Urinary titin is not an early biomarker of skeletal muscle atrophy induced by muscle denervation in mice

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Short Report

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Abstract

Early detection of skeletal muscle atrophy is important to prevent further muscle weakness. However, there are few non-invasive biomarkers for skeletal muscle atrophy. Recent studies have reported that the N-terminal fragment (N-titin) of titin, a giant sarcomeric protein, is detected in the urine of patients with muscle damage. In this study, we hypothesized that urinary N-titin would be a potential early biomarker of skeletal muscle atrophy in mice caused by sciatic nerve denervation. Male mice were randomly divided into control and denervation groups, and urinary N-titin levels were assessed daily for 9 days using an enzyme-linked immunosorbent assay system. Despite reduced titin protein levels in atrophic muscles 10 days after denervation, cleaved N-titin fragments were not increased in the urine of mice with denervation-induced muscle atrophy. Furthermore, we found no uptake of Evans blue dye from the extracellular space into the cytoplasm in atrophic muscles, suggesting that the sarcomeric membrane is intact in those muscles. The present results suggest that cleaved N-titin in the urine is not suitable as an early biomarker of skeletal muscle atrophy.

Background

Skeletal muscle atrophy due to microgravity [1], disuse [2], cancer [3], and aging [3] directly leads to muscle weakness and is strongly associated with reduced quality of life [4]. Therefore, the early detection of muscle atrophy is important to its prevention and reduction. Imaging methods such as dual-energy X-ray absorption measurement (DXA) and computed tomography (CT), walking speed tests, and 6-min walking tests are used to detect muscular atrophy [5]. However, defining muscle atrophy using the above-mentioned tests is not always easy. A simple and reliable screening tool for the diagnosis of muscle atrophy is very important and needed [6]. Biomarkers could serve as objective, early indicators of the biological process of muscle atrophy [7]. Several studies have suggested that inflammatory biomarkers such as C-reactive protein, interleukin-6, tumor necrosis factor-a [8], and irisin [9] could be useful for the detection of muscle atrophy due to aging. Recent studies have also demonstrated that the Wnt antagonist frizzled-related protein (FRZB) is a biomarker for denervation-induced muscle atrophy in amyotrophic lateral sclerosis (ALS) [10] and creatinine in spinal muscular atrophy (SMA) [11]. However, these biomarkers have not been widely applied to diagnose muscle atrophy, because they were not specific to muscle atrophy, showed weak association with clinical outcomes, and were invasive due to the required blood sampling from patients [12]. Therefore, it is important to identify non-invasive and specific biomarkers that can detect the early stage of muscle atrophy.

Titin is a giant sarcomeric protein that is involved in muscular passive tension and viscoelasticity. According to previous reports [13], titin is one of the earlier myofibril proteins whose expression is decreased by muscle atrophy. Importantly, the N-terminal titin fragment (N-titin) in urine was recently identified as a useful non-invasive biomarker for muscle damage such as that seen in Duchenne muscular dystrophy (DMD) [14, 15]. Urinary N-titin was also demonstrated to be increased in muscle damage due to non-alcoholic fatty liver disease [16] and in healthy volunteers engaged in endurance exercise [17][18] by using the enzyme-linked immunosorbent assay (ELISA) system [14]. Furthermore, we
found that urinary N-titin is increased in patients after cardiac surgery [19]. Nakanishi et al. recently reported that urinary N-titin levels were increased 10- to 30-fold in non-surgical adult patients staying in the intensive care unit (ICU) compared with normal levels, and that they were correlated with decreases in patient muscle mass [20]. According to Nakanishi et al.’s report [20], urinary N-titin could be a potential candidate as an early biomarker of skeletal muscle atrophy. However, there are no reports directly investigating whether urinary N-titin is increased with muscle atrophy using highly sensitive ELISA system. In the present study, we hypothesized that urinary N-titin would be a good indicator for detecting muscle atrophy in the early stage. To evaluate our hypothesis, urinary N-titin was measured in mice that underwent denervation of the sciatic nerve.

**Methods**

**Animals**

Experiments were performed after obtaining approval from the Animal Experiment Committee of the Jikei University School of Medicine. Male C57BL/6J mice were purchased from Nihon CREA. Mice were allowed free access to a pelleted laboratory animal diet and tap water. At 10 weeks of age, mice were randomly divided into two groups: a control (CON) group and a denervated (DEN) group (n = 6 each group). Mice in the DEN group underwent sciatic nerve transection surgery as previously described [21]. Briefly, mice were anesthetized using isoflurane, and a small incision was made in the posterior aspect of both hindlimbs to expose the sciatic nerve at the level of the femoral trochanter. At least 5.0 mm of the sciatic nerve was excised using small operating scissors. The skin was then closed with surgical glue. Mice in the CON group were also anesthetized using isoflurane and a small incision was made in the posterior aspect of both hindlimbs, which was then closed.

**Urine Sample Collection And Measurement Of N-titin**

Mice were individually housed in metabolic cages during the experimental period. For urine samples, pooled urine was used every 24 h pre-operatively and post-operatively from day 1 to day 9. Collected urine samples were stored at -20°C until analysis. The urinary levels of N-titin were measured using the ELISA system (#27602 Mouse Titin N-fragment Assay Kit, Immuno-Biological Laboratories) according to the manufacturer’s instructions. To avoid the effects of urinary filtrations, the value of N-titin concentrations was corrected by the value of creatinine and is shown by the following creatinine ratio: (N-titin/Cr) = N-titin (pmol/L)/creatinine (mg/mL). Urine creatinine concentration measurement was carried out with Lab Assay™ Creatinine (Wako Pure Chemical Industries). The samples were analyzed in duplicate and averaged for each measurement.

**Tissue Preparation**
Mice were sacrificed by cervical dislocation on post-operative day 10. The body and wet muscles were weighed. The tibialis anterior (TA) and soleus (SOL) muscles were collected using standard dissection methods. These muscles were frozen in liquid nitrogen for RNA and protein analysis.

**Real-time Polymerase Chain Reaction (PCR) Analysis**

RNA isolation from the TA and SOL muscles in the CON and DEN groups and cDNA synthesis were performed as described [22]. Atrogin-1, Muscle RING Finger-1 (MuRF1), and titin expression levels were measured by real-time polymerase chain reaction (PCR) using the SYBR Premix Ex TaqII (TAKARA). The expression levels of these genes were normalized to those of 18S rRNA.

**Detection Of Titin By Electrophoresis**

Detection of titin by electrophoresis
Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the detection of titin was conducted as per a previous study [23]. Protein samples of the TA and SOL muscles in the CON and DEN groups were prepared by Laemmli’s buffer system [23]. The preparations were then solubilized and electrophoresed on 2% polyacrylamide slab gel that included 0.5% agarose. Finally, the gels were stained with Coomassie brilliant blue (CBB). These gels were created as per Tatsumi and Hattori’s method [24]. The quantification of titin levels was carried out by the National Institutes of Health Image J software.

**Western Blot Analysis**

Protein was extracted from the TA and SOL muscles in the CON and DEN groups. Western blot analysis was performed as previously described [25]. The primary antibodies used in this study were as follows: anti-connexin 43 (abcam) and anti-GAPDH (Cell Signaling Technology). The band intensities of the target proteins were analyzed using the National Institutes of Health Image J software and normalized by the band intensity of GAPDH.

**Myofiber Damage Evaluation**

For Evans blue dye (EBD)-injected mice, animals were interperitoneally injected with a 1% EBD solution at a dose of 50 mg/kg on post-operative day 5 and sacrificed by cervical dislocation 24 h after EBD injection. The TA and SOL muscles from the CON and DEN groups were sampled and then frozen in isopentane cooled by liquid nitrogen for myofiber damage evaluation. Cryosections of 8 mm thickness were cut from these muscles. Each cryosection was observed under a fluorescence microscope at 10× magnification using BZ-9000 (KEYENCE) to estimate the area of myofiber.

**Immunohistochemistry**
Cryosections of 8 mm thickness were cut from the TA and SOL muscles. Immunohistochemistry was performed as described previously [26]. Sections were stained with polyclonal rabbit antibodies directed against connexin 43 (abcam), and Alexa 488-conjugated goat anti-rabbit IgG antibody (Invitrogen) was used as a secondary antibody. Fluorescence images were obtained using a BZ-9000 fluorescence microscope (KEYENCE).

**Statistical analysis**

All data are presented as means ± standard error of the mean (SEM). Statistical differences were assessed by an unpaired t-test or one-way repeated measures analysis of variance (ANOVA). A p value < 0.05 was considered statistically significant.

**Results And Discussion**

**Titin was decreased at the protein level in muscles with denervation-induced atrophy**

Ten days after sciatic nerve denervation, the weights of the TA and SOL muscles were 0.68- and 0.62-fold smaller in the DEN group compared with those in the CON group, respectively (Fig. 1A), as previously reported [27]. The body weights were not different between the CON (24.8 ± 0.5 g) and DEN groups (25.0 ± 0.4 g). According to previous reports [13], titin is one of the earlier myofibril proteins whose expression is decreased by sciatic nerve denervation. Therefore, we examined the expression levels of titin mRNA and protein in the TA and SOL muscles. Although the titin expression of the TA and SOL muscles was not significantly different at the mRNA level between the two groups (Fig. 1B), the expression levels of titin protein in the TA and SOL muscles were significantly decreased in the DEN group (Fig. 1C and D). This result indicated that the downregulation of titin in muscle atrophy after denervation occurred at the post-transcriptional level, which is consistent with the results of previous studies [13]. Because titin protein is known to be degraded by E3-ubiquitin ligase such as MuRF1, we next examined the mRNA expression levels of muscle-specific E3-ubiquitin ligases. Quantitative PCR analysis revealed that MuRF1 and atrogin-1 mRNAs were significantly increased in the denervated TA and SOL muscles (Fig. 2A and B). Although we have not examined the MuRF1 protein level, MuRF1 is known to be upregulated [27] and its activity is also enhanced [13] from the early stage of denervation. Therefore, it is suggested that the expression level of titin in protein is decreased due to protein degradation through E3-ubiquitin ligase such as MuRF1 from the early stage after denervation.

**Muscle Atrophy Did Not Increase Urinary N-titin Levels After Sciatic Nerve Denervation**

Titin protein is also known to be cleaved by calpain and matrix-metalloproteinase-2 when protease activity is increased in pathological conditions such as oxidative stress [28, 29]. Previous studies have
demonstrated that cleaved titin fragments can be detected in the striated muscle that is associated with muscle damage in urine [14, 15, 18]. Furthermore, a recent study also demonstrated that the urinary titin level increased 10- to 30-fold compared with the normal level in nonsurgical, critically ill patients [20]. The authors suggested that the increased urinary titin levels reflected lower limb muscle atrophy. Therefore, we examined whether sciatic nerve denervation–induced muscle atrophy is associated with the increase in cleaved titin fragments in urine. In the present study however, we found that urinary N-titin/Cr levels were not different at any time point 10 days after the operation (Fig. 3), which is not consistent with the results of the previous study by Nakanishi et al. [20]. It should be noted that urinary N-titin/Cr levels were significantly increased in both CON and DEN groups on post-operative day 1 when compared to the level before operation, although there was no significant difference between DEN and CON groups (Fig. 3). Urinary N-titin levels were then rapidly decreased day by day and returned to the pre-operative levels around 7 days after the operation. By the 9th post-operative day, the levels of urinary N-titin were not different between the DEN and CON groups. Therefore, we think that the transient increase in urinary N-titin/Cr levels immediately after the surgical operation could be due to muscle damage by surgical incision.

To confirm the effectiveness of the ELISA kit (#27602 Mouse Titin N-fragment Assay Kit; Immuno-Biological Laboratories) used in this experiment, we examined urinary N-titin/Cr in dystrophin and utrophin double-knockout (dKO) mice that exhibit severe muscular dystrophy compared to the most common murine DMD model, mdx mice [30]. We found that urinary N-titin/Cr levels in the dKO mice increased 15-fold compared with that in wild-type mice (Fig. S1). In contrast, the degree of N-titin/Cr increase was approximately 100-fold higher in DMD patients than in healthy volunteers according to the results of the ELISA kit used for human urinary titin measurement (#29501 Human Titin N-fragment Assay Kit, Immuno-Biological Laboratories) [14]. These results suggest that the sensitivity of the ELISA kit used in this experiment may be lower than that of the ELISA kit for measuring muscle damage in DMD patients [14] and ICU patients [20]. Next, we used another ELISA Kit (#SEB667 ELISA Kit for Titin, Cloud-Clone Corp.) provided by a different company to test the sensitivity for measuring mouse urinary titin using the same samples. We found that urinary N-titin/Cr levels in denervation-induced atrophy mice were very low, even at the first post-operative day and were not significantly different from those in wild-type mice (Fig. S2A). This result is consistent with our previous reports (31). Furthermore, the urinary N-titin/Cr levels in the dKO mice were increased only 6-fold compared with that in wild-type mice (Fig. S2B), suggesting that the sensitivity of the #SEB667 ELISA Kit is much lower than that of the #27602 Mouse Titin N-fragment Assay Kit.

**Sarcomeric Membrane Was Intact In Muscles With Denervation-induced Atrophy**

Serum creatine kinase, which is commonly used as a marker of muscle damage in muscle disease [26, 32], is not elevated in atrophic muscles [33], which is consistent with the present findings. To clarify the reason why fragmented titin did not leak into the urine due to muscle atrophy, the stabilization of the
muscle membrane was examined using EBD. EBD uptake was not observed in the TA and SOL muscles in the CON and DEN groups, indicating that the sarcolemma was stabilized even after denervation-induced muscle atrophy (Fig. 4A). Because previous studies have demonstrated that small molecules such as EBD are permeabilized via connexin 43 hemichannels on the sarcolemma of fast myofibers [34] and that the expression of connexin is increased in atrophic muscle induced by both denervation and dexamethasone [35], we analyzed the expression levels of connexin 43 in denervated muscle. The expression levels of connexin 43 in the TA and SOL muscles were significantly increased in the DEN groups (Fig. 4B and C), which is consistent with the results of previous studies. Connexin hemichannels can release or take up only the monovalent cation, Ca\(^{2+}\), and molecules with a molecular weight below 1.5 kDa [36]. Because the molecular weight of the N-titin fragment is approximately 21.4 kDa, the N-titin fragment cannot go through the connexin hemichannels even though the connexin 43 is upregulated in atrophic muscle. Therefore, we think that the N-titin fragment did not leak into the blood and urine to a detectable level even though it was degraded and fragmented by muscle atrophy. Another possibility is that the amount of titin released in the urine is small because muscle atrophy due to denervation occurs only in the hindlimb muscles whereas muscle damage in DMD patients occurs throughout the whole body. Our study also suggests that connexin 43 upregulation is not sufficient to induce EBD leakage from sarcolemma of atrophic muscles.

**Conclusion**

We found that the cleaved N-titin fragment was not increased in the urine of mice with skeletal muscle atrophy 10 days after sciatic nerve denervation, even though the expression levels of titin protein were decreased. Therefore, the urinary N-titin fragment is not suitable for detecting the early stage of skeletal muscle atrophy.

** declarations**

**Availability of data and materials**

The data underlying this article will be shared upon reasonable request to the corresponding author.

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Not applicable.

**Author contributions**

J. T. and S. M. conceived of the study. J. T. and S. M. designed the experiments. J. T. and S. M. wrote the manuscript. J. T. performed the experiments. All authors discussed the results and implications and commented on the manuscript at all stages of its development.

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**Ethics approval and consent to participate**

The animal experiments were performed in accordance with the National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals). All animal studies were approved by the Institutional Animal Care and Use Committee of the Jikei University.

**Consent for publication**

Not applicable.

**Competing interests**

All authors declare no competing interests.

**References**


Figures
Figure 1

The effects of muscle atrophy induced by denervation on muscle weight and titin expression levels.

(A) The weight in the TA and SOL muscles of the CON and DEN groups on post-operative day 10. (B) Quantification of real-time PCR products for titin expression in the TA and SOL muscles of the CON and DEN groups on post-operative day 10. (C) Typical SDS-PAGE image showing titin expression in the TA and SOL muscles of the CON and DEN groups. (D) Quantification of titin in the TA and SOL muscles of the CON and DEN groups on post-operative day 10. Data are presented as the means ± standard error of the mean (SEM). **p < 0.01 and ***p < 0.001 by an unpaired t-test.
Figure 2

The effects of muscle atrophy induced by denervation on the mRNA expression levels of muscle atrophy–related gene.

Quantification of real-time polymerase chain reaction (PCR) products for MuRF1 (A), and atrogin-1 (B) expression in the TA and SOL muscles of the CON and DEN groups on post-operative day 10. Data are presented as means ± standard error of the mean (SEM). *p < 0.05, **p < 0.01 and ***p < 0.001 by an unpaired t-test.
**Figure 3**

The effects of muscle atrophy induced by denervation on urinary N-titin/Cr values.

Time course changes in urinary N-titin/Cr levels. Open circles: CON group. Closed circles: DEN group. Data are presented as means ± standard error of the mean (SEM). **p < 0.01 (0 days vs. 1 day after the operation in the CON group) and ### < 0.001 (0 days vs. 1 day after the operation in the DEN group) by one-way repeated measures analysis of variance (ANOVA).
Figure 4

The effects of muscle atrophy induced by denervation on the stabilization of muscle membrane and expression levels of connexin 43.

(A) Evans blue staining in the TA and SOL muscles of the CON and DEN groups on post-operative day 6. The TA muscle of *mdx* mice is shown as a positive control. Scale bar: 100 µm. (B) Immunohistochemical staining of connexin 43 in the TA and SOL muscles. Scale bar: 50 mm. (C) Western blot of connexin 43 in the TA muscle. The bar graph shows relative connexin levels compared to those of GAPDH. Data are presented as means ± standard error of the mean (SEM). n = 6/group. *p < 0.05 by an unpaired t-test.

Supplementary Files

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- MuscleatrophyandtitinSupplementfigureVer.12forJPS.pdf