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INVITED REVIEW

Titin mutations and muscle disease

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Abstract

The introduction of next-generation sequencing technology has revealed that mutations in the gene that encodes titin (TTN) are linked to multiple skeletal and cardiac myopathies. The most prominent of these myopathies is dilated cardiomyopathy (DCM). Over 60 genes are linked to the etiology of DCM, but by far, the leading cause of DCM is mutations in TTN with truncating variants in TTN (TTNtvs) associated with familial DCM in ~ 20% of the cases. Titin is a large (3–4 MDa) and abundant protein that forms the third myofilament type of striated muscle where it spans half the sarcomere, from the Z-disk to the M-line. The underlying mechanisms by which titin mutations induce disease are poorly understood and targeted therapies are not available. Here, we review what is known about TTN mutations in muscle disease, with a major focus on DCM. We highlight that exon skipping might provide a possible therapeutic avenue to address diseases that arise from TTNtvs.

Keywords Titin · Dilated cardiomyopathy · Mutations · TTNtv · Exon skipping

Introduction

Titin is a giant myofilament that extends from the Z-disk (N-terminus) to the M-band (C-terminus) region of the sarcomere and is encoded by the *TTN* gene [11, 37, 43, 44, 69]. Due to its enormous size, TTN has been insufficiently analyzed in the past. However, recent whole genome sequencing studies revealed that TTN is a major human disease gene [13, 26, 45, 56, 74, 75, 96, 98, 99]. Many titin mutations are also linked to neuromuscular diseases [20, 26, 87, 89, 98], but this review mainly focuses on the role of titin in cardiomyopathies where TTNtvs have been studied most.

The human titin gene contains 364 exons, of which 363 exons are coding exons. Titin has a maximum molecular mass of ~ 4200 kDa [11, 69] and has a modular domain composition consisting of immunoglobulin (Ig) and fibronectin type III (FnIII) domains and unique sequences [69, 106] (see Fig. 1 supplemental Table S1). Titin provides passive stiffness to the striated muscle sarcomere and modulates active contractile force [4, 9, 16, 18, 33–36, 42, 50, 73, 79, 104]. Titin's N-terminus is embedded in the Z-disk and acts as a mechanosensor [65]. The I-band region of titin functions as a molecular spring and is the main determinant of cardiac myocyte elasticity in cardiac muscles [25, 42, 75, 77, 113, 117]. It comprises three distinct elements, the tandem Ig segment, the PEVK region (rich in proline, glutamic acid, valine, and lysine residues) and the N2B element, containing the extensible N2B unique sequence (N2B-Us) [11, 55, 69]. The spring elements can be posttranslational modified, altering their elastic behaviors [8, 49, 53, 54, 58, 59, 92, 121]. In addition to providing elasticity, these segments also interact with signaling proteins and have been proposed to function as mechanosensor complexes [43, 67, 77, 81, 88, 95, 114] with mouse models that genetically target individual spring elements supporting such roles [15, 23, 47, 61, 93, 94]. The A-band segment of titin contains 178 Ig and Fn3 domains and is functionally inextensible [16, 69, 106]. The A-band segment contains the so-named I/A zone, D-zone, C-zone, and M-band regions (supplemental Table S1). The IA zone is near the ends of the

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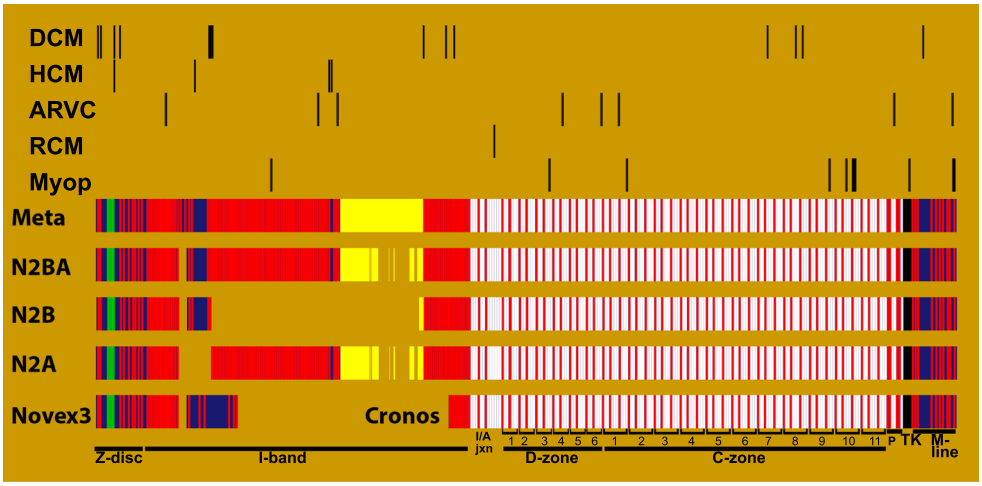

Fig. 1 Titin isoforms and mapped disease-associated missense mutations. Titin isoforms assembled from the metatranscript, cardiac N2BA, cardiac N2B, skeletal muscle N2A, Novex3 and Cronos transcripts (from top to bottom). See text for details. Missense mutations causing DCM, HCM, ARVC, RCM and myopathy are shown by vertical lines mapped on the protein domains where they occur. Missense mutations downloaded from the TITINdb (http://fraternalilab.kcl.ac.uk/TITINdb/), see Laddach et al. [71]. Domain colors: red: Ig domains, white: Fn domains, green: Z-repeats, yellow: PEVK sequence, blue: unique sequences

thick filaments and is striking in that the regular domain patterns of Ig and FnIII domains is broken with a stretch of 6 FnIII domains that is found preceding the D zone. It has been suggested that the unique domain composition of the IA zone reflects an alteration in titin–myosin interaction that is critical for the termination of the thick filament [14]. However, a mouse model in which titin's IA junction was targeted revealed that deleting the IA junction does not alter thick filament length [48]. In the D-zone region of the A-band, Ig and FnIII domains form 6 repeats, each containing 7 domains and in the C-zone 11 Ig and FnIII domains form super-repeats, each containing 11 domains [69]. The C-zone region of titin likely plays a role in anchoring MyBP-C [31], regulating actomyosin interaction [82], and regulating the thick filament length [103]. Titin's M-band region contains the serine/threonine kinase (TK) domain and is involved in numerous signaling pathways [19, 39, 83, 90, 91, 115, 116]. Moreover, exon 363 (Mex5), coding for is7 domain in the M-band region, is differentially spliced and gives rise to is7+ and is7- titin isoforms [21, 66].

Extensive mRNA splicing results in distinct titin isoforms [11, 70] (Fig. 1). In the heart, three titin isoform classes are present: fetal cardiac titin (3.5–3.6 MDa), adult N2BA (~ 3.3 MDa), and adult N2B (~ 3.0 MDa) isoforms [11, 69, 72]. An important titin splicing factor is RBM20. Deficiency in RBM20 is leading to increased expression of large N2BA-type titin isoforms in the adult heart [50, 61, 79, 80]. In addition to full-length titins, isoforms that are not full-length also exist (Fig. 1). Novex-3 titin, a ~ 700 kDa titin isoform is found in cardiac and skeletal muscle [11, 46, 64]. The 3′ end of novex-3 contains the stop codon polyadenylation signal and functions as an alternative C-terminus, resulting in a truncated titin isoform [11]. Unlike full-length titin isoforms, novex-3 is too short to reach the A-band region [11, 96]. Recently, an alternative start site has been identified in the titin gene that is predicted to results in expression of cronos titin, a ~ 2000 kDa isoform that lacks the Z-disk and most of the I-band domains but contains the A-band and M-line domains [123]. The functions of novex-3 and cronos titin have not been established. Due to alternative splicing, adult full-length cardiac isoforms differ in the length of their tandem and PEVK segments in the I-band and their stiffness varies accordingly [11, 17, 117] [32]. The adult full-length cardiac isoforms (N2B and N2BA) are co-expressed at the level of the half sarcomere [105]; their expression ratio is approximately 50:50 in humans [84, 85] but can vary in disease states [84, 85, 118–120].

Titin gene mutations as a cause of cardiomyopathies

Cardiomyopathies are diseases that cause primary abnormalities in the heart muscle [57]. The most common type is dilated cardiomyopathy (DCM) with a prevalence of up to ~ 1:250 [57, 99]. DCM is characterized by left ventricular dilation and systolic dysfunction [57]. Recent landmark sequence studies in large patient cohorts revealed that mutations in the titin gene (TTN) are responsible for ~ 20% of all DCM cases [56, 96, 99]. Many of the DCM-causing TTN mutations are heterozygous truncating variants (TTNtv) that include frameshift, nonsense, and essential splice site mutations and are over-represented in the A-band segment of titin [56, 96], see Fig. 1. Moreover, TTNtvs show a high penetrance after the age of 40 years and there is a possibility that secondary stressors are needed to develop DCM phenotype [27, 56]. Titin missense mutations

are also likely to contribute to a small fraction of DCM [13, 38] and they are a rare cause of hypertrophic cardiomyopathy (HCM) and of arrhythmogenic right ventricular dysplasia [10, 16, 56, 75, 102] (Fig. 1).

TTNtv-induced DCM

Epidemiology and penetrance of TTNtv DCM is the most common indication for heart transplantation and is associated with TTNtv in ~20% of DCM cases [56, 57, 96, 99]. Surprisingly, 1–3% of the general population has a TTNtv but the overwhelming majority does not present a cardiac phenotype, and thus, the genotype-phenotype relationship of TTNtvs is uncertain [5–7, 56, 99]. Clearly, it is important to focus on the underlying mechanisms of TTNtv-induced DCM.

Localization of TTNtv TTNtv are predominantly found in the A-band region of titin and show a position-dependent manner with increasing disease severity closer to the C-terminus [56, 60, 96, 99]. Recently, TTNtv-induced DCM has also been associated with Z-disk, I-band, and M-band exons in a small subset of patients [99]. The position-dependent effect might be explained by TTN exon usage in left ventricular tissue, characterized by the relative incorporation of exons into titin transcripts, termed proportion spliced-in (PSI) [96]. Constitutively expressed exons have high PSI values, whereas exons that are subject to alternative splicing show low PSI scores [27, 96]. Therefore, titin's A-band exons that have high PSI scores and are incorporated in all titin isoforms are most affected by TTNtvs [27, 60, 96]. Notably, exons in the I-band region where intense alternative splicing occurs have low PSI values [96]. Consequently, I-band exons with TTNtv can be excluded from the transcript without resulting in a frameshift, acting as a natural 'exon-skipping' mechanism [77, 96]. Hence, it has been suggested that TTNtv can be tolerated in the healthy population because the majority of the mutations fall in I-band exons that are subject to alternative splicing [60, 96]. It has also been proposed that the upregulation of cronos titin [24], a novel titin isoform driven by an internal promoter (Fig. 1), could rescue the effects of truncating mutations that localize proximal to its internal I-band promoter [24, 123]. Truncating variants in the novex-3 exon that functions as an alternative C-terminus occur equally in patients with DCM and in healthy controls [96, 99, 110]. Although currently there is lack of evidence for pathogenicity of novex-3 titin mutations [96], whole-exome sequencing technologies are enabling the identification of novel rare cardiomyopathy-causing titin truncating variants [101] and it is possible that in future studies novex-3 titin truncating mutations will be shown to play a role in the pathomechanism of some cardiomyopathies [22, 64].

Currently, there is much uncertainty about the exact mechanism by which titin truncating mutations lead to a cardiac phenotype. Multiple mechanisms have been proposed to explain TTNtv-induced DCM: haploinsufficiency, poison-peptide/dominant-negative mechanism, and perturbation of cardiac metabolism and signaling.

Haploinsufficiency Schafer et al. developed 2 rat strains and modeled a proximal and distal TTNtv mutation and their RNA-seq study revealed a profound nonsense mediated mRNA decay (NMD) of the allele with TTNtv, indicating haploinsufficiency [99]. However, protein gels did not reveal truncated titins, suggesting that either no truncated proteins are produced or that they are produced but rapidly degraded [99]. Moreover, total protein levels of full-length titin appear not different, suggesting an upregulation of the wild-type allele, consistent with the transcript findings of the Schafer study [99]. To study the effect of titin deficiency, Radke et al. generated a conditional KO mouse model with progressive postnatal loss of the complete titin protein achieved by removing exon 2 (E2-KO) [94]. Results showed that titin deficiency leads to sarcomere disassembly and atrophy in striated muscle and eventually DCM. Overall, these animal studies suggest a need to further investigate the haploinsufficiency mechanism in DCM patients with TTNtvs.

Poison peptide mechanism Another possible mechanism by which TTNtv can induce DCM is the poison peptide/dominant-negative mechanism. A limited amount of truncated protein has been found in induced pluripotent stem cell (iPSC) cardiomyocytes derived from patients with TTNtv [60]. Additionally, heterozygous TTNtv mutant iPSC-s have fewer myofibrils and show sarcomere disorganization [60]. A recent study by Schick et al. also demonstrates defects in sarcomere assembly in patient-derived iPSC cardiomyocytes [100]. In a large DCM patient cohort, Roberts et al. found that TTNtv containing transcripts are not subjected to NMD and no changes in the protein expression levels of major titin isoforms are detectable, suggesting the possible role of poison peptide/dominant-negative mechanism in TTNtv-related DCM [96].

Perturbation of cardiac metabolism and signaling It is known that mTORC1, which functions as a nutrient/energy sensor and controls protein synthesis, is activated in DCM patients [99, 122]. It is of interest therefore to determine whether distinct molecular pathways are associated with TTNtv-based DCM. Indeed, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis suggests altered cardiac metabolism in TTNtv rats, independently of the position of the truncation [99]. Furthermore, biochemical analysis revealed a shift from fatty acids toward glycolysis, similar to those seen in the failing heart that may be adaptive [99]. The levels of metabolites that can activate mTOR are also increased in TTNtv rats

[99]. Interestingly, major signaling pathways, involving transforming growth factor-β, vascular endothelial growth factor, and mitogen-activated protein kinases, that are critically important to cardiomyocyte function, are diminished in iPS-derived cardiac cells containing TTNtv [60, 110]. Additionally, Verdonschot et al. found that all components of the mitochondrial electron transport chain are significantly upregulated in patients with TTNtv, leading to pronounced cardiac alterations in mitochondrial function [109]. In accordance with these alterations, Zhou et al. found decreased oxygen consumption rate, elevated reactive oxygen species (ROS) levels and increased mitochondrial protein ubiquitination in rat hearts with TTNtv, indicating mitochondrial dysfunction caused by TTNtv [2]. Additionally, TTNtv hearts show increased mTOR phosphorylation and impaired autophagy function [2]. Interestingly, mutated iPSC cardiomyocytes, derived from DCM patients with TTNtv, show attenuated response to isoproterenol, $[Ca^{2+}]_{out}$ and angiotensin II. Furthermore, mutated cells display a longer recovery period after caffeine administration [100]. These changes suggest altered function of calcium-handling proteins, such as SERCA, phospholamban (PLB), and calsequestrin [100]. Overall, the importance of changes in cardiac metabolism and calcium handling in DCM caused by TTNtv warrant further investigation, including whether these changes develop directly from the truncating mutation or, more likely, are secondary effects.

Is a TTNtv sufficient to induce a phenotype or are modifying effects required?

Not all individuals that carry a TTNtv develop DCM and a multifactorial disease model has been proposed where multiple factors contribute to the development of a TTNtv-based phenotype [27, 99]. In this model, a second genetic variant and/or environmental stressor is needed, as a 'second or third hit', to uncover the effects of the TTNtv.

Sex differences Interestingly, the onset of DCM is ~ 40 years and the penetrance of TTNtv is sex dependent [30, 56]. The median age of onset in males is estimated to be 28 years and 56 years in females [30]. In addition, women carrying TTNtv mutations have a better prognosis than men [30, 56]. Further studies are needed to establish whether the sex dependence might be more related to the link between titin phosphorylation and increased oxidative stress [12, 30] and whether the cardioprotective effects of estrogen in premenopausal women contribute to sex-related differences [62, 76].

Peripartum cardiomyopathy TTNtv have also been linked to peripartum cardiomyopathy (PPCM) where the distribution of truncating variants in PPCM is similar to that found in DCM [108, 112]. PPCM can also be a manifestation of familial DCM and TTNtv in PPCM patients is a possible prognostic factor for low recovery rate [108, 112].

Second mutation Often additional rare truncating variants or other pathogenic cardiomyopathy genes are present in TTNtv carriers that can increase the severity of DCM or can be associated with an earlier onset of the disease [51, 56, 86, 97].

Environmental factors Although the onset of TTNtv-induced DCM is ~ 40 years [56], environmental insults, such as chemotherapy, can induce *pediatric-onset* DCM cases [28]. Furthermore, TTNtv can be associated with a more severe form of chemotherapy-induced cardiomyopathy (CCMP). Recently, it has been reported that patients with TTNtv have a prevalent genetic predisposition for alcoholic cardiomyopathy and an even more impaired ejection fraction can be observed in TTNtv-induced DCM patients with alcohol abuse [110]. Therefore, alcohol is an additional environmental risk that can contribute to a more severe outcome of TTNtv-associated DCM. Finally, Gramlich et al. showed that hemodynamic stress caused by angiotensin II or isoproterenol can induce a more severe phenotype in heterozygous TTNtv mice compared to control litter mates [40]. This finding suggests that hypertension, a common risk factor for heart disease and stroke [52], results in a more severe form of DCM in patients with TTNtv [40].

In summary, many additional genetic and environmental factors can influence the outcome of an existing TTNtv. In most of the cases these stressors can unmask the effects of TTNtv or induce an even more severe DCM phenotype.

Comparison between TTNtv- and TTNtv+ DCM

To date, there are contradictory observations in patient populations about the symptoms and differences between DCM patients with (TTNtv+) or without (TTNtv-) mutations. Furthermore, as discussed above, there is much debate about the genotype-phenotype relationship of TTNtv in DCM, as truncating titin mutations can be found in 1–3% of the general population [5, 7, 56, 99]. Herman et al. showed no significant differences in clinical manifestations between TTNtv+ and TTNtv- subjects, including the risk of major cardiac events [56]. Similarly, others reported that TTNtv+ does not appear to be associated with worse prognosis and DCM patients with TTNtv are unaccompanied by conduction disease [30]. Although, Verdonschot et al. found more life-threatening arrhythmias in TTNtv+ patients associated with enhanced interstitial myocardial fibrosis, the survival rate was similar between TTNtv+ and TTNtv- patients at long-term follow-up [109]. Interestingly, recent whole-exome sequencing studies

by Ahlberg et al. identified TTNtv as a major genetic contributor to atrial fibrillation [3]. A new zebrafish model that contains a TTNtv mutation displays increased fibrosis and altered sarcomere structure in the atria. Moreover, TTNtv+ zebrafish show electrophysiological defects that could potentially develop into arrhythmia [3]. Comparing TTNtv+ and TTNtv- DCM patients, Roberts et al. observed more severely impaired left ventricular (LV) function, lower stroke volumes, and more sustained ventricular tachycardia in TTNtv+ patients [96]. Furthermore, patients with TTNtv are at higher risk to more adverse cardiac events, as death, cardiac transplant, or LV assist device [96]. Overall, it is still uncertain whether or not patients with TTNtv have more severe symptoms compared to TTNtv- DCM patients.

Patients with DCM caused by TTNtv respond to standard DCM therapies [63] and long-term prognosis is similar to that of patients without TTNtvs [29, 109]. Recovery from TTNtv-associated PPCM is also possible with proper and careful medical assistance [68]. Based on the metabolic changes in TTNtv+ humans and animal models, mTOR pathway modulation with metformin or 'rapalogues' (rapamycin analogues) could serve as a potential treatment for TTNtv-induced DCM [2, 110]. Zhou et al. observed that the mTORC1 inhibitor rapamycin is able to rescue the attenuated autophagy in rat hearts containing TTNtv mutations [2]. Additionally, research groups are focusing on exon-skipping approaches to cure TTNtv-associated DCM. Most TTN exons can be deleted while keeping the reading frame intact. The deletion of a large TTN exon induced by antisense oligonucleotides has been accomplished [41], but it is currently uncertain how well the absence of exons is tolerated or whether it might lead to a cardiac phenotype at some stage of life. An exon-skipping therapeutic strategy has already been approved by the Food and Drug Administration (FDA) for use in Duchenne muscular dystrophy [1, 110], and the hope is that similar exon-skipping approaches are feasible and be beneficial in TTNtv patients as well. In summary, exon skipping has the potential to cure TTNtv-induced DCM but much research is required first, particularly focused on possible off-target effects that might occur.

Conclusions and perspectives

It is now well established that TTN is a major human disease gene that causes multiple neuromuscular and cardiac diseases [13, 20, 26, 56, 74, 75, 89, 96, 98, 99]. Most studies are currently focused on TTNtv that cause dilated cardiomyopathy [56, 96, 99]. Even though TTNtv mutations are likely to affect ribosome activity [99], sarcomeric organization [40, 60], and alter cardiac metabolism [99, 109], a clear genotype-phenotype correlation is often lacking. Indeed, 1–3% of the general population has a TTNtv, and it has been proposed that additional genetic and/or environmental stressors might be needed to unmask the effects of TTNtv [40, 78, 97, 108, 110, 111]. Although TTNtv+ patients present more life-threatening arrhythmias associated with enhanced interstitial myocardial fibrosis, the survival rate is similar between TTNtv+ and TTNtv- patients at long-term follow-up [29, 109]. In addition, TTNtv-associated DCM patients respond well to standard DCM therapies [63]. Mimicking natural skipping of exons with low PSI scores [77, 96], exon skipping with antisense oligonucleotides could provide a more specific treatment option for patients with DCM caused by TTNtv. Clearly, more research is required into the pathomechanism by which TTNtv mutations induce DCM and into the possibility of exon skipping as a therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any primary studies with human participants or animals performed by any of the authors.

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