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Point mutation and polymorphism in Duchenne/Becker Muscular Dystrophy (D/BMD) patients

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Abbreviations: DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; Mb, megabases; kb, kilobases; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; m-PCR, multiplex polymerase chain reaction; SSCA, single strand conformation analysis; HA, heteroduplex analysis; EMG, electromyography; Pm, promoter; MDE, mutation detection enhancement; LMP, low melting agarose; EDTA, ethylene diamine tetraacetic acid; TBE, tris borate EDTA

Abstract

Duchenne and Becker muscular dystrophies (D/BMD) are caused by mutations in the dystrophin gene. Two-thirds of patients have large intragenic deletions or duplications and the remaining one-third have point mutations, small deletions or insertions. Point mutations are more difficult to detect due to the enormous size (2.4 Mb) of the gene and its large transcript (14 kb). In the present study, a total of 50 DNA samples from unrelated D/BMD (38 DMD and 12 BMD) patients who did not show intragenic deletions by multiplex PCR, were analyzed for detection of point mutations. Single stranded conformation analysis and heteroduplex analysis observed electrophoretic mobility shifts in one (BMD) and two (DMD and BMD) patients, respectively. The mobility shift and heteroduplexes were observed in exon 17 in all of the three patients. Sequencing of the amplified PCR products revealed a nucleotide change (-37 g to t) in the intronic region in two of the patients while a C2268T substitution in the exonic region in one. Mutation database search for D/BMD mutations showed the nucleotide substitution in the exonic region as a novel change in the human dystrophin gene, which was not reported earlier. It resulted in an amino acid transition from threonine to methionine in the 687th position of the dystrophin protein. This novel substitution has been included in the mutation database of Leiden muscular dystrophy pages (http://www.dmd.nl) in the rare polymorphism/mutation category. The substituted nucleotide segregated with the disease phenotype in the family suggesting that it can be directly used for carrier detection and prenatal diagnosis without identification of disease causing mutation.

Keywords: Duchenne/Becker muscular dystrophy, point mutation, polymorphism, dystrophin gene

Introduction

Duchenne/Becker muscular dystrophies (D/BMD) are the most frequent muscle diseases in children caused by mutations arising in the gene encoding dystrophin, a cytoskeletal protein. It is estimated to affect 1 in 3,500 newborn males worldwide (Emery, 1991). About 30-35% cases of D/BMD are assumed to result from microdeletions, microinsertions or substitutions of one or more nucleotide(s). Several independent studies have reported small mutations in the dystrophin gene (Kiliman *et al.*, 1992; Nigro *et al.*, 1992, 1994; Roberts *et al.*, 1992; Tuffery *et al.*, 1993, 1995, 1996; Kneppers *et al.*, 1995; Sitnik *et al.*, 1997; Eranslan *et al.*, 1999; Wibawa *et al.*, 2000). These are randomly distributed throughout the dystrophin gene (http://www.dmd.nl).

Till date, 370 sequence variations have been identified in the human dystrophin gene and 177 mutations were found to be responsible for the disease (http://www.dmd.nl). Most of the point mutations lead to premature translational termination due to nonsense (34%), frameshift (33%), splice site (29%) and missense (4%) mutations in the dystrophin gene (Rininsland and Reiss, 1994; Roberts *et al.*, 1994; Barbieri *et al.*, 1995; Gardner *et al.*, 1995; Prior *et al.*, 1995; Winnard *et al.*, 1995).

In the course of point mutation analysis in D/BMD patients, several sequence variations have been reported which do not cause any change in the gene size but cause minor substitutions. It is difficult to predict whether the observed change is a mutation or a rare polymorphism (Tuffery *et al.*, 1992; Saad *et al.*, 1994, 1997, 1998; Todorova and Daneili, 1997; Chen *et al.*, 1998).

In our laboratory more than 400 D/BMD patients have been registered so far. In about one third of patients, no detectable deletion was observed using m-PCR and Southern blotting, and was therefore presumed to have point mutations (Singh *et al*., 1997). In order to look for point mutations in the dystrophin gene, six exons of the dystrophin gene (5 proximal and 1 central regions) were screened by using Single Strand Conformation Analysis (SSCA) and Heteroduplex Analysis (HA) in 50 unrelated non-deletional D/BMD patients. Bands shifted in SSCA and HA were further analyzed by sequencing.

Materials and Methods

Patients

A total of 50 clinically confirmed unrelated non-deletional D/BMD patients (38 DMD and 12 BMD) from North India were subjected to point mutation analysis. Clinical diagnosis was based on EMG, Gower's sign, Pradhan's sign and calf hypertrophy (Sinha *et al*., 1992; Pradhan and Mittal, 1995). The deletion analysis was performed by m-PCR using 25 pairs of primers (Pm, 3, 4, 6, 8, 12, 13, 17, 20, 21, 22, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 55, and 60) of the dystrophin gene (Chamberlain *et al*., 1988; Beggs *et al*., 1990; Sinha *et al*., 1992; Singh *et al*., 1997). Mutation analysis was carried out using six exonic regions with flanking intronic sequences (Exons 4, 6, 8, 12, 17 and 44) in two triplex PCRs (Exons 6, 8, 17 and Exons 4, 12, 44).

Single Strand Conformation Analysis (SSCA)

The selected exons were amplified in two m-PCR with DNA samples of all 50 patients and two normal male individuals as controls. Five μl PCR product (80-100 ng) of each patient and normal male individual were diluted with equal volumes of denaturing buffer (98% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95°C and loaded on a 0.5 X MDE™ (Mutation detection enhancement) gel (20×40 cm, 0.5 mm thick) prepared according to FMC Bioproducts protocol. The samples were run at a constant power of 6 W for 10 h at room temp in 0.6 X TBE with or without 5% glycerol. The bands were visualized by silver staining.

Heteroduplex Analysis (HA)

The selected exons were amplified in two m-PCR with DNA samples of the patients and two normal male individuals as controls. Five μl of PCR product (80-100 ng) of each patient was mixed with an equal amount of control product, denatured at 95°C for 5 min and reannealed at 37°C for 1 h. Five μl (30-35 ng) of reannealed fragments were mixed with double dye (30% sucrose, 0.05% bromophenol blue and 0.05% xylene cyanol) and loaded onto a 1X MDE gel (20×40 cm, 1 mm thick). Electrophoresis was carried out at a constant voltage of 600V for 8 h at room temperature in 0.6 X TBE and the bands were visualized by silver staining.

DNA Sequencing

The PCR products were purified from low melting point (LMP) agarose and subjected to manual and automated sequencing. Manual sequencing of the purified PCR product was carried out using di-deoxy thermosequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences). The sequencing gel was electrophoresed at 1700 V for 4 h, fixed for 10 min in 10% acetic acid (v/v) containing 10% methanol (v/v), vacuum dried at 80°C for one hr and exposed to X-ray film (X-Omat™, XK-5) for 24-36 h at -80°C. The autoradiograms were developed and read carefully.

Automated sequencing (Applied Biosystems model 373A) of the purified PCR products was carried out using FS-Dye termination cycle sequencing ready reaction kit (Perkin Elmer, USA). At the completion of the reaction, the volume of reaction mix was raised to 100 μl and phenolized once with phenol: chloroform (68:14). The aqueous layer was precipitated with ethanol and the pellet was washed with 70% ethanol and dried. It was then dissolved in 5 μl of formamide loading buffer, denatured and loaded onto a sequencing gel. Before loading the samples, the background fluorescence was measured and scan area was cleaned to get a uniform base line for all the sample lines. The samples were electrophoresed for 16 h at 40 W. The sequence was obtained as an electropherogram. The nucleotide sequence so obtained was analyzed using PC-Gene software rel. 17.0 (Intelligenetics, USA). The BLAST program was used for analysis of nucleotide sequences.

Results

Out of 50 unrelated D/BMD patients analysed for point mutation detection, the electrophoretic mobility shift was observed in three patients: one by SSCP and two by HA. All three changes were observed in exon 17 (Figures 1, 2). Sequencing of the amplified PCR products confirmed the nucleotide alteration in all the three patients (Figures 3-5).

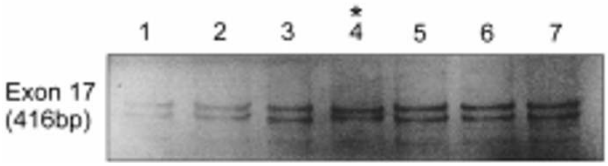

Figure 1. SSCA gel pattern of exon 17. Lanes 1 and 7, normal male controls. Lanes 2-6, D/BMD patients. Lane 4, mobility shift in MD-138 patient.

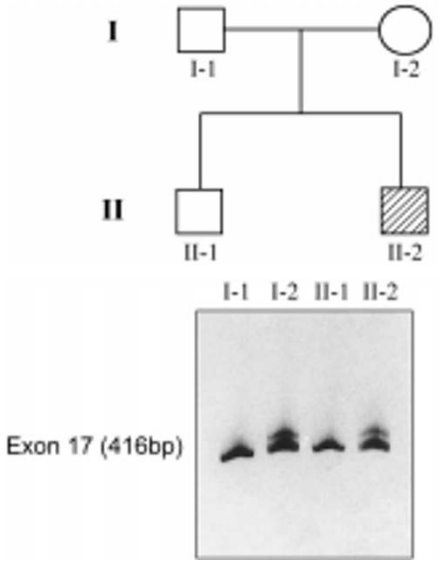

Figure 2. Pedigree and heteroduplex formation of exon 17 of MD-28.

SSCA showed a band shift only in exon 17 in one of the patients, MD-138 (BMD) (Figure 1). In the remaining 49 patients, no band shift was observed in any of the exons analysed. The manual dideoxy sequencing of exon 17 showed a substitution in the intronic region (-37 g → t) in MD-138 when compared to the normal male control (Figure 3).

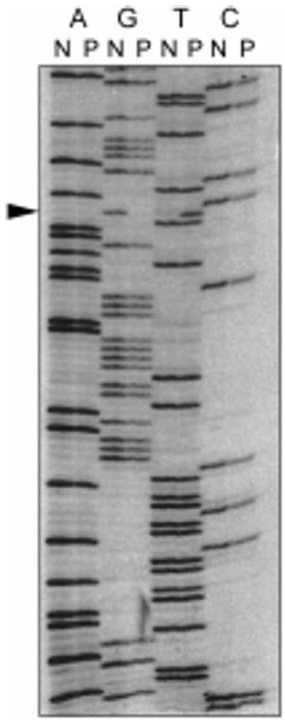

Figure 3. Manual DNA sequencing of exon 17 in control (N) and MD-138 (P). The arrow indicates the position of the nucleotide substitution (-37 g to t).

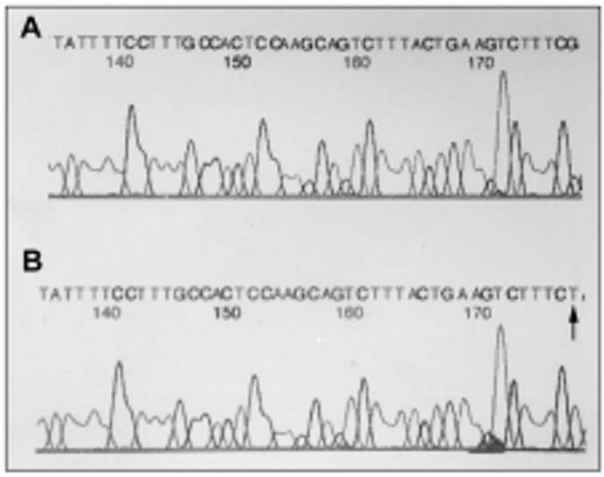

Figure 4. Automated DNA sequencing of exon 17 with flanking region (A) normal male control and (B) MD-70. The arrow indicates the nucleotide substitution (-37 g to t) in intron 16 of the dystrophin gene at 2201 position.

The heteroduplex formation was observed in exon 17 in two patients, MD-28 and MD-70 (one DMD and one BMD respectively) (Figures 2). Automated DNA sequencing of exon 17 using fluorescent dideoxy dye termination showed a substitution in the intronic region (-37 g → t) in the BMD patient, MD-70 (Figure 4). In case of MD-28, HA was further carried out in the DNA samples of mother, father and unaffected brother. In the mother, a heteroduplex formation was observed as in the proband (Figure 2). Automated DNA sequencing showed a substitution (transition) in the exonic region at 68th nucleotide position of exon 17 (C2268T) of the dystrophin gene. Sequencing the PCR products of father, unaffected son, mother and normal male control revealed that the father and son had no change in the sequence but the mother was heterozygous for the change (Figure 5). In the remaining patients, no heteroduplex formation was observed in the other exons. The results of BLAST searches are shown in Figure 6.

Discussion

Several independent investigators have used different methods to search for point mutations in the dystrophin gene in 30% of D/BMD patients. SSCA (Orita *et al.*, 1989; Glavac and Dean, 1993) and HA (Glavac and Dean, 1995) are mutation detection techniques that rely on detecting changes in the physical properties of DNA caused by the presence of sequence changes. These methods have shown an efficiency of 7-27% depending on the conditions and exons analysed (Soto and Sukumar, 1992; White *et al.*, 1992; Kneppers *et al.*, 1995; Eranslan *et al.*, 1999). Prior *et al.* (1995) screened around 80% of the dystrophin coding sequences for small mutations by using HA in 158 patients and identified mutations in 29

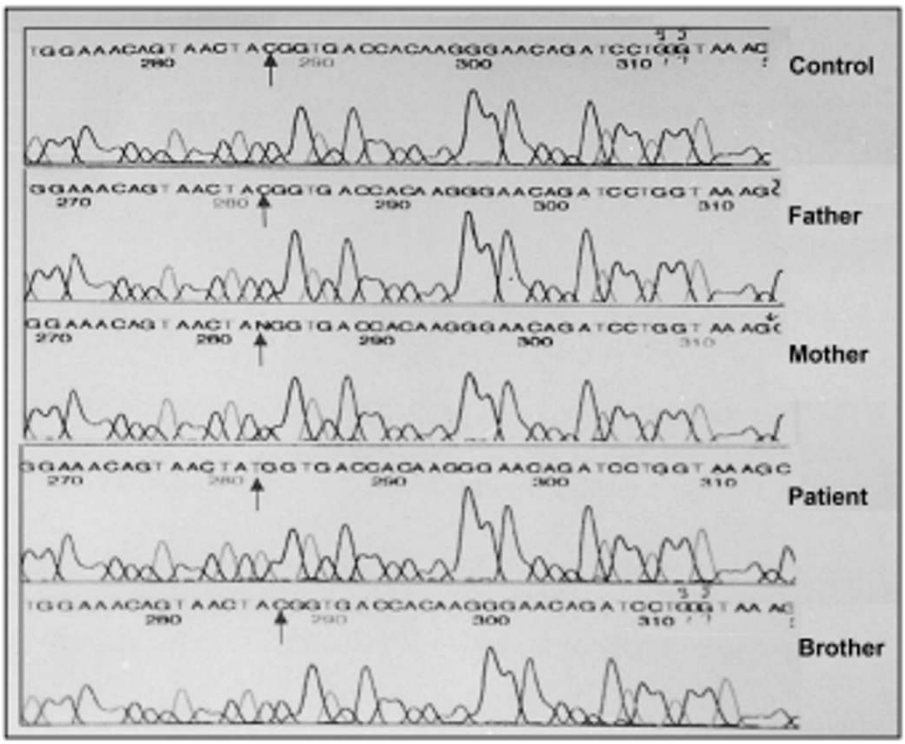

Figure 5. Automated DNA sequencing of exon 17 of normal male control and members of family, MD-28. The arrow indicates the nucleotide substitution C2268T in the exonic region of the patient, the heterozygous condition in the mother and normal sequence in the father and unaffected brother.

Figure 6. BLAST searches. (A) Homology of the sequenced exon 17 of patient MD-28 with a normal human dystrophin sequence. (B) Comparison of the above sequence with human, chicken and murine dystrophin exon 17.

of them. They concluded that in many of the DMD and majority of BMD, small mutations lie in the non-coding regions of the gene. Eranslan *et al.* (1999) screened 18 deletion-prone exons (25.5% of the coding region) of the dystrophin gene by using non-isotopic multiplex SSCA in 56 unrelated non-deletional D/BMD patients. They

identified 5 diseases causing mutations and 6 polymorphisms in Turkish D/BMD patients.

In the present study, sequencing showed a nucleotide substitution of G to T in the intronic region (2201-37G/T) in two patients (MD-138 and MD-70) and a nucleotide substitution in the exon 17 (C2268T) in one (MD-28). The observed intronic nucleotide change in the dystrophin gene has been reported earlier by many independent investigators (Kiliman *et al*., 1992; Tuffery *et al*., 1992; Nigro *et al*., 1994; Prior *et al*., 1995; Sitnik *et al*., 1997). They have observed this not only in D/BMD patients but also in normal individuals, thereby constituting a rare polymorphism. The possibility of it being involved in the pathogenesis of the disease is ruled out since it is away from the splice site.

In MD-28 the nucleotide substitution (C2268T) in the exon 17 resulted in an amino acid alteration from threonine to methionine at the 687th position of the dystrophin protein. The sequence homology analysis using BLAST depicted that the substituted nucleotide is present in chicken and murine dystrophin gene. Therefore, this change does not appear to be conserved across species. Thus, it suggests that this change may not be responsible for the disease but appears to be a polymorphism. In the family of MD-28, the mother was found to be heterozygous for the C2268T nucleotide substitution. The sequence analysis showed that the substituted nucleotide was inherited with the disease phenotype.

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