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Review

Titin in muscular dystrophy and cardiomyopathy: Urinary titin as a novel marker

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ABSTRACT

Titin, encoded by the gene *TTN*, is the largest human protein, and plays central roles in sarcomeric structures and functions in skeletal and cardiac muscles. Mutations of *TTN* are causally related to specific types of muscular dystrophies and cardiomyopathies. A developed methodology of next generation sequencing has recently led to the identification of novel *TTN* mutations in such diseases. The clinical significance of titin is now emerging as a target for genetic strategies. Titin-related muscular dystrophies include tibial muscular dystrophy, limb-girdle muscular dystrophy, Emery-Dreifuss muscular dystrophy, hereditary myopathy with early respiratory failure, central core myopathy, centronuclear myopathies, and Salih myopathy. Truncation mutations of *TTN* have been identified as the most frequent genetic cause of dilated cardiomyopathy. In this review article, we highlight the role of titin and impact of *TTN* mutations in the pathogenesis of muscular dystrophies and cardiomyopathies. Recently, a novel sensitive sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of the urinary titin N-terminal fragments (U-TN) has been established. We discuss the clinical significance of U-TN in the diagnosis of muscular dystrophies and differential diagnosis of cardiomyopathies, as well as risk stratification in dilated cardiomyopathy.

1. Introduction

Titin, encoded by the gene *TTN*, is the largest human protein (4200 kDa), composed of 34,350 amino acids mapped on chromosome 2q31 [1]. Titin is the third most abundant striated muscle protein as a main component of the sarcomeric organization in myocytes, including skeletal and cardiac muscles [1,2]. The huge size and complex structure of titin provide architectural support and maintenance during contraction [2,3]. Titin is essential for the development, elasticity, and signaling in sarcomeres as well as stabilization of the thick filament [2,3]. Muscular dystrophy (MD) is a group of genetic diseases that cause progressive weakness and loss of skeletal muscle mass. MD is predominantly considered to be a skeletal muscle pathology; however, some MD patients develop cardiac complications [4]. Mutations of *TTN* are related to specific types of MD and cardiomyopathies. The developed methodologies of next generation sequencing (NGS) allow the numbers of genes to be sequenced one at a time [5]. NGS can identify the novel *TTN* mutations responsible for the MD diseases, as well as cardiomyopathies, despite the huge size of its coding gene. In contrast, a large number of missense and truncation variants of *TTN* have also been reported in the general population [6]. Thus, the clinical interpretation of *TTN* variants remains a challenge. In this review, we provide a current overview of the role of titin in human MD and cardiomyopathies. We also introduce a novel assay of titin fragments in urine, which is a useful marker for the diagnosis of MD and cardiomyopathies, as well as risk stratification in dilated cardiomyopathy (DCM).

2. Molecular characteristics and physiological function of titin

TTN contains 363 coding exons in humans [1]. The titin protein spans half a sarcomere from *Z*-line to M-line. The protein consists of four structural and functional regions; the N-terminal Z-disk, I-band, A-band, and C-terminal M-band regions [1,2] (Fig. 1A and B). It also contains the immunoglobulin (Ig)-like domain, fibronectin type 3- (FN-III)-like domain, PEVK-rich domain (comprised of proline [P], glutamate [E], valine [V], and lysine [K]), and titin kinase domain [1] (Fig. 1C). The *Z*-disk contains seven Ig domains and structurally interacts with myofibrillar and sarcomeric proteins, providing myofibrillar assembly, stability and signaling [1]. I-band is composed of the Ig domain and PEVK-rich domain with splicing of the skeletal N2A region and cardiac N2B region. The extensible I-band gradually lengthens and

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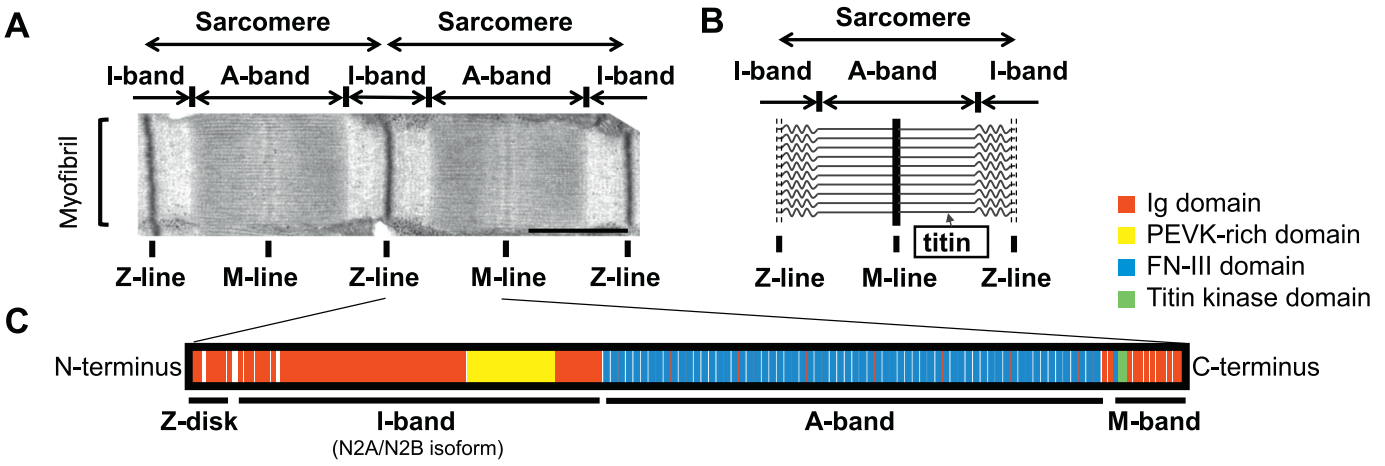

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	Z-disk	I-band	A-band	M-band
Muscular dystrophies		Centronuclear myopathy	TMD Hereditary myopathy with early respiratory failure	TMD Limb-girdle muscular dystrophy Emery-Dreifuss muscular dystrophy Central core myopathy Salih myopathy
Cardio-myopathies	HCM Left ventricular noncompaction	DCM HCM Arrhythmogenic right ventricular cardiomyopathy	DCM Restrictive cardiomyopathy	

Fig. 1. Schematic diagrams of titin, its domains and the titin-related diseases due to *TTN* mutations.
A, Electron micrograph of a cardiomyocyte from the heart tissue section. The structures of two sarcomeres are presented. Scale bar, 1 μm. B, Schematic figure showing the layout of titin in the sarcomere. C, Schematic diagram of the domains of titin from the N-terminus to the C-terminus. Ig indicates immunoglobulin-like; PEVK, proline, glutamate, valine, lysine; and FNIII, fibronectin type 3-like. D, *TTN* mutations identified in muscular dystrophies and cardiomyopathies in the different regions. TMD indicates tibial muscular dystrophy; HCM, hypertrophic cardiomyopathy; and DCM, dilated cardiomyopathy.

develops passive tension when the sarcomere is stretched during diastole. The A-band, which mainly consists of Ig and FN-III domains, is inextensible and functions as a stable anchor binding to myosin. The M-band contains a titin kinase domain and Ig domains and plays a key role in the structural integrity of the sarcomere during myofibrillogenesis. The overlap of titin's N- and C-terminus forms a continuous filament system along the full length of the myofibril. Titin isoforms are controlled during postnatal development and expressed in a tissue-specific manner [7]. Multiple splicing events, particularly in the I-band region, have been reported [7]. Interactions of titin with other proteins are enormously varied and many putative ligand proteins have been reported [8,9]. Detailed description of the molecular features of titin have been reviewed [2,3,10], but here it is essential to understand how *TTN* mutations in different regions are involved in human diseases.

3. *TTN* mutations and cardiac involvement in muscular dystrophies

MD is the inherited disease caused by the defects in certain genes. Although genetic variations have been found in most MDs, identification of the precise cause is a complex task. Routine analysis of *TTN* is especially challenging because of its large size and complexity. Muscle disorder due to problems with the titin protein is known as titin myopathy or titinopathy. This is caused by gene mutations in *TTN*; however, the severity and affected muscles are variable due to the positions and types of the mutations. Currently, NGS methodologies have enabled the identification of new *TTN* mutations responsible for MD. It becomes apparent that the mutations in a single gene can produce a wide range of phenotypes. Here, we provide a review of the various types of MD caused by *TTN* mutations.

Tibial muscular dystrophy (TMD) is a mild adult-onset slowly progressive myopathy that affects the anterior compartment muscles of the lower legs [11]. TMD is the first described human titinopathy, and is most commonly observed in Finland [11]. Mutations, including insertion-deletion or missense, were identified in exon 363 in the M-band in *TTN* among European families [12]. The Finnish founder mutation (FINmaj) is known as an 11-bp insertion-deletion mutation exchanging four amino acids in the exon 363 and last exon [13]. Novel mutations in Mex (M-line exon) 6 and exon 340 in the A-band were also identified in homozygosity or compound heterozygosity [13]. In addition, eight European patients with more complex and severe phenotypes resulting in young or early adult onset recessive distal titinopathy have been reported with novel frameshift mutations and a missense mutation in the A-band in *TTN* [14].

Limb-girdle muscular dystrophy (LGMD) is a severe childhood onset disease that causes weakness and wasting of the proximal muscles, such as in the shoulders, upper arms, pelvic area and thighs [15]. The subtypes of LGMD mostly demonstrate cardiac complications, including cardiomyopathy in LGMD2I, atrioventricular block, and DCM in LGMD1B (known as laminopathies caused by mutations of *LMNA* mutations) [15]. There was a reported case of a French family with autosomal dominant late-onset distal myopathy showing a nonsense mutation in the last exon [16]. Recently, a novel mutation 107788T > C (W35930R) in *TTN* was identified in four Chinese individuals by NGS [17].

Emery-Dreifuss muscular dystrophy (EDMD), also known as humeroperoneal muscular dystrophy, is a progressive muscle-wasting disorder defined by early contractures of the Achilles tendon, spine, and elbows [18]. EDMD is caused by mutations in nuclear membrane proteins, such as *LMNA* and *EMD* [19]. Cardiac involvement in EDMD patients is very common with atrial arrhythmias, atrioventricular conduction abnormalities, and DCM [19]. Three EDMD patients without

cardiomyopathy exhibited new truncating mutations in the C-terminal parts of Mex1–3 in *TTN* [20].

Hereditary myopathy with early respiratory failure (HMERF) is a dominantly inherited muscle disease characterized by slowly progressive proximal weakness and respiratory involvement at onset. HMERF in several northern European families has been associated with a 274375T > C (C30071R) mutation in the A-band of *TTN* [21]. A novel G30150D mutation was also identified in the highly conserved in the A-band of *TTN* [22]. Cardiac implications have been reported as conduction abnormalities and cardiomyopathy in HMERF patients with *TTN* missense mutations [23].

Central core myopathy (CCM) is the most common form of inherited non-dystrophic muscle disorder in childhood with delayed motor development and systemic muscle weakness, sometimes associated with respiratory failure [24]. The cardiac involvement in the CCM was regarded unusual, but recently, novel *TTN* mutations in the M-band were identified by whole-exome sequencing in CCM patients with cardiac diseases, including atrial and/or ventricular septal defects and DCM [25].

Centronuclear myopathy is a rare inherited muscle disorder that often presents in infancy with weakness and hypotonia, defined by centralized nuclei in muscular fibers [26]. *TTN* truncating mutations and nonsense mutations were identified in the I-band [27,28].

Salih myopathy is an early-onset myopathy with fatal cardiomyopathy [29]. This is a novel titinopathy characterized by slowly progressive skeletal muscle weakness that becomes apparent in early infancy and delays the development of motor skills, typically accompanied by a form of DCM [29]. Homozygous *TTN* deletion mutations were identified in the M-band (Mex1 and Mex3) in five patients with Salih myopathy [30] (Fig. 1D).

Among the several forms of MD, the cardiac muscle is similarly affected, and the cardiac complications contribute to mortality [31], although the incidence, severity, and pathogenesis of the cardiac involvement differs between the types of MD. For example, patients with Duchenne muscular dystrophy (DMD), which is caused by the absence of dystrophin, display cardiac dysfunction, typically developing DCM [32]. Likewise, MD patients with *TTN* mutations exhibit cardiac complications, mainly cardiomyopathy, whereas no cardiomyopathy has been observed among TMD patients. Cardiac involvement in MD patients appears to vary among the types of diseases and *TTN* mutations [33].

4. *TTN* mutations in primary cardiomyopathies and the role of titin isoforms

Cardiomyopathies are referred to as diseases of the cardiac muscle associated with mechanical and electrical dysfunction [34]. Primary cardiomyopathies include DCM, hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC) and are diagnosed based on the exclusion of secondary cardiomyopathies. The genetic etiology is demonstrated in these primary cardiomyopathies, usually with incomplete penetrance and variable expressivity. The *TTN* mutations have been implicated in the pathogenesis of the cardiomyopathies without the involvement of skeletal muscle [35]. Until routine analysis by NGS has been applied, only a few *TTN* mutations have been documented in the primary cardiomyopathies. NGS technologies enabled comprehensive mutation analysis to identify the new *TTN* mutations, which are causally related to the cardiomyopathies. In 2012, Herman et al. used NGS for *TTN* in a large number of patients and demonstrated that the *TTN* mutation is the most common genetic cause of DCM, whereas it is rare in HCM [6].

DCM is defined by dilatation and impaired contraction of the left ventricle, which leads to heart failure, and is a major cause of heart transplantation [34]. The role of *TTN* mutations in the pathogenesis of DCM has been largely reported [10]. *TTN* truncating variants were identified by means of NGS as the most prevalent genetic cause, including nonsense variants, frameshift insertions and deletions and canonical splice-disrupting variants [6]. The frequency of *TTN* mutations was significantly higher in subjects with DCM (27%) than in the control subjects (3%). *TTN* variants associated with DCM were largely observed in the A-band and not commonly found in the I-band, presumably with dominant negative/gain-of-function mechanisms. There were no mutations in the *Z*-disk or M-band regions [6] (Fig. 1D). No significant differences were found between the subjects with and those without *TTN* truncating mutations regarding the left ventricular end-diastolic dimensions, left ventricular ejection fraction (LVEF) and the rate of cardiac death [6,36]. Adverse events occurred earlier in male *TTN* mutation carriers than in female carriers [6], and *TTN* truncating variant-positive DCM patients showed more severely impaired LVEF, lower stroke volumes and adverse cardiac events than in *TTN* truncating variant-negative DCM patients [37]. The distance from the N-terminus of *TTN* was significantly correlated with LVEF and stroke volume [37]. In contrast, it has been reported that the *TTN* mutation-related DCM was less severe at presentation and more treatable with standard pharmacotherapy for heart failure [38]. Thus, the clinical interpretation of *TTN* mutations remains to be elucidated. In addition to *TTN*, it has been reported that the mutations of the genes encoding proteins that interact with titin, such as *ANKRD1* and *TCAP*, were identified in DCM patients [39,40].

HCM is characterized by left ventricular hypertrophy and myocardial disarray [34]. Most HCM patients show an autosomal dominant trait and mutations in different genes, especially encoding the sarcomere proteins. Although previous reports showed that missense mutations were identified in HCM patients in the *Z*-disk and I-band regions in *TTN* [41,42], the frequency of the *TTN* truncating variants did not significantly differ between HCM patients (1%) and the control subjects (3%) [6]. The pathogenic role of the *TTN* variants in HCM remains to be determined. RCM is characterized by restrictive filling of the ventricles with preserved biventricular systolic function. A *TTN* missense variant (22862A > G) within the FN-III domain in the I-band and A-band junction was observed in six RCM individuals within a family [43]. ARVC is characterized as a symptom of ventricular arrhythmia due to fibrofatty replacement of the right ventricle with preserved left ventricular function. Among seven different probands with an ARVC phenotype, eight *TTN* rare variants were identified in the I-band region [44]. Additionally, it has been reported that the A178D missense mutation of *TTN* affecting a conserved residue in the second Ig domain in the *Z*-disk was observed in familial cardiomyopathy with features of left ventricular noncompaction, showing prominent left ventricular trabeculae and a thin compacted layer [45] (Fig. 1D). As the genotype-phenotype correlation remains to be determined, functional investigations are required to understand the mechanisms by which *TTN* mutations lead to the specific types of cardiomyopathies.

The two main titin isoforms are N2A and N2B in the I-band region (Fig. 1C) [1]. Skeletal muscles express the N2A isoform, while alternative splicing results in the cardiac-specific isoforms of N2B and N2BA, which contain unique elements of both the N2B and N2A. These cardiac N2B and N2BA isoforms are co-expressed in the sarcomere at different ratios depending on the developmental stage and disease state [46]. The N2BA/N2B ratio determines passive stiffness, which is increased in heart failure [46]. The isoform switching is regulated by the splicing factor RNA-binding motif protein-20 (RBM20) [47]. A loss of function mutation in the *RBM20* causes pathological titin isoform expression [47]. *RBM20* mutations are present in 1.9–3% of individuals with idiopathic DCM [48], suggesting that the post-transcriptional regulation of titin is linked to the pathogenesis of DCM and heart failure.

5. Utility of the urinary titin fragments for diagnosis of MD and cardiomyopathies

The N- and C-terminal fragments of titin were most frequently

Table 1
Clinical usefulness of urinary titin fragment in muscular dystrophies and dilated cardiomyopathy.

	Cut-off value of U-TN/Cr (pmol/mg/dl)	AUC or c-statics	Sensitivity, %	Specificity, %	Reference
Diagnostic strategy					
DMD	3.84	1.0	100	100	Ref No. [51]
DMD/Becker muscular dystrophy	3.52	0.99	98.9	100	Ref No. [51]
MD with cardiomyopathy	8.7	0.92	100	82	Ref No. [53]
Prognostic strategy in DCM					
Cardiac mortality at 1 year	8.3	0.73	75	76	Ref No. [54]
All-cause mortality at 1 year	8.3	0.78	73	78	Ref No. [54]

DMD indicates Duchenne muscular dystrophy; MD, muscular dystrophy; DCM, dilated cardiomyopathy; U-TN/Cr, urinary titin N-terminal fragments normalized by urinary creatinine and AUC, area under the curve.

detected among the titin-derived peptides in MD patients by comprehensive proteome studies [49]. Recently, the measurements of urinary titin N-terminal fragments (U-TN) of 200 residues have been established by using a sandwich enzyme-linked immunosorbent assay (ELISA) [50]. The titin N-terminal fragments, which are cleaved by calpain-3, are excreted into the urine via glomerular filtration [49]. U-TN indicates the sarcomere damage in the myocytes and loss of muscle mass. It has been reported that U-TN is a useful marker for the diagnosis of patients with DMD and Becker muscular dystrophy (BMD) [51]. DMD and BMD patients are both X-linked recessive disorders caused by mutations of the *DMD* gene. In DMD patients, U-TN normalized by urinary creatinine (U-TN/Cr) was 700-fold higher than in healthy controls [51]. The analysis of the receiver operating characteristic curve (ROC) demonstrated that the area under the curve (AUC) for U-TN/Cr was 1.0, and when the cut-off value of U-TN/Cr was set at 3.84 pmol/mg/dl, both sensitivity and specificity were 100% (Table 1). In addition, the U-TN/Cr was significantly higher in DMD than in BMD patients, but BMD patients showed significantly higher U-TN/Cr than in the normal controls. AUC for U-TN/Cr was 0.99 in all DMD and BMD patients with 98.9% sensitivity and 100% specificity at the cut-off value of 3.52 pmol/mg/dl. Thus, U-TN could be a useful tool for the screening of MD. It should be clarified whether and how U-TN can serve as a biomarker for monitoring disease progression of MD. Due to low prevalence of MD diseases, extensive clinical research is needed to elucidate whether differential U-TN level can be observed among the types of MD and to clarify how U-TN can serve as the management of MD patients.

It is important to detect early cardiac involvement in MD patients because cardioprotective medical treatments can delay the onset and progression of cardiac dysfunction and symptoms of heart failure in those patients [52]. We reported for the first time that U-TN is a useful biomarker for the differential diagnosis of cardiomyopathies [53]. The level of U-TN/Cr in MD patients was significantly higher than that in patients with other cardiomyopathies including DCM, HCM, sarcoidosis, amyloidosis, Fabry disease, and in the control subjects (Fig. 2). ROC analysis showed that the U-TN/Cr identified MD with 100% sensitivity and 82% specificity with a cut-off value of 8.7 pmol/mg/dl. AUC of U-TN/Cr was superior to that of other markers such as creatinine kinase, B-type natriuretic peptide (BNP) and troponin I. Furthermore, we reported that applying the U-TN could be a useful predictor of DCM patients [54]. We followed up 102 DCM patients over a mean period of 1167 days. The patients were divided into three groups based on the U-TN/Cr: first (U-TN/Cr < 3.35 pmol/mg/dl), second (3.35 ≤ U-TN/Cr < 7.26 pmol/mg/dl), and third (7.26 pmol/mg/dl ≤ U-TN/Cr) tertiles. Cardiac and all-cause mortality were progressively increased from the first to the second and the third groups, indicating that U-TN/Cr can effectively identify high-risk patients with DCM. The ROC analysis revealed that the U-TN/Cr (cut-off value of 8.3 pmol/mg/dl) predicts cardiac mortality at 1 year with 75% sensitivity and 76% specificity, and all-cause mortality at 1 year with a sensitivity of 73% and specificity of 78%. The C-statistics demonstrated that the prognostic value of U-TN/Cr was comparable to those of BNP and troponin I and superior to that of LVEF for cardiac mortality. Regarding all-cause mortality, the prognostic value of U-TN/Cr was comparable to that of BNP, and superior to those of troponin I and LVEF, which are generally known as important prognostic markers of DCM. Thus, U-TN/Cr could be a novel marker for the differential diagnosis in cardiomyopathies, as well as for risk stratification in DCM patients. Larger population-based studies are required to strengthen the significance of U-TN, and future works will clarify whether U-TN can be a powerful tool for monitoring for disease progression and therapeutic management in DCM patients.

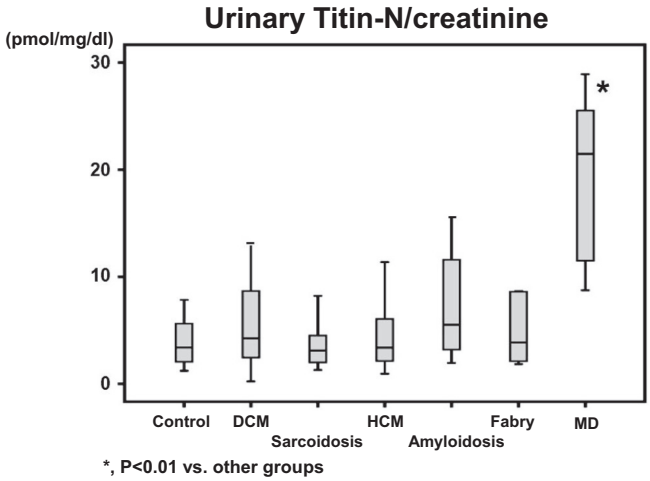

Fig. 2. Comparisons of urinary levels of titin-N/creatinine ratio among study subjects (permitted from reference No. 53).
*, $P < .01$ vs. other groups.
DCM indicates dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; and MD, muscular dystrophy.

6. Summary and conclusions

Titin plays central roles in skeletal and cardiac sarcomere structures and functions and is associated with the cause of MD and cardiomyopathies. NGS technologies provide comprehensive DNA mutation analysis and identify new mutated sites of *TTN* that are responsible for such diseases. A single protein of titin can lead to a variety of diseases of the skeletal and/or cardiac muscles. *TTN* sequencing can be adopted in diagnostic laboratories and for familial screening. In contrast, as *TTN* truncating variants have been found in healthy people, further mechanistic insights of the genotype-phenotype correlation are required. In addition to the new sequence, a novel ELISA method for the detection of the urinary titin has been introduced. Quantification of U-TN is useful for the clinical diagnosis of DMD and BMD, and MD with cardiomyopathy, or risk stratification of DCM patients. U-TN was superior to or comparable with known other biomarkers, such as BNP and troponin I for diagnosing the MD with cardiomyopathy, and for predicting

the prognosis in the DCM. Further analysis is needed to elucidate whether and how the U-TN can be useful in other diseases, such as heart failure and types of myopathies. In conclusion, titin is an emerging target for genetic stratification and a diagnostic or prognostic biomarker, as well as a potential therapeutic target for MD and cardiomyopathies.

Potential conflicts of interest

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