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Brief papers

A novel splice site mutation in a Becker muscular dystrophy patient

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Abstract

A Becker muscular dystrophy patient was found to have a single base substitution at the 5′ end of intron 54. This single base substitution disrupts the invariant GT dinucleotide within the 5′ donor splice site and was shown to cause an out of frame deletion of exon 54 during mRNA processing. This is predicted to produce a truncated dystrophin protein which is more consistent with a DMD phenotype. However, small quantities of normal mRNA are also transcribed and these are sufficient to produce a reduced amount of normal molecular weight dystrophin and give rise to a milder BMD phenotype. This indicates that a single base substitution at an invariant dinucleotide of the splice site consensus sequence may still allow read through of the message and allow the production of some normal protein. This shows that there are a greater number of possible intronic mutations that can lead to a mild phenotype and it also underlines the importance of performing cDNA analysis when screening for small gene alterations in the BMD patient population.

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Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are allelic X linked neuromuscular disorders characterised by progressive muscle weakness and severe skeletal muscle degeneration. The DMD gene encodes a large 427 kDa cytoskeletal protein, dystrophin.[1] The causative mutations in 70% of affected boys are large deletions or duplications of one or more dystrophin gene exons,[2–5] while the remaining 30% of affected subjects presumably have smaller alterations (nucleotide substitutions, small deletions, or small insertions) which are more difficult to detect owing to the very large size of the gene. The majority of the small mutations identified in these muscular dystrophy patients have resulted in premature termination of protein translation, giving rise to unstable dystrophins and the more severe DMD phenotype.[6] Unfortunately, since these types of mutation do not produce dystrophin, they provide little or no information about the structural and functional domains of the protein. On the other hand, mutations identified in the less severely affected BMD patients often maintain the translational reading frame and allow for the synthesis of a correctly localised dystrophin protein. For this reason, it becomes important to identify small gene alterations in BMD patients. The BMD small mutations provide insight into the mechanisms involved in generating the milder phenotype.

Although there have been numerous reports of DMD point mutations, at present there are few published BMD point mutations.[6] Roberts *et al*[7] reported a G to C transversion at the 3′ end of intron 56 of the dystrophin gene. This single base substitution caused partial inactivation of the splice acceptor site of exon 57, resulting in activation of a cryptic splice acceptor site located 18 bp downstream from the normal one, which maintained the translational reading frame. A similar mutation that affected normal splicing was reported by Hagiwara *et al*.[8] This involved a G to T substitution at position −1 of the 5′ splice site of intron 13 and resulted in the production of a dystrophin protein with an in frame deletion of exon 13. Finally, Wilton *et al*[9] described a C to A base change at position +3 of intron 19 which resulted in the deletion of exon 19 from the dystrophin mRNA. Since this mutation affected a base in the consensus sequence that is only 60% conserved,[10] some normal mRNA was also observed. Thus, the reported BMD mutations are consistent with the reading frame rule, which proposes that deletions or duplications causing BMD allow for the production of dystrophin because the translational reading frame is maintained.[11] We now report a splice site mutation, which by sequence analysis was predicted to produce a severely truncated protein and was therefore more consistent with a DMD mutation. However, the mutation allowed for the synthesis of a reduced amount of normal molecular weight dystrophin and gave rise to a milder BMD phenotype.

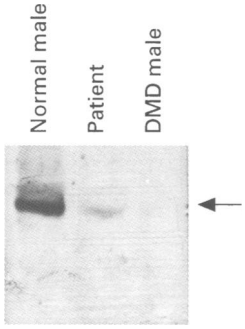

Figure 1 Western blot analysis of dystrophin from normal male muscle samples, the BMD patient, and a DMD negative control. The arrow indicates normal dystrophin at 427 kDa. The western blot was stained with the antibody Dys-2.

Materials and methods

RNA ANALYSIS

Total RNA was isolated from 30 mg of a muscle biopsy sample obtained from an unaffected person and from the affected patient with TRIzol™ reagent (BRL). A total of 1·5 µg of total RNA was reverse transcribed into cDNA using 0·5 µg/µl oligo $dT_{(12-18)}$ (BRL) and 200 U Superscript II reverse transcriptase (BRL) in a 20 µl reaction containing 20 mmol/l Tris-HCl (pH 8·4), 50 mmol/l KCl, 2.5 mmol/l $MgCl_2$, 0·5 mmol/l dNTPs, and 10 mmol/l DTT. The template and oligo dT primer were initially incubated at 70°C for 10 minutes to enhance priming and the reverse transcription reaction was then performed at 42°C for 50 minutes.

Seminested PCR was carried out using the prepared cDNA as template. The first round reaction was performed in a 100 µl volume containing 20 mmol/l Tris-HCl (pH 8·4), 50 mmol/l KCl, 2 mmol/l $MgCl_2$, 10 ng of each primer (53F: 5′-GAAAGAATTCAGAATCAGTGGGATG-3′, 59R: 5′-TCGAGGTGATCTTGGAGA-3′ and 2·5 U *Taq* DNA polymerase (BRL) for 30 cycles of 94°C for one minute, 50°C for two minutes, and 72°C for three minutes with an initial denaturation step at 94°C for five minutes. An aliquot was removed from the first round PCR product and used as a template in the second round reaction. This was carried out as described above except that 0·2 mmol/l dNTPs and 5 U *Taq* DNA polymerase (BRL) were added to the reaction mix. The nested primer set included the forward primer, 53F, and a reverse primer, 57R (5′-GTACATCGTTCTGCT-3′). The PCR products were then separated on a 2·5% agarose gel and visualised with ethidium bromide. The purified products were sequenced using the ds DNA Cycle Sequencing System (BRL). Primers were end labelled with gamma [^{32}P]-ATP. Product from the sequencing reaction was analysed using a 5% denaturing polyacrylamide gel.

ANALYSIS OF GENOMIC DNA

PCR amplification and sequence analysis were accomplished following the same procedure as described previously.[12] The oligonucleotide primers used for PCR amplification and sequence analysis were: 54F: 5′-GTTTGTCCTGAAAGGTGGGTTAC-3′ and 54R: 5′-TTATCGTCTTGAACCCTCCCAAG-3′.

PROTEIN ANALYSIS

The immunocytochemical and western blot procedure for dystrophin were the same as that described in detail previously.[13 14]

Results and discussion

A muscle biopsy specimen was obtained from a BMD patient for dystrophin protein analysis. Western blot analysis using both an N-terminal antibody and the C-terminal antibody specific to the last 17 amino acids of the dystrophin C-terminus (Dys-2, Nova-Castra Labs, Newcastle upon Tyne, UK), indicated the presence of an apparently normal sized but reduced dystrophin, approximately 10% of normal (fig 1). Since there was no evidence of a small gene deletion or duplication, by multiplex PCR and Southern blotting, we analysed DNA for the presence of point mutations and cDNA for the presence of splice type mutations. The DNA was isolated from the patient's muscle biopsy and was screened by a heteroduplex analysis approach,[15] while muscle cDNA was analysed for splice type mutations as indicated by the presence of aberrant bands using primer sets spanning the dystrophin message.

All cDNA PCRs from the patient's muscle resulted in normal sized products, except for the product spanning nucleotides 7871-8735 (numbering as in ref 16). The RT-PCR product from the patient sample yielded two bands: one band of the expected size (864 bp) but present at approximately 10% of the normal control, as well as a more intensely staining product of reduced size (~ 700 bp). The abnormal band was gel purified and sequenced and was found to lack nucleotides 8081–8236, which corresponds to exon 54 (fig 2). The deletion of exon 54 results in a translational frameshift with the new reading frame terminating in exon 55. Therefore, in addition to a stable aberrantly spliced mRNA that has a frameshifting deletion of exon 54, the BMD patient transcribes small quantities of normal mRNA that are sufficient to produce the approximately 10% levels of dystrophin observed on the western blot.

Sequence analysis of genomic DNA amplified using primers flanking exon 54 showed a T to C transition at position +2 of the 5′ splice site of intron 54. This single base substitution disrupts the GT dinucleotide within the 5′ donor splice site,[17 18] causing the deletion of exon 54 during mRNA processing. For normal splicing to occur, the splicing system requires introns to begin with GT and end with AG. With few exceptions,[19] these dinucleotides are invariant and required for correct splicing. Since this was an isolated case of BMD and given the high frequency of germline mosaicism in Duchenne muscular dystrophy, the possibility of somatic mosaicism for the point mutation exists. However, sequence analysis from the muscle biopsy showed only the abnormal sequence. It is therefore unclear how the splicing apparatus was able to recognise the mutant splice donor site and still produce some normal message. In comparison, the BMD mutation described by Wilton *et al*[9] involved a base outside the highly conserved GT dinucleotide and therefore resulted in some normal transcript.

A scoring system was devised by Shapiro and Senapathy[20] to determine optimal splice site sequences based on the frequency of each nucleotide in normal splice sequences. The wild type sequence at the 5′ donor site of intron 54 (AG:GTATGA) scores 81% using this method, indicating a high degree of similarity to the actual consensus sequence (AG:GTA/CAGT) established by Mount.[18] The mutated sequence at this site (AG:GCATGA) scores ~64% and since normally used splice site sequences have a score > 70, the point mutation at the invariant

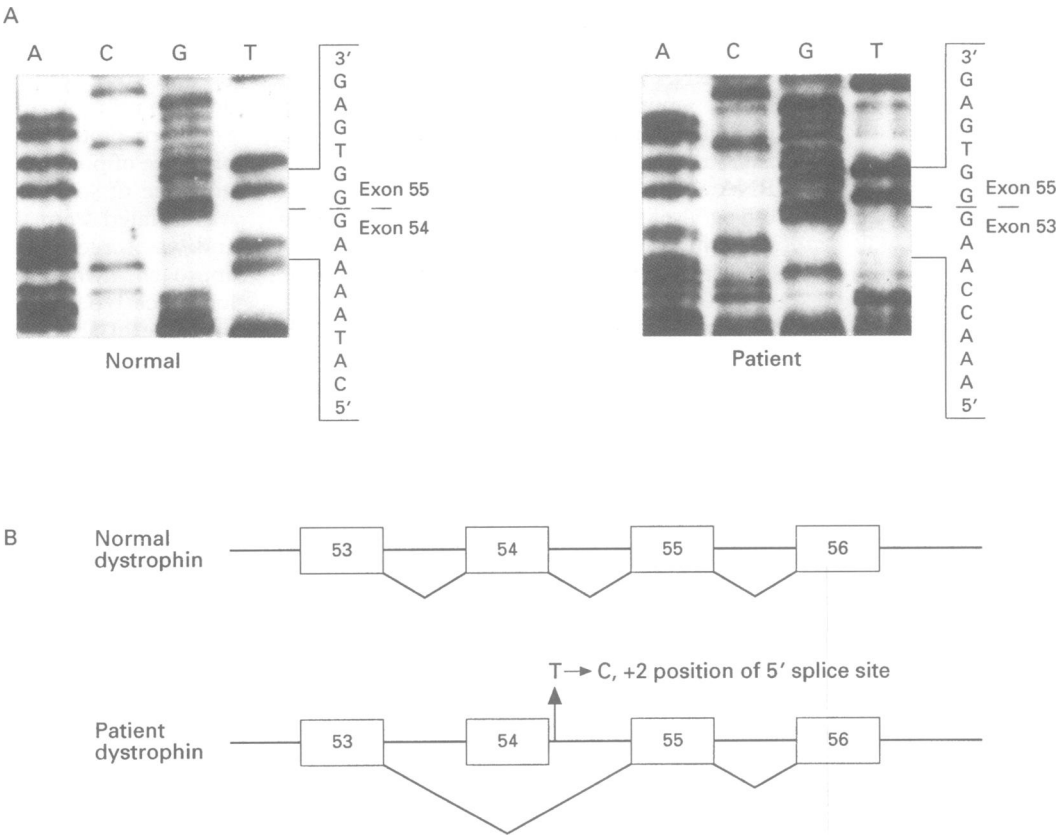

Figure 2 Dystrophin cDNA sequence analysis. (A) Portions of the sequencing gel with a normal control on the left and the BMD patient on the right are shown. A comparison of the nucleotide sequences of the two PCR products showed that the patient sample is deleted for exon 54, and that exon 53 is precisely joined to exon 55. (B) The two PCR products represent normally spliced (above) and misspliced (below) dystrophin mRNA. Exon 54 has been skipped owing to the T to C transition at the donor splice site of intron 54. Exons are represented by open boxes while solid lines represent introns.

GT dinucleotide is predicted to prevent normal mRNA splicing.

This patient had a mild phenotype and was shown to produce a low level of dystrophin. The patient is at present 9 years old and can walk well on both his heels and toes. There is only mild slowing when he runs and he can rise from a squat position with only minimal hand support on his knee. Thus, the low abundance of normal transcript was sufficient to synthesise enough dystrophin to give rise to the milder phenotype. It has been observed in other genetic diseases that variations outside the highly conserved 5′ GT or 3′ AG dinucleotides often result in the production of both abnormal and normal spliced mRNA transcripts. We have shown that a point mutation at the invariant dinucleotide of the consensus sequence may still allow read through of the mutation and production of some normal transcript. This increases the number of possible intronic mutations that can occur in the dystrophin gene, which allow for the synthesis of sufficient levels of dystrophin to produce the milder phenotype. This may also account for the fact that very few BMD mutations have been identified using genomic DNA analysis. Intron mutations that affect splice sites, branch sites, or create cryptic splice sites may be responsible for producing the reduced amount of dystrophin and the milder phenotype in many of the BMD patients. Strategies based on cDNA analysis will therefore be most successful in finding more BMD small mutations.

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