Splicing mutations in DMD/BMD detected by RT-PCR/PTT: detection of a 19AA insertion in the cysteine rich domain of dystrophin compatible with BMD

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Abstract

We have used an RNA based mutation detection method to screen the total coding region of the dystrophin gene of a Duchenne and a Becker muscular dystrophy patient in whom DNA based mutation detection methods have so far failed to detect mutations. By RT-PCR and the protein truncation test (PTT) we could identify point mutations in both cases. DMD patient DL184.3 has a T→A mutation in intron 59 at position -9, creating a novel splice acceptor site for exon 60. As a result seven intronic bases are spliced into the mRNA, causing a frameshift and premature translation termination 20 codons downstream. Since this patient had died and only fibroblasts were available, we applied MyoD induced myodifferentiation of stored fibroblasts to enhance muscle specific gene expression. With the results of this mutation analysis, prenatal diagnosis could subsequently be performed in this family. BMD patient BL207.1 carries a G→C mutation at position +5 of intron 64, disrupting the splice donor consensus sequence and activating a cryptic splice donor site 57 bp downstream. The inclusion of these 57 intronic bases in the mRNA leaves the reading frame open and results in the insertion of 19 amino acids into the cysteine rich domain of dystrophin. Interestingly, this insertion in a part of the dystrophin considered to interact with the dystrophin binding complex of the sarcolemma is apparently compatible with mild BMD-like clinical features. Both mutations reported are missed by analysis of multiplex PCR products designed for deletion screening of the coding region. Extrapolation from existing point mutation detection efficiencies by DNA and RNA based methods emphasises that RNA based methods are more sensitive and that most of the remaining undetected mutations may affect splice or branch sites or create cryptic splice sites. (*J Med Genet* 1996;33:935-939)

Key words: Duchenne muscular dystrophy; Becker muscular dystrophy; protein truncation test.

Duchenne and Becker muscular dystrophy (DMD, BMD) are allelic, degenerative, X

linked neuromuscular disorders caused by mutations in the dystrophin gene.¹ Mutations causing a frameshift are considered to cause DMD while mutations leaving an open reading frame generally cause BMD.² About two-thirds of the mutations consist of large deletions or duplications, which are concentrated in two "hot spots",^{3,4} and can be detected by a simple multiplex PCR of exons in the deletion hot spots.^{5,6}

Screening for the remaining one-third of mutations is often done by SSCP or heteroduplex analysis using multiplex PCR products.⁷⁻¹¹ Until now, no clustering of small mutations has been found.¹² Although there is a higher concentration of mutations 3' of exon 55, this is consistent with the high number of CGA codons which are more likely to mutate to a TGA.¹¹ Strikingly, assuming a random distribution of point mutations over the coding region, the percentage of point mutations identified so far by DNA based studies is far below the percentage expected.⁷⁻¹¹ Screening between 20% and 80% of the coding region showed only 2-18% mutations.

One possible explanation is that the deletion hotspots covered by the multiplex PCR commonly used for point mutation screening are relatively sparse in point mutations. More likely, however, the remaining mutations are splicing mutations located outside the coding region usually screened. Indeed, 14 out of the 36 multiplex primers are either located inside an exon or cover or flank intron-exon boundaries and thus do not permit the detection of mutations affecting splice sites.

Using RNA based mutation detection methods, a much higher percentage of mutations is identified. By screening the total coding region, mutations have been found in seven out of seven patients (100%)¹³ and 16 out of 22 (73%).¹⁴

We present a study in which the entire dystrophin gene of a DMD and a BMD patient was screened for mutations. For this purpose we performed RT-PCR followed by a protein truncation test (PTT), that is, in vitro transcription/translation of RT-PCR products.¹⁵ Not all parts of the dystrophin gene of patient DL184.3 could be amplified on RNA isolated from fibroblasts (the patient has died and no RNA from peripheral blood lymphocytes was available). Therefore, we performed MyoD transfection and myodifferentiation on his fibroblasts. Fibroblast were transfected with a

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MyoD gene (a regulator of myogenesis) in a retroviral vector and selected for vector derived neomycin resistance. Induction of myogenesis by transfer to low serum conditions increases the amount of dystrophin transcripts in RNA from these cells.¹⁶ Subsequently, in both the DMD and the BMD patient, a mutation was identified which interferes with normal splicing. The site of the mutation was in both cases located in an intron and would not have been identified with the currently used DNA based methods.

Material and methods

CELL CULTURE AND FORCED

MYODIFFERENTIATION

Fibroblasts of patient DL184.3, kindly provided by Dr W Kleyer, Cellbank Rotterdam, were grown in DMEM (Gibco-BRL, UK) without phenol red, supplemented with 10% FCS. They were infected with a MyoD containing retrovirus and differentiation was induced as previously described.¹⁶ The efficiency of differentiation was tested by immunohistochemical detection of desmin, titin, and the appearance of cell fusion. No dystrophin could be detected, confirming the clinical diagnosis of DMD.

MUTATION DETECTION

For patient DL184.3 total RNA was isolated from myodifferentiated cell pellets using RNA-sol (Cinna/Biotech Laboratories, USA). For patient BL207.1 peripheral blood lymphocytes were isolated from total blood using histopaq (Sigma, USA), RNA was isolated, and RT-PCR was performed as previously described.¹⁶ The protein truncation test (PTT) was performed as previously published.¹⁵ Sequence analysis was performed using a PCR product sequencing kit (USB, USA). To confirm the presence of mutations detected, genomic DNA was amplified using a primer in the intron sequences and sequenced as described.

Results

DMD patient DL184.3 had a very severe phenotype. He was wheelchair bound by the age of 5 and died at the age of 13 owing to breathing difficulties. No mutation was found using multiplex PCR analysis. The only material available was cultured fibroblasts. By RT-PCR on RNA isolated from these fibroblasts we failed to amplify the total coding region of the dystrophin. Therefore we enhanced expression of the dystrophin gene in the patient's fibroblasts by MyoD transfection and myodifferentiation.^{16,17} In these fibroblasts we observed expression of desmin and titin and the occurrence of cell fusion, all indicative of a successful myodifferentiation. As expected for a DMD patient, no dystrophin was detected. RNA isolated from these cells was used for RT-PCR reactions that amplified the dystrophin coding region in 10 partly overlapping segments. All products were of the expected length, excluding gross rearrangements in the

Figure 1 RT-PCR and PTT products of patients and normal subjects. (A) The RT-PCR products from primer set 5CD of a normal control (lane 1), DMD patient DL184.3 (lane 2), and the slightly larger product derived from BMD patient BL207.1. M is a 100 bp marker. The sizes of several marker bands are given in base pairs. (B) Protein truncation test products from primer set 5CD of a normal control (lane 1), the truncated product of DL184.3 (lane 2), and the slightly larger product of BL207.1 (lane 3). The sizes of the marker proteins are indicated in kDa.

dystrophin gene. Subsequently, the fragments were analysed by the in vitro transcription translation protein truncation test (PTT).¹⁵ The PCR product derived from base pairs 8827–10124 of the dystrophin cDNA¹⁸ showed a 15 kDa truncated protein instead of the expected 50 kDa protein (fig 1). Sequence analysis of this PCR product showed a 7 bp insertion between exons 59 and 60 leading to a frameshift and a translation stop downstream. Database analysis indicated that this insertion was derived from the 3' end of intron 59. No other sequences were visible in the cDNA, which confirmed the absence of normally spliced transcripts. Computer inspection of the sequence around this position showed that a TG sequence located immediately upstream of the inserted intronic sequence, when mutated to AG, would create a novel splice acceptor site (fig 2). This novel splice site would have a Shapiro/Senapathy splice acceptor site score¹⁹ of 70.3, while the wild type sequence has a score of 73.2. In addition, the occurrence of an AG sequence within 10 bases upstream of a wild type splice acceptor site would predict the latter to be inactivated.¹⁹ Sequence analysis of genomic DNA of this patient confirmed the presence of the postulated T→A mutation at this position.

Shortly after obtaining this result we experienced the value of our continued effort to identify the mutation in each DMD/BMD family. Before we identified the mutation in this family, the carrier risk of the patient's female relatives could only be determined by haplotype analysis. Since one-third of DMD mutations are new, there is 66% risk for the mother to be a carrier. Since she has normal CK levels this risk decreases to about 30%. Since no mutation was found with five cDNA probes tested, her risk further decreases to about 20%. The sister of the patient (DL184.4, fig 3) shared the maternal allele; therefore her carrier risk was also 20%. The mother's twin sister (DL184.7) shared a part of this haplotype

Figure 2 Normal and altered splice sites. (A) Schematic drawing of the splicing that occurs in DMD patient DL184.3 where the 3' part of intron 59 is spliced into the mRNA. (B) Schematic drawing of the splicing that occurs in BMD patient BL207.1 where the 5' part of intron 64 is spliced into the mRNA. (C) The wild type sequences of both intron-exon boundaries aligned with the mutated sequences found in the patients and the RNA and protein sequences.

Figure 3 Pedigree of family DL184. The arrows indicate the index case and his cousin requesting prenatal diagnosis. Haplotyping was performed using the markers p782, pXUT23, p87-15, p754-11, and OTC. In DL184.7 a recombination between p782 and p754-11 was found, while DL184.9 had a recombination between pXUT23 and p87-15.

(owing to recombination) and her carrier risk was estimated to be 10%. Sister DL184.6 had inherited the other haplotype and could be excluded from being at risk. On identification of the mutation we could determine that both the patient's mother (DL184.2) and his sister (DL184.4) did not carry the mutation. Soon afterwards, a cousin of the patient (DL184.11) became pregnant and requested prenatal diagnosis. As the mutation had now been identified and the patient carried a proven new mutation, we could exclude her as a DMD carrier, thus precluding the need for chorionic villi or amniotic fluid sampling.

Similarly, no mutation could be found in BMD patient BL207.1. Western blot data showed a decreased amount of apparently normal sized dystrophin indicative of a diagnosis of BMD. RT-PCR on RNA isolated from his peripheral blood lymphocytes showed that the product covering base pairs 8827–10124 of the dystrophin cDNA¹⁸ had a slightly increased size when compared to controls (fig 1). On PTT analysis the protein product was longer. This showed that the product maintained an open reading frame (fig 1), in agreement with a Becker phenotype.² cDNA sequencing showed an insertion of 57 bases derived from the 5' end of intron 64 directly adjacent to exon 64 in the reading frame. It also showed a G→C point mutation located in the intronic insertion, five bases downstream of exon 64. Genomic sequencing of the intron (unpublished wild type sequence kindly provided by Dr R Roberts) confirmed the G→C mutation and did not show any other mutation. No other sequences were visible in the cDNA, indicating the absence of normally spliced transcripts. Because of the mutation, the Shapiro/Senapathy score of the 3' end of exon 64 decreased from 76.8 to 63.2,¹⁹ thereby activating a cryptic splice site with a score of 77.0, located 57 bp downstream in intron 64 (fig 2).

Discussion

Two mutations affecting splicing have been found in the dystrophin gene. The mutation identified in Duchenne patient DL184.3 results

in a truncated protein while the mutation in Becker patient BL207.1 results in a slightly larger protein. Western blot analysis of a muscle biopsy from BL207.1 showed a reduced amount of apparently normal sized dystrophin (data not shown). The latter is plausible since the inclusion of 19 amino acids does not significantly alter the length of a 427 kDa protein. The decreased amount of dystrophin suggests, besides a possibly less efficient splicing of the cryptic splice site causing a reduced amount of mRNA, that the enlarged protein may be somewhat less stably anchored to the membrane than the normal dystrophin and probably less functional. The 19 inserted amino acids, NYWPVFEDLDTMSLLRIKM, consist of nine hydrophilic and 10 hydrophobic amino acids of which three are acidic and two basic, which makes it a neutral insertion without particular 3D folding properties. The insertion is located in the cysteine rich domain of the dystrophin, which binds to the dystrophin binding glycoprotein complex.²⁰ Considering the mild BMD phenotype, this insertion is apparently without any major disruptive effect on the function of dystrophin. While we cannot formally exclude the existence of yet another mutation causing the BMD phenotype an extensive search for deletions or point mutations has not yielded any results. Moreover, if another BMD mutation exists this would imply that the insertion itself is entirely inconsequential. Considering both these points, the more plausible hypothesis for the cause of the BMD phenotype is the 19AA insertion. Thus, this insertion is at least compatible with mild dystrophic function and possibly, but unlikely, with normal function, but clearly, against expectation, does not cause severe DMD.

The mutation also effects the Dp140, Dp116, and Dp71 dystrophin transcript,²¹⁻²³ expressed in brain, cortex, peripheral nerves, and other tissues. BL207.1 is mentally retarded, which is in agreement with the results of Lenk *et al*,²⁴ who reported that a large proportion of patients with a mutation in the Dp71 gene region are mentally retarded.

The mutation in DL184.3 would not have been identified using multiplex PCR followed by SSCP or heteroduplex analysis, since the primers for the amplification of exon 60 are located within the exon and thus cannot detect mutations affecting the splice sites. Of the primers used in the multiplex sets,^{5,6} 14 out of 36 will not detect intronic splice site mutations; four are located within exons (forward and reverse primers of exons 52 and 60), eight cover the intron-exon boundary (forward primers of exons 6 and 47, reverse primers of exons 6, 8, 13, 17, 43, and 47), and two are intronic but within 1 bp of the intron-exon boundary (forward primer of exon 44 and reverse of exon 45). This means that mutation detection methods using multiplex PCR miss 40% of the mutations affecting splice sites.

Recently we have identified another example where primer location prevented the detection of a disease causing mutation.¹⁶ In DMD patient DL90.3, we identified a CGA→TGA nonsense mutation at base pair 6492 (exon 43)

Table 1 Mutations found in studies using analysis of multiplex PCR products. In the first column the number of patients tested is indicated, in the second column the number of mutations found, and in the third column the number of splice site affecting mutations. The last column contains references. Bottom line: summary of data

Patients	Point mutations	Splice	Ref
60	1	1	8
70	6	1	9
29	1	0	25
110	3	0	26
269	11	2	Total

Table 2 Mutations found in studies using RT-PCR methods. In the first column the number of patients tested is indicated, in the second column the number of mutations found, and in the third column the number of splice site affecting mutations. The last column contains references. Bottom line: summary of data

Patients	Point mutations	Splice	Ref
22	16	1	14
1	1	1	27
1	1	1	28
7	7	1	13
1	1	1	29
1	1	1	30
2	1	1	31
3	3	2	Our studies
38	31	9	Total

of the coding region using RNA based mutation screening. This mutation was missed using multiplex PCR/SSCP analysis, since it is located at the end of exon 43 and covered by the reverse primer used to amplify the exon.

Although the multiplex products cover 20% of the dystrophin coding region, DNA based methods only detect mutations in 4% of non-deletion patients (table 1). RNA based methods, screening the total coding region, identify mutations in 82% of the patients (table 2). The number of mutations identified by RNA based methods^{12-14 16 27 29-31} (this study) is still limited; however, seven mutations out of 31 found (23%) are located in that 20% of the coding sequence which is covered by multiplex PCR. This suggests that this region is not particularly sparse in point mutations.

Of the mutations identified with multiplex based PCR material, 18% affect splice sites (table 1), while in RNA based mutation screening studies as much as 29% of the mutations affect splicing (table 2). As suggested by Prior *et al*,¹¹ many mutations missed in DNA based studies might be located outside the coding region, comprising mutations affecting splice sites or branch sites or creating or activating cryptic splice sites. If all primers in the multiplex set were designed to amplify the splice sites as well as the exons (as now only 60% of primers do), the percentage of splice mutations detected by analysis of multiplex products should probably increase to about 30%, which is close to the 29% identified by RNA based detection methods. Finally, even by RNA based methods, still only 82% of the mutations are detected. For the missing 18% several explanations have been postulated¹⁴: (1) the phenotype might not be caused by mutations in the dystrophin gene; (2) large duplications can be missed with the techniques used; (3) PTT has not yet been extensively evaluated and possibly some chain terminating mutations

could be missed; and (4) mutations might be located outside the coding region, that is, in regions controlling expression levels, RNA stability, or in the promoter region of the gene. We want to point out that another possible reason for missing mutations affecting splicing is that they produce transcripts too large to be detected by RT-PCR. Indeed, since most splice site mutations are leaky and produce low levels of correctly spliced products, normal transcripts covering the mutation site will be detected as the only product and the mutation will be missed. We are currently studying a potential example of such an event: a BMD patient with an intron 17 derived insertion in a portion of his dystrophin RNA. No mutation can yet be detected, and the altered transcript might just represent a splice alternative generated by an intronic mutation that produces transcripts too large to amplify.

In conclusion we believe, as emphasised by the mutations identified in this report, that many of the remaining elusive DMD/BMD mutations will be located outside the coding region and affect splicing.

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