	f	41	–	+	+	–	–	+	+	2	5	~0.6
F6-III**	f	15	–	–	–	–	–	+	+	2	14	~1.4
F7-I	m	45	+	+	+	–	–	+	+	3	5	~0.3
F8a-I	m	26	+	–	+	–	+	+	+	3	5	~4.0
F8b-I	m	33	+	–	+	–	+	+	+	3	12	~4.0

Patient: (F) family 1–8; (a), (b) siblings; (I, II, III) 1st, 2nd, 3rd generation; \* affected son of patient F6b-II; \*\* affected daughter of patient F6c-II; (m) male; (f) female; (+) present; (–) absent; Age, current age in years; Cogn. imp., cognitive impairment; cardiac involv., cardiac involvement; motor imp., motor impairment; EMG, electromyography; MDRS, muscular disability rating scale; kb, kilobase.

them had an amplification size of ~3.0 kb or more, except patient F6-II who had an expansion of only ~1.1 kb. Fourteen (78%) of the 18 DM-patients had cardiac abnormalities (Table 1). Almost half (44%) of the patients showed cognitive impairment and cataract, while diabetes was seen in only 2 of 18 DM-patients. The largest triplet repeat expansion observed was ~6.0 kb in patient F3-I, (Table 1), a case of classical DM with juvenile/adult onset. We performed a statistical analysis on the 18 DM-patients and found a significant correlation between the size of amplification of the CTG trinucleotide repeat and the severity of muscular disability according to the MDRS (Spearman  $r = 0.51$ ;  $p = 0.029$ ). There was also a significant correlation observed between the size of the trinucleotide expansion and the SSS (Spearman  $r = 0.538$ ;  $p = 0.0259$ ) (Fig. 3). Furthermore, the correlation between the two scores used, MDRS and SSS, was statistically significant ( $r = 0.4885$ ;  $p = 0.0466$ ).

## Discussion

This study on a small group of Austrian DM-patients confirms previous observations in other populations on the behavior of the unstable CTG trinucleotide repeat (2, 9, 21–23, 30–32). Up to now, DM has not been investigated on a molecular level in Austrian patients. In our study, the allele distribution in healthy controls is very similar to that observed by Davies et al. (17). Thirteen alleles

of different repeat lengths were observed, the most common of which had 5, 12, 13 and 14 repeats. We could not detect any alleles with 15, 16 and 21 repeats which Davies et al. (17) found in Europeans. In our study, however, the number of controls analyzed might have been too small to detect such repeats. It is noteworthy that in the patient group the non-expanded allele was found to have only up to 14 repeats, whereas in healthy control subjects six alleles were found to be longer than 14 repeats (13.4%). The allele frequencies of two allele groups, one with alleles of 14 or fewer CTG repeats (0.87) and the other with alleles with greater than 14 CTG repeats (0.13), found in our

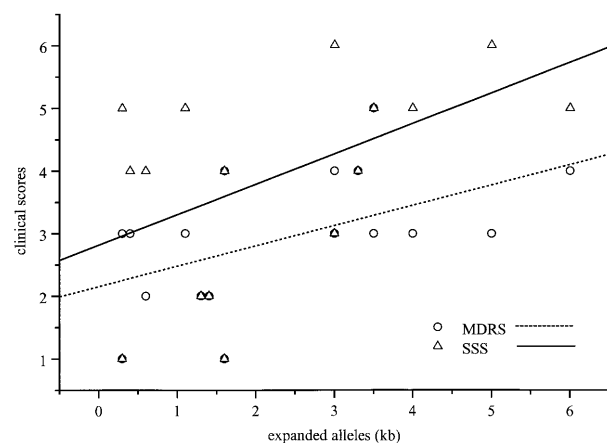


Fig. 3. Correlation between CTG trinucleotide expansion (in kb) and clinical disease scores (MDRS, SSS).

control subjects are almost identical to those described by Davies et al. (17) for Europeans (frequency of 0.85 and 0.15, respectively). In the present study all patients were heterozygous for the DM-mutation, we observed no homozygous cases as reported by Cobo et al. (33) and Martorell et al. (34). Up to now it was assumed that the homozygous state was so severe that it would lead to intrauterine death (1). These authors could, however, demonstrate that DM-patients homozygous for the trinucleotide repeat mutation are viable and affected in a similar way to heterozygous patients. This finding indicates that the DM-phenotype is truly dominant.

The largest normal allele observed in our DM-patients had 14 repeats suggesting that a larger copy number within the normal range may somehow predispose to progression towards a 'premutation state'. The fragile X syndrome, for example, is caused by an unstable CGG trinucleotide repeat. Studies of these affected families indicated that the size of the repeats during the 'premutation state' is a major risk factor for transition from premutation to full mutation (35, 36). Imbert et al. (19) suggested that the length of the CTG repeat in the DM gene is a main driving force for DM mutations. These authors and others (37) speculate that larger normal alleles (more than 19 repeats) may be unstable and thus be prone to mutation in successive generations.

Upon transmission of the expanded CTG repeat we observed both expansion and contraction. Expansion of the CTG repeat length between generations was not generally accompanied by increased severity of the phenotype in the next generation. The largest contraction seen in our families studied (~2.5 kb) was associated with very little changes in the clinical phenotype even taking into account the age of the affected offspring. These findings are in accordance with previous observations in other groups of patients (20, 38–46). We observed, however, no reverse mutation to a normal-sized allele in our DM-patients as has been reported previously (38, 41, 42, 47). Monckton et al. (45) clearly demonstrated that both germline and somatic variations can contribute to the differences between generations observed in DM pedigrees. Especially, in light of the age-dependent somatic variability, the comparison of the allele sizes in the leukocyte DNA of the parents and the child may not necessarily reflect the actual germline length difference between the progenitor allele inherited by the parent and that transmitted to the child. These authors suggest that caution should be used when attempting to infer germline processes by comparing parent and offspring leukocyte allele sizes.

Because of a wide variation in the range and severity of symptoms, the diagnosis of this neuromuscular disorder can be problematic. Patients are often divided into three groups according to the severity of clinical symptoms and the age at onset of the disorder (48). The mildest form is often difficult to diagnose. In this group only symptoms such as cataract, baldness, minimal or absent muscle involvement can be seen in middle- or old-age. The classical form with juvenile or adult onset is phenotypically variable and shows pareses, myotonia, progressive muscle wasting, testicular atrophy and cardiac conduction defects. The most severe form, congenital DM (CDM), frequently fatal after birth, is associated with generalized muscular hypotonia, mental retardation and talipes (1). Sometimes it is difficult to distinguish between these three clinical categories especially in juvenile, mildly affected cases. In the latter case, a genetic analysis is often the only way to prove the diagnosis DM. Patients F1-I and F5-III in this study are a typical example for such cases.

The DM-mutation is known to display pronounced heterogeneity in somatic cells (26, 49–51). In general, affected tissue, such as skeletal muscle shows greater expansion than blood (49, 50, 52–54) and therefore it was argued that muscle DNA analysis may be more informative than leukocyte analysis in predicting progression of the disease. Zatz et al. (54), however, found no significant correlation between the size of the trinucleotide expansion in muscle and the clinical symptoms. Even though it was demonstrated that the repeat length in peripheral blood cells continues to enlarge throughout adult life, it does not correlate with progression of the disease (55) reaffirming lymphocyte DNA testing as a reliable means for DM-mutation assessment. In this context it might be noteworthy that few reports demonstrate the limits of lymphocyte CTG repeat number analysis in predicting genotype–phenotype correlations in DM-patients, because in some patients clinical severity of the disease does not correlate with the triplet repeat number found in peripheral leukocytes (56, 57).

In general, the trinucleotide repeat number correlates positively with most of the individual clinical symptoms found in DM-patients, including muscle impairment (22), cardiac dysfunction (58, 59), and gonadal dysfunction (60). However, Jaspert et al. (22) found that other symptoms such as cataract, myotonia and gastrointestinal dysfunction were not correlated with the trinucleotide repeat size. In accordance with Gennarelli et al. (23) we clearly demonstrate that the (CTG) trinucleotide repeat number in lymphocyte DNA corre-

lates significantly with the clinical status of the disease. It is, however, important to point out that in individual cases, the clinical status of a patient cannot be predicted on the basis of the number of trinucleotide repeats.

In conclusion, we could show that in a sample of 18 Austrian DM-patients a correlation exists between the size of the CTG trinucleotide repeat expansion and the clinical disease scores (MDRS and SSS).

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