ONLINE MUTATION REPORT

Multiple exon skipping and RNA circularisation contribute to the severe phenotypic expression of exon 5 dystrophin deletion

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Deletion and duplication of one or more exons in the dystrophin gene account for 70% of patients with Duchenne and Becker muscular dystrophies (DMD and BMD) and other allelic clinical entities such as raised serum creatine kinase and X linked dilated cardiomyopathy (XLDC).¹ The severity of the resulting phenotype can be generally predicted by whether these mutations lead to translation frame disruption and premature termination of protein synthesis.² Nevertheless, the occurrence of severely affected patients with in frame deletions as well as mild phenotypes associated with frameshift, indicate that factors other than the frame disruption should contribute to the clinical severity. Exceptions to the “frame rule” are found in about 8% of patients with mutations occurring both at the 5′ and 3′ end of the dystrophin gene,^{1,3,4} although they seem to predominate in the 5′ region.⁴ Despite extensive clinical, immunocytochemical, and transcriptional studies, the basis of genotype-phenotype correlation in these “atypical” cases remains controversial and its clarification will surely provide relevant

information about normal and abnormal dystrophin function. Several reports suggest a role for alternative splicing in altering the clinical phenotype by modulating the editing of the translation reading frame.^{3,5,6} Patients with BMD carrying the frameshift deletions of exons 3–7 and 45 show alternative splicing phenomena theoretically restoring the reading frame.^{3,7} However, the relevance of these events in contributing to a milder phenotype is still unclear.^{3,8,9} Supporting the bridging role of the dystrophin splicing machinery as active modulator between genotype (deletion mutation) and protein production, cell specific somatic exon skipping has been documented in skeletal muscle revertant fibres in DMD.¹⁰

Production of circular RNA molecules, resulting from the adjoining of the donor splice site of a 3′ exon to the acceptor site of a 5′ exon, represents a peculiar splicing behaviour described to date in a limited number of human genes, dystrophin included.^{11–15} These circular RNAs represent scrambled transcripts supposed to have originated as a corollary of alternative splicing events.¹⁶ Nevertheless, the “exon skipping circular RNA” hypothesis cannot account for all circular molecules, and their origin as well as their function remains unclear.^{15,16} Some circular dystrophin RNAs are transcribed at low levels in normal human skeletal muscle; however, their production has been found to be abolished in tissues from patients with DMD carrying deletions in the 5′ region of the gene.¹⁵

MATERIALS, METHODS, AND RESULTS

To elucidate novel splicing mechanisms underlying unexpected phenotypes in dystrophin deletions, we studied two previously described patients with DMD (HH2 and HH4 in Muntoni *et al*.¹; clinical follow up of patient HH2 allowed us to reclassify him as a DMD phenotype) carrying the rare isolated deletion of exon 5, which would be predicted to result in a mild phenotype according to the frame model. The definition of the deleted genomic region and the cloning of intronic junction fragments were achieved in both patients by a long range polymerase chain reaction (PCR) walking approach with primers amplifying overlapping intronic regions of 1.5–2 kb (the sequence and nucleotide position of oligonucleotides for introns 4 and 5 used are available on request). The detailed genomic configuration of the patients analysed is described in fig 1A. Sequence analysis of junction fragments showed a clear intronic junction in patient HH2, whereas an insertion of an 18 bp palindrome was present in patient HH4 (sequence: GAGAATTTTAAATCTC).

Key points

- The “frame rule”, linking protein expression and clinical severity to disruption of the coding frame, represents the largely accepted mechanism explaining genotype-phenotype correlations in dystrophinopathies. Nevertheless, exceptions to this model account for at least 8% of patients with deletions and duplications in the dystrophin gene.
- We have defined the intronic breakpoint regions in two patients with Duchenne muscular dystrophy carrying the in frame isolated deletion of dystrophin exon 5, representing a known example of “exception to the rule” dystrophin mutation.
- Transcription analysis in skeletal muscle from one patient showed a complex RNA configuration, combining an unfavourable exon skipping event (involving exon 6 and leading to an out of frame transcript) with the production of scrambled, circular RNA molecules. Circularisation of RNA specifically involved the in frame transcripts (retaining exon 6) with the consequence of depletion of the functional messenger. We also documented abundant in frame exon 9 skipping.
- This peculiar splicing behaviour leading to a defect in the in frame messenger RNA and in critical protein domain, might represent the pathogenic background underlying the severe clinical impact of the rare exon 5 deletion. The circular molecule formation focuses attention on the role that RNA scrambling might have in contributing to the clinical severity in dystrophin deletions.

Abbreviations: BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; RT, reverse transcription; PCR, polymerase chain reaction; XLDC, X linked dilated cardiomyopathy

Figure 1 Genomic configuration in the two patients with deleted exon 5. (A) Schematic diagram of the deleted/maintained intronic regions in the two analysed patients with deleted exon 5. Nucleotide positions are referred to the genomic clone including dystrophin exons 3–9 (GenBank accession number AL096699). In patient HH4, the deleted region spans 3846 bp and involves both introns to a similar extent (1766 bp in intron 4 and 1987 bp in intron 5). In patient HH2, the deleted region is larger (7784 bp) and removes the greater part of intron 5 (4555 bp). A common deleted region was identified, spanning 3.8 kb and including exon 5. A 2 kb region in intron 5, flanking exon 6, was maintained in both patients. RepeatMasker analysis of breakpoint regions (HGMP, <http://www.hgmp.mrc.ac.uk-GCG> package) showed that three out of four intronic breakpoints lie within repetitive elements (LINE1, Alu, LTR). (B) VISTA plot of the alignment between human and mouse exon5-intron5-exon6 sequences. Human and mouse query sequences were obtained from GenBank database (accession number AL096699) and the Mouse Genome Browser database (<http://genome.ucsc.edu/>) respectively and aligned by VISTA program using default parameters. Both the identified intronic CNSs are located in the intron 5 region commonly retained in the patients with DMD. CNS1 spans 265 nucleotides (from nt 152472 to nt 152207 of accession number AL096699) and shows a 78.5% identity; CNS2 is 116 bp long (from nt 151939 to nt 151823 of accession number AL096699) and is characterised by 75.8% identity. (C) Clustering within CNS1 and CNS2 of several sequences with high homology with previously reported splicing silencer motifs (disclosed by FASTA analysis). ISS1¹⁷; HIVtat3, α-TM and IgM¹⁸; B5, B9, and A2¹⁹; HIVenv.²⁰

Transcription analysis of the dystrophin canonical messenger was performed on skeletal muscle from patient HH2 by RT-PCR with oligonucleotides spanning from exon 2 to exon 18 (primer sequences are available on request). Different combinations of reverse primers, coupled with forward primers on exons 2 and 4, disclosed a complex RNA configuration with multiple transcripts (fig 2A). Sequence analysis of the amplified fragments from 2For-7Rev, 2For-8Rev, 2For-9Rev reactions led to the characterisation of two main RNA populations. The higher molecular weight transcript corresponded to the expected in frame dystrophin messenger with exon 4 joined to exon 6, resulting from the genomic deletion ($\Delta 5$ transcript). The additional identified transcript carried an exon 4-exon 7 junction, owing to the skipping of exon 6, and was predicted to disrupt the dystrophin reading frame ($\Delta 6$ transcript). In the PCR reaction 2For-8Rev, an additional product was detectable, corresponding to an out of frame exon2-exon8 joined transcript. In the light of the occurrence of an exon 6 skipping event in the skeletal muscle of patient HH2, both exon 6 and intronic 3' splice site consensus sequences were sequenced (1296 bp from nt 152014 to nt 150718 of GenBank accession number AL096699). This analy-

sis allowed us to exclude the existence of nucleotide variations which could have been responsible for the exon 6 skipping. When reverse primers were positioned on exons 10, 12, 16, and 18, the prevalent amplification products were splicing variants in which exon 9 was skipped (fig 2A). Exon 9 skipping occurred in both the $\Delta 5$ and the $\Delta 6$ transcripts and most of the dystrophin messenger from the skeletal muscle of patient HH2 lacked exon 9. In the PCR reaction 2For-16Rev, the occurrence of the out of frame transcript with the exon2-exon 8 junction was confirmed.

PCR amplifications by using couples of oligonucleotides located within exon 2-exon 7 and exon 2-exon 10 on eight adult normal skeletal muscle cDNAs failed to identify any alternative splicing products, ruling out the possibility that transcripts lacking exons 6 and 9 represent a significant proportion of the dystrophin messenger in normal muscle (fig 2B).

To clarify whether changes occurred in the use of the dystrophin promoters in the skeletal muscle of the patient, oligonucleotides specific for muscle (M), brain (B), and Purkinje (P) exons 1 were coupled with a 7Rev primer. The two expected transcripts ($\Delta 5$ and $\Delta 6$) were driven exclusively

A

B

C

Figure 2 Multiple exon skipping events occurring in skeletal muscle from patient HH2. (A) Reverse transcription (RT) PCR analysis along the dystrophin transcript from exon 2 to exon 18 showing the occurrence of multiple alternative splicing events in skeletal muscle from patient HH2. Total RNA was isolated from a biopsy specimen by the method of Chomczynsky and Sacchi.²¹ RT was performed as previously described.²² Amplification reactions were performed using ExTaq polymerase (Takara) and conditions were 94°C for one minute, 64°C for 45 seconds, and 68°C for one minute for five cycles; 94°C for 30 seconds, 63°C for one minute, and 68°C for one minute 30 seconds for 30 cycles. All the PCR fragments were gel purified using an QIAquick purification kit (QIAGEN) and sequenced. Exonic junctions are indicated as well as the occurrence of exon 9 skipping (unmarked fragment in the 2For-16Rev reaction represents a non-specific PCR product). Molecular weight marker VI (Roche Biochemicals). (B) RT-PCR analysis with the couples of primers exon 2For/exon 7Rev and exon 2For/exon 10Rev simultaneously performed on normal adult skeletal muscle and on skeletal muscle from patient HH2. No exon skipping events are visible in the control muscle (lane C). N=negative control. Molecular weight marker VI (Roche Biochemicals). (C) RT-PCR using muscle promoter exon 1 primer coupled with exon 7Rev and exon 10Rev primers which shows that alternative transcripts detectable in skeletal muscle from patient HH2 are driven by the muscle promoter. Molecular weight marker VI (Roche Biochemicals).

by the muscle promoter (fig 2C). By using muscle exon 1 primer coupled with a reverse oligonucleotide on exon 10, all alternative transcripts were detected, all being driven by the dystrophin M promoter (fig 2C).

In summary, our results show that in skeletal muscle from the patient with exon 5 deletion in the dystrophin gene, the dystrophin messenger RNA underwent multiple exon skipping events, variably affecting the reading frame, and all these transcripts were driven by the dystrophin muscle promoter. The out of frame exon 6 skipping is likely to be of pathogenic importance, its occurrence never being described in normal conditions in skeletal muscle despite the propensity of the 5' region of the dystrophin gene to undergo alternative splicing.²³ This exon skipping event could have been favoured by the new genomic configuration created by the deletion; in fact, a novel fusion intron was formed and this event could weaken the exon 6 definition process. In both patients with DMD analysed, the 2 kb at the 3' end of intron 5 were preserved and fused with different upstream sequences belonging to intron 4. Comparative analysis between human and mouse dystrophin intron 5, performed by the VISTA alignment program (<http://www-gsd.lbl.gov/VISTA/>), showed that this 2 kb region contained the only two intron 5 sequences that are evolutionarily highly conserved between

humans and mice (fig 1B). Both are unique sequences as shown by RepeatMasker analysis and are located 1484 bp and 1117 bp from the 3' splice site, respectively. RT-PCR analysis on these two conserved intronic regions showed that they were not transcribed either in normal human skeletal muscle or in the skeletal muscle of patient HH2. Interestingly, several sequences with high homology to known splicing silencer motifs (disclosed by FASTA analysis: HGMP, <http://www.hgmp.mrc.ac.uk-GCG> package) were clustered within the dystrophin region containing the two CNSs (fig 1C). The clustering of motifs in a CNS supports a possible functional role that they may exert on the adjacent splice sites. We propose that the configuration of the novel fusion intron may favour exon 6 skipping. Supporting this possibility there is evidence that multiple splicing motifs scattered along introns can cooperate in controlling distant splice sites.²⁴ The relation between CNSs containing splicing motifs and the pathogenesis of the dystrophinopathy needs to be further investigated.

Although exon 6 skipping resulted in an out of frame mRNA, it involved only a proportion of the dystrophin transcript in the skeletal muscle of patient HH2 and therefore other phenomena probably contribute to determine his severe muscular involvement. To explore the contribution of scrambled RNA molecules to the patient's in frame transcripts, we

Figure 3 Production of scrambled dystrophin RNAs in skeletal muscle from patient HH2. (A) RT-PCR with primers exon 7For coupled with primer exon 2Rev showing the occurrence of a 181 bp scrambled product in skeletal muscle from patient HH2. The PCR approach as well as the PCR product obtained are also shown. Sequence analysis of the high molecular weight unmarked fragment showed that it includes exon 7-exon 2 flanked by primer concatamers. The simultaneous amplification of a normal adult skeletal muscle cDNA (lane C) failed to detect any product. N=negative control. Molecular weight marker VI (Roche Biochemicals). (B) Sequence analysis of the exon 7-exon 2 transcript showing that the 3' end of exon 2 is correctly joined to the 5' end of exon 7. (C) Hybridisation analysis evaluating the exon content of the scrambled RNA molecules produced in skeletal muscle from patient HH2. RT-PCR products exon 3For/exon 2Rev obtained both from a control skeletal muscle and skeletal muscle from patient HH2 were hybridised with three different probes detecting dystrophin exons 6, 7, and 4. Two hybridisation signals were detected by using exon 6 and exon 7 probes, whereas exon 4 probe identified only the upper molecular weight product. No hybridisation signals were detected in the normal adult skeletal muscle. A schematic representation of the exon content in the scrambled products is shown.

analysed the skeletal muscle of patient HH2 for the production of circular RNAs, previously described in dystrophin RNA.¹⁵ By using a forward primer on exon 7 and a reverse primer on exon 2, in 35 cycles of PCR, we were able to obtain and to directly sequence a single scrambled product in which the 3' end of exon 7 was correctly joined to the 5' end of exon 2 (fig 3A, B). No other scrambled molecules including exons distal to exon 7 were detectable in this PCR reaction. The simultaneous amplification of control skeletal muscles failed to detect any product (fig 3A). To define the exon content of this circular RNA molecule, a PCR with a forward primer on exon 3 and a reverse primer on exon 2 was performed on cDNA both from patient HH2 and from a control skeletal muscle. The PCR products were hybridised with three different exonic probes, designed on exons 6, 7, and 4. The hybridisation pattern showed that two types of scrambled molecules, owing to exon 4 skipping, were produced in skeletal muscle from patient HH2. Remarkably, both transcripts included exon 6. No scrambled products were detectable in the control skeletal muscle (fig 3C). The results obtained exclude the possibility that circular molecules extending beyond exon 7 are produced in the patient's skeletal muscle.

Thus, search for scrambled splicing products disclosed that part of the in frame dystrophin transcript, retaining exon 6, is not produced as a linear molecule but is converted into circular molecules, this event probably accounting for a further depletion in functional messenger.

DISCUSSION

The mechanism leading to circular splicing products has not been clarified, although an unusually large exon size (as for *NCX1* gene exon 2¹⁴) as well as the existence of complementary intronic regions (as for rat *Sry* gene²⁵) may probably favour RNA circle formation. Moreover, at least for the *ETS-1* gene, only specific exons participate in the formation of circular RNAs.¹² In the case of the dystrophin gene, several circular RNAs have been described to occur at a low level in normal skeletal muscle but they are not detectable in muscle from patients with DMD carrying deletions of certain 5' exons.¹⁵ Isolated deletion of dystrophin exon 5 behaves differently, preserving, and to some extent increasing, the possibility of circular molecules being formed. However, RNA circularisation occurs only when exon 6 is spliced into the dystrophin transcript, whereas exon 6 skipping seems to hamper the formation of the correspondent circular product. How exon 6 inclusion can affect RNA circularisation is not clear but it is interesting to underline that 5' dystrophin deletions that have been described to abolish production of circular RNAs involve exon 6 alone or together with flanking exons (3–6, 4–6, and 6 in Surono *et al*¹⁵). The mechanism leading to the juxtaposition of the exons is not clear but the interaction between transacting factors (SR and hnRNPs proteins) bound to different exons has been proposed.²⁶ It is conceivable that different exon (or different splice sites) content in pre-mRNA may affect the propensity to juxtaposition defects and circular RNA formation. On this basis

and considering our results we could speculate that the RNA circularisation might be influenced by the nucleotide composition/length of adjacent exons. This might imply that some exons (such as dystrophin exon 6) are required for circularisation. As a corollary of this hypothesis, we can exclude the fact that exon 5 as well as exon 4 have relevance in this phenomenon, because we described circular molecules lacking these exons. Notably, at variance from exon 5, exon 4 is known to be often skipped in normal adult skeletal muscle.²⁷ As far as we know, this is the first report showing that scrambled RNA products can be formed even in the absence of both constitutive and alternative spliced exons.

Despite the detrimental transcriptional behaviour with an exon skipping event leading to an out of frame transcript and exon scrambling events, the skeletal muscle of the patient is still able to produce an amount of in frame messenger. This finding is in keeping with the residual expression of dystrophin noticed in this patient.⁴ However, most of the residual in frame dystrophin transcripts skips exon 9 and this further unexpected splicing behaviour probably negatively affects the overall function of the protein produced.

Dystrophin transcript lacking exon 9 has been previously reported to be very abundant in tissues with ectopic dystrophin expression such as lymphocytes, but rare in normal skeletal muscle as well as in other tissues requiring a functional protein.²⁸ Dystrophin exon 9 codes for the first hinge domain of the protein, located between the N terminal and the rod domains, and is thought to confer flexibility to the membrane associated network of dystrophin.¹ The dystrophin N terminal region is the site of interaction with F actin and three actin binding sites (ABS 1–2–3) have been defined within the primary sequence of the protein, with the intermediate one encoded by exon 5 (reviewed by Corrado *et al*²⁹). The crystal structure of the human dystrophin actin binding region has been also recently determined, being composed of calponin homology domains occurring in tandem (CH1-CH2), formed from a scaffold of α helices and assembled into an antiparallel dimer.³⁰ In the light of the complex tertiary arrangement of the dystrophin N terminal region, it has been proposed that removal of large portions (as in exon 5 deletions) could have a deleterious effect on CH domain folding and function.³⁰ Nevertheless, how dystrophin binds actin has not been clearly defined and an extended lateral association between dystrophin and the actin filament has been proposed with a novel actin binding activity identified in the rod domain.³¹ Thus, dystrophin-actin interaction extends beyond the N terminal domain and involves regions of the protein distal to the hinge region encoded by exon 9. Moreover, a missense mutation has been described in dystrophin exon 9, associated with XLDC phenotype.³² This evidence suggests a possible critical function for the dystrophin region encoded by exon 9 and makes it conceivable that the simultaneous absence in the prevalent in frame transcript of exon 5 and exon 9 could magnify the general impairment of the dystrophin actin binding domain.

In conclusion, in the patient analysed, the in frame genomic deletion of a single dystrophin exon in the 5' portion of the gene was far more devastating than could have been predicted by the sole genomic configuration. The genomic rearrangement subverted the canonical splicing leading to adverse exon skipping and exon scrambling events. We propose that the lack of exons 5, 6, and 9 in the transcript as well as the increased production of circular RNAs have deleterious effects on both the availability of the in frame products and on the structure of important protein domains involved in the abridging function. These multiple effects probably contribute to the severe phenotype that is commonly associated with this in frame deletion mutation.

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