exons (Fig. 1e), suggesting that higher regulatory mechanisms as well as the known *cis*-control elements regulated splicing. We were unable to develop a strict set of rules to explain the observed exon skipping events.

The induction of the ribosomal read-through of nonsense mutations by gentamicin and other drugs has recently been reported as a novel method to drive dystrophin expression in DMD patients carrying nonsense mutations in the associated gene (Mankin & Liebman, 1999). Our results show that alternative rescue transcripts arising from secondary splicing alterations may be present in patients with nonsense mutations. As these transcripts may have semi-functional expression, it is important to carry out pre-analysis of dystrophin mRNA before treatment with drugs that induce ribosomal read-throughs in order to obtain an accurate assessment of the effects of the treatment.

The transformation of out-of-frame mRNA transcripts into in-frame messages through exon skipping leads to the production of semi-functional internally deleted dystrophin protein. Such a technique is a promising approach to the treatment of DMD. Individual differences in splicing despite identical mutations (Table 2) suggest that this type of treatment may have unpredictable outcomes. The therapy, however, could be effective nonetheless. In a previous report, we induced the skipping of exon 19 in a DMD patient carrying a deletion in exon 20 using antisense oligonucleotides against an splicing enhancer sequence in exon 19; this treatment lead to the production of an in-frame dystrophin mRNA and detection of dystrophin-positive skeletal muscle cells (Takeshima et al., 2006). The optimal target exon sequences for this type of therapy, however, remain unknown. Our current results suggest that mutation sites that induce exon skipping may be candidate target sites for antisense oligonucleotide treatment of patients with DMD (Surono et al., 2004).

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Letter to the Editor

Wide ranges of serum myostatin concentrations in Duchenne muscular dystrophy patients

Dear Editor,

Duchenne muscular dystrophy (DMD), a common inherited myopathy that affects approximately 1 in 3500 males, is characterized by progressive muscle wasting due to a deficiency in muscle dystrophin. DMD progresses with a rather uniform pattern of muscle weakness; i.e., DMD causes affected individuals to lose their ability to walk by the age of 12 y, and patients succumb during their twenties due to either respiratory or cardiac failure. The deficiency in dystrophin is caused by translational reading frame shift or nonsense mutations in the dystrophin gene [1]. However, the existence of a modifying gene has been suggested by the identification of unusually mild DMD phenotypes [2–4].

Although some phenotypic variability may arise due to environmental factors, such as diet or exercise, genetic components are likely to contribute to this variability. Myostatin, also known as growth and differentiation factor 8 (GDF8), is a muscle-specific secreted peptide that limits muscle growth [5,6]. However, genotyping of the myostatin gene failed to disclose any nucleotide changes that behaved as a phenotypic modifier of DMD [7]. Remarkably, blocking endogenous myostatin has been shown to result in anatomic, biochemical, and physiologic improvements in the dystrophic phenotype of *mdx* mice, a mouse model for DMD, including particularly prominent enlarged fiber diameters and greatly reduced fatty fibrosis [8–10]. These results suggest that blocking endogenous myostatin is a potential strategy to treat DMD [11]. We examined the hypothesis that serum myostatin is increased in DMD, thereby enabling treatment by myostatin blockage.

Forty-one DMD patients followed at Kobe University Hospital were enrolled in this study. All but 1 of the mutations in the dystrophin gene were found to introduce premature stop codons in the dystrophin mRNA: 24 cases with mutations that induced a translational reading frame-shift due to exon deletion or duplication; 5 cases with nonsense mutations; 7 cases with exon mutations involving one or a few nucleotides deleted or inserted; 3 cases with intron mutations that induced splicing errors; and one case with an abnormal chromosome (Table 1). The subjects' ages ranged from 1 to 22 y (average: 8.3 y). Regular clinical checkups, including determination of serum creatine kinase (CK) concentrations, were performed at the outpatient clinic. All protocols were approved by the ethics committee of the Kobe University School of Medicine. Blood samples were taken after written informed consent was obtained from all patients, and serum was separated using a clinical centrifuge.

Serum myostatin was measured using the Human Myostatin ELISA (Prodomain Specific) kit purchased from BioVendor Laboratory Medicine, Inc. (Bmo, Czech Republic). The upper limit of determination was 50 ng/ml, and normal adults have serum concentrations of 0.19 to 9.02 ng/ml (BioVendor Laboratory Medicine, Inc.). The Pearson product-moment coefficient was calculated to quantify the relationship.

Serum myostatin concentrations in DMD patients ranged from 1.1 to >50 ng/dl (Table 1). Remarkably, 13 samples were >50 ng/ml, and the lowest concentration was 1.1 ng/ml (Table 1). Though age differences were examined in 2 conditions either including or deleting 13 samples with >50 ng/ml, no significant correlationship between age and serum myostatin concentration was found (Fig. 1). We next examined whether concentrations of myostatin were related to the type or location of mutations in the dystrophin gene. Though serum myostatin concentrations were compared based on their mutation types (exon deletion/duplication or others), no clear difference between two groups was revealed (Fig. 1). There found no significant difference in serum myostatin concentration between patients with mutation in the 5′ and 3′ regions of the dystrophin gene (Table 1).

Considering that myostatin is an inhibitor of muscle growth, cases with high serum myostatin concentrations were predicted to present rather severe phenotypes. Transgenic overexpression of myostatin in mice was shown to result in cachexia [12]. However, the ages when DMD patients with high myostatin concentrations became wheelchair-bound were not different from those of patients with low myostatin concentrations, and signs of muscle weakness appeared mostly between ages 4 and 5. Furthermore, serum CK concentrations were not significantly lower in DMD cases with high myostatin concentrations than in those with low concentrations (Table 1). This indicates that serum myostatin did not modify the DMD phenotype even though blocking endogenous myostatin has been shown to result in improvements in the dystrophic phenotype of *mdx* mice [8–10]. In this study, we measured myostatin that reacted with a monoclonal antibody recognizing the prodomain of myostatin. Considering that myostatin is secreted as an inactive propeptide and is cleaved to produce the active form, further studies would be required to measure active or latent myostatin individually.

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Expression of the myostatin gene has been examined previously in skeletal muscle by measuring mRNA and protein concentrations [13]. Serum myostatin concentration has been determined by the Western blot analysis in a patient with a mutation in the myostatin gene, disclosing an absence of myostatin [14]. Furthermore the Western blot analysis disclosed that serum myostatin concentration was lower than that of rat [14]. But no further study has been conducted on serum myostatin concentrations. Our results disclosed a wide range in serum myostatin concentrations in DMD patients. Though myostatin blockage is attracting attention as a novel target for increasing muscle growth in cases of DMD [11], our results suggest that myostatin blockage therapy would only be effective in DMD cases involving high serum myostatin concentrations. Therefore, myostatin blockage therapy should be applied carefully as a treatment for DMD.

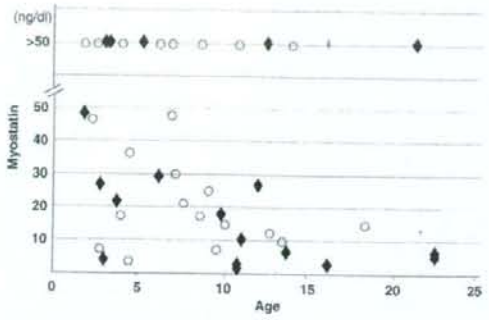

Fig. 1. Serum myostatin concentrations and patients' ages. Serum concentrations of myostatin (vertical axis) are plotted versus patient age (horizontal axis). Open circles and black diamonds represent exon deletion/duplication and other mutations, respectively.

Table 1
List of DMD patients

Case	Age	Mutation	CK (IU/L)	Myostatin (ng/ml)
749	21	ex45–50del	603	>50
643	14	ex57G8460A	3296	>50
765	13	ex51del	2219	>50
225	11	ex41C5899T	2848	>50
300	8	ex2dup	11,560	>50
761	7	ex63 c.9262delA	10,300	>50
344	6	ex46–49dup	20,008	>50
394	5	ex53–54del	22,680	>50
579	4	ex27 3613delG	27,160	>50
767	3	ex51del	10,492	>50
764	2	ex36 5071–2insA	25,040	>50
715	2	c.8669–1G>C	32,700	>50
581	2	ex46–51del	26,488	>50
755	1	ex46–52del	18,640	49.2
434	6	ex25 AGAA3347–50del	20,527	47.8
651	2	ex41C5899T	9860	46.7
736	4	ex2–7dup	8259	35.8
294	6	int17 2168G+1C	27,718	30.2
502	6	ex17del	15,500	30
763	2	ex44del	27,647	28.2
112	12	ex48–50del	2221	25.9
343	8	ex46–49dup	6119	24
570	3	ex46–53del	6389	22.5
481	6	46Y,inv(X)(p21.2 q28)	12,635	20.4
427	10	ex46–48del	8954	18.5
536	7	?	11,760	17.9
414	4	ex38 5434–7delTTCA	22,004	16.7
145	18	ex56dup	1209	15.4
401	10	ex70 C10171T	2528	14.1
13	12	ex2dup	3828	11.6
67	11	ex45–52del	2804	9.9
58	13	ex5 G354A	4669	9.8
453	22	ex10–44del	2899	7
453	22	ex10–44del	3345	6.6
436	9	ex39 T5561del	9160	6.6
641	2	int4+3insGT	16,165	6.3
788	13	ex46–52del	1726	6.2
759	4	ex45–48dup	13,402	3.4
689	2	ex8–24del	25,920	3.2
H.H.	16	ex48–50del	1436	2.3
701	10	int45 6641+1G>A	7121	1.9
107	10	ex51–54del	4497	1.1

DMD patients are listed according to their serum myostatin concentrations. Detail mutation of the dystrophin gene is described in addition to their age and serum CK concentration. One question mark indicates an unrevealed mutation (case 536) and one has an abnormal chromosome (case 481).

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