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TECHNICAL NOTE

A sandwich ELISA kit reveals marked elevation of titin N-terminal fragment levels in the urine of *mdx* mice

Taku Shirakawa[1,2] | Ayumu Ikushima[3] | Nobuhiro Maruyama[4] | Yoshinori Nambu[5] | Hiroyuki Awano[5] | Kayo Osawa[6] | Kei Nirasawa[7] | Yoichi Negishi[7] | Hisahide Nishio[1] | Shoji Fukushima[3] | Masafumi Matsuo[1,2]

[1]Research Center for Locomotion Biology, Kobe Gakuin University, Kobe, Japan

[2]KNC Department of Nucleic Acid Drug Discovery, Faculty of Rehabilitation, Kobe Gakuin University, Kobe, Japan

[3]Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Kobe, Japan

[4]Diagnostic & Research Reagents Division, Immuno-Biological Laboratories Co., Ltd, Fujioka, Japan

[5]Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan

[6]Department of Medical Technology, Faculty of Health Sciences, Kobe Tokiwa University, Kobe, Japan

[7]Department of Drug Delivery and Molecular Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan

Correspondence
Masafumi Matsuo, Research Center for Locomotion Biology, Kobe Gakuin University, 518 Arise, Ikawadani, Nishi, Kobe 651-2180, Japan.
Email: matsuo@kobe-u.ac.jp

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Abstract

The *mdx* mouse is a model of Duchenne muscular dystrophy (DMD), a fatal progressive muscle wasting disease caused by dystrophin deficiency, and is used most widely in preclinical studies. Mice with dystrophin deficiency, however, show milder muscle strength phenotypes than humans. In human, the introduction of a sandwich enzyme-linked immunosorbent assay (ELISA) kit revealed a more than 700-fold increase in titin N-terminal fragment levels in the urine of pediatric patients with DMD. Notably, the urinary titin level declines with aging, reflecting progression of muscle wasting. In mouse, development of a highly sensitive ELISA kit has been awaited. Here, a sandwich ELISA kit to measure titin N-terminal fragment levels in mouse urine was developed. The developed kit showed good linearity, recovery, and repeatability in measuring recombinant or natural mouse titin N-terminal fragment levels. The titin N-terminal fragment concentration in the urine of *mdx* mice was more than 500-fold higher than that of normal mice. Urinary titin was further analyzed by extending the collection of urine samples to both young (3–11 weeks old) and aged (56–58 weeks old) *mdx* mice. The concentration in the young group was significantly higher than that in the aged group. It was concluded that muscle protein breakdown is active and persistent in *mdx* mice even though the muscle phenotype is mild. Our results provide an opportunity to develop DMD treatments that aim to alleviate muscle protein breakdown by monitoring urinary titin levels.

KEYWORDS
biomarker, Duchenne muscular dystrophy, ELISA, *mdx* mouse, titin, urine

1 | INTRODUCTION

Duchenne muscular dystrophy (DMD) is an inherited muscle disease, affecting approximately 1 in nearly 5000 live-born males.[1,2] DMD is caused by mutations in the *DMD* gene that result in dystrophin deficiency in skeletal muscle. DMD is characterized by progressive muscle wasting. Initial muscle weakness appears around the age of 4–6 years, and independent walking is lost by the age of 12 years. Most patients die before 30 years of age.

Although the life expectancy of DMD patients is increasing due to benefits from multidisciplinary medical care,[3–5] the establishment of treatments for DMD has been a main subject of DMD research. For decades, most DMD research has used the *mdx* mouse as the best preclinical model, which is characterized by muscle dystrophin deficiency caused by a nonsense mutation in exon 23 of the *DMD* gene.[6] Even though dystrophin is deficient in skeletal muscle, adult *mdx* mice do not exhibit changes characteristic of human DMD. Namely, *mdx* mice do not show severe muscle weakness, weight loss, and accumulation of fat and fibrosis in skeletal muscle until almost 2 years of age.[7] Additionally, *mdx* mice have a nearly normal lifespan. These results indicate pathophysiological differences between humans and model mice. The phenotypic difference suggests that milder protein degradation occurs in *mdx* mice than in humans.

Titin is the third most abundant protein after actin and myosin and interconnects actin and myosin in sarcomeres of skeletal muscle. It is the largest protein, weighing nearly 2900 kDa, and acts as a molecular spring during the relaxation-contraction cycle.[8] The clinical significance of titin is largely unknown, except for titinopathy, which is caused by mutations in the *TTN* gene.[9,10] Recently, proteomics analysis revealed a series of degraded titin fragments in the urine of patients with DMD, and a western blot assay revealed both titin N- and C-terminal fragments in the urine using antibodies against the respective regions.[11] This is compatible with the finding that breakdown products of titin were identified in skeletal muscles of DMD patients by western blot assay.[12] Thus, titin breakdown is a part of muscle wasting in DMD. In the urine of *mdx* mice as well as humans, western blot assays revealed titin N- and C-terminal fragments.[11]

We introduced a sandwich enzyme-linked immunosorbent assay (ELISA) kit to measure human titin N-terminal fragment levels in urine.[13] Using this kit, we measured urinary titin levels in DMD and showed that urinary titin levels in children with DMD were more than 700-fold higher than those in normal children.[14] Consistent with our findings, Robertson et al. also developed an ELISA system and reported strongly elevated titin N-terminal fragment levels in urine samples obtained from DMD patients.[15] Notably, we have disclosed that the urinary titin levels of DMD patients declined with aging.[14] This decline matched with the progression of muscle wasting. As a result, the urinary titin N-terminal fragment level was concluded to be a good biomarker of DMD.

To investigate muscle protein breakdown in *mdx* mice, it is necessary to establish a highly sensitive ELISA system enabling the determination of titin N-terminal fragment levels in both normal and *mdx* mice. Here, a sandwich ELISA system was developed for the quantification of titin N-terminal fragment levels in mouse urine. Using this system, the urinary titin level was first quantified in normal mice, and a remarkable elevation of urinary titin levels was revealed in *mdx* mice. Notably, the elevation of urinary titin levels persisted over 56 weeks of age. Measurement of urinary titin levels may facilitate the development of DMD treatments to alleviate muscle protein degradation using *mdx* mice.

2 | MATERIALS AND METHODS

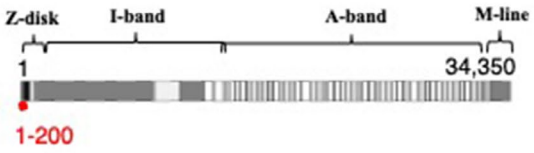
2.1 | Animals and urine collection

Pairs of C57BL/10ScSn-Dmd^{mdx}/Jic mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) through CLEA Japan, Inc. (Tokyo, Japan) and further bred in the vivarium at both the School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, and the Faculty of Pharmaceutical Science, Kobe Gakuin University. C57BL/10 (wild type) mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). All animals were housed in a temperature-regulated facility in individually ventilated and pathogen-free cages in rooms with a 12 h/12 h light/dark cycle, and supplemented ad libitum with food and water according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the Guideline of Experimental Animal Care issued by the Prime Minister's Office of Japan. The protocol of the present study was approved by the Committee of Animal Use and Welfare of Tokyo University of Pharmacy and Life Sciences (P20-55), and the Faculty of Pharmaceutical Science, Kobe Gakuin University (A20-14). Efforts were made to minimize the burden and distress of the animals. For mouse urine sample collection, mice were held over a hydrophobic surface to induce urination that could be acquired as droplets as described previously.[15] The samples were decanted into a plastic tube with no additives and immediately cooled on ice. After that, the samples were stored at −20°C until analyzed. All samples were tested in duplicate in a blinded manner.

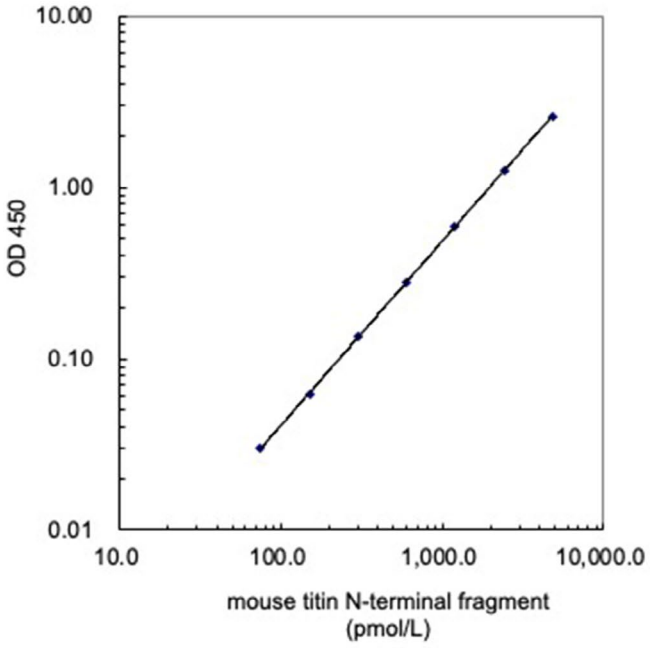
2.2 | Mouse ELISA kit

The mouse Titin N-Fragment Assay Kit-IBL is an ELISA system to measure mouse urinary titin N-terminal fragment levels that was developed by the Immuno-Biological Laboratories Co. Ltd. (Fujioka, Japan). This sandwich ELISA is composed of 2 antibodies to the human titin N-terminal fragment (Figure 1A), 144A2 and 151A1. 151A1 is used as a capture antibody, and horseradish peroxidase (HRP)-conjugated 144A2 Fab′ is used as a detection antibody. These antibodies have higher affinity for the mouse titin N-terminal fragment than those used in the human Titin N-Fragment Assay Kit-IBL (Immuno-Biological Laboratories Co. Ltd.).[13]

(A)



(B) Linearity test



(C) Intra-Assay precision

QC	Measured Values (pmol/L)	SD	CV (%)	n
H	2072.7	54.1	2.6	24
M	629.9	24.9	3.9	24
L	260.2	15.5	3.4	24

(D) Inter-assay precision

QC	Measured Values (pmol/L)	SD	CV (%)	n
H	2110.2	88.6	4.2	7
M	623.0	38.7	6.2	7
L	268.8	14.1	5.3	7

(E) Recovery test

Sample Value (pmol/L)	Additive Value (pmol/L)	Theoretical Value (pmol/L)	Measured Values (pmol/L)	Recovery (%)
1162.6	2400	3525.1	3422.6	96.1
	600	1725.1	1629.1	92.4
	150	1275.1	1262.3	96.2

FIGURE 1 Validation of the mouse titin N-terminal fragment assay kit using recombinant titin fragment. A, Location of an immunogenic fragment in human titin. Human titin consists of 34 350 amino residues and 4 domains (Z-disk, I-band, A-band and M-line). A fragment consisting of 1–200 amino residues was used for immunization to produce antibody (red bar). B, Standard curve for calculating the mouse titin N-terminal fragment levels. The linearity range for the mouse titin N-terminal fragment is 75–4800 (pmol/L). C, Results of the intra-assay precision study. The results were obtained from analysis of 24 replicates of each QC sample. D, Results of the inter-assay precision study. The results were derived from analysis of 7 replicates of each QC sample. E, Results of the recovery test

2.3 | Standard of the mouse titin N-terminal fragment

The mouse titin N-terminal fragment 1–200 amino acid expression vector, which is FLAG-tagged at the N-terminus in pcDNA3.1(+), was transfected into COS-1 cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA). The concentration of mouse titin N-terminal fragment in the conditioned medium was determined by quantification using human Titin N-Fragment[13] as the indicator. The conditioned medium was used as the standard protein.

2.4 | Validation of the kit

To assess the intra- and inter-assay precision of the mouse ELISA kit, 3 quality controls (QCs) of the standard (high, mid, and low) were prepared and evaluated. For validation of the kit to measure natural titin N-terminal fragment levels, stock solutions of *mdx* mouse urine were prepared using the appropriate assay sample diluents.

2.5 | Determination of creatinine levels

Urinary creatinine (Cr) concentrations were measured using an assay kit (LabAssay Creatinine, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.6 | Statistical analysis

The Shapiro-Wilk normality test was used to determine whether a dataset was normally distributed. When the p value was <.05, the dataset was deemed to have a non-normal distribution. Findings in the 2 groups were compared using Student's t-tests when the data were distributed normally; otherwise, Mann-Whitney U tests were used. Findings in 3 or more groups were compared by one-way analysis of variance (ANOVA) for normally distributed data; otherwise, Kruskal-Wallis tests were used. Values of $p < .05$ were considered statistically significant in all analyses. All statistical analyses were

performed using GraphPad PRISM 9.1 (GraphPad Software, San Diego, USA).

2.7 | Ethics

Animal experimentation was conducted in accordance with the Japanese guidelines. All animal care and experimental procedures were approved by Tokyo University of Pharmacy and Life Sciences (P20-55) and by the Animal Experimentation Ethics Committee of Kobe Gakuin University (A20-14).

3 | RESULTS

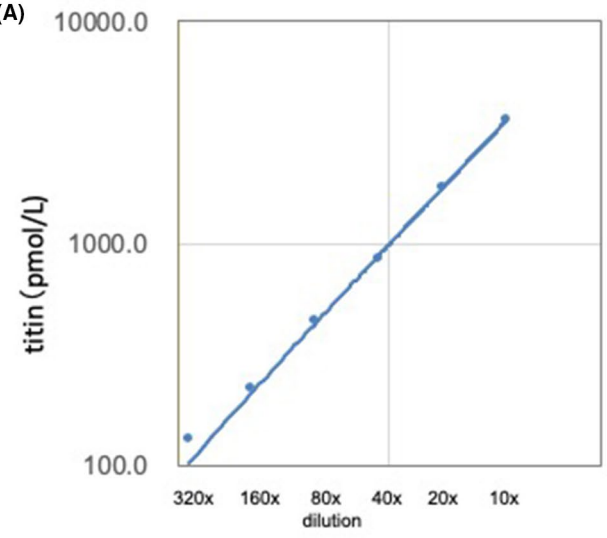
3.1 | Validation of the mouse ELISA kit using the mouse titin standard

The mouse Titin N-Fragment Assay Kit-IBL, a sandwich ELISA kit to measure mouse titin N-terminal fragment levels in urine, was validated using the standard mouse titin N-terminal fragment solution. A dose-response curve for the standard mouse titin N-terminal fragment solution was constructed (Figure 1B). The curve showed excellent linearity ($R^2 = 1$) when plotted on a log/log scale over a range of 75–4800 pmol/L. The precision of the assay was determined using 3 spiked QC controls (high [H], middle [M], and low [L]). The intra-assay precision exhibited coefficients of variation (CVs) of 2.6%, 3.9%, and 3.4% for H, M, and L, respectively (Figure 1C). Additionally, the inter-assay results for the CV were 4.2%, 6.2%, and 5.3% for H, M and L, respectively (Figure 1D). Furthermore, in the recovery validation test, the recovery rates were nearly 100% at a 5-fold dilution (Figure 1E).

3.2 | Validation of the mouse ELISA kit using *mdx* mouse urine

The performance of the mouse Titin N-Fragment Assay Kit-IBL was evaluated using a mixture of *mdx* mouse urine samples. To assess the influence of factors in the urine samples, a linearity test was performed. Urine samples were mixed to obtain enough volume for dose-response curve construction. The curve showed excellent

(A)



(B) Intra-assay precision

	Avr (pmol/L)	SD (pmol/L)	CV (%)	n
normal	4644.5	275.8	5.9	8
mdx	2175950.9	145201.7	6.7	8

(C) Inter-assay precision

	Avr (pmol/L)	SD (pmol/L)	CV (%)	n
normal	3817.5	364.6	9.6	7
mdx	2071348.5	247077.9	11.9	7

(D) Recovery test

	Control (pmol/L)	Additive (pmol/L)	Theoretical (pmol/L)	Result (pmol/L)	Recovery (%)
1	8926.9	8531.4	17458.3	18052.7	107.0
2		4265.7	13192.6	12813.1	91.1
3		2132.5	11059.4	10860.3	90.7
4		1066.3	9993.2	9881.5	89.5

FIGURE 2 Validation of the mouse titin N-terminal fragment assay kit using urine samples from *mdx* mice. A, The linearity of measured values for serial dilutions of the mixture of urine samples. A clear linear line was created by dilution of the mixed urine ($R^2 = 1$). The linearity range for the mouse titin N-terminal fragment is 112.5–3600 (pmol/L). B, Results of the intra-assay precision study. C, Results of the inter-assay precision study. The results were obtained on 4 separate months. D, Results of the recovery test

linearity ($R^2 = 1$) from a dilution ratio of 10-fold to 320-fold in *mdx* mouse urine (Figure 2A). The precision of the assay was determined using normal and *mdx* mouse urine. The intra-assay precision exhibited CVs of 5.9% and 6.7% in normal and *mdx* mice, respectively (Figure 2B). The inter-assay precision was determined by measuring the same sample at an interval of 4 months (Figure 2C). The obtained CVs were 9.6% and 11.9% in normal and *mdx* mice, respectively. From the standpoint of precision, the newly established ELISA system was reliable. For the recovery test, samples with different concentrations of titin were measured and the difference between the measured concentrations and the theoretical concentrations was calculated to estimate their recovery rates. The recovery rates were nearly 100% (Figure 2D).

3.3 | High urinary titin levels in *mdx* mice

To examine applicability for the measurement of titin N-terminal fragment levels in the urine of normal mice, 9 urine samples obtained from mice aged 5–8 weeks were analyzed by the developed kit. The titin concentrations were normalized by creatinine (Cr) contents because urine volume and components are affected by physiological conditions such as changes in water and food intake, dehydration, sweating, and physical actions. Thus, data were shown as titin/Cr ratio (pmol/mg Cr). The concentration ranged from 5.5 to 27.4 pmol/mg Cr, and the median (95% confidence interval [CI]) urinary titin concentration was 8.4 (6.8–14.6) pmol/mg Cr (mean ± standard deviation [SD]; 10.8 ± 6.8 pmol/mg Cr). The lowest concentration was in the range of linearity. Next, the concentration was measured in urine of *mdx* mice. In total, 7 urine samples were collected from *mdx* mice aged 5–8 weeks and analyzed for titin and Cr levels. The median (95% CI) was 4555.4 (3827.9–8059.7) pmol/mg Cr (range 3627.9–8059.7 pmol/mg Cr, and mean ± SD 5326.5 ± 1721.8 pmol/mg Cr). Compared with the normal urinary titin level, the urinary titin level of *mdx* mice was elevated more than 500-fold ($p < .001$) (Figure 3).

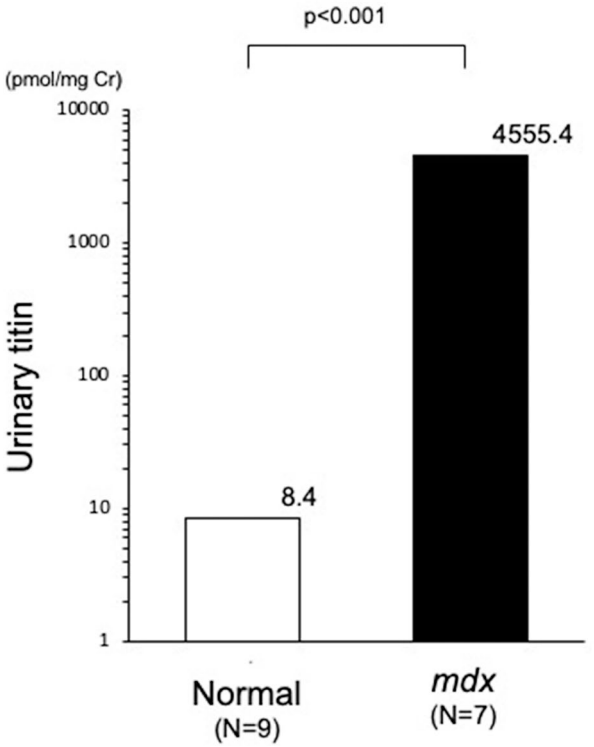


FIGURE 3 Urinary titin N-terminal fragment concentrations in the urine of mice. The concentrations of urinary titin were plotted on a logarithmic scale. The median in normal and *mdx* mice was 8.4 and 4555.4 pmol/mg Cr, respectively. There was a significant difference between normal and DMD mice ($p < .001$)

3.4 | Persistent and marked elevation of urinary titin levels in *mdx* mice

Urinary titin levels of *mdx* mice were further examined by extending the collection of urine samples to both young (3–11 weeks, $n = 104$) and aged (56–58 weeks, $n = 39$) *mdx* mice. From 3 to 11 weeks of age, titin levels ranged from 1014.1 to 18 696.8 pmol/mg Cr. From 56 to 58 weeks of age, the levels ranged from 729.7 to 15 632.4 pmol/mg Cr, indicating persistent hypertitinuria. Then, the levels of urinary titin were compared between the 2 groups (Figure 4A). There was a significant difference between the young and aged groups (4214.2 [3652.4–5211.8] versus 1278.5 [1133–1451.6], median [95% CI] pmol/mg Cr). A plot of median urinary titin for each age by week shows no significant week-by-week changes in either group (Figure 4B,C). In the young group, the highest value was observed at 7 weeks of age, showing a significant difference from the median observed at 3 weeks of age ($p < .05$). Considering that mice 50 weeks of age correspond to humans 40 years of age,[16] this suggested that DMD patients surviving more than 40 years should have hypertitinuria. However, our results from a human study showed that the urinary titin concentration reached a nearly normal level in aged patients.[14] Therefore, persistent hypertitinuria in aged *mdx* mice appears to be remarkably different from that in humans. It was concluded that muscle protein breakdown persists in *mdx* mice, even in the presence of a mild phenotype. This suggested that *mdx* mice maintain muscle volume despite active protein breakdown.

4 | DISCUSSION

The developed mouse Titin N-Fragment Assay kit-IBL showed good performance in the measurement of titin N-terminal fragment levels in mouse urine, enabling the determination of the fragment concentration in normal mice. Notably, the urinary titin N-terminal fragment level in *mdx* mice was more than 500-fold higher than that in normal mice. The fold change was similar to that observed in child patients with DMD.[14] In humans, studies on urinary titin have been accelerated by the introduction of an ELISA kit to measure human titin N-terminal fragment levels (the human titin N-fragment ELISA

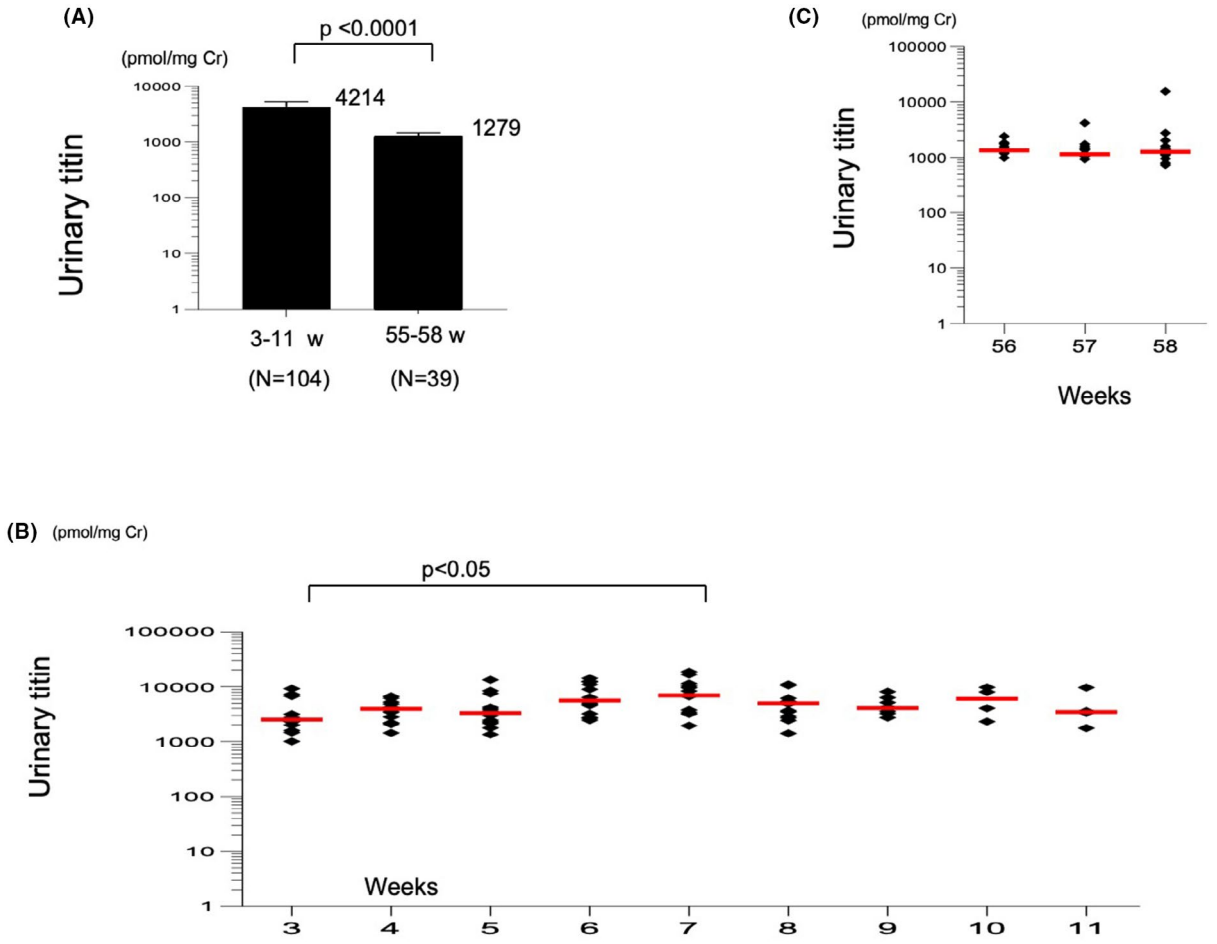


FIGURE 4 Persistent elevation of urinary titin levels in *mdx* mice. A, The titin concentrations compared between the young (3–11 weeks old) and aged (56–58 weeks old) groups. There was a significant decrease in urinary titin levels with aging. B, Weekly determinations of urinary titin concentrations from 3 to 11 weeks of age. Concentrations of urinary titin are plotted against weeks of age. Bars in red represent median. C, Weekly determinations of urinary titin concentrations from 56 to 58 weeks of age. Concentrations of urinary titin are plotted against weeks of age. Bars in red represent median

kit).[14,17–22] As a result, high urinary titin levels have been identified in association with not only diseases[23] but also physical activities.[23] Clinically, urinary titin has been nominated as a biomarker for catabolism of neonates[24] and muscle atrophy of patients in intensive care unit.[20,25] Similar examinations could be performed in mice using the developed kit in the future.

A high urinary titin level in the *mdx* mice was observed at 3 weeks of age. The time of this elevation is consistent with the pathological finding that skeletal muscles of *mdx* mice undergo startling necrosis between 3 and 6 weeks of age.[26] Persistent hypertitinuria in *mdx* mice lasted over 56 weeks of age, and this persistence was compatible with the pathological finding that no apparent fiber loss was observed in aged *mdx* mice.[27] These results indicated that the *mdx* mouse suffers from severe muscle myofibrillar protein breakdown, as observed in humans. However, the *mdx* mouse shows mild muscle phenotypes different from those of DMD patients.[28] The mild phenotypes of *mdx* mice are produced by replacement of degenerated muscle fibers with new muscle fibers provided by robust regeneration activity.[27] Persistent hypertitinuria was thought to result from a continuous supply of titin molecules in muscle provided by regeneration.[27] In *mdx* mice, therefore, the persistence of hypertitinuria could be a marker of regenerative activity.

Studies on DMD have mainly focused on the development of treatments and employed the *mdx* mouse as a model animal. The serum creatine kinase level has been used as a biomarker in evaluating treatment effects. However, the determination of creatine kinase levels requires blood sampling, which hampers frequent repeat examinations. In contrast, the determination of urinary titin levels is noninvasive, making frequent measurements possible. Furthermore, the urinary titin level reflects the degree of muscle protein breakdown. Although the cardinal symptom of DMD is muscle wasting, muscle protein breakdown has rarely been examined. The development of an ELISA kit to measure mouse titin levels made it possible to estimate protein breakdown directly and frequently in *mdx* mice. In particular, drugs aiming to alleviate protein degradation can be developed by monitoring urinary titin levels in adult *mdx* mice. This may enable the identification of novel drugs for DMD therapy.

There were several limitations in this study. First, this study was preliminarily conducted to examine the feasibility of the developed

kit. Hence, a small number of urine samples from DMD model mice were analyzed. The current results strongly suggest the need to conduct a large-scale study. Second, the effect of physical activities on the urinary titin level was not analyzed. This analysis may reveal the mechanistic load that leads to titin breakdown. Third, this study was not designed to include mouse models other than DMD. Therefore, it was not concluded whether abnormalities in urinary titin levels are observed in mouse models of other muscle diseases.

5 | CONCLUSIONS

The mouse titin N-terminal fragment assay kit-IBL showed good performance in determining titin N-terminal fragment levels in mouse urine. Muscle protein breakdown is extremely active and persistent in DMD mice even though the muscle phenotype is mild. Our results provide an opportunity to develop DMD treatments that aim to alleviate muscle protein breakdown by monitoring urinary titin levels of *mdx* mice.

CONFLICTS OF INTERESTS

N.M. is an employee of Immuno-Biological Laboratories Co., Ltd. M.M. discloses being employed by Kobe Gakuin University, which received funding from KNC Laboratories Inc. M.M. further discloses being a scientific adviser for Daiichi-Sankyo Co. and JCR Pharma Co. The other authors have no conflicts of interest relevant to this article to disclose.

AUTHOR CONTRIBUTIONS

Conceptualization, Y. Ne. and M.M.; methodology, T.S., K.O., and N.M.; validation, Y. Ne. and S.F.; in-vestigation, A.I. and K.N.; data curation, Y. Na. and H.A.; writing—original draft preparation, T.S. and N.M.; writing—review and editing, H.N. and M.M.; project administration, M.M.; funding acquisition, M.M. All authors have read and agreed to the published version of the manuscript.

ORCID

Masafumi Matsuo https://orcid.org/0000-0001-6747-7194

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