Presymptomatic diagnosis of myotonic dystrophy

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Abstract

The discovery of an expanded (CTG)_n repeat sequence in myotonic dystrophy (DM) has greatly improved our ability to detect DM gene carriers who have few or none of the classical signs of this disorder. We report here our experience with two such groups of gene carriers. We used a PCR based protocol that should be especially sensitive to small increases in CTG triplet number which might escape detection by conventional Southern blot analysis. Our analyses show that on 100 non-DM chromosomes the number of CTG triplets ranged from five to 37. We then studied 17 obligate gene carriers aged 55 years and over who showed no muscle weakness. All of the gene carriers in this group showed a relatively small increase in the number of CTG triplets (52 to 90 CTG triplets) with limited somatic mosaicism. We subsequently studied 11 subjects (aged 19 to 36 years) who had previously been identified as gene carriers by genetic linkage studies, but who lacked diagnostic signs. In this prospectively studied group, nine subjects showed an expanded allele, confirming the earlier prediction from linked genetic markers. The other two subjects had only two normal alleles and no expanded allele. Revision of the clinical data casts doubt on the original diagnosis of DM in their families. Preferential amplification of the normal non-expanded allele was noted in three asymptomatic gene carriers in this study (as well as in two of their clinically affected relatives). We caution that, at least in our hands, the DM mutation can be confidently excluded by this PCR based method only if both normal alleles have been identified. If an expanded allele is not seen, and only a single normal allele is visualised on 6% PAGE, confirmatory testing with linked genetic markers or conventional Southern blotting of the (CTG)_n repeat is advocated.

(*J Med Genet* 1992;29:780-4)

Myotonic dystrophy (DM) shows a wide range of clinical expression in affected subjects.¹ Severity of the disease is at least partly correlated with the number of CTG trinucleotide repeat units found in the 3' untranslated region of a protein kinase gene located in band q13.3 of chromosome 19.²⁻¹⁰ There is a marked tendency for an increase in CTG triplet number

from one generation to the next, which is paralleled by earlier onset of the disease in successive generations,^{11,12} a phenomenon known as anticipation. Genealogical studies in DM families¹¹⁻¹⁵ suggest that in earlier generations additional asymptomatic gene carriers may have been present, presumably with only a minor expansion of the CTG motif. However, a premutation not involving expansion of the CTG trinucleotide repeat is another possibility. This situation is highly reminiscent of the CCG repeat expansion seen in the fragile X syndrome.¹⁶⁻¹⁸ Our previous study using linked DNA markers has suggested that a considerable proportion of sibs and offspring of DM patients are asymptomatic in early adulthood.¹⁹ Therefore, asymptomatic subjects may not be confined to the early generations of a DM family.

We studied these issues in detail by assaying the CTG triplet number in 17 obligate carriers of the DM gene as defined by pedigree analysis and genealogical studies. We further studied a group of asymptomatic offspring of DM patients who had been identified previously as probable gene carriers by the application of closely linked DNA markers.

Methods

Families were ascertained through the DNA diagnostic service in our department. As from January 1992, this service has performed diagnosis with linked DNA markers in 164 DM families, of which 76% were referred by other genetic departments. Care was taken to ensure that all families met previously defined diagnostic criteria.²⁰ For the purposes of this study, we selected 13 families that contained one or more obligate gene carriers aged 55 and over who showed no muscular weakness. Obligate gene carriers were identified by pedigree analysis or through genealogical studies that linked independently ascertained pedigrees. There were 14 males and three females in this group, ranging in age from 55 to 92 years (mean 69.8 years). Among 17 obligate gene carriers, seven had undergone cataract surgery. The other 10 were considered asymptomatic. EMG and slit lamp examination had been performed in six out of 10. Typical lenticular opacities were noted in three out of six. EMG was normal in all six. In addition, 11 subjects from nine families were restudied, as our previous study¹⁹ had indicated a high probability of carrying a DM mutation in spite of normal results on clinical examination. We used data from 50 normal Dutch subjects (100 chromosomes) who had married into DM

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Received 8 June 1992.
Revised version accepted 30 July 1992.

Figure 1 Distribution of allele sizes for 100 non-DM chromosomes. Allele sizes are given as the number of CTG triplets.

families to establish the normal range of CTG triplet number for controls.

Chromosomal DNA was isolated from peripheral blood.²¹ Genetic marker systems flanking the DM mutation have been described previously.²²⁻²⁶ Using a recently described PCR assay,⁷ we tested the expansion of the CTG repeat in genomic DNA. The CTG repeat was amplified with flanking primers 406 and 409 reported by Mahadevan *et al.*⁷ The resulting DNA fragments were resolved on 1% and 4% agarose gels. A Southern blot, made from the 1% gel on Gene Screen Plus, was screened with a ³²P end labelled (CTG)₁₀ oligonucleotide as a probe, and the hybridising fragments were subsequently visualised by autoradiography. Alleles containing up to approximately 140 CTG triplets could also be identified on a 6% polyacrylamide/7 mol/l urea sequencing gel after ³²P end labelling of one of the amplification primers.

Results

NORMAL CONTROLS

Data from 100 normal chromosomes are summarised in fig 1. Allele lengths were found to range from five to 37 CTG triplets. The most common alleles in this sample were five, 13, and 14 CTG triplets (38, 22, and 15% respectively).

OBLIGATE CARRIERS

All of the 17 obligate gene carriers (14 males and three females) showed an expanded allele with multiple bands and weaker signals on 6% polyacrylamide gels, different from normal alleles at this locus (five to 37 CTG triplets). Allele size (estimated from the strongest signal among these multiple bands) ranged from 52 to 90 CTG triplets (fig 2). The apparent instability of the expanded allele could represent an in vitro artefact of the PCR reaction. However, in view of the strikingly different appearance of the 37 and 52 CTG triplet signals, it is more likely that the CTG repeat shows somatic mosaicism in vivo above a threshold of approximately 40 to 50 CTG triplets. With

Figure 2 CTG repeat analysis of obligate DM gene carriers (lanes 1-5, 7, 8, 10-12, and 14-17) by 6% denaturing polyacrylamide gel electrophoresis. Expanded PCR amplified alleles range from 52 to 76 CTG triplets. The larger alleles show up less well, probably owing to differences in the efficiency of the PCR reaction. Lane 7 represents a 72 year old male who shows bilateral cataract as his only clinical feature. This man has an expanded allele of 74 CTG triplets and a normal allele of 37 CTG triplets.

one exception (II.7, fig 3), CTG repeats were found to be larger in offspring than in these obligate carriers, confirming that the expanded repeat sequence was unstable.

A striking example of anticipation is shown in fig 3. In this family, two subjects (I.3 and II.7), aged 90 and 53 respectively, are asymptomatic on full examination, including EMG and slit lamp examination, except for a few typical lenticular opacities in I.3. A third subject (I.1) is also completely normal at the age of 85, but no EMG has been performed. These obligate heterozygotes showed CTG triplet numbers of 52, 60, and 52 respectively, based on results of 6% PAGE (lanes 16, 15, and 17, fig 2). The 1% agarose Southern blot of the PCR product shows the expected band of approximately 0.2 kb for these subjects. Above this band, a smear can be detected, which is frequently observed for alleles of this size, and which may be the result of heteroduplex formation of normal and expanded alleles, rather than somatic mosaicism. Symptomatic disease has been diagnosed in the descendants of these obligate carriers (III.1, 2, 3, fig 3). These

Figure 3 Southern blot analysis of PCR amplified CTG repeat sequence run on a 1% agarose gel in a DM family. Transmission of DM through three clinically asymptomatic subjects (I.1, I.3, II.7), aged 90 years, 85 years, and 53 years, who show only mild expansion in this assay. CTG triplet numbers for these obligate carriers (determined from 6% polyacrylamide gel electrophoresis) were 52, 52, and 60 respectively. Clinically affected subjects (II.4, II.5, III.1, III.2, III.3) show more extensive expansion of the CTG repeat.

subjects all have expanded alleles exceeding 200 CTG triplets.

PROBABLE GENE CARRIERS IDENTIFIED BY LINKAGE ANALYSIS

An expanded CTG repeat was found in nine of 11 asymptomatic offspring, who carried the high risk haplotype for closely linked DNA markers (table). These subjects ranged in age from 19 to 36 years. In four of the nine the size of the expanded allele fell within the range previously observed for asymptomatic (or cataract only) subjects aged 55 years and older. In the other five asymptomatic DM gene carriers, allele sizes were found to be considerably larger, more than 200 CTG triplets).

In the final two cases, a clinical diagnosis of DM in a key relative was not supported by the DNA studies. In the first family, the 34 year old proband had received a clinical diagnosis of myotonic dystrophy on the basis of electromyographical myotonia, typical multicoloured cataract, and muscle weakness (albeit in an unusual distribution for DM). This subject carries two normal alleles for the CTG trinucleotide repeat. Electromyographical myotonia and multicoloured lens opacities were reported for her father, who also showed a normal number of CTG triplets on both chromosomes. Either this family shows an unusual form of the disease, not associated with expansion of the CTG repeat, or the original clinical

diagnosis was incorrect. If the latter applies, the sister, who carries the same haplotype for markers flanking the DM locus (subject 8 in the table) is probably not at risk for the disease. In the other family, several affected subjects showed a characteristic smear of expanded repeats. However, in a female in whom DM had been diagnosed based on mild muscular weakness and electromyographical myotonia, an expanded allele was not found. This puts the clinical diagnosis in doubt for this woman and indicates that her son (subject 6 in the table) is probably not at risk for DM.

PREFERENTIAL AMPLIFICATION OF THE NORMAL ALLELE

An expanded allele was not initially detected in three subjects with the primer pair (406 and 409 from Mahadevan *et al.*⁷) used in this study in spite of multiple (up to five) attempts. These subjects had previously been predicted to be gene carriers by linkage analysis. Since only a single normal allele was seen on 6% polyacrylamide gel electrophoresis, we assumed that an abnormal allele was present but undetectable. By choosing another primer combination (primer 406 from Mahadevan *et al.*⁷ and a new primer GGC.ACA.GAA.GCC.CGG.CCC.ACC, nt position 11–32) the presence of an expanded allele was confirmed in two subjects. In the third subject an expanded allele could only be shown by direct restriction analysis and Southern blotting of the genomic fragment containing the CTG repeat sequence.

Discussion

We show here that asymptomatic carriers of the DM gene mutation can be diagnosed by PCR based analysis of the CTG trinucleotide repeat. This method is especially helpful in determining the origin of the mutation when both parents are clinically unaffected and the family history is negative.²⁷

Among 17 cases aged 55 years and over without muscle weakness, none showed an allele exceeding 90 CTG triplets. Larger alleles may not be compatible with absence of muscular symptoms in middle and old age. However, more data are needed before predictions are possible for individual cases.

Our results confirm the paucity of new mutations in DM suggested by genealogical studies,^{11–15} since subjects identified as obligate gene carriers always carried an expanded allele. This is consistent with the results of studies showing linkage disequilibrium in various populations.^{28–30} In fact, the finding of linkage disequilibrium in DM is difficult to explain, unless we assume that either the penetrance of the DM gene is virtually zero for many generations as suggested by the anticipation model, or that carriers of the DM mutation have increased fecundity, or both. The finding that all of the asymptomatic obligate carriers defined by pedigree analysis or genealogical studies showed only a small increase in CTG triplet number (52 to 90 CTG triplets) supports the anticipation model of DM. In

Results of CTG analysis in 11 asymptomatic subjects considered to be at high risk of carrying a DM mutation by linked DNA markers.

Subject*	Age	Sex	Affected parent	Clinical examination†	CTG analysis‡	Remarks
1	19	M	Father	Normal	Expanded smear (> 200 CTG triplets)	Mental retardation
2	19	M	Father	Atypical lens changes. Equivocal clinical myotonia/normal EMG	Expanded smear (> 200 CTG triplets)	
3	33	M	Father	Normal§	Expanded smear (> 200 CTG triplets)	
4	33	F	Father	Normal	Approximately 74 CTG triplets	
5	24	F	Mother	Normal	Approximately 60 CTG triplets	
6	29	M		Normal	Normal	Not at risk. Diagnosis in relatives in doubt
7	36	F	Father	Slight ptosis. Atypical lens changes§	Expanded smear (> 200 CTG triplets)	
8	28	F		Normal	Normal	Not at risk. Diagnosis in relatives in doubt
9	33	M	Mother	Normal	Approximately 61 CTG triplets	
10	38	M	Mother	Normal	Approximately 88 CTG triplets	
11	21	M	Father	Normal	Expanded smear (> 200 CTG triplets)	

* Subjects at high risk were identified in a previous study of 139 asymptomatic offspring of DM patients.¹⁹

† Clinical examination included EMG, neurological examination, and slit lamp examination. Non-specific signs (such as colourless opacities or mental retardation) were found in three out of nine (33%), compared to 24 out of 128 (19%) at those at low risk.

‡ Based on results of 1% agarose gel electrophoresis and 6% polyacrylamide gel electrophoresis of PCR amplified products.

§ After DNA marker analysis, repeated EMG examination was performed, and myotonia was diagnosed.

|| After DNA marker analysis, DM was diagnosed in a son.

spite of anticipation, the DM mutation may occasionally remain clinically silent, even among sibs and offspring of symptomatic cases. In five of our patients, (aged 19, 19, 21, 33, and 36 respectively) careful clinical examination including EMG and slit lamp examination failed to show any definite sign of DM. This may appear surprising since each showed an expanded allele which clearly exceeded 200 CTG triplets (subjects 1, 2, 3, 7, and 11 in the table). Follow up studies are required to establish whether these subjects will develop muscular symptoms in later life. So far, repeated EMG studies (performed after the results of the DNA analysis were known) have shown myotonic discharges in two of them (subjects 3 and 7, table). Neither clinical myotonia nor significant muscle weakness was detected on re-examination. In the other asymptomatic cases, CTG triplet numbers of 60, 61, 74, and 88 were within the range found for obligate gene carriers without muscular symptoms after the age of 55.

We currently estimate the chance that a clinically normal sib or offspring of a DM patient carries a mutated gene to be 7.8% for those aged between 20 and 39 years of age. This figure is very similar to our previous estimate in the same sample of DM families (8.3%).¹⁹ We have now excluded two subjects because of unreliable diagnosis, while we had formerly anticipated the reclassification as low risk of two subjects because of genetic recombination with the marker loci.¹⁹ In addition, our study confirms that at the molecular level anticipation is the rule in DM, since in our sample of nine asymptomatic offspring we always found a CTG triplet number equal to or exceeding that of the affected parent. Finally, we do not know the explanation for the family in which expansion of the CTG repeat was not detected in either clinically affected member. Such families have been noted by others.⁷ It remains to be seen whether

the results in these families are the result of diagnostic error or allelic mutation. The existence of allelic mutations, not causing expansion of the CCG repeat sequence, has been suggested for the fragile X syndrome.³

Finally, we want to stress that in subjects with only a single normal band by PCR, the DM mutation should not be considered excluded, because of the possibility of preferential amplification of the normal allele. In such instances, additional studies are required, using either alternative primer combinations, conventional Southern blotting of genomic fragments, or linked genetic markers. Only when such studies confirm the presence of normal alleles only, can the consultant be reassured that future offspring will not be at risk.

We wish to thank Drs van de Biezenbos, van Essen, Snoek, Sterringa, Niermeijer, Busch, Höweler, De Die-Smulders, and Hennekam for providing additional clinical information. We thank Marcel Nelen and Hennie Bruggenwirth for skilful technical assistance. This study was supported by the American Muscular Dystrophy Association, the Prinses Beatrixfonds, Grant 87-2694, and by Grant Ro 389/15-4 from the Deutsche Forschungsgemeinschaft.

- 1 Harper PS. *Myotonic dystrophy*. 2nd ed. London: Saunders, 1989.
- 2 Harley HG, Brook JD, Rundle SA, *et al.* Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. *Nature* 1992;355:545-6.
- 3 Aslanidis C, Jansen G, Amemiya C, *et al.* Cloning of the essential myotonic dystrophy region and mapping of the putative defect. *Nature* 1992;355:549-51.
- 4 Buxton J, Shelbourne P, Davies J, *et al.* Detection of an unstable fragment of DNA specific to individuals with myotonic dystrophy. *Nature* 1992;355:547-8.
- 5 Brook JD, McCurrach ME, Harley HG, *et al.* Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell* 1992;68:799-808.
- 6 Fu YH, Pizzuti A, Fenwick R, *et al.* A sequence scanning method identifies an unstable triplet repeat in myotonic dystrophy. *Science* 1992;255:1256-8.

- 7 Mahadevan M, Tsilfidis C, Sabourin L, *et al.* Identification and characterization of an unstable trinucleotide repeat at the myotonic dystrophy locus. *Science* 1992;255:1253-5.
- 8 Jansen G, Mahadevan M, Amemiya C, *et al.* Characterisation of the myotonic dystrophy (DM) region predicts multiple protein kinase isoform encoding mRNAs that differ at their 3' ends. *Nature Genet* (in press).
- 9 Harley HG, Rundle SA, Reardon W, *et al.* Unstable DNA sequence in myotonic dystrophy. *Lancet* 1992;339:1125-8.
- 10 Tsilfidis C, MacKenzie AE, Mettler G, Barceló J, Korneluk RG. Correlation between trinucleotide repeat length and frequency of severe congenital myotonic dystrophy. *Nature Genet* 1992;1:192-5.
- 11 Höweler CJ, Busch HFM, Geraedts JPM, Niermeijer MF, Staal A. Anticipation in myotonic dystrophy: fact or fiction. *Brain* 1989;112:779-97.
- 12 Fleischer B. Ueber myotonischer Dystrophia mit Katarakt. *Graefes Arch Klin Ophthalmol* 1918;96:91-133.
- 13 Mathieu J, De Braekeleer M, Prévost C. Genealogical reconstruction of myotonic dystrophy in the Saguenay-Lac Saint-Jean area (Quebec, Canada). *Neurology* 1990;40:839-42.
- 14 Klein D. La dystrophie myotonique (Steinert) et la myotonie congénitale (Thomsen) en Suisse: étude clinique, génétique, et démographique. *J Genet Hum* 1958;7(suppl):1-328.
- 15 Lotz BP, Van der Meyden CH. Myotonic dystrophy. Part I. A genealogical study in the northern Transvaal. *S Afr Med J* 1985;67:812-4.
- 16 Verkerk AJMH, Pieretti M, Sutcliffe JS, *et al.* Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* 1991;65:905-14.
- 17 Fu YH, Kuhl DPA, Pizzuti A, *et al.* Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell* 1991;67:1047-58.
- 18 Rousseau F, Heitz D, Biancalana V, *et al.* Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. *N Engl J Med* 1991;325:1673-81.
- 19 Brunner HG, Smeets HJM, Nillesen W, *et al.* Myotonic dystrophy. Predictive value of normal results on clinical examination. *Brain* 1991;114:2303-11.
- 20 Griggs RC, Wood DS. The Working Group on the Molecular Defect in Myotonic Dystrophy. Criteria for establishing the validity of genetic recombination in myotonic dystrophy. *Neurology* 1989;39:420-1.
- 21 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215.
- 22 Brunner HG, Smeets H, Lambermon HMM, *et al.* A multipoint linkage map around the myotonic dystrophy locus on chromosome 19. *Genomics* 1989;5:589-95.
- 23 Korneluk RG, MacKenzie AE, Nakamura L, Dube I, Jacob P, Hunter AGW. A re-ordering of human chromosome 19 long-arm DNA markers and identification of markers flanking the myotonic dystrophy locus. *Genomics* 1989;5:596-604.
- 24 Smeets HJM, Hermens R, Brunner RG, Ropers HH, Wieringa B. Identification of variable simple sequence motifs in 19q13.2-qter: markers of the myotonic dystrophy locus. *Genomics* 1991;9:257-63.
- 25 Jansen G, de Jong PJ, Amemiya C, *et al.* Physical and genetic characterization of the distal segment of the myotonic dystrophy area on chromosome 19q. *Genomics* 1992;13:509-17.
- 26 Harley HG, Walsh KV, Rundle S, *et al.* Localization of the myotonic dystrophy locus to 19q13.2-19q13.1 and its relationship to twelve polymorphic loci on 19q. *Hum Genet* 1991;87:73-80.
- 27 Smeets HJM, Nelen MR, Los F, *et al.* Myotonic dystrophy: prenatal diagnosis using direct mutation analysis. *Lancet* 1992;340:237-8.
- 28 MacKenzie AE, MacLeod HL, Hunter AGW, Korneluk RG. Linkage analysis of the apolipoprotein C2 gene on human chromosome 19 reveals linkage and linkage disequilibrium in a French-Canadian population. *Am J Hum Genet* 1989;44:140-7.
- 29 Nokelainen P, Alanen-Kurki L, Winquist R, *et al.* Linkage disequilibrium detected between dystrophin myotonia and APOC2 locus in the Finnish population. *Hum Genet* 1990;85:541-5.
- 30 Harley HG, Brook JD, Floyd J, *et al.* Detection of linkage disequilibrium between the myotonic dystrophy locus and a new polymorphic DNA marker. *Am J Hum Genet* 1991;49:68-75.
- 31 Nakahori Y, Knight SLJ, Holland J, *et al.* Molecular heterogeneity of the fragile X syndrome. *Nucleic Acids Res* 1991;19:4355-9.