

DNA amplification of the CTG repeats sequence in the molecular characterisation of Myotonic Dystrophy

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Abstract

Myotonic dystrophy (DM) results from an expansion of a (CTG)_n repeat at the 3' untranslated region of the myotonin-protein kinase gene. DNA amplification by PCR followed by oligonucleotide hybridisation and DNA analysis with probes have been used to detect the increase in the size of the expanded myotonic dystrophy-specific fragment. Using these techniques, we evaluated 6 DM subjects and family members of 3 of these subjects. The normal family members showed the normal amplified band, whereas affected patients and carriers gave both a normal and expanded DNA fragment. It was not possible to determine the number of repeats in the expanded PCR bands as the expanded alleles were observed as DNA streaks after oligonucleotide hybridisation. DNA analysis with restriction enzyme *Eco* RI and the p5B1.4 probe produced normal bands of 9.8 and 10.8 kb. Affected patients and carriers produced the normal 9.8 kb band and a larger (>10.8 kb) DM-specific fragment, except in one of the carriers where a smaller 10.5 kb band was observed. The size of the expanded alleles in our study ranged from 11.2-15.2 kb. The number of CTG repeats within this group of adult-onset DM patients did not correlate with the severity or age of onset of disease. There was also wide variation in the number of CTG repeats among affected siblings within the same family. Nevertheless, there was clearly an increase in the number of CTG repeats with successive generations consistent with the phenomenon of "anticipation". Carriers with the expanded alleles could not be identified from the clinical examination, illustrating the importance of DNA analysis for the purpose of genetic counselling.

Key words: myotonic dystrophy, molecular genetics, Singapore

INTRODUCTION

Myotonic Dystrophy (DM) is one of the most prevalent inherited forms of progressive neuromuscular disorder among adults, with an average incidence of 1:8000 in Europeans and 1:20,000 in the Japanese.^{1,2} A correlation has been observed between the severity and age of onset of the disease.^{3,4,16,17,18} Individuals with DM exhibit a wide range of symptoms ranging from progressive muscle weakness with myotonia, baldness, characteristic facial features, cardiac arrhythmia, infertility, and cataracts to endocrine disorders.^{5,6,7} The molecular basis of DM has recently been mapped to the 3' untranslated region of the myotonin-protein kinase gene on chromosome 19q13.2-13.3.^{8,9,10} Patients with DM were observed to possess a variable expansion of an unstable DNA fragment at this region. The unstable site has been identified as a CTG trinucleotide repeat and DM results from an expansion of the CTG repeat in

this region.^{3,11} Normal individuals were reported to have CTG repeats of 5-35 copies, mildly affected patients showed CTG repeats of more than 50 copies and severely affected patients had repeats of more than 2000 copies.⁶ The DM mutation is mitotically unstable demonstrating the phenomenon of "anticipation" i.e. increases in the length of the CTG repeats through successive generations. This is reflected clinically in more severe and earlier onset of disease in subsequent generations. Molecular studies indicated that congenitally affected newborn patients had the longest repeat sizes coupled with an earlier age of onset of disease.^{6,10} However, DM occurs as a severe neonatal form only when transmission is from the carrier mother.

Molecular characterisation of DM is currently carried out by DNA amplification using the polymerase chain reaction¹² with oligonucleotide hybridisation^{13,14} and by the Southern Blotting technique with gene probes.^{4,10}

In our study, molecular characterisation of the CTG repeat in 6 DM patients and family members of 3 of these patients was carried out using both PCR with oligonucleotide hybridisation, and Southern Blotting with the p5B1.4 gene probe.

MATERIALS AND METHODS

Patients: Six patients diagnosed clinically to be suffering from DM were included in the study. These patients had typical features of DM with myotonia detected on needle electromyography. Family members of 3 of these patients were also available for DNA analysis.

DNA extraction: Peripheral blood samples (10 ml) were collected in EDTA tubes and stored at -70°C . Blood samples were thawed at 37°C and resuspended in cold Tris-EDTA (10 mM Tris pH 8.0/10 mM EDTA.) The white blood cells (WBC) were pelleted by centrifugation for 10 minutes at 8000 rpm. Repeat washings with TE were continued until WBC pellets were clear. The WBC were then resuspended in cold TE 10/10, 10% sodium dodecyl sulphate and proteinase K and incubated overnight at 37°C . DNA was purified with phenol-chloroform-isoamylalcohol extraction and precipitated with 4M sodium chloride in 2 volumes of ice-cold ethanol. Precipitated DNA was washed in 100% cold ethanol and vacuum dried before resuspension in sterile double distilled water. DNA concentration was measured spectrophotometrically at OD_{260} .

Oligonucleotide primers: Oligonucleotide primers used for amplification of the CTG trinucleotide repeat sequence were 101:5'-CTTCCCAGGCCTGCAGTTTGC-CCATC-3' and 102:5'-GAACGGGGCTCGAAGGGTCCTTGTAGC-3'(15).

DNA amplification using the polymerase chain reaction: DNA amplification was carried out in 25 μl reactions with 1 microgram DNA, 10 pmol primers, 200 mM dNTPs and 0.5 units of Taq Polymerase.⁵ The PCR mixture was first denatured at 98°C for 2 min. DNA amplification was then carried out for 35 cycles at 98°C for 1 min and 72°C for 5 min with a final extension of 20 min at 72°C .

Gel electrophoresis of amplified DNA products and Southern Blotting: Amplified DNA (10 μl)

was electrophoresed in 1% agarose. DNA was visualised under ultra-violet illumination after ethidium bromide staining. Amplified DNA was transferred to Hy-Bond N membranes by the Southern Blotting technique.

Hybridization with oligonucleotide (CTG)₁₀ probe: An oligonucleotide consisting of 10 repeats of the trinucleotide (CTG) was synthesised and used as a probe. The (CTG)₁₀ repeat was labelled at the 5' end with T4 polynucleotide kinase using [$g\text{-}^{32}\text{P}$] dCTP.¹⁵ Membranes were prehybridised for 1 h at 42°C . Hybridised membranes were washed once at room temperature for 10 min and for 20 min at 51°C . The membranes were then exposed to X-ray films (Kodak X-Omat AR, Kodak) at -70°C overnight.

DNA analysis: Genomic DNA was digested with the restriction enzyme Eco RI according to manufacturer's recommendations. Digested DNA was electrophoresed in 0.8% agarose overnight and then transferred to Hy-Bond N membranes by the Southern Blotting technique. The gene probe p5B1.4 (a kind gift from Prof L J Garey, University London, UK) was labelled with [$a\text{-}^{32}\text{P}$]dCTP using the Mega Prime labelling kit. Membranes were prehybridised overnight at 42°C and then hybridised with the labelled probe for 24 h. Membranes were then washed at room temperature for 10 min followed by a stringent wash at 52°C for 20 min. Bands were visualised after autoradiography with X-ray films at -70°C .

RESULTS

Clinical characteristics: There were 5 males and 1 female Chinese subjects. The age at diagnosis ranged from 22 to 46 years with a mean age of 32. The index case in family A was a 30 year-old male. He presented with diffuse muscle weakness and atrophy at the age of 27. Both parents and a 31 year-old sister did not exhibit any clinical features of the disease. Examination of his other siblings revealed an 18 year-old brother and a 28 year-old sister with clinical characteristics of the disease, albeit less severe in terms of muscle atrophy. The index case in family B was a 23 year-old male. He presented with diffuse muscle weakness and atrophy. Both parents and 2 sisters were normal clinically. The index case in family C was 22 year-old at presentation. He was referred from the military service because of failure to fulfil

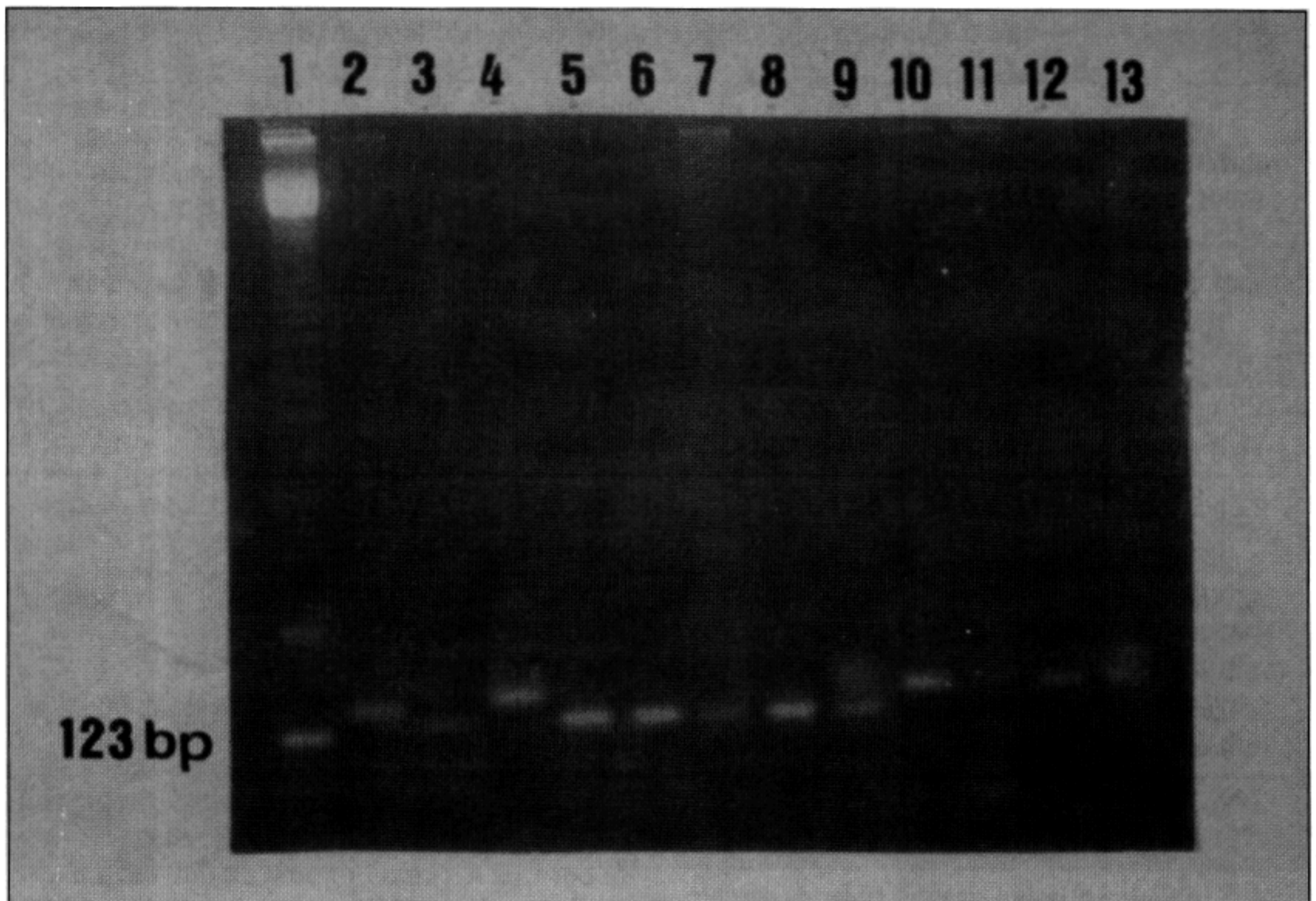


FIG. 1: Agarose gel electrophoresis of PCR products. Lane 1 - Molecular weight marker, Lanes 2 and 3 - DM patients 1&2; Lanes 4-9 - Family A, father, mother, propositus, brother, 2nd sister, and 1st sister; Lane 10-12 - Family B, Father, mother, propositus, sister

his physical-training standard. Apart from myotonia and mild weakness of his masseter muscles, he had no atrophy or weakness of his limb muscles. Both his parents and an 18-year-old brother were normal clinically. However, the other 24 year-old brother like him had myotonia clinically. The other 3 individuals studied, presented with muscle weakness and atrophy in late adulthood.

DNA amplification by PCR: The PCR produced three classes of results: (a) subjects with a normal and an expanded allele, (b) subjects with two normal alleles and (c) subjects with a single normal allele, as shown in Figure 1. After gel electrophoresis of PCR products, the normal allele(s) was seen as a distinct band(s) while the expanded allele was seen as a distinct streak of DNA in only one affected patient (Fig. 1, lane 7).

Oligonucleotide hybridization: Oligonucleotide hybridisation with the oligonucleotide (CTG)₁₀ probe was carried out on the DNA after Southern Blotting. After hybridisation, all affected patients and their carrier parent (father or mother) showed

a normal and an expanded allele while normal subjects showed only the normal band(s) (Fig. 2). In lanes 2 and 3, a normal and an expanded band were amplified from DNA of two affected patients. Lanes 4 -9 represent PCR products from Family A with three affected children and one normal child. DNA from the father (lane 4) and the three affected children (lanes 6,7,8) amplified one normal allele and an expanded allele, while DNA from the unaffected mother and sister (lanes 5,9) amplified only the normal allele. Lanes 10 -13 represent DNA from Family B with one affected and one normal child. Expanded PCR bands were observed in the mother and affected son (lanes 11,12) while the father and daughter showed only normal bands (lanes 10,13). Members of family C showed similar results, with expanded PCR bands observed for the carrier father and his three sons (data not shown).

DNA analysis: DNA analysis was carried out to confirm the results derived from oligonucleotide hybridisation and to determine the molecular weights of the expanded bands. Hybridisation of DNA digested with restriction enzyme Eco

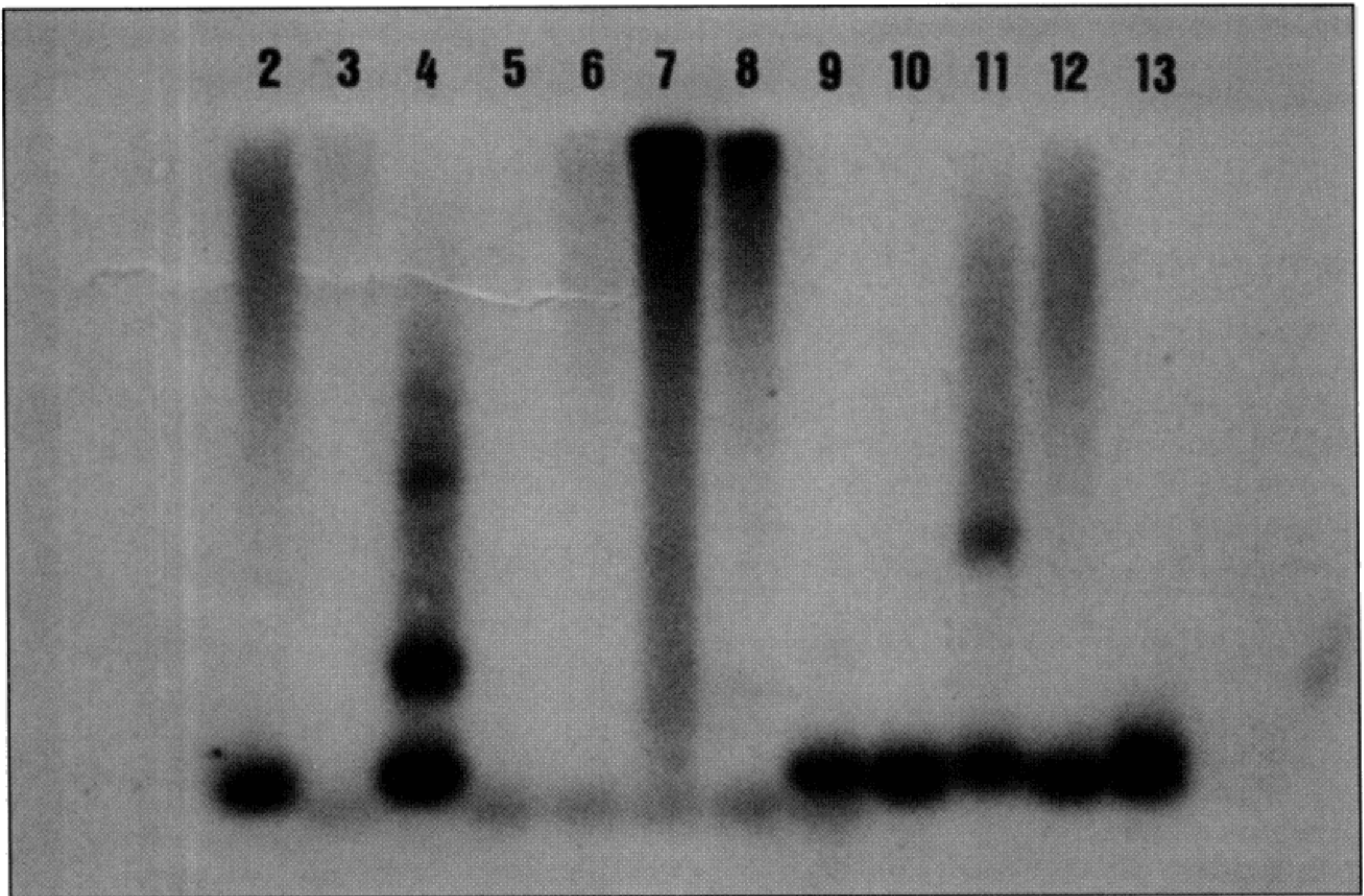


FIG. 2: Results of hybridisation of electrophoresed PCR products using (CTG)₁₀ oligonucleotide probe. Lanes 2 and 3 - DM patients 1&2'; Lanes 4-9 - Family A, father, mother, propositus, brother, 2nd sister, and 1st sister; Lane 10-12 - Family B, father, mother, propositus, sister.

RI with the p5B1.4 probe produced normal bands of 9.8 and 10.8 kb (Fig. 3). All carriers and patients with DM showed a single normal allele accompanied by a larger myotonic dystrophy-specific band, except for the mother

in Family B (lane 11) where the normal 9.8 band was observed with a smaller 10.5 kb band. The normal bands in Family B appeared slightly larger in molecular weight (lanes 11,12,13) in the autoradiograph, and this was due to

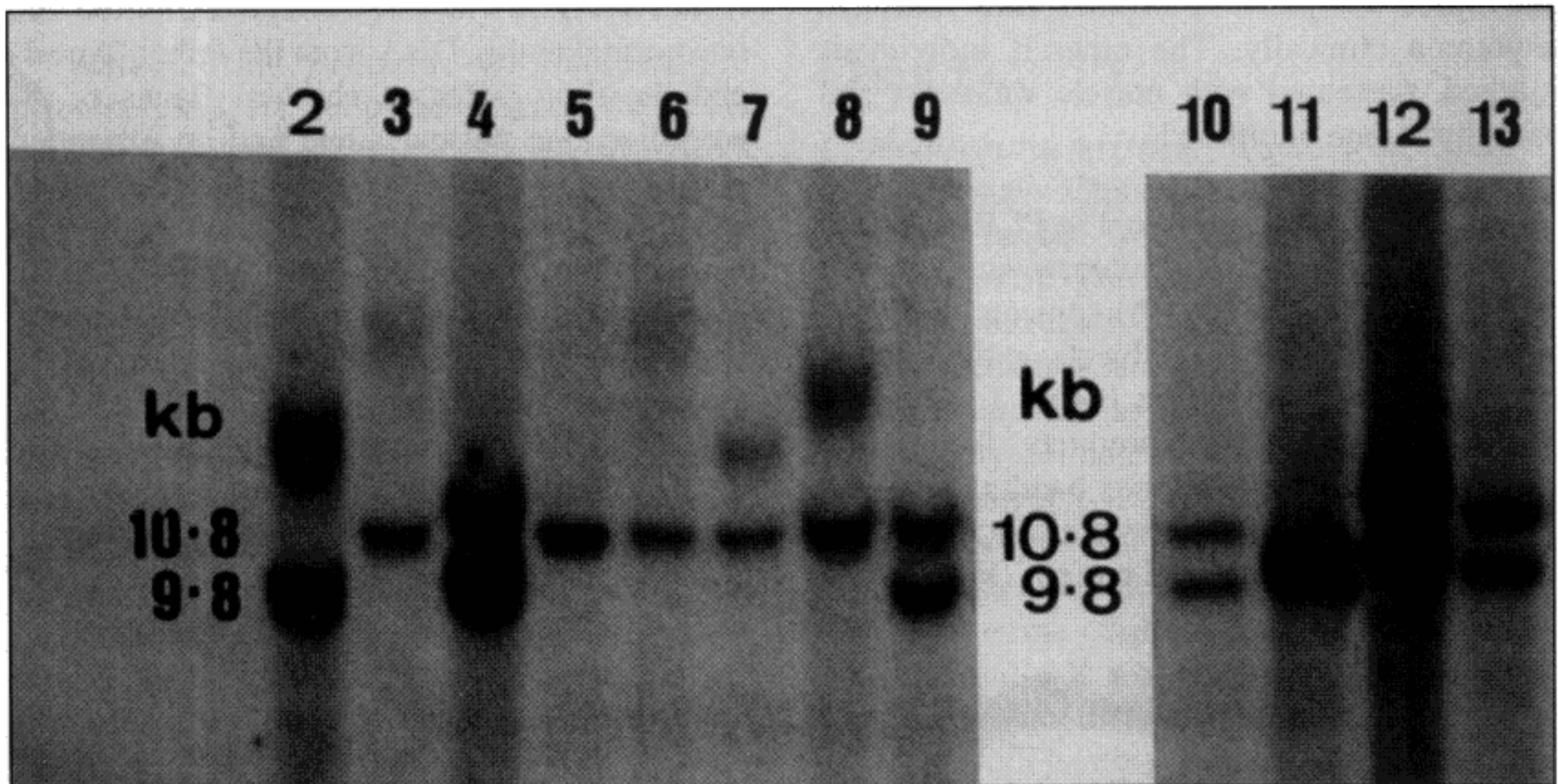


FIG. 3: Restriction fragment length polymorphism analysis of genomic DNA using p5B1.4 gene probe. Lanes 2 and 3 - DM patients 1&2'; Lanes 4-9 - Family A, father, mother, propositus, brother, 2nd sister, and 1st sister; Lane 10-12 - Family B, father, mother, propositus, sister.

overloading of DNA in the wells. The expanded (>10.8 kb) myotonic dystrophy-specific band varied in size in the affected patients and carriers. Members of Family C (not shown in Figure 1-3) showed the presence of expanded alleles in the father and his three sons. Table 1 summarises the data on the clinical phenotype, PCR analysis and RFLP analysis of the 3 families studied.

All 6 patients with DM had disease onset after the age of 20 (Table 1). In this group of patients, the length of the trinucleotide repeats varied from 1.5-5.7 kb (500-1900 repeats). Within this small group there was no correlation between the number of trinucleotide repeats and the age of onset or severity of disease. However for the individual families, there was clearly an increase in the number of CTG repeats for the successive generation. Transmission was maternal in 1 family and paternal in the other 2. These carriers with the expanded allele were clinically normal. In the 2 families where other siblings were also affected, the number of CTG repeats among siblings within the same family varied from 466 to 1466 in Family A to 1466 to 1900 in Family C. However, the index cases in both families had larger alleles compared to

their less severely affected siblings.

DISCUSSION

Molecular analysis of the DM patients and their family was carried out by PCR with oligonucleotide hybridisation and by DNA analysis to detect expansions in the DM gene and to determine the size of the DM-specific fragment.

In Family A, the asymptomatic father was detected as the carrier of DM as he clearly showed an expanded PCR band (Fig. 2, lane 4). The parents of Family A possessed normal PCR bands of different molecular weights (Figure 1, lanes 4,5). The 3 affected children inherited the expanded allele from their father and the normal allele from their mother as their normal bands were of the same molecular weight as their mother's (Fig. 1, lanes 5,6,7 and 8). In the case of family B, the parents produced normal PCR bands of similar molecular weight. The mother was concluded to be the carrier solely from the oligonucleotide hybridisation study, which clearly showed the expanded band (Fig. 2, lane 11). In family C, the father and his 3 sons also

TABLE 1: Relationship between the phenotype and age of onset with the result of DNA amplification of the CTG repeats sequence.

Subjects	Family relationship	Age	Phenotype	PCR & Probe	RFLP alleles (kb)
1	Nil	47	DM	+	9.8/12.4 (1.5)
2	Nil	37	DM	+	10.8/15.2 (4.4)
3	Nil	38	DM	+	10.8/13.0 (2.2)
4	Index case (Family A)	27	DM	+	9.8/15.2 (4.4)
5	Brother (Family A)	15	DM	+	9.8/12.2 (1.4)
6	Sister (Family A)	25	DM	+	10.8/13.8 (2.0)
7	Sister (Family A)	28	Normal	-	9.8/10.8
8	Mother (Family A)	52	Normal	-	10.8/10.8
9	Father (Family A)	60	Carrier	+	9.8/11.2 (0.4)
10	Index case (Family B)	23	DM	+	10.8/11.5 (1.7)
11	Sister (Family B)	21	Normal	-	9.8/10.8
12	Mother (Family B)	46	Carrier	+	9.8/10.5 (0.7)
13	Father (Family B)	48	Normal	-	9.8/10.8
14	Index case (Family C)	22	DM	+	10.8/16.5 (5.7)
15	Brother (Family C)	24	DM	+	10.8/15.2 (4.4)
16	Brother (Family C)	18	Carrier	+	9.8/11.2 (0.4)
17	Mother (Family C)	45	Normal	-	9.8/10.8
18	Father (Family C)	49	Carrier	+	9.8/11.2 (0.4)

Figures in parenthesis denotes expansion in kilobases

DM: Dystonia Myotonica

PCR: Polymerase chain reaction

RFLP: Restriction fragment length polymorphism

showed the expanded bands (not shown).

DNA analysis was also performed on 3 patients whose family members were not available. The 3 DM patients clearly showed normal 9.8 and 10.8 kb bands and the expanded DM-specific fragment of between 12.4 and 15.2 kb. In our study of the 3 families, we were not able to determine clinically which parent carried the DM gene. In Family A, the father was found to be the carrier as his DNA showed the expanded DM allele with oligonucleotide hybridisation and the size of the allele was 11.2 by gene analysis. The mother in Family A only showed normal alleles. The 3 affected children clearly showed the expanded DM-specific fragment of 15.2 kb, 12.2 kb and 13.8 kb. The unaffected sister showed only the normal 9.8 and 10.8 kb bands. Using PCR and oligonucleotide hybridisation the mother in Family B was found to have an expanded PCR product high suggestive of a carrier. However, gene analysis (carried out twice) did not show any expansion but instead produced the normal 9.8 kb band and a smaller band of 10.5 kb. We are not able to explain the 10.5 kb band. The affected son showed the 10.8 kb band and an expanded 11.5 kb fragment. In family C, the father was found to be a carrier by PCR and RFLP analysis.

In assessing the impact of increased number of CTG repeats on the severity and clinical onset of the disease, Redman et al¹⁶ have devised categories for age of onset including congenital (1 year), juvenile (1-20 years) and adult onset (>20 years). They found a relationship between increasing repeat length and earlier clinical onset of disease although overlap of clinical categories existed. Other authors have also observed that the size of the CTG repeats correlated statistically not only with the age-of-onset but also the clinical severity of the disease.^{16,17,18} Since all 6 of our patients had their onset of disease after the age of 20, it was not possible to correlate the onset of disease with the length of the CTG repeat according to the broad age bands used by other investigators. However, within this small group of adult-onset DM patients, no relationship was observed between the size of the CTG repeats and the age of onset or clinical severity of disease. The patient diagnosed at age 46, for example, had CTG repeat length of 1.5 kb while another patient diagnosed at age of 22 had repeat length of 5.7 kb. The inability to pinpoint the onset of disease is a potential source of error in such analysis. Other investigators have also emphasised limitation in the ability to predict disease severity based on the length of the CTG

repeats within arbitrary defined age groups.^{16,17,18} In the 3 families in this study, there was a tendency for an increase in the length of the CTG repeat in successive generation. The CTG repeats ranged from 0.4 to 0.7 kb in asymptomatic members of the first generation with the expanded alleles, while their affected offsprings have CTG repeat length varying from 1.4 to 5.7 kb. This phenomenon of genetic anticipation results in an increase in the severity of the disease in successive generations. In the 2 families with other affected siblings, the length of the CTG repeats showed considerable variation even with the same families. In agreement with the study of Hunter et al¹³, sibs with the larger alleles tended to show earlier onset of disease.

In conclusion, it is clear that for DM, DNA analysis for the unstable trinucleotide repeats, is required for the identification of carriers for accurate genetic counselling and prenatal diagnosis. The phenomenon of genetic anticipation, whereby disease manifestations increase in severity with successive generations, was also observed in this small series.

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