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ORIGINAL ARTICLE

Deletion of exons 3–9 encompassing a mutational hot spot in the *DMD* gene presents an asymptomatic phenotype, indicating a target region for multiexon skipping therapy

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Few cases of dystrophinopathy show an asymptomatic phenotype with mutations in the 5' (exons 3–7) hot spot in the *Duchenne muscular dystrophy* (*DMD*) gene. Our patient showed increased serum creatine kinase levels at 12 years of age. A muscle biopsy at 15 years of age led to a diagnosis of Becker muscular dystrophy. The patient showed a slight decrease in cardiac function at the age of 21 years and was administered a β-blocker, but there was no muscle involvement even at the age of 27 years. A deletion of exons 3–9 encompassing a mutational hot spot in the *DMD* gene was detected, and dystrophin protein expression was ~15% that of control level. We propose that in-frame deletion of exons 3–9 may produce a functional protein, and that multiexon skipping therapy targeting these exons may be feasible for severe dystrophic patients with a mutation in the 5′ hot spot of the *DMD* gene.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is a lethal X-linked disorder caused by loss of the *DMD* gene that encodes the sarcolemmal protein dystrophin.[1] In cases where the open reading frame is disrupted by a mutation (that is, out-of-frame mutation), dystrophin is not expressed, resulting in a severe phenotype, DMD. However, when the open reading frame is preserved despite the presence of a mutation (that is, in-frame mutation), a truncated but still functional dystrophin is expressed, leading to the more benign phenotype, Becker muscular dystrophy (BMD). To date, 96% of DMD and 92% of BMD are explained by the so-called reading frame theory.[2] In addition, 5′ (exons 3–7) and 3′ (exons 45–55) mutational hot spots have been detected in the *DMD* gene.[2,3]

Recently, an exon skipping strategy using antisense oligonucleotides such as phosphorodiamidate morpholino oligomers (PMOs) or 2O-methylated antisense oligonucleotides was found to restore dystrophin expression and, consequently, skeletal muscle function.[4–6] In this approach, the restoration of the *DMD* open reading frame results in a conversion of dystrophinopathy from a severe to a milder form. We previously reported that three unrelated patients with a deletion of exons 45–55 covering one of the hot spots showed very mild skeletal muscle involvement and could ambulate late in life.[7] Another study described 15 patients with the same deletion mutation who exhibited a very mild or asymptomatic phenotype.[8] These observations suggest that multiexon skipping targeting exons 45–55 may allow treatment of not only DMD but also severe BMD cases with deletions in this region.[8,9] We recently treated *mdx*52 mice—which lacked exon 52 of the murine *DMD* gene and presented dystrophic changes similar to conventional *mdx* mice—by multiexon 45–55 skipping therapy via intravenous injection of mixtures of 10 vivo-PMOs, and demonstrated that this approach could restore dystrophin expression at the sarcolemma and lead to recovery of muscle function.[9,10]

Here, we present a case of a 27-year-old male with deletion of exons 3–9 exhibiting an asymptomatic phenotype. Based on our report, we propose that multiexon skipping therapy targeting exons 3–9 may be feasible for DMD or severe BMD patients with a mutation in the 5′ hot spot.

PATIENTS AND METHODS

Case

The patient, now 27 years old, was born to healthy parents. His maternal great grandfather showed muscle atrophy and died of unknown cause at 50 years of

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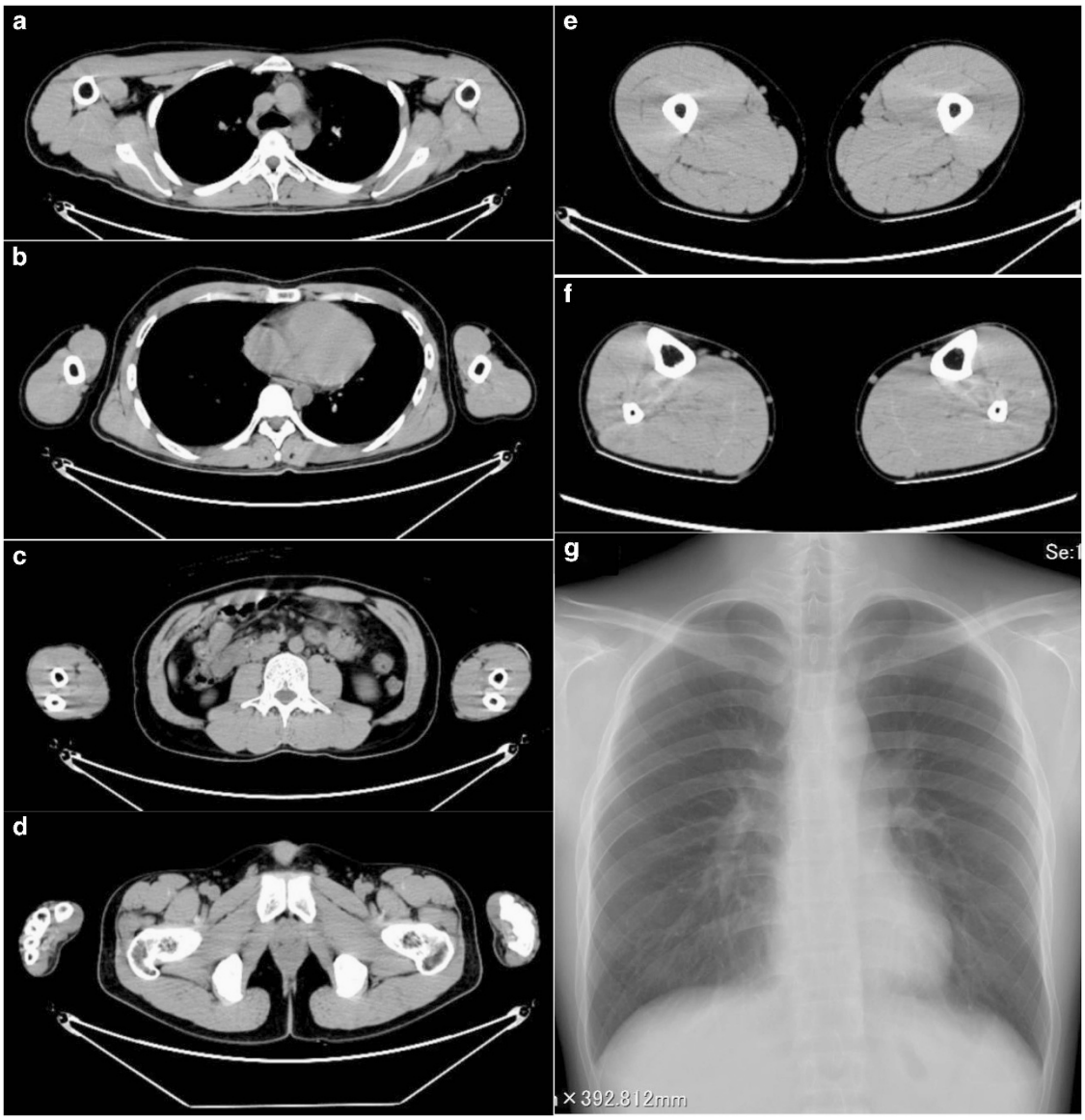

Figure 1 Whole body muscle computed tomography and chest X-ray of the patient. (**a**) Upper limb-girdle level. (**b**) Proximal upper limb level. (**c**) Distal upper limb and lumbar level. (**d**) Lower limb-girdle level. (**e**) Proximal lower limb level. (**f**) Distal lower limb level. None of the skeletal muscle levels were affected. (**g**) Chest X-ray of the patient; no abnormalities were observed.

age. The patient's developmental milestones during infancy were normal. At the age of 11 years, his school physical examination revealed high serum aspartate aminotransferase level and he was recommended to a nearby hospital for further examination; liver dysfunction was not excluded. At 12 years of age, he suffered from acute enterocolitis and blood examination revealed a high serum creatine kinase level of 1474 IU l^{-1} (normal range: 62–287 IU l^{-1}), although he did not display any muscular atrophy, weakness or developmental delay. At the age of 15 years, he underwent a muscle biopsy, and a histological analysis by hematoxylin–eosin staining revealed minor variations in fiber size but no necrotic or regenerating fibers, no cellular infiltration and no increase in fibrous tissue. Immunohistochemistry using antibodies against C-terminal dystrophin showed weakly positive reactions in the sarcolemma, and multiplex PCR revealed a deletion of exons 3–8. The patient was diagnosed as having BMD. During junior high and high school, he played baseball and excelled at sports. At 21 years of age, he did not show any cardiac symptoms nor did he become easily fatigued, but the echocardiogram revealed a slight decrease in the left ventricular ejection fraction (45%), and the patient was therefore administered the β-blocker carvedilol.

At 27 years of age, the physical examination revealed normal manual muscle test scores and there was no evidence of calf hypertrophy, and serum creatine kinase level (217 U l^{-1}) was within the normal range (47–272 U l^{-1}). Surprisingly, the whole skeletal muscle computed tomography scan revealed no muscle atrophy and no fatty infiltration (Figures 1a–f). Regarding cardiac involvement, the N-terminal pro brain-type natriuretic polypeptide level was 13.6 pg ml^{-1} (normal: $<$125.0), the chest X-ray was normal (Figure 1g) and the electrocardiogram showed typical sinus rhythm with no evidence of a narrow and deep Q wave in limb leads or increase in R/S ratio in the right precordial leads (V_{1-2}) that are frequently observed in dystrophinopathies.[11] The echocardiogram showed no chamber size dilation or thinning or hypokinesis of left ventricular wall motion; left ventricular ejection fraction and fractional shortening were 55% and 31.96%, respectively, indicating a favorable response to carvedilol treatment (30 mg per day). Overall, the patient was a well-adjusted member of society. Thus, we have molecular biologically and pathologically reevaluated in this patient.

Methods

After obtaining informed consent, genomic DNA was extracted from white blood cells using the Gentra Puregene Blood Kit (Qiagen, Valencia, CA, USA). Molecular analysis of intragenic deletions and duplications in the *DMD* was performed by multiplex ligation-dependent probe amplification analysis (MLPA) for DMD using SALSA MLPA KIT P034/035 DMD /Becker (MRC-Holland, Amsterdam, The Netherlands). Total mRNA was extracted from the frozen skeletal muscle biopsied previously and complementary DNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Reverse transcription-PCR was performed using intra-exonic primers for *DMD* exons 2 and 10, respectively; designed by Leiden Muscular Dystrophy data pages (http://www.DMD.nl/), and followed by direct sequencing analysis. Total protein was also extracted from the frozen sample and western blot analysis of dystrophin was conducted by a standard procedure using anti NCL-DYS2 (Leica Biosystems, Newcastle, UK). Furthermore, we performed the immunohistochemistry of previously biopsied skeletal muscles by standard procedures using anti-C-terminal dystrophin antibody NCL-DYS2 (Leica Biosystems) and anti-α-sarcoglycan antibody NCL-a-SG (Leica Biosystems).

RESULTS

MLPA revealed a deletion spanning exons 3–9 of the *DMD* gene (Figure 2a) that was also confirmed by the direct sequencing of amplified complementary DNA derived from skeletal muscle tissue (Figure 2b). Western blot analysis of dystrophin revealed that the patient's dystrophin level was expressed as ~15% of that in the control (Figure 2c). The histopathological analysis by hematoxylin–eosin staining revealed minor variations in fiber size but no necrotic or regenerating fibers, no cellular infiltration and no increase in fibrous tissue (Figure 3a). Immunohistochemistry of dystrophin and α-sarcoglycan showed weakly positive reactions of the sarcolemma (Figure 3b). Taking these results together with his clinical data, we

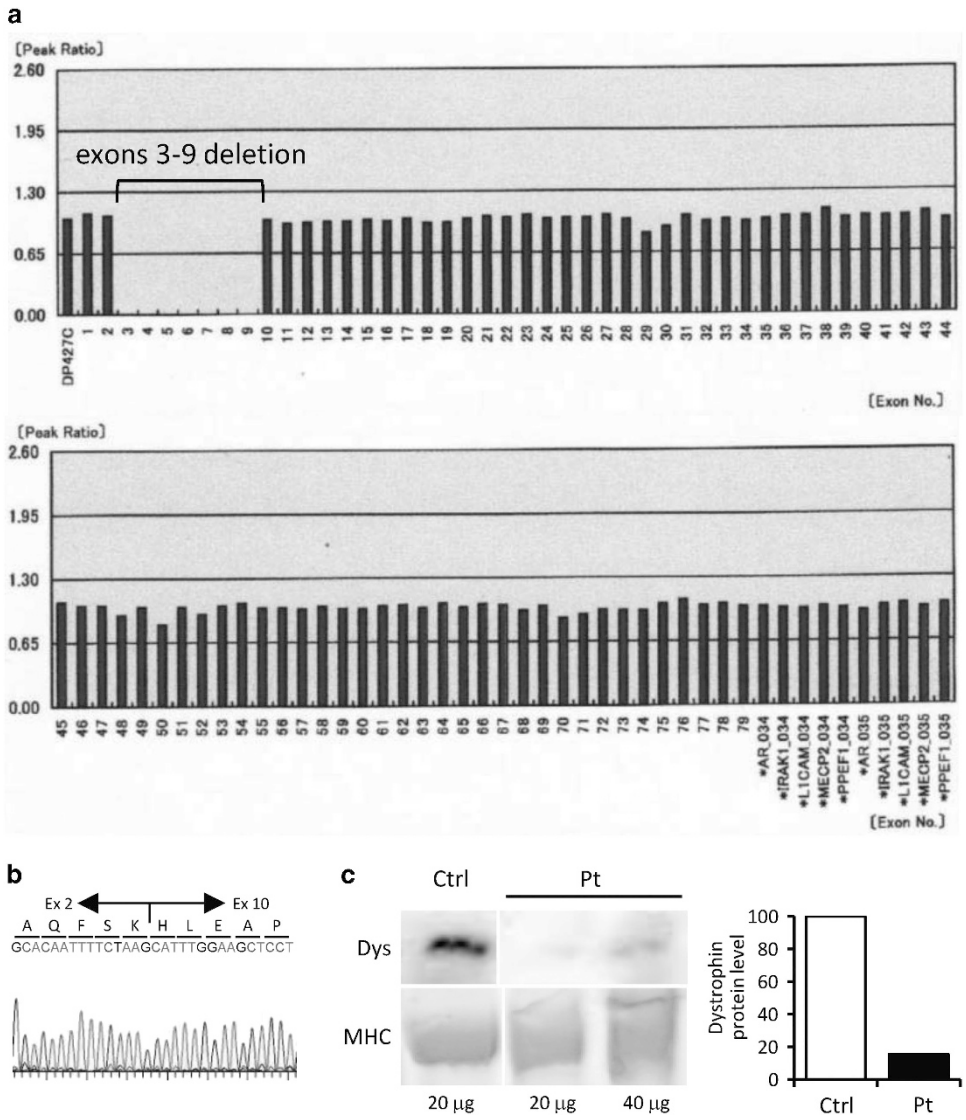

Figure 2 Molecular biological analyses of the patient. (**a**) Multiplex ligation-dependent probe amplification analysis of the *DMD* gene using the patient's genomic DNA revealed the absence of exons 3–9. (**b**) Direct sequencing of patient muscle complementary DNA (cDNA). The nucleotide sequence of exon 10 directly followed exon 2. Amino acid sequence information is shown with the nucleotide sequence. (**c**) Western blotting of dystrophin (NCL-Dys2; upper panels) and Coomassie Brilliant Blue staining of myosin heavy chain (MHC; lower panels) in the control (Ctrl) and our patient (Pt). Semiquantitative analysis of dystrophin level relative to that of MHC. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

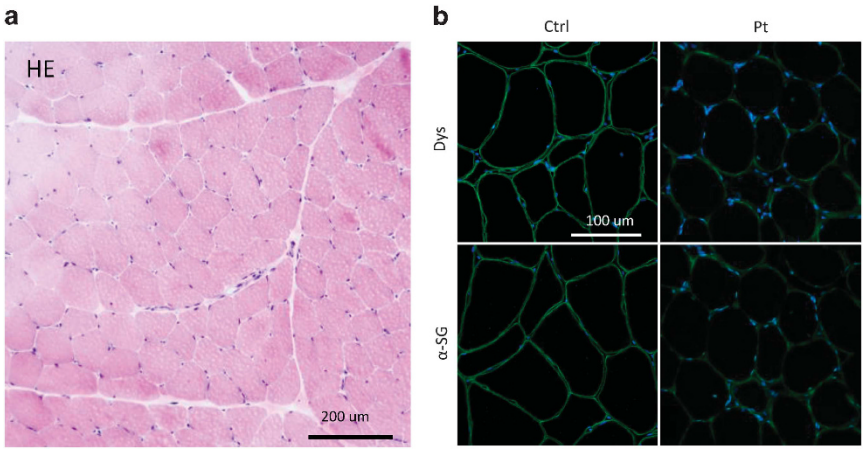

Figure 3 Muscle pathological analyses of the patient. (**a**) Hematoxylin–eosin (HE) staining of skeletal muscle tissue from the patient. Bar, 200 μm. (**b**) Immunohistochemistry of dystrophin (NCL-Dys 2; upper panels) and α-sarcoglycan (NCL-α-SG; lower panels) expression in a control (Ctrl; neurogenic muscular atrophy) and our patient (Pt). Bar, 100 μm.

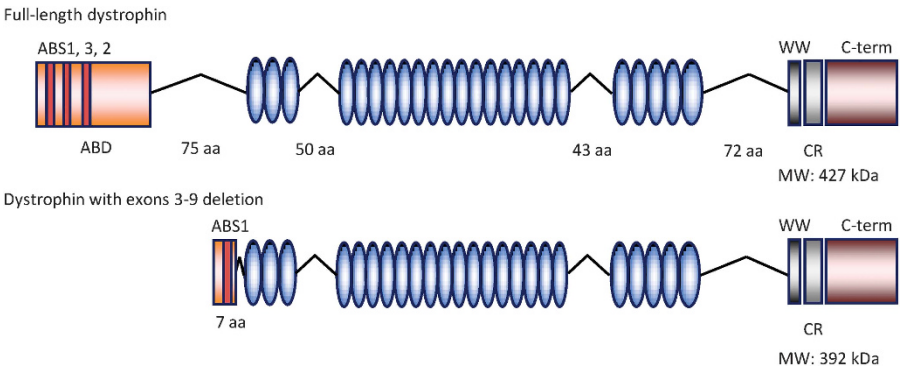

Figure 4 Schematic of full-length dystrophin (upper) and truncated dystrophin with exon 3–9 deletion (lower). ABD, actin-binding domain; ABS, actin-binding site; CR, cysteine-rich domain; MW, molecular weight; WW, WW domain. The truncated protein lacks ABS2 and ABS3 and part of the hinge 1 region.

found that the patient is asymptomatic for dystrophinopathy despite having the *DMD* deletion of exons 3–9.

DISCUSSION

The present report describes a patient with asymptomatic dystrophinopathy with deletion of exons 3–9 in the *DMD* gene. In the Leiden Open Variation Database (LOVD), Leiden Muscular Dystrophy Pages, Duchenne Muscular Dystrophy (http://www.dmd.nl/nmdb/home.php?select_db=DMD), 12 patients with a deletion of exons 3–9 were registered as having high creatine kinase ($n=3$), BMD ($n=5$), BMD/DMD ($n=2$), intermediate type ($n=1$) and DMD ($n=1$), with the exception of two DMD patients with registered errors that we confirmed. Furthermore, in the universal mutation database UMD-DMD (http://www.umd.be/DMD/W_DMD/index.html), three BMD patients with a deletion of exons 3–9 were registered (two were included in LOVD). In addition to these patients, our present case and another reported case[12] make a total of 15 patients who have a deletion of exons 3–9. Of these, 11 patients showed the BMD phenotype or were asymptomatic with high creatine kinase; however, detailed clinical descriptions were shown only in our case and in another case previously reported.[12] The other patient was a competitive badminton player until his early 60s, and was symptomatic until his mid-60s. At the age of 67 years, he became aware of progressive weakness and wasting of his lower limb muscles without intellectual and heart impairment, and was subsequently diagnosed with BMD by muscle pathology and molecular genetic analyses.

It has been proposed that the presence of alternative transcripts in which the original mutation had been modified, or a milder phenotype, may be evidence of actin-binding potential in a different region of the dystrophin molecule.[12] There are three actin-binding sites (ABS) in the N-terminal domain (ABS1–3) (Figure 4), and in our patient, dystrophin with an exon 3–9 deletion lacked ABS2 and ABS3 but not ABS1. There are some reports that ABS1 is critical for the actin-binding ability of dystrophin,[13–15] and transgenic *mdx* mice—a DMD model—expressing dystrophin lacking ABS2 and ABS3 display a mild Becker phenotype.[16] Therefore, retention of ABS1 may be important for maintaining dystrophin function in skeletal muscle.

In our patient, dystrophin expression was ~15% of the level seen in normal controls. A level of at least 30% is required to avoid muscular dystrophy in cases in which there are mutations in the 5' region of the *DMD* gene.[17] However, one study reported that asymptomatic individuals had dystrophin levels >40% with mutations in the rod domain.[18] The discrepancy in these findings may be explained by the mutational regions. For instance, in- or out-of-frame mutations in the 5' actin-binding domain lead to low levels of dystrophin expression (10–20%) and are associated with more severe BMD phenotypes.[19,20] The dystrophin protein level in our patient was comparable to that reported for severe BMD with a mutation in the 5' actin-binding

domain, implying that in-frame deletion of exons 3–9 may produce a protein that is structurally stable, although in low quantities. In other words, the expression level of 15% may be sufficient to maintain muscle integrity and avoid damage if the protein is functional. On the other hand, it should be considered that some modifier genes or potential epigenetic factors might affect the severity of the asymptomatic dystrophinopathies.[21]

Our patient had no cardiac symptoms at 21 years of age, but showed a slight decrease in left ventricular ejection fraction by echocardiography. Cardiac dysfunction is associated with mutations in the 3' hot spot that may cause a structural defect in the dystrophin protein, such as loss of the spectrin-like domain. However, this may not apply to our patient, as exons 3–9 do not encode the rod domain. We speculate that ABS2 and/or ABS3 are important for maintaining the mechanical functioning of cardiac muscle. However, our patient showed no cardiac symptoms even at 27 years of age, and his cardiac function was stable under daily administration of carvedilol, indicating that regular echocardiographic evaluation and early administration of a β-blocker can prevent the progression of cardiac symptoms.

Our findings demonstrate that dystrophinopathy with deletion of exons 3–9 of the *DMD* gene may show an asymptomatic phenotype even at later stages, although there may be slight cardiac involvement. This finding gives rise to another important suggestion, as this deletion completely covers the hot spot in exons 3–7. This region has been considered a therapeutic target by exon skipping in the cells carrying an exon 2 duplication, and this study showed that multiexon skipping of exons 2–7 to generate a BMD-like dystrophin transcript could only be induced efficiently with PMOs.[22] If multiexon skipping of exons 3–9 was possible, this could potentially convert ~7% of DMD patients with mutations in the 5' hot spot based on the UMD-DMD database[23] to an asymptomatic phenotype. Dystrophic dogs could be used to test the efficacy of this multiexon skipping strategy in preclinical studies. Golden retriever muscular dystrophy[24,25] and beagle-based canine X-linked muscular dystrophy in Japan ($CXMD_J$)[26] models show more severe muscle involvement, similar to DMD, than mouse models. Dystrophic dogs have a point mutation at the intron 6 splice acceptor site in the canine *DMD* gene, resulting in the skipping of exon 7 and a premature stop codon in exon 8 that prevents dystrophin from being expressed. Along with our collaborators, we reported that three PMOs targeting exons 6 and 8 that convert an out-of-frame into an in-frame mutation can restore dystrophin expression in whole body skeletal muscle, thereby improving muscle pathology and function in $CXMD_J$.[27] A preclinical study for exon 3 − 9 skipping therapy will be feasible in the dystrophic dogs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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