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Brief Reports

Receiver operating curve analyses of urinary titin of healthy 3-y-old children may be a noninvasive screening method for Duchenne muscular dystrophy

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ABSTRACT

Background: Duchenne muscular dystrophy (DMD) is a progressive, fatal muscle wasting disease. Early detection of DMD by mass screening may enable the early treatment of these patients. We have reported that urinary titin concentration, an indicator of severe muscle wasting, is a diagnostic biomarker for DMD.

Methods: Urinary titin concentrations were measured in healthy 3-y-old children and, by comparison with concentrations in 4 DMD patients, and validated as a screening biomarker for DMD. Urine samples were obtained from 100 healthy Japanese children, 52 boys and 48 girls, and their urinary titin concentrations measured by ELISA.

Results: The mean ± SD urinary titin concentration was 1.5 ± 2.5 nmol/l, and the mean urinary titin concentration normalized to creatinine was 2.2 ± 4.1 pmol/mg creatinine, with no differences between boys and girls. Histograms and box-and-whisker plots showed that almost all titin and normalized titin concentrations were in narrow ranges, with one outlier in common. Receiver operating characteristic curve analysis showed that titin and normalized-titin concentrations from healthy 3-y-olds were completely separate from those of 3-y-old DMD patients.

Conclusions: These findings indicate that urinary titin may be an excellent non-invasive biomarker to screen for DMD.

1. Introduction

Duchenne muscular dystrophy (DMD) is the most common inherited muscle disease in childhood, affecting approximately 1 in 3500–6000 live-born males [1, 2]. DMD is characterized by a progressive muscle wasting, with initial muscle weakness occurring around age 4–6 y, loss of ambulation by age 12 y, and eventually death by late 20s and early 30s. Multidisciplinary medical care has increased the life expectancy of these patients [3–5], with new drugs such as ataluren and eteplirsen modifying the severity of DMD [6].

Early identification of DMD patients may enable proper care before severe muscle damage occurs [7, 8]. Although newborns in some areas in the world are screened for DMD by measuring serum creatine kinase concentrations [8, 9], these trials have had drawbacks, including high false positive rates and the invasiveness of the procedure [10]. One highly efficient manner now under development is to specifically measure the MM isoform of creatine kinase [11].

Titin is the third most abundant protein in the sarcomere, after actin and myosin, and functions as a molecular spring during the relaxation-contraction cycle [12]. Although titin is the largest protein in the human body, proteomics analysis disclosed titin fragments in the urine of DMD patients [13]. Using ELISA, we previously analyzed urinary titin concentrations in a large cohort of DMD patients, finding that urinary titin alone is a sufficient and non-invasive diagnostic biomarker for DMD [14]. Especially, urinary titin concentration was found to be strongly elevated in presymptomatic 3-y-old DMD patients [14].

In Japan, maternal and child health laws require children to undergo periodic health checkups, performed free by the municipality, with urinalysis being mandatory for 3-y-olds at their health checkup [15]. This provides a suitable opportunity to screen urine samples for DMD by measuring urinary titin concentrations. In addition, age 3 years is considered as a proper time to detect DMD, as guidelines recommend

Abbreviations: DMD, Duchenne muscular dystrophy; Cr, creatinine

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that DMD patients start steroid treatment at age 4–6 y [1, 16, 17].

2. Urine samples and methods

2.1. Urine samples

Urine samples were collected from consecutive 3-y-old Japanese boys and girls who underwent health checkups at a single health care center. Samples nearly 2 ml in volume and obtained after anonymizing personal information except sex were decanted into plastic tubes with no additives, immediately cooled on ice, and stored at −20 °C until analyzed.

2.2. Determination of titin in urine

Urinary titin was measured using an enzyme-linked immunosorbent assay (ELISA) system (Titin-N Fragment Assay Kit-IBL; Immuno-Biological Laboratories Co. Ltd.), as described [18]. Urinary creatinine (Cr) concentrations were measured using an assay kit (LabAssay Creatinine, Wako Pure Chemical Industries, Ltd.). All samples were tested in duplicate in a blinded manner, and the average of the values was obtained.

2.3. Statistical analysis

Shapiro-Wilk normality test was used to determine if a data set was normally distributed, with a $p < .05$ indicating that the data set was non-normally distributed. Differences between groups were determined using Mann-Whitney *U* tests.

Receiver operating curves (ROC) were generated by plotting true positivity (sensitivity) versus false positivity (1 - specificity) at various cutoff points [19], and the area under the curve (AUC) was calculated using GraphPad PRISM 7.02 (GraphPad Software). An AUC of 1.0 was defined as a perfect discrimination between 2 curves, and an AUC of 0.5 defined as random distribution. A p values $< .05$ was considered statistically significant. All statistical analyses were performed using GraphPad PRISM 7.02 (GraphPad Software).

2.4. Ethics

The study protocol was approved by the ethics committees of both Kobe Gakuin University and Kobe City.

3. Results

A total of 102 urine samples were collected. Two were considered non-analyzable because of low urine volume, whereas the remaining 100 urine samples, from 52 boys and 48 girls, were tested (Table 1).

Table 1
Urinary titin and creatinine concentrations and normalized titin concentrations in Japanese children.

	Total	Boys	Girls	P (B vs G)
Number	100	52	48	
Titin (nmol/l)				
Mean ± SD	1.5 ± 2.5	1.8 ± 3.4	1.1 ± 0.7	0.15
Median (range)	0.9 (0.1–24.8)	1.1 (0.1–24.8)	1.0 (0.2–3.1)	
Creatinine (mg/dl)				
Mean ± SD	68.4 ± 33.0	73.0 ± 35.4	63.4 ± 29.6	0.18
Median (range)	66.2 (7.5–197.1)	70.8 (13.1–197.1)	62.4 (7.5–36.2)	
Normalized Titin (pmol/mg Cr)				
Mean ± SD	2.2 ± 4.1	2.8 ± 5.7	1.7 ± 0.8	0.46
Median (range)	1.8 (0.2–42.3)	1.7 (0.2–42.3)	1.8 (0.4–5.3)	

The mean ± SD urinary titin concentration in these samples was 1.5 ± 2.5 nmol/l, and the median and range were 0.9 and 0.1–24.8 nmol/l, respectively. Mean ± SD urinary titin concentrations were similar in boys and girls (1.8 ± 3.4 vs 1.1 ± 0.7 nmol/l). A histogram was constructed, with sample concentrations separated into 1 nmol/l bins (Fig. 1A bottom). Of the 100 samples, 98 were within a narrow range of urinary titin concentrations, from 0.1 to 3.9 nmol/l. The other two samples, with urinary titin concentrations of 6.6 and 24.8 nmol/l, were defined as outliers with the value of > 3 standard deviations (Table 2). A box-and-whisker plot was also generated (Fig. 1A top). Two samples were also defined as outliers.

When urinary titin concentrations were normalized to urinary Cr concentrations (Table 1), the mean ± SD concentration of normalized titin was 2.2 ± 4.1 pmol/mg Cr, and the median concentration was 1.8 pmol/mg Cr (range, 0.2–42.3 pmol/mg Cr). Mean ± SD normalized urinary titin concentrations were similar in boys and girls (2.8 ± 5.7 pmol/mg Cr vs 1.7 ± 0.8 pmol/mg Cr). Construction of a histogram showed that 99 of the 100 samples were within a narrow range of normalized titin concentrations (Fig. 1B bottom), whereas the other, with normalized titin concentrations of 42.3 pmol/mg Cr. A box-and-whisker plot disclosed an narrow range with one exceptionally high normalized titin (Fig. 1B top). This abnormal one was defined as an outlier (Table 2). Only one sample was considered an outlier for both titin and normalized titin concentrations, suggesting that this child had a pathological condition. According to the Shapiro-Wilk normality test, neither titin nor normalized titin concentrations were normally distributed in all samples ($p < .01$ each).

We had previously measured urinary titin concentrations in two 3-y-old DMD patients [14]. Urinary titin concentrations were also measured in two other 3-y-old DMD patients (manuscript in preparation). Thus, titin concentrations were compared in these 4 urine samples from DMD patients and the 100 healthy 3-y-olds without DMD. The median titin concentration in the four DMD patients was 428.0 nmol/l and the median normalized titin concentration in these 4 children was 1240.5 pmol/mg Cr. Statistical comparisons between these 2 groups resulted in non-overlapping differences for both titin and normalized-titin concentrations (Fig. 2).

To further validate the significance, ROC curves were generated for urinary titin concentrations obtained from the four DMD patients and the 100 controls (Fig. 2). The AUC for urinary titin was 1.00 ($p = .0007$), characteristic of an ideal test [20], and suggesting that urinary titin would be an accurate diagnostic biomarker for DMD in 3-y-old children. Since only males are affected by DMD, ROC curve was generated for urinary titin concentrations obtained from the 4 DMD patients and 52 male controls. Again, the AUC for urinary titin was 1.00 ($p = .0009$).

4. Discussion

We found that urinary titin levels were similar in boys and girls. As exercise-induced titin fragmentation occurs in normal individuals [21], we thought it likely that urinary titin concentrations would be distributed widely in children. However, histograms of titin and normalized titin concentrations showed narrow range distributions with few outliers, indicating that exercise-induced titin fragmentation is not a major contributor to urinary titin in children. In particular, one subject, classified as an outlier on both non-normalized and normalized titin concentrations, was regarded as having pathological muscle damage. No further examination of this subject was possible, however, because their information had already been anonymized, preventing their identification.

We observed a clear difference between the 100 non-DMD controls and the 4 DMD patients (median; 1.8 pmol/mg Cr and 1240.5 pmol/mg Cr, respectively). ROC curve analysis showed excellent sensitivity and specificity for detecting DMD, as the AUC was 1.0 [19]. This finding indicated that urinary titin would be an excellent screening biomarker