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ARTICLE

Identification of point mutations in Turkish DMD/BMD families using multiplex-single stranded conformation analysis (SSCA)

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Small mutations are the cause of the disease in one third of cases of Duchenne and Becker muscular dystrophy (DMD/BMD). The identification of point mutations in the dystrophin gene is considered to be very important, because it may provide new insights into the function of dystrophin and direct information for genetic counselling. In this study, we have screened 18 deletion-prone exons (25.5% of the coding region) of the dystrophin gene by using a modified non-isotopic multiplex single-stranded conformation analysis (SSCA). Mutations responsible for the disease phenotype could be identified in five out of 56 unrelated DMD/BMD patients without detectable deletions. Two of these mutations, 980–981delCC and 719G > C, are novel mutations which have not been described previously. Four of the five mutations, including 980–981delCC detected in this study are found to be nonsense or frameshift mutations leading to the synthesis of a truncated dystrophin protein. The missense mutation, 719G > C, causing the substitution of highly conserved alanine residue at 171 with proline in the actin binding domain of the dystrophin, is associated with a BMD phenotype. This study also revealed the presence of six polymorphisms in Turkish DMD/BMD patients.

Keywords: DMD/BMD; point mutations; multiplex single-stranded conformation analysis (SSCA); nonsense mutation; missense mutation

Introduction

Duchenne, and its milder allelic form Becker, muscular dystrophy (DMD/BMD) are X-linked recessive neuromuscular disorders resulting from mutations in the gene encoding the muscle protein, dystrophin.[1]

The underlying defects in DMD/BMD are partial deletions in approximately 50–60% of all DMD and 85% of BMD cases.[1] These deletions, clustered in the central and near the 5' end of the dystrophin gene can easily be detected by two separate multiplex gene amplification systems.[2,3] A small proportion of mutations (6%) are duplications.[4]

Small mutations, including point mutations or micro-deletions/insertions, are the cause of the disease in the remaining DMD/BMD cases, therefore it is very important to find the underlying defect in these group of patients. 203 different mutations have been identified and reported to the Muscular Dystrophy (Point) Mutation Database so far.[5] Most of these mutations were found to be unique to a patient and no hot spot region could be identified.

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The majority of DMD small mutations are nonsense mutations resulting in the premature termination of translation, giving rise to unstable dystrophins leading to severe DMD phenotype. Mutations causing the mild BMD phenotype may maintain the translational reading frame and result in the synthesis of either correctly localised dystrophin protein with slightly altered function or normal protein at reduced levels. The determination of point mutations within this gene represents a challenge due to its large size and complexity. Since 1991, when the first nonsense mutation in the dystrophin gene was reported by Bulmann *et al*[6] using western blot analysis and direct sequencing, a great effort has been put forward to develop techniques that will enable the systematic identification of point mutations.[7–10]

Single-stranded conformation analysis (SSCA) technique is one of the most commonly used approaches for the screening of the point mutations and/or polymorphisms in the gene of interest. Single or multiplex PCR products corresponding to the various exons of the dystrophin gene including exon–intron boundaries were analysed on long non-denaturing gels by SSCA technique and resulted in the identification of point mutations within this gene.[11–13]

In the framework of this study, we have screened 18 deletion-prone exons of the dystrophin gene by using a modified non-isotopic multiplex SSCA. We have identified five different pathogenic mutations, two of which are novel, and six polymorphisms in 56 non-deletion DMD/BMD patients.

Methods

Patients

56 unrelated DMD/BMD patients who did not have any detectable deletions were selected for the screening of point mutations by SSCA analyses. Normal males were included in the study as controls.

PCR Conditions

Each patient was screened for 18 exonic regions of the dystrophin gene using primers described for multiplex gene amplification systems.[2,3] Exons 3, 4, 6, 8, 12, 13, 17, 19, 43, 44, 47, 50, 51, 52 and 60 were amplified either as triplex (A–C) or duplex sets (D–F) for each patient. Exons to be analysed together on a single SSCA gel were carefully selected according to their size and the location of their single-stranded patterns on the selected gel system. The promoter region (Pm), exon 45 and 48 (G–I) were amplified separately due to their large size (Table 1). Amplifications were performed as described previously.[2,3] A 1–2 μl aliquot of each PCR mix was mixed with 5 μl of 98% formamide, 10 mM NaOH and 0.05% BPB and XC dyes. The samples were denatured at 85°C for 5 min and cooled on ice prior to loading on to the SSCA gels.

SSCA for Screening

Electrophoresis was performed using DGGE apparatus (DGGE-2000, CBS Scientific Company Inc, USA) with a constant circulation of buffer between lower and upper chambers. Optimum electrophoresis conditions for each set (A–I) used for the screening of the small mutations within the dystrophin gene were determined carefully and are summarised in Table 1. The C% of 2.6 (a ratio of 37.5:1 acrylamide/bisacrylamide), the concentration of glycerol (10%) and the voltage (500 V) were kept constant for all sets.

The gels were silver stained and each patient presenting an abnormal pattern when compared with normal individuals was sequenced using Omnibase cycle sequencing kit (Promega, USA). The reactions were carried out as described by the manufacturer using α-^{35}S-dATP for labelling and analysed on 6% sequencing gel.

Results

In the framework of this work, 56 unrelated DMD/BMD non-deletion patients were selected for the screening of point mutations in 18 exons of the dystrophin gene. A modified SSCA technique was established for the screening of point mutations in Turkish DMD/BMD patients.

Each patient was screened by SSCA analysis for point mutations within 18 exons of the dystrophin gene by using the sets A–I. Figure 1 shows the multiplex SSCA of a panel of individuals using set A comprising exons 19, 8 and 44 of the dystrophin gene. Different migration patterns were detected in four DMD patients in exons 19 and 8. Sequencing of exon 19 in patients 1 and 2 (lanes 2 and 5 respectively) revealed the presence of base substitutions, namely 2510 C>T and 2516

Table 1 Optimum conditions for each selected SSCP set. The C% value (2.6), running buffer (1×TAE), temperature (20ºC) and the voltage (500V) were kept constant for each set

Name	Exon numbers	Product sizes (bp)	Gel conc (%)[a]	Time (h)
A	19, 8, 44	459, 360, 268	8	7
B	17, 12, 13	416, 331, 238	8	7
C	3, 50, 60	410, 271, 139	8	8
D	51, 6	388, 202	8–10	10
E	43, 4	357, 196	10	10
F	47, 52	181, 113	10	10
G	45	547	5–6	6
H	48	506	6	6
I	Pm	535	6	6

[a]Non-denaturing gels containing 10% glycerol (C%=2.6).

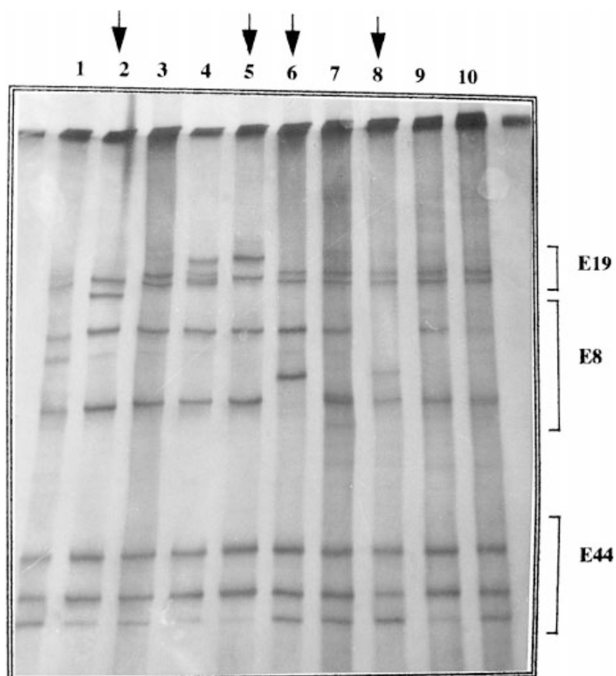

Figure 1 *The SSCA of Turkish DMD/BMD patients by using set A comprising exons 19, 8 and 44. Lanes 1 and 10: normal male controls, Lane 2: Patient 1, Lane 3: Mother of patient 1, Lane 4: Mother of patient 2, Lane 5: Patient 2, Lane 6: Patient 3, Lane 7: Patient 4, Lane 8: Patient 5, Lane 9: Patient 6. Big arrows indicate the patients with abnormal SSCA patterns for exon 19 and small arrows indicate the patients with abnormal SSCA patterns for exon 8.*

A > T, leading to the formation of stop signals at codons 768 (R768X) and 770 (K770X).

In order to identify their carrier status the mothers of both patients were also included in the SSCA. The presence of the same abnormally migrating band in addition to the normal one (lane 4) indicated that the mother of patient 2 was the carrier of the mutation. The SSCA showed the absence of the aberrant exon 19 band in the mother of patient 1 (lane 3). This observation suggested the non-carrier status of this mother. However, germline mosaicism could not be excluded for this mother.

Sequencing of exon 8 in patient 3 (lane 6) indicated the presence of a base substitution, 932 C > T, which also creates a stop signal at codon 242 (Q242X). The sequence analysis of the same exon in patient 4 (lane 8) showed that the shift in the migration pattern was caused by a novel two basepair deletion (–CC) at position 980–981 (980–981delCC). This novel mutation causes a frameshift and creates a stop signal at codon 259 of exon 8 (P258–2X259) in this DMD patient.

SSCA analyses of Turkish DMD/BMD patients using set D, comprising exons 51 and 6, displayed an abnormal pattern in exon 6 of a BMD patient. Sequence analysis of exon 6 in this patient showed the presence of a substitution, G to C at nucleotide position 719 (719G > C), changing alanine to proline (A171P). This nucleotide variation, which was first described in the framework of this study, creates a restriction site for Mnl I enzyme. The mother and sister of this patient was also found to be carriers of this mutation (Figure 2).

The screening of 56 patients using sets B, E, H and I, comprising exons 17, 12 and 13; 43 and 52; 45 and 48, respectively, revealed the presence of six polymorphisms. The frequency of each polymorphism among the screened population of DMD/BMD patients was calculated either according to restriction enzyme analysis or from the direct observation of SSCA patterns. Mutations and polymorphisms identified in this study are summarised in Table 2.

Discussion

In one third of DMD/BMD cases the disease is caused by small mutations and since 1991, 203 different mutations have been reported to the DMD point mutation database so far.

In the present study 25.5% of the coding region of the dystrophin gene was screened using a modified multiplex SSCA. Mutations responsible for the disease

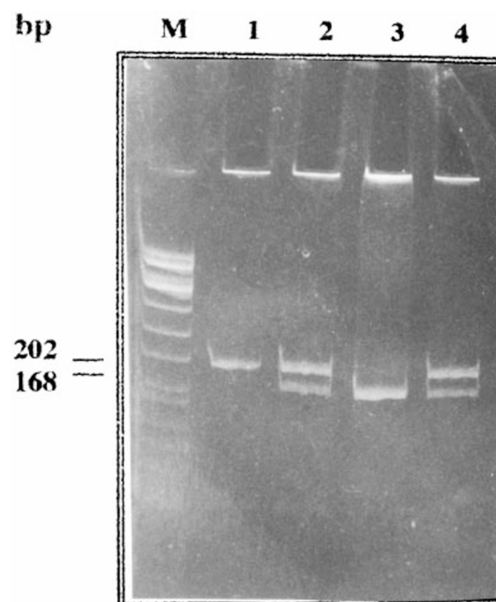

Figure 2 *Mnl I digestion of exon 6 of the dystrophin gene. M: Marker DNA (φX 174/Hinf I), Lane 1: Control DNA, Lane 2: Mother, Lane 3: Patient, Lane 4: Sister*

Table 2 Mutations and polymorphisms identified in Turkish DMD/BMD non-deletion patients using SSCA technique

Exon No 1	*Name*	*RE site*	*Effect on the translation*	*Phenotype*	*Observed frequencies*	*References*
Mutations						
6	719G>C	+Mnl I	A 171P	BMD	0.02 (1/56)	This study
8	980-981 del CC	–	P 258–2 X 259	DMD	0.02 (1/56)	This study
8	932 C>T	–	Q 242 X	DMD	0.02 (1/56)	14–16
19	2510 C>T	–	R 768 X	DMD	0.02 (1/56)	12, 15, 17
19	2516 A>T	–	K 770 X	DMD	0.02 (1/56)	18
Polymorphisms						
13	1691–72 t/c	+Dde I	none	–	0.21 (12/56)	5, 19, 20
13	1691–67 a/t	–	none	–	0.04 (2/56)	5, 19, 20
17	2201–37	–Taq I	none	–	0.45 (25/56)	5, 11, 12, 14, 19
43	6326–60 ins/at	–	none	–	0.14 (8/56)	5
45	6779 C/T	–	Arg 2191 Trp	–	0.13 (7/56)	12, 16
48	7304 A/C	–Mse I	Lys 2366 Gln	–	0.50 (28/56)	7, 19, 21, 22

phenotype could be identified in five out of 56 unrelated DMD/BMD patients included into this study. Two of these mutations are novel mutations which were not described previously.

Four among the five mutations detected in this study are found to cause the premature termination of the dystrophin synthesis and associated with the severe DMD phenotype as expected. 99.8% of point mutations reported so far leading to DMD phenotype were found to be nonsense mutations or frameshift mutations causing a premature termination of the dystrophin. The mutation 2510C > T was already described in three DMD patients, 932C > T in one IMD and two DMD patients and 2516A > T in one DMD patient. The frameshift mutation, 980–981delCC, first described in this study was also identified in one DMD patient.

A novel missense mutation in exon 6 causing a substitution of alanine at position 171 with proline was described in a BMD patient. The identification of the sequence variation uniquely in the affected members and identified carriers of the family, and its absence in normal males and non-carrier females, indicated the possible role of this variation in the pathogenesis of the disease in this family. The amino acid alanine at position 171 of the dystrophin protein is highly conserved in the dystrophin of human, mouse and chicken. Furthermore investigation of the NH_2 terminus actin-binding domains of the structurally related proteins showed the conservation of this residue in human utrophin, α-actinin, β-spectrin, flamin and ABP-120 (Figure 3). These observations provided indirect evidence for the pathogenic nature of this variation.

Elucidation of the precise sequences required for the binding of F-actin is considered to be very important for the understanding of the pathogenesis of DMD and for the development of therapeutic approaches for this disease. It has been reported that dystrophin has been shown, *in vitro*, to have at least three actin binding sites, namely ABS 1–3, in the NH_2 terminal actin-binding domain of this protein.[24,25] However, the identification of a missense mutation in exon 3 of the dystrophin gene which does not reside in any of these binding sites

dysH 153 Q V N V I N F T T S W S D G L A L N **A** L I H S 175
dysM 153 Q V N V I N F T S S W S D G L A L N **A** L I H S 175
dysC 157 Q V N V I N F T S S W S D G L A F N **A** L L H S 179
UtrH 169 Q V N V L N F T T S W T D G L A F N **A** V L H R 192
SPCB 192 H V N V T N F T S S W K D G L A F N **A** L I H K 216
AAC3 177 N V N V Q N F H T S W K D G L A L C **A** L I H R 201
ABP120 143 K V V V N N F T D S W C D G R V L S **A** L T D S 165
filH 155 Q L P I T N F N R D W Q D G K A L G **A** L V 175

Figure 3 *Alignments of the partial amino acid sequence of dystrophins and NH_2 terminus actin-binding domains of the structurally related proteins; human dystrophin (dysH); mouse dystrophin (dysM); chicken dystrophin (dysC); human utrophin (utrH); human beta spectrin (SPCB); human alpha actinin-skeletal muscle isoform (AAC3); actin-binding protein 120 (ABP120); human filamin (filH). The sequences were aligned with the aid of a protein database similarity search program, PSI-BLAST.*[23]

suggested that amino acid 54 may also be important for the function of the protein possibly due to interaction with actin.[26] The missense mutation identified in the present study, resulting in a substitution of alanine for a proline at position 171 which does not reside in any of these three actin-binding sites indicated the importance of this residue possibly in the actin binding function of the protein.

The fact that this missense mutation is associated with the BMD phenotype indicates that this mutation may only reduce the stability or the function of the protein. Further studies are required to clarify the effect of 719G > C on the biological function and the severity of the mutation. However, it should be kept in mind that other genetic factors may also be important in the creation of a milder phenotype in this patient.

A mutation detection rate of 9.6% was observed as expected in this study by the analysis of almost one fourth of the coding region of the dystrophin gene in the present study. The non-radioactive multiplex SSCA system optimised for screening 18 exons seems to be a sensitive and reproducible approach for the rapid screening of pathological mutations. The design and optimisation of the multiplex systems to screen other exons of the dystrophin gene needs to be established and further studies using these systems will possibly reveal other mutations responsible for the DMD/BMD phenotype.

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