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An X:Autosome Translocation Stabilizes Truncated Dystrophin: Implications for Lack of Truncated Dystrophins in Duchenne Muscular Dystrophy

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Abstract

We report a 5-year-old girl with clinical symptoms of typical *Duchenne* muscular dystrophy in males. The girl showed dramatic elevations of serum creatine kinase, and muscle biopsy histopathology consistent with a severe dystrophic myopathy. Cytogenetic analysis revealed an X:22 translocation (46,X,t [X;22] [p21.2;11.2]). Dystrophin immunofluoresence studies showed strong membrane immunostaining of dystrophin with antibodies directed against the amino terminus of the protein, but vastly reduced immunostaining with carboxyl-terminal antibodies. Immunoblot studies showed a major immunoreactive protein of approximately 350 kDa at approximately 20% levels. Nested RT-PCR analysis of the dystrophin mRNA in the patient's muscle showed the RNA to be positive for primers covering the first 85% of the dystrophin coding sequence, and negative for the carboxyl-terminal 15%. Taken together, our data suggests that the translocation breakpoint occurs towards the 3' end of the gene. The translocated dystrophin gene is still expressed into a truncated dystrophin protein associated with the plasma membrane. Our results are consistent with the translocation resulting in a more stable abnormal dystrophin mRNA.

Key words

DMD, female – X:autosome translocation – Dystrophin

Introduction

Duchenne muscular dystrophy is a common X-linked recessive lethal muscle disorder caused by dystrophin deficiency (6, 7). The disease generally affects males, however, rare females have been found who manifest symptoms as heterozygous carriers of the disease (2, 10). All symptomatic female carriers studied to date have shown skewed X inactivation: the mutant dystrophin gene is used by the majority of muscle cells, while the normal gene is inactivated through "skewed" lyonization (13). Many of the most severe manifesting carriers have been found to have X:autosome translocations involving the dystrophin gene at Xp21 (2, 3, 4). The translocation is thought to result in preferential inactivation of the normal, intact X chromosome. This would be expected to lead to nearly complete dystrophin deficiency in muscle, and a *Duchenne* muscular dystrophy phenotype. In this report we present clinical, morphological, biochemical, cytogenetic, and molecular RT-PCR findings in a girl with a translocation at Xp21 and classic *Duchenne* muscular dystrophy clinically. We show the unusual finding of a truncated dystrophin protein which is retained at the sarcolemma in the girl's muscle fibers.

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Case report

The proband is a 5-year-old girl born after a normal pregnancy and delivery, although she had an Apgar score of 6. Her motor milestones were delayed; she began to walk at 15 months and was clumsy. There was slight improvement in walking during her 2nd year, but during the 3rd year she had increasing difficulties in climbing stairs and rising from the floor. At age 5 years, clinical examination showed hypertrophy of the calves and quadriceps muscles, and marked weakness of hip girdle musculature (Fig. 1). Tendon reflexes were present. Contractures of Achilles tendons, lumbar lordosis, and a waddling gait were observed.

Biochemical tests revealed serum CK activity of 5888 IU (n. 0–34), aldolase 196 U (n. 3–8), SGOT 299 U (n. 5–40), and SGPT 445 (n. 7–56). Electromyographic examination of biceps and quadriceps muscles showed a myopathic picture, with short, polyphasic motor unit potentials of low amplitude. Conduction velocity in peripheral nerves was normal. Heart studies (ECG) showed complete block of the right HIS branch. Intelligence quotient (*Termann-Merrill* scale) was 74. The mother's serum CK was normal (23 IU).

Cytogenetic analysis using high resolution banding techniques showed a reciprocal translocation involving chromosomes 22 and X (46X t[X:22] [p21.2; q11.2]). Karyotypes of both parents were normal.

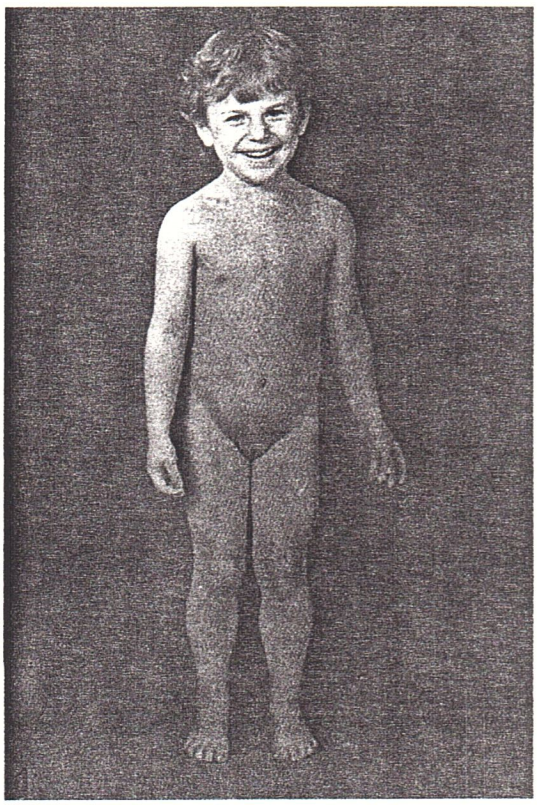

Fig. 1 Proband at 5 yrs.

Materials and methods

Muscle biopsy analysis

The muscle biopsy was obtained from quadriceps femoris muscle. Serial sections for light microscopy were stained according to standard techniques.

Dystrophin analysis was done with affinity purified sheep polyclonal antibodies directed against the amino-terminal (60kd) (6), central rod domain (30kd) (6) and carboxylterminus (d10) (10). Biotinylated second antibodies, and streptavidin Texas Red were used to visualize immune complexes, as previously described (8). Dystrophin immunoblotting was done using a 1:500 dilution of a monoclonal antibody directed against the central rod domain (Dy4/6D3) (12) (kindly provided by Dr. *Louise Anderson)*, using 3.5%–12.5% gradient SDS-PAGE gels, and chemiluminescence (ECL, Amersham).

RT-PCR analysis of dystrophin RNA

Total RNA was isolated from 0.15 g of the patient's muscle biopsy and from two control muscle biopsies. Biopsy fragments were homogenized with a *Brinkman* Polytron homogenizer in guanidinium thiocyanate homogenization buffer (17) and RNA pelleted through a CsCI cushion. The pellet was rinsed with cold 70% ethanol, resuspended in 200 µl TE and 1% SDS, 20 µl sodium acetate [2M] (pH 5.2) and 400 µl of ice-cold ethanol. The RNA was stored as an ethanol precipitate at –20 °C until used. RNA integrity was verified by 1% agarose gel electrophoresis, and concentration determined by OD 260.

The cDNA was synthesized by incubation of 10 µg of total RNA at 42 °C for 1 hr in a 50 µl reaction containing 800 ng oligo dT, 25 units of AMV reverse transcriptase (BM), 10 µl 5X of reverse transcriptase buffer (BM), 4 µl 2.5 mM dNTPs and 20 units RNase inhibitor (BM).

All 10 sets of nested PCR primers were used for RT-PCR analysis of the patient and control dystrophin mRNAs, as previously described (16). The first amplifications of cDNA were carried out as follows: one microliter of the RT product was mixed with a 2.5 µl mixture containing 2.5 µl 10XPCR buffer (*Perkin Elmer*), 200 ng forward outer primer, and 200 ng of the reverse primer, 2.5 units Amplitaq Taq polymerase (*Perkin Elmer Cetus*). The PCR conditions were: denaturation at 93 °C for 3 min, then 25 cycles 93 °C 30 s, 53 °C 30 s, and 65 °C 4 min with a final 1 min 94 °C 10 min 65 °C extension cycle. For the second round of nested PCR, 1 µl of the PCR products were added to a 25 µl mixture containing 2.5 µl 10XPCR buffer (*Perkin Elmer*) 200 ng of nested forward and 200 ng reverse primers and 2.5 units Taq polymerase (*Perkin Elmer Cetus*). PCR was repeated as above for 30 cycles.

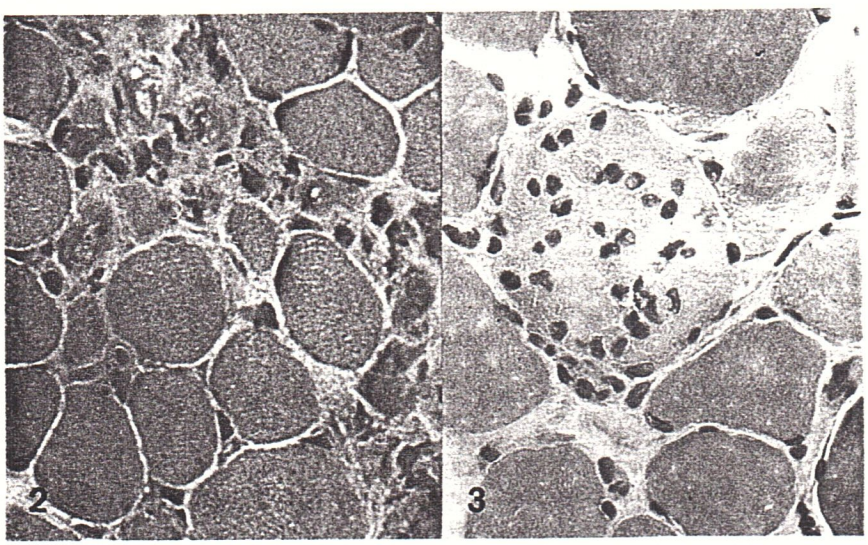

Fig. 2 Transverse section from quadriceps femoris muscle showing an increased variability in size of fibres. (H + E, 448 x).

Fig. 3 Necrotic fibre undergoing phagocytosis. (H + E, 448 x).

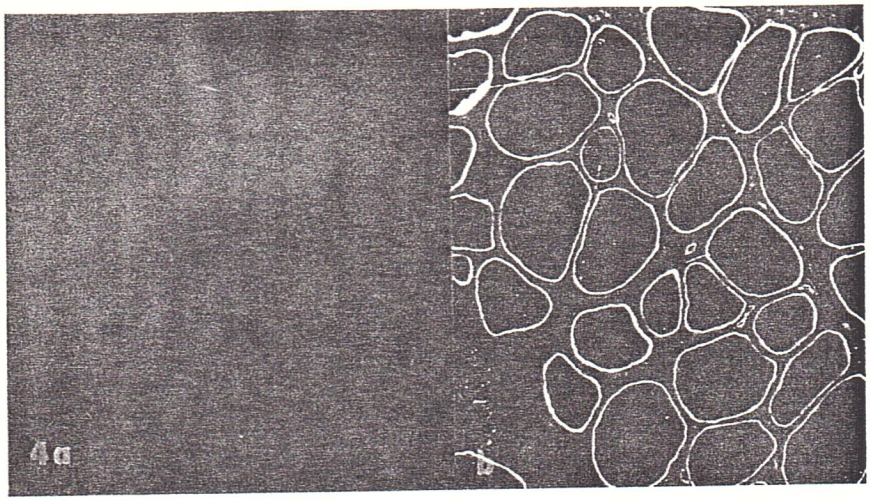

Fig. 4 Immunohistochemical localization of dystrophin in patient muscle biopsy. Shown is extremely faint immunostaining with an antibody reacting with the cysteine-rich domain and the carboxyl-terminal region of the rod domain (d10) (Panel A). This antibody probably cross-reacts with a small number of epitopes contained in the truncated dystrophin produced by this girl. Antibodies raised against the amino-terminal portion of the rod domain (60kd; Panel B) shows strong cross-reaction with the truncated dystrophin which is immunolocalized at the plasma membrane.

Sets of 40 oligonucleotides used for nested RT-PCR were as previously described (16).

Results

The muscle biopsy showed an increased variability in the size of fibres (Fig. 2). Splitting as well as necrotic muscle fibers were observed (Fig. 3). Groups of basophilic fibers with internal nuclei were noted. There was a slight increase of endomysial connective tissue.

In spurr-embedded sections, numerous darkly stained, rounded, large fibers had wedge-shaped areas of rarefication (Fig. 5). Electron microscopic examination demonstrated defects of the plasma membrane associated with pathological alterations in the underlying fiber region, with focal areas of sarcolemmal discontinuity in both necrotic and non-necrotic muscle cells (Fig. 6).

Immunocytochemical analysis with 60 kd and 30 kd antibodies directed against the first 30% of the dystrophin protein showed positive dystrophin staining around most myofibers (Fig. 4b), however, the staining was clearly less intense than in normal controls. The antibody raised against the carboxyl-region of dystrophin (d10) showed a nearly complete absence of immunostaining (Fig. 4a). Quantitation of immunostaining patterns in 569 myofibers showed 1% to be completely positive with antibodies directed against the carboxyl cystein-rich domain (d10), 1.6% to be partially positive, and 97.4% to be nearly completely negative. This analysis suggested the presence of truncated dystrophin lacking the carboxyl terminus but which showed very slight reaction with the d10 antibody raised against the cystein-rich region.

Immunoblot analysis of the proband's muscle with a central rod domain monoclonal antibody showed a

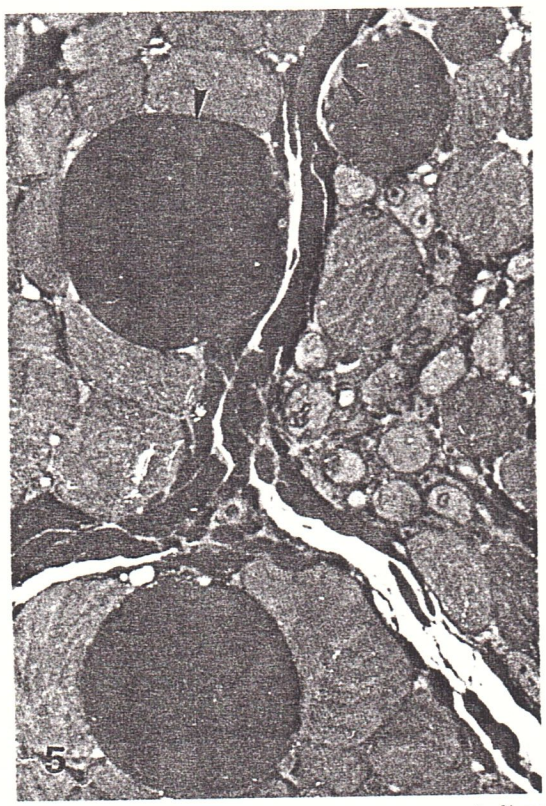

Fig. 5 Electron microscopy shows opaque muscle fibres with wedge-shaped areas of rarefication (arrow). Spurr resin, 1120 x.

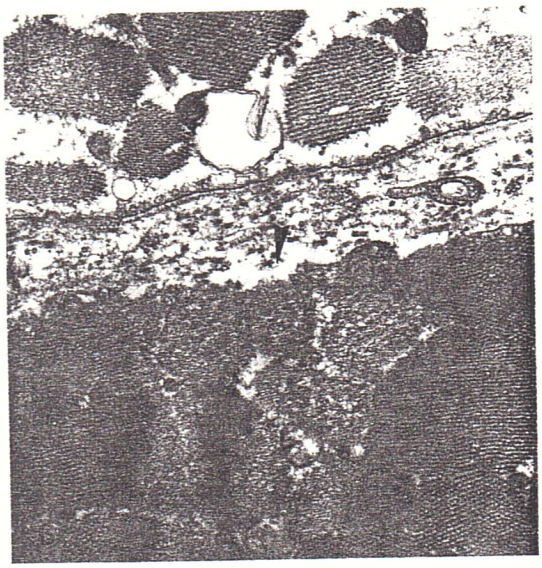

Fig. 6 Focal plasma membrane defects (arrow) in a nonnecrotic fibre. Spurr resin, 30,000 x.

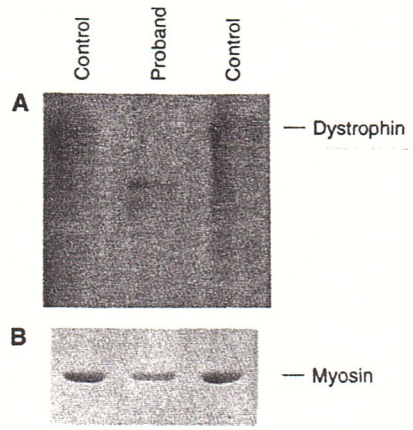

Fig. 7 Immunoblot analysis shows persistence of a truncated dystrophin protein in the female patient with an X:22 translocation. Shown is immunofluorescence using a polyclonal antibody directed against the carboxyl cystein rich domain (d10) (Panel 4a), and with a polyclonal antibody directed against the amino-teminal portion of the rod domain (60kd) (Panel 4b). Extremely faint reaction was observed with the carboxyl domain antibodies, while strong immunoreactivity was observed with the amino domain antibodies. This result was consistent with the production of a truncated dystrophin in the patient's muscle.

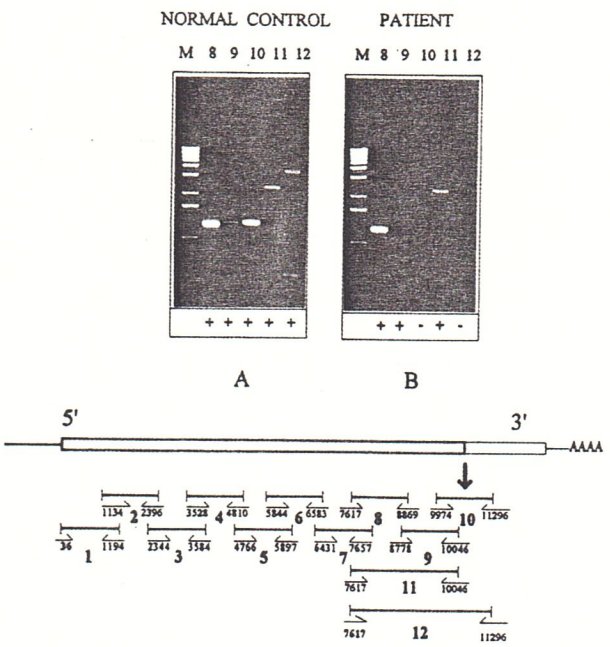

Fig. 8 Nested RT-PCR analysis of the dystrophin mRNA in RNA from the female translocation patient's muscle biopsy. Shown are nested RT-PCR results for a normal control biopsy from the translocation female discussed in this report. All regions tested positive for the presence of dystrophin mRNA in the control biopsy. In the translocation patient, PCR products were not observed 3′ of position 10,000 bp on the cDNA. This result was consistent with the presence of a truncated RNA corresponding to the truncated protein observed on immunoblotting.

dystrophin protein of very abnormal molecular weight (~350 kDa), and reduced quantities (~20% of normal levels) (Fig. 7). Immunoblotting was done four separate times, with similar results. No full-length dystrophin protein from the normal nontranslocated gene was detectable despite long exposures of the blots. This result was consistent with expression of a truncated dystrophin protein from the translocated dystrophin gene. The detection of full-length dystrophin in 1% of fibers by immunofluorescence is consistent with the absence of full-length dystrophin by immunoblot: 1% of dystrophin is below the resolution of immunoblotting.

To determine if the dystrophin RNA in the patient's muscle was indeed lacking the carboxyl-terminal regions of the gene, a series of 12 nested RT-PCR experiments were done using total RNA isolated from muscle biopsy from the patient and a control (Fig. 8). The normal control biopsy gave the expected RT-PCR products for all 12 amplified segments of the dystrophin RNA. The proband's biopsy showed the expected PCR products for 10 segments of the dystrophin mRNA (those including primers 5′ to 10,046 bp in the cDNA sequence). Two segments (segments 10 and 12; Fig. 6) failed to amplify (Fig. 8). This result suggests that the translocation breakpoint is soon after 10,046 bp in the dystrophin mRNA sequence.

Discussion

We have shown biochemical and molecular data which indicates that dystrophin lacking the carboxyl-terminal domains can associate with the sarcolemmal membrane. This female patient bears some molecular homology to other male patients which have similarly been found to produce low levels of dystrophin lacking the carboxyl terminus, yet still associate with the plasma membrane (5, 9, 15). It is important to note that the abnormal truncated dystrophin in this patient may only appear to associate with the plasma membrane, and could instead possibly simply associate with the actin cytoskeleton near the plasma membrane, as previously discussed (15).

Most male *Duchenne* dystrophy patients are predicted to produce truncated dystrophin proteins, however, the truncated proteins are rarely observed by immunoblotting or immunofluorescence, and if present occur at very low levels. An important unresolved question in research in the dystrophinopathies is the molecular basis for the apparent instability of the abnormal truncated proteins. This "down regulation" could occur at the RNA level: mRNA stability has been shown to be determined, in part, by specific 3′ untranslated sequences (1), and it has also been hypothesized that mRNA molecules with premature stop codons (nonsense mutations) are targeted for degradation by specific mechanisms within the cell. Alternatively, the truncated protein may be unable to associate with the different components of the membrane cytoskeleton, and thus be degraded at the protein level. We have studied the dystrophin protein profiles of three different translocation carriers, and two have shown high levels of severely truncated dystrophin protein: the X:21 patient in this report (320 kDa, 20%); and an X:3 translocation we have previously described (260 kDa, 46 ± 13%) (14). An X:12 translocation female we have previously reported did not show significant truncated dystrophin (18). This current report is the only study to

characterize the translocation breakpoint by RT-PCR studies, and we found that the molecular weight of the truncated dystrophin was indeed consistent with the truncated dystrophin mRNA present in the patient's muscle. Thus, we can conclude that the translocation removes the normal 3′ end of the dystrophin gene, but the truncated gene is still expressed into mRNA and protein.

The persistence of the truncated dystrophin protein at high levels in 2 of 3 translocation patients, and the virtual absence of such truncated dystrophin proteins in the vast majority of boys with *Duchenne* muscular dystrophy, suggests that there is a fundamental difference between post-transcriptional regulation in the two types of mutant genes (translocation vs. frameshift/nonsense). At the protein level, the truncated dystrophin from the translocated genes should be quite similar to that of frameshift/nonsense mutations in *Duchenne* boys. Thus, we can not envision why the truncated dystrophin proteins should be differentially regulated in the translocated girls vs. frameshift/nonsense *Duchenne* boys. Similarly, it is difficult to imagine how transcription or translation should be differentially regulated in the two types of mutations. This leaves mRNA stability as the primary site of down-regulation of the mutant genes. The frameshift/nonsense mutations common in *Duchenne* dystrophy boys result in mRNA molecules with extremely long 3′ untranslated regions: premature termination of translation has indeed been found to be a signal for mRNA degradation. On the other hand, the translocations in the female patients likely result in a truncated mRNA encoding a truncated dystrophin protein; this should permit translation to continue to near the 3′ end of the RNA. Thus, the translocations may permit the truncated mRNA and protein to escape the down-regulation mechanism for mRNA destabilization which presumably occurs in *Duchenne* boys. Alternatively, there may be sequences in the normal dystrophin 3′UTR that are responsible for regulating the stability of the dystrophin mRNA: these would be present in *Duchenne* boys, but lacking in the translocation females. This model would suggest that such sequences, if they exist, destabilize the mRNA in *Duchenne* boys with frameshift mutations. Girls with translocations would lack these sequences, and escape the destabilization mechanism.

While our data is consistent with mRNA degradation as the mechanism for loss of truncated dystrophin proteins in male *Duchenne* patients, this should be confirmed by study of the sequences attached to the dystrophin mRNA in our female translocation patients downstream of the translocation breakpoint. If our hypothesis is correct, then the non-translated region 3′ of the translocation should be quite short in sequence length. We attempted to amplify the unique sequence using oligo-dT as the reverse primer, however, this was unsuccessful. Northern blot experiments would also resolve this question, however, the small amount of muscle biopsy tissue does not permit this type of analysis.

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