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Carrier detection by microsatellite analysis of Duchenne/Becker muscular dystrophy in Hungarian families

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Summary

Duchenne and Becker muscular dystrophies are among the most severe and frequent inherited disorders. Being still incurable, medical treatment is concentrated on the carrier diagnosis of the members of the affected families. Here we report the results of the studies of 151 members of 41 Hungarian families, obtained with multiplex PCR amplification of 18 exons as well as the muscle specific promoter region, and haplotype analysis of two polymorphic $(CA)_n$ repeat microsatellite loci in introns 45 and 49 of the dystrophin gene. The analysis of 15 deletion-type families revealed a frequency of new mutations not differing significantly from that in the other regions of Europe. We also compared the allele distributions of the two microsatellites in randomly selected normal individuals and affected family members. The allele distribution of STRP45 shows interesting differences between the two populations.

INTRODUCTION

Duchenne/Becker muscular dystrophies (D/BMD) are amongst the most common and severe X-linked disorders, with an incidence of approximately 1 in 3500 new-born males (Roberts, 1995). In most cases deletions in the dystrophin gene have been shown (den Dunnen *et al.* 1987; Hu *et al.* 1990). Due to the giant size of the gene, new mutations were reported in one third of the patients from different populations (Roberts, 1995). The determination of carriers is an important aspect in providing prenatal diagnosis and genetic counselling to families with D/BMD patients. The detection of carrier status of at-risk family members is problematic however, because of the insufficient number of informative markers. The methods are usually based on haplotype or dosage analysis of polymorphic markers, or short tandem repeat polymorphisms (STRPs), Southern blots or quantitative PCR (Mao & Cremer, 1989; Hu *et al.* 1990; Clemens *et al.* 1991).

Here we report the analysis of family members of Hungarian D/BMD patients. The original molecular diagnosis of the patients was made by multiplex PCR (Beggs et al. 1990; Chamberlain *et al.* 1990). The analysis of the carrier status of the individuals was carried out using two short tandem repeat polymorphic markers within introns 45 and 49 of the dystrophin gene (Clemens *et al.* 1991). The loss of heterozygosity of these markers indicates carrier status. Since microsatellite analysis has been successfully used to compare genetic diversity among populations (Brinkmann *et al.* 1998), we have also investigated if these STRP markers exhibit any population-specific characteristics.

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This is the first attempt to estimate the rate of new mutations in D/BMD in this part of Europe. This is particularly relevant, since the region's environment is considered to be more contaminated than other parts of Europe, and therefore a higher than 'normal' mutation rate would be expected.

materials and methods

Patients and controls

The diagnosis of our DMD and BMD patients was based on the clinical symptoms, the highly elevated serum creatine phosphokinase (CPK) values, myopathic changes in electromyography and on muscle biopsy. Blood samples from 50 unrelated, randomly chosen new-born children were used as controls.

Isolation of genomic DNA from peripheral blood

Blood samples were collected from the patients and family members on the basis of informed consent. DNA was prepared from leukocyte nuclei with slight modifications of the method described by Woodhead *et al.* (1986). Briefly, 4 ml peripheral blood was anticoagulated with 1 ml of 5% EDTA and haemolysed by freeze-thawing. White blood cells were lysed with the addition of 45 ml lysis buffer (0.32 M sucrose, 5 mM $MgCl_2$, 10 mM TRIS-HCl pH: 7.4, 1% Triton-X100). Nuclei were pelleted by centrifugation, washed in PBS twice and resuspended in 1 ml solution of 75 mM NaCl, 50 mM EDTA. Nuclei were lysed and digested with the addition of 100 μl 10% SDS and 22 μl 25 mg ml^{-1} Proteinase K (Merck) (1% and 0.5 mg ml^{-1} final, respectively) overnight at 55 °C. DNA was phenol extracted, precipitated with ethanol, and dissolved in TE.

Multiplex PCR

For the detection of deletions in the dystrophin gene, the multiplex PCR methods described by Beggs *et al.* (1990) and Chamberlain *et al.* (1990) were used.

Detection of STRPs

The oligonucleotide primers used for PCR amplification of STRP45 (DMD-45/A and DMD-45/B) and STRP49 (DMD-49/A and DMD-49/B) were described by Clemens *et al.* (1991). Primers for the internal amplification control CFTR8 were the following: CFTR8F: 5′-TCTATCTCATGTTAATGCTG-3′; CFTR8R: 5′-GTTTCTAGAGGACATGATC-3′. The amplification was carried out in 50 μl total volumes, containing 80 ng genomic DNA, 10 pmol each of forward and reverse primers for the STRP45 or STRP49, and 20 pmol of each primer for the CFTR8 STRP, 200 μM Σ dNTP, 10 mM Tris-HCl, pH:8.5, 50 mM KCl, 1.5 mM $MgCl_2$, 0.3 μCi ^{32}P[α-dCTP] and 3U Taq polymerase. The amplification was carried out in the following cycles: 94 °C, 4 min → 53 °C, 45 sec → 72 °C, 30 s: 1 ×; 94 °C, 30 s → 53 °C, 45 s → 72 °C, 30 s: 26 ×; 94 °C, 30 s → 53 °C, 45 s → 72 °C, 4 min: 1 ×; 4 °C. 2.5 μl product was mixed with equal amount of sequencing dye, denatured at 80 °C for 2 min, run on 6% denaturing polyacrylamide sequencing gel and autoradiographed. The sizes of alleles were determined by comparison with control sequencing reactions of plasmids with known sequences, electrophoresed on the same gel.

Statistical analysis

Sum of Gaussian curves was fitted to the observed data with robust method according to the equation:

$$\sum_{i=1}^{3} \frac{k_i}{s_i} \times \exp\left(-0.5 \times \frac{x - m_i}{s_i}\right)^2,$$

where m and s are the mean and standard deviation values, k/s is the height and i is the number of the peaks. The goodness of fit was assessed by the Kolmogorov–Smirnov goodness of fit method (Zar 1984).

results and discussion

In the studies presented here, 151 members of 41 families with one or more D/BMD patients were involved. Based on clinical and diagnostic

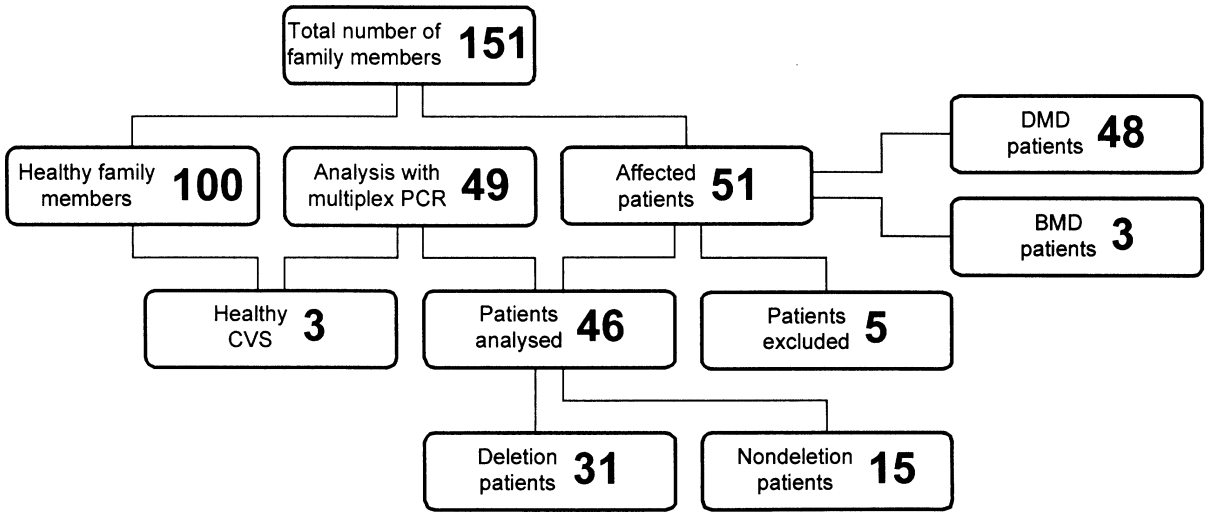

Fig. 1. Flowchart summarising the distribution of the individuals included in this study, and the results of the multiplex PCR analysis. Due to the earlier death of five patients they were not involved in the multiplex PCR analysis, the genetic counselling of their family members based on STRP analysis.

symptoms, out of 51 patients, 48 showed Duchenne and 3 showed Becker allelic forms of the disease. We also had two symptomatic female carriers, in one family the mother, in another family the sister of the DMD patient showed the symptoms. This finding is well known in the literature (Kowalewski *et al.* 1966; Barkhaus & Gilchrist, 1989; Shigihara-Yasuda *et al.* 1992).

Multiplex PCR analysis

In order to detect gene deletions, 19 intragenic regions, spanning 18 exons as well as the muscle specific promoter, were amplified in multiplex PCR reactions. Forty-four D/BMD patients and five chorion biopsy samples from male foetuses were involved in the multiplex PCR studies (Fig. 1). In three chorion biopsy samples it was possible to rule out the disease. In two cases from one family the foetus proved to be affected, and on the genetic counselling the parents decided to abort the pregnancies (mother D/303 in Fig. 6). Thirty-one out of 46 affected patients (67.4%) showed deletions, while 15 showed no deletions in the regions studied. The distribution of the exon deletions (Fig. 2) is in good agreement with the studies published by others (Roberts, 1995), with most deletions falling into the 3′ deletion hot spot region, between exons 45 and 52.

STRP analysis

Out of the 151 family members, 138 were involved in the STRP studies. In 78 cases both STRPs (in intron 45 and 49, respectively) were analysed, while in 60 cases only the STRP in intron 45 was used. Both STRPs were used in families, where deletion of either marker was expected. Results of the STRP analysis are summarised in Fig. 3.

The data obtained from 34 family members of 13 families did not result in informative polymorphic profile; mutations causing the dystrophic phenotype fell out of the region studied. As it has already been reported, STRP analysis can provide suitable information for carrier detection in the absence of DNA from an affected boy (Richards *et al.* 1992). Similarly, we tried to trace back the mutations in five patients of three families from the STRP profile of the relatives (they were not involved in the multiplex PCR analysis either, see Fig. 1). Neither STRP markers were found to be deleted in the relatives, presumably, because the mutation did not hit these regions. The STRP profiles, therefore, proved to be non-informative; no reliable genetic advice could be given to the families.

The analysis was informative in 28 families, providing useful STRP profiles for either estab-

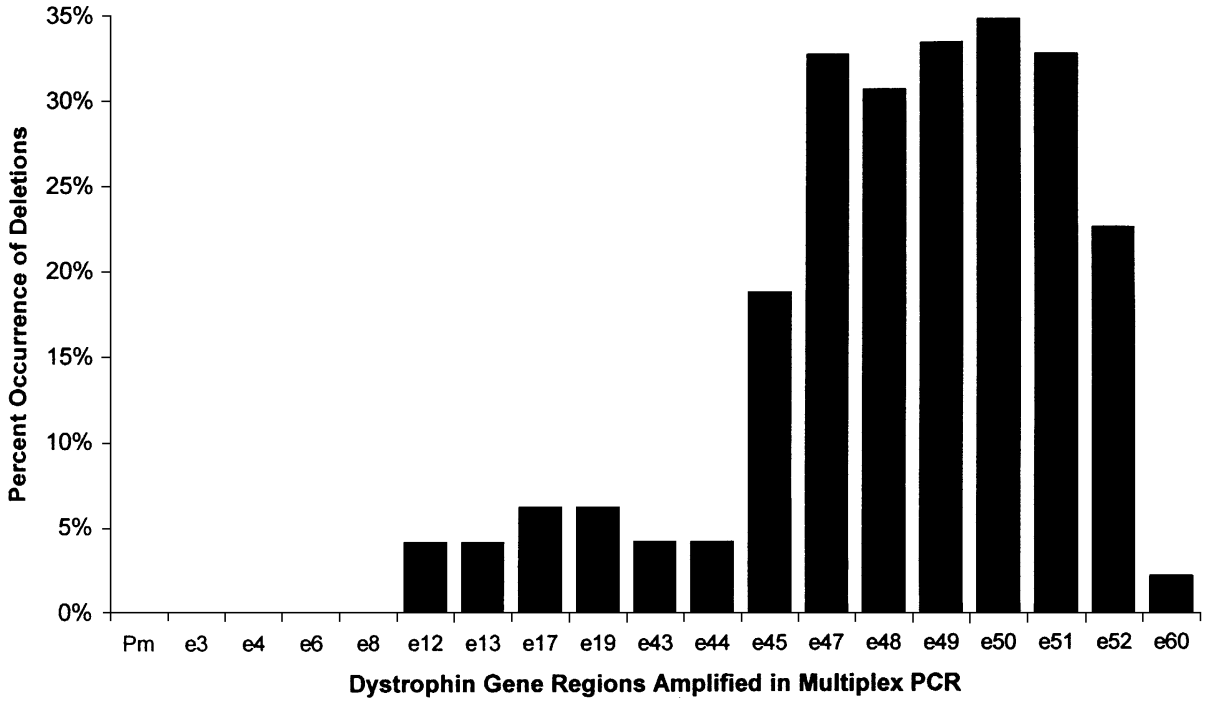

Fig. 2. Frequency of deletions in the 19 studied regions of the DMD gene in Hungarian families. Pm: muscle specific promoter, e3-e60: exons.

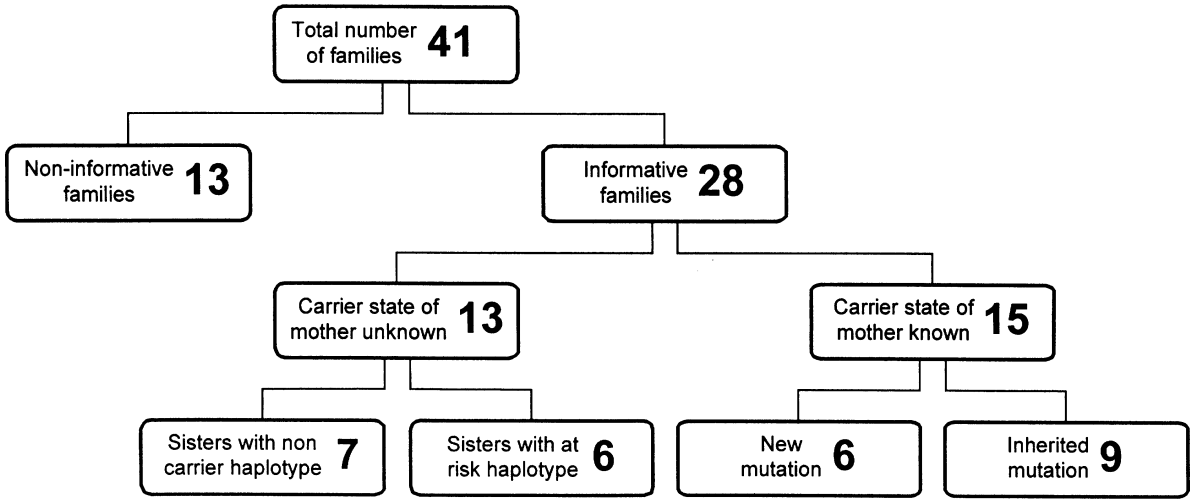

Fig. 3. Flowchart summarising the distribution of families analysed with STRP45 and STRP49. A family is considered to be informative if either the carrier state of the mother can be proven, or the potential carrier status of sisters of either the mother or the affected patient can be determined.

lishing the origin of the mutation, or tracing potential carriers in the affected families.

Twenty-one probands of 15 families had deletion-type mutations that affected either or both STRPs. In nine families the affected 15 patients inherited the mutation from their mothers who were themselves carriers (10 carrier mothers), while in six families (6 patients) the mother proved to be non-carrier having new mutation. Thus, the new mutation rate is 37.5% (6 new mutation out of 16 mothers). This figure, though the number of patients is not high, is not very much different from the one third ratio of new mutations observed in other geographical

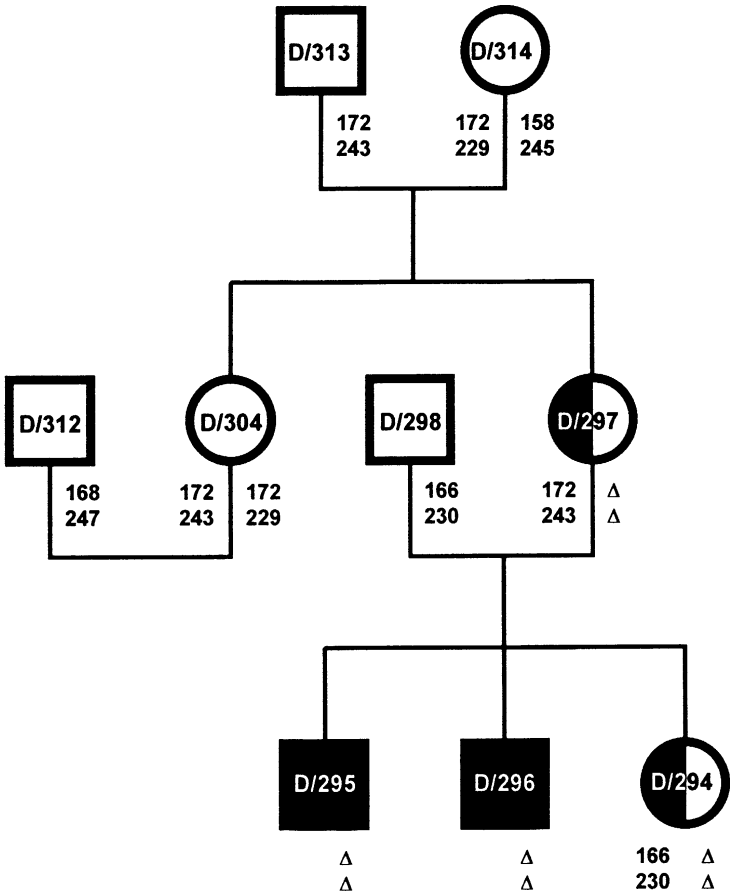

Fig. 4. Pedigree of a DMD family with mutation originated from the grandmother. The two affected males showed deletions between e45 and e52, that also affected the two STRP markers. Mother D/297, as well as chorion biopsy D/294 showed loss of heterozygosity of the two STRP markers. In the case of grandmother D/314, STRP analysis indicated intact chromosomes, therefore this grandmother must be the origin of a new mutation. The numbers below the symbols of each family members indicate PCR fragment sizes as alleles; upper number: STRP45, lower number: STRP49, Δ: deletion.

regions (Roberts, 1995), except for data from Northern Indian patients where higher frequency of new mutations was reported (Sinha *et al.* 1996).

In a few families it was possible to determine the origin of the mutation of the mother, because the grandparents of the probands were alive and their DNA could be analysed. As an example, the family tree in Fig. 4 shows a mutation that was developed in the maternal grandmother resulting in a carrier mother. The sister of the carrier mother does not show the deletion haplotype.

The pedigree in Fig. 5 shows a different mutational origin. Though DNA samples from the maternal grandparents were not available, the haplotype of the grandparents can be traced back based on the STRP profile of the carrier mothers. The origin of the mutation therefore can be explained with the asymptomatic carrier state or germline mosaicism of the grandfather; both cases have already been reported by others (Hennekam *et al.* 1989; Claustres *et al.* 1990). However, the trivial possibility of non-paternity can also be a plausible explanation.

The family tree in Fig. 6 shows a typical familial inheritance. Mother D/303 sought genetic counselling having had a deletion type brother, as were shown with multiplex PCR analysis. Her first two pregnancies resulted in two boy foetuses with the same multiplex PCR deletion pattern. She requested to abort both pregnancies. Third time her foetus proved to be a carrier daughter, whom she delivered successfully. Her sister also sought genetic advice

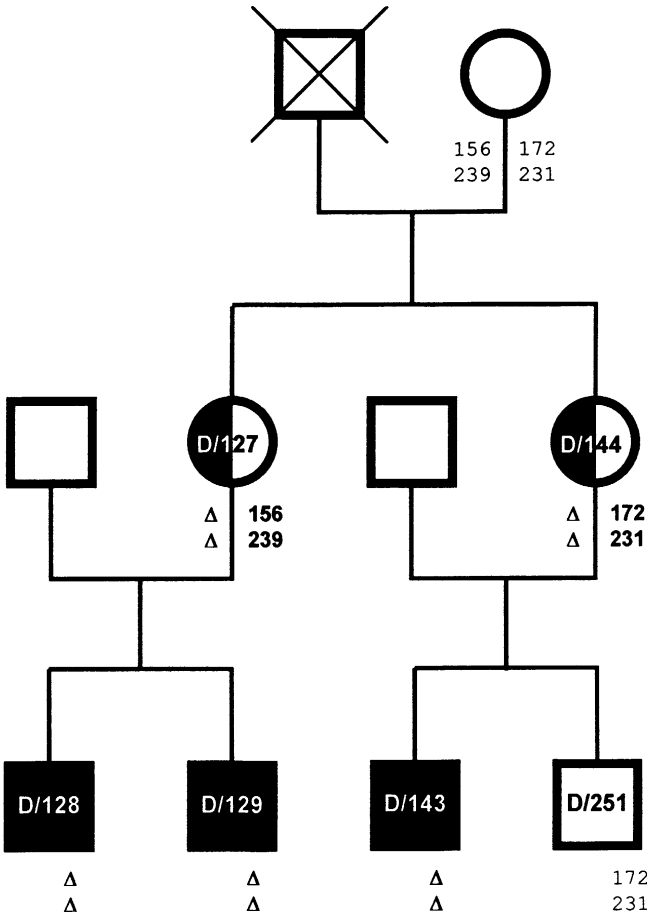

Fig. 5. Pedigree of a DMD family with the grandfather as proposed mutational origin. Multiplex PCR analysis indicated deletions between e45 and e50 in all three affected males, while chorion biopsy D/251 showed no deletions, proved to be healthy. Mothers D/127 and D/144 are hemizygous, therefore carriers, for the two STRP markers. Haplotype of the grandmother can be traced back from that of the mothers (thin faced numbers), in this case the origin of the mutation must be the grandfather.

during her pregnancy. In this case chorion biopsy showed a full trisomic chromosome set, that was further supported by the STRP analysis. This pregnancy ended with spontaneous abortion. With the STRP analysis later, the non-carrier status of this sister, and the carrier state of the grandmother were shown.

In 13 families the STRP markers studied were not hit by deletion event, therefore it was not possible to show where the mutation occurred. However, based on STRP profiles five sisters of seven families proved to be unlikely carriers, because their STRP haplotypes were different from that of the affected brothers. In this family group we made four prenatal diagnoses that resulted in two healthy boys, a non-carrier daughter, and a non-informative haplotype. Interestingly, in two families the haplotypes of the family members (carrier mothers and maternal grandmothers) showed that the chromosomes with the haplotypes linked to the disease must have come from the maternal grandfathers. Unfortunately, due to the lack of DNA samples from the grandfathers no further investigation was possible. In the other family group (6 families), seven sisters showed the same haplotype as the affected patients, therefore they were considered being at-risk carriers.

In two cases, the multiple PCR and STRP analyses provided contradictory results. One patient showed deletions of e45, e47, e48, e49 and e50. Contrary to this, amplification of the STR in intron 45 (STRP45) was successful, STRP49 was deleted. Similarly, another patient showed

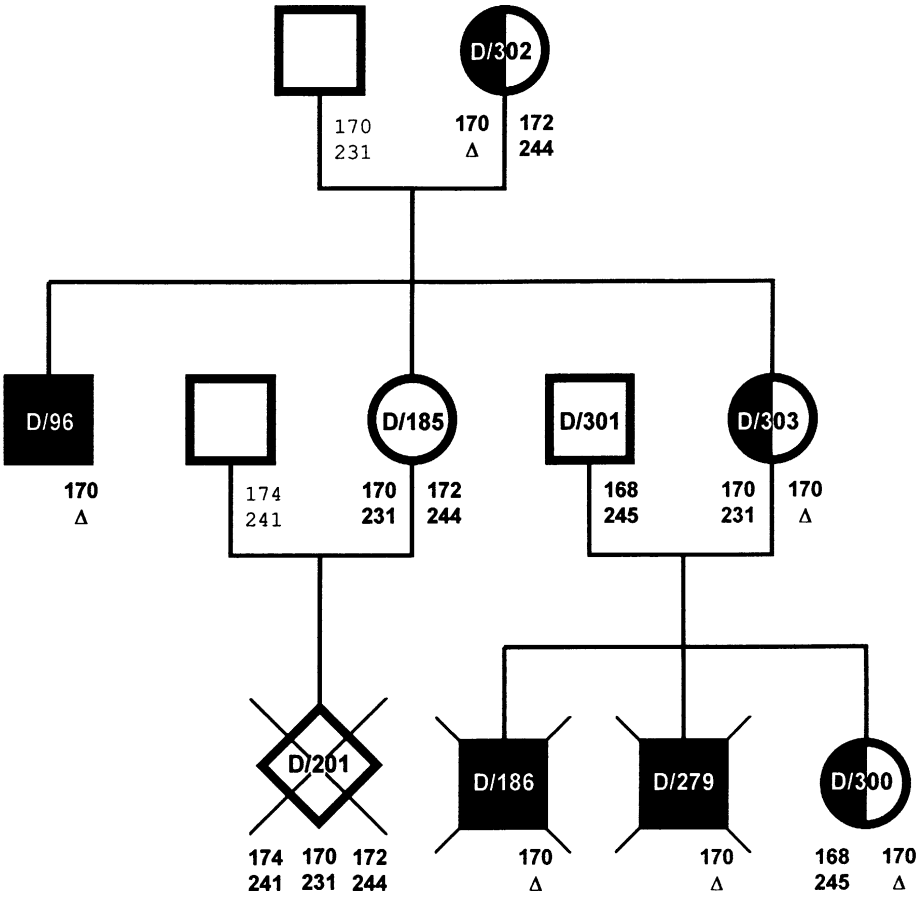

Fig. 6. A typical family tree showing inheritance of the disease through generations. The affected male, D/96 had a deletion type mutation between e47 and e52. Chorion biopsies D/186 and D/279 showed the same deletion pattern. Haplotype analysis revealed carrier status of D/302 and D/303, as well as chorion biopsy D/300. This family was also hit by the spontaneous abortion of D/201 who was trisomic for the full chromosome set. Thin-faced numbers indicate proposed STR haplotypes.

deletions of e49, e50, e51 and e52, while amplification of the STR in intron 49 (STRP49) succeeded. Both cases can only be explained with double recombination in the affected regions, which has also been reported by others (Morrone *et al.* 1997).

Distribution of STRP alleles in Hungarian populations

Since we had a relatively high number of families with D/BMD, it seemed appropriate to analyse the pattern of distribution of the polymorphisms in introns 45 and 49 and compare with those of healthy, randomly selected controls. Figure 7 shows the allele distributions of STRP45 originated from the analysis of 201 chromosomes from members of families with D/BMD patients, compared with those of 58 chromosomes of randomly selected Hungarian, and 57 chromosomes of American Caucasian healthy individuals (Clemens *et al.* 1991). There is a biphasic distribution with a smaller peak at 158 bp and a characteristic second peak at 174 bp in healthy Hungarian individuals, while in the case of members of families with muscular dystrophy probands this second peak is at 172 bp. The second peak is accompanied by a shoulder at 166 in the affected group, while this shoulder is at 168 bp in the control group. The distribution of the control group is also different from that published for the American Caucasian population, where the second peak is at 172 bp, the shoulder is at 168 bp.

These direct observations were also confirmed by statistical analysis. Three component normal

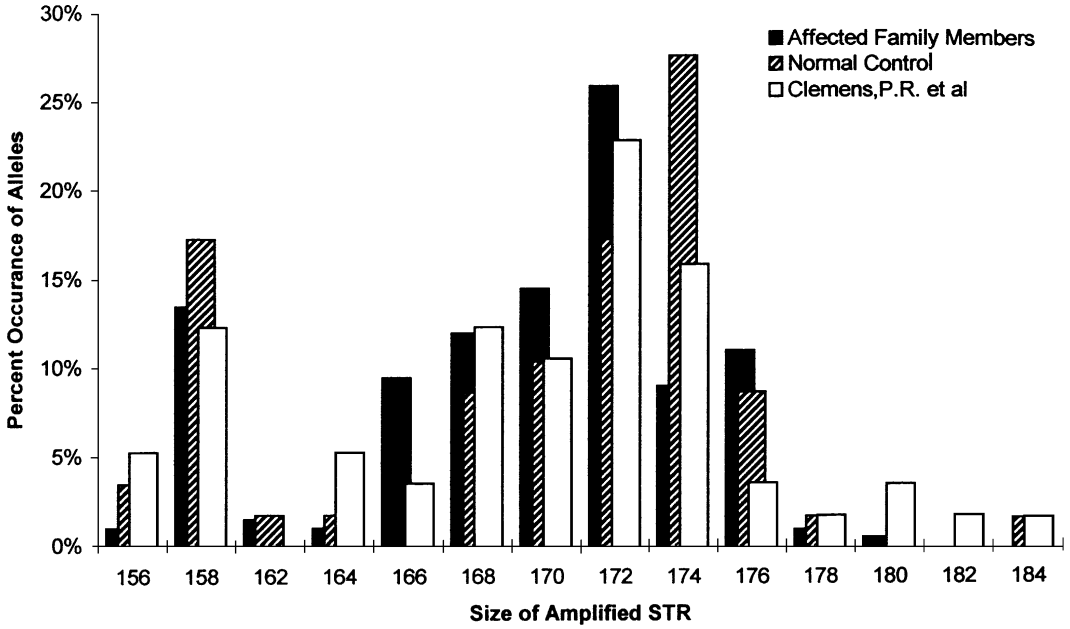

Fig. 7. Comparison of STRP45 allele distributions among members of families with D/BMD patients, randomly selected Hungarian and American Caucasian healthy individuals (Clemens *et al.* 1991). In the 164–178 bp range the distribution pattern of the affected family members shows a one-repeat shift towards the lower repeat number categories compared to the healthy Hungarian controls. Interestingly, the most frequent allele is 172 bp in the North-American population, similarly to the Hungarian affected family members. A secondary peak at 158 bp can be observed in all of the three different populations.

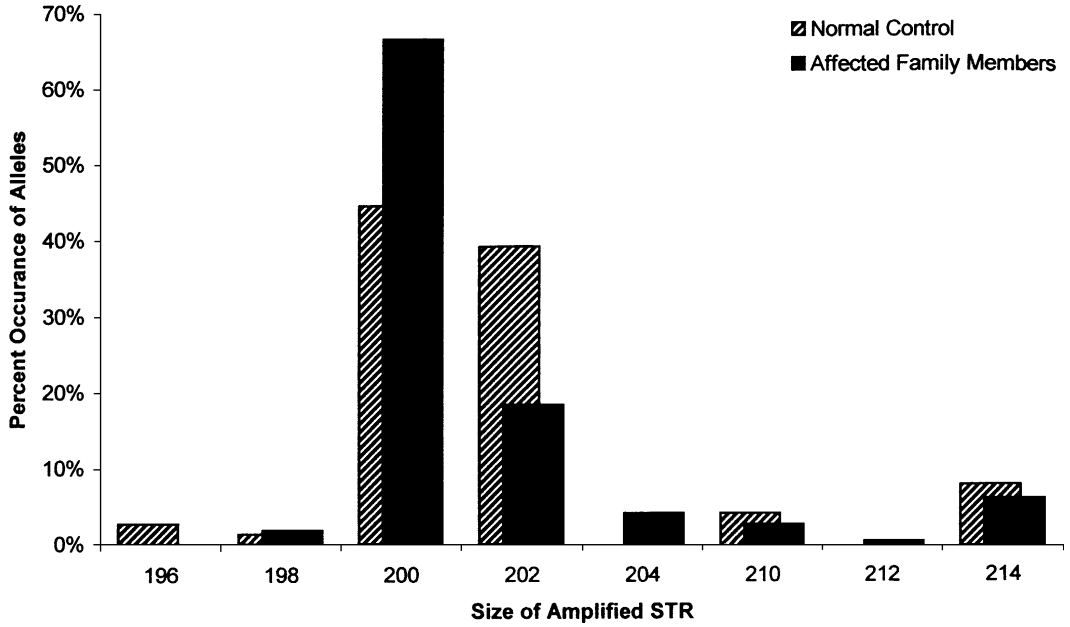

Fig. 8. Distribution of CFTR8 alleles in randomly selected normal controls and individuals of families affected with D/BMD. The most frequent alleles are 200 and 202 bp long in both the affected and the randomly selected normal populations. However, the one-repeat shorter allele seems to be preferred in the affected family members, similarly to the one-repeat shorter STRP45.

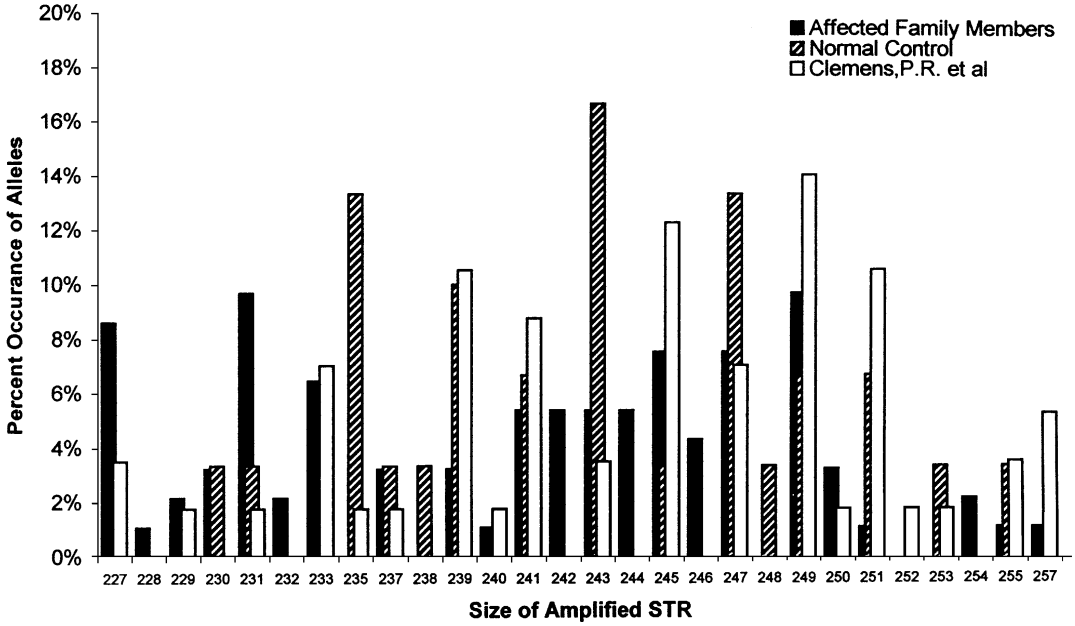

Fig. 9. Distribution of STRP49 alleles of members of families affected with D/BMD, randomly selected Hungarian and American Caucasian healthy individuals (Clemens *et al.* 1991). Unlike in the case of STRP45, the sizes of the amplified STRP49 fragments are distributed more-or-less evenly, and randomly. No characteristic differences between the allele distribution of the affected and normal control individuals can be observed. The allele distribution of the Hungarian and North-American control populations do not differ significantly either.

distribution curves were fitted to the observed data (not shown). According to Kolmogorov–Smirnov goodness of fit analysis the fitted curves were in very good agreement with the observed values ($0.5 < p$). The hypotheses, if the fitted allele distributions are the same, were tested with the Kolmogorov–Smirnov goodness of fit method. Probability values show that the allele distributions of the healthy and affected group differ significantly ($0.002 < p < 0.005$), as well as the Hungarian and American Caucasian healthy control groups ($0.01 < p < 0.02$), while there is no significant difference between the allele distributions of the affected Hungarian and healthy American Caucasian control groups ($0.5 < p$).

In order to prove that smaller repeat number polymorphisms could really be connected to D/BMD more data are needed. Interestingly, the STRP profile in intron 8 of the CFTR gene (which was used throughout this study as an internal control for successful amplification) shows preference of the shorter (200 bp) versus the longer (202 bp) most frequent alleles in the 272 chromosomes of the at-risk family members, while such a preference does not exist among the 74 chromosomes of the controls (Fig. 8). One component Gaussian distribution curves were fitted to the values of both sets of data. Though the Kolmogorov–Smirnov goodness of fit test showed low accuracy of the fits ($0.005 < p < 0.01$ for the normal control, and $0.005 < p < 0.01$ for the affected family members) better fit could not be achieved. However, the test, if the two allele distributions are the same showed significant difference ($P < 0.001$ with both the observed and fitted distributions).

Preference of shorter alleles cannot be recognised in the case of STRP49. As depicted in Fig. 9, it would be very hard to fit a relatively simple distribution curve to any group of data that would support this conclusion.

In the study presented here, we summarised the results of the carrier detection and prenatal diagnosis of 41 families with 151 individuals. The environment of this part of Europe is considered to be more contaminated than other parts of the continent, therefore one would have expected higher frequency of new mutations and higher

incidence of the disease. Our data support neither expectations, moreover, the cases that appeared to be extraordinary at first sight have already been reported by others as well. The very interesting finding is, however, that in families affected by D/BMD, in at least two loci, one repeat shorter microsatellites occur at a higher frequency than in the normal control individuals. The significance of this finding remains to be elucidated.

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