TTN GENE'S VARIANTS AS POTENTIAL MARKERS ASSOCIATED WITH MUSCLE TISSUE'S DISFUNCTIONS AND PHYSICAL PERFORMANCE

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Abstract

A titin, encoded by a *TTN* gene, is a third most abundant sarcomere component. Although, this myofilament plays a wide range of key roles in muscle tissue such as structural, developmental, mechanical, and regulatory functions, it is a usually missed aspect of the muscle properties formation. At first, the *TTN* gene variants was described in development of skeletal and cardiac muscle diseases. Recently, the gene is also considered a very promising genetic marker for sport performance which may underlie differences in the potential to be an elite athlete. The aim of the present study is to provide the comprehensive update of the titin protein and the *TTN* gene variants role in formation of skeletal and cardiac muscle properties. We review function and structure of the protein, the gene, and the isoforms, as well as molecular mechanisms, disease-causing mutations, associated phenotypes, and their implications for human health, physical performance, adaptive changes of muscles in response to training, and injury risk.

Key words: *sport genetics, titin, muscle performance, exercise, injury risk, human health*

Introduction

Human skeletal muscles occupy 40%–50% of whole body mass and play a key role in locomotion and body metabolism, with individual differences in the muscle structural and functional properties being linked to ability to perform aerobic or anaerobic exercise, and consequently athlete status. The basis of this heterogeneity is determined by diversity of a combination of morphological and neural factors, as well as a range of adaptive changes of muscles in response to training program. The mechanisms that influence the muscle performance is multifactorial and can be affected by various factors such as initial strength, training status, diet, and genetics (Blaauw et al., 2013; Suchomel et al., 2018).

Muscle stiffness, described as the ratio between changes in force and muscle deformation, is one of key factors influenced muscle performance - increased tissue stiffness can enhance force transmission (Rieder et al., 2015; Suchomel et al., 2018; Maciejewska-Skrendo et al., 2020). Therefore, the muscle length and cross-sectional area, the forces applied, and the structures within the muscle such as actin, myosin, titin, and connective tissue affect muscle strength and related traits for example rate of force development and power (Baumgart et al., 2000; Suchomel et al., 2018; Maciejewska-Skrendo et al., 2020). However, a usually missed aspect of the muscle structural and functional properties formation is the role of a main sarcomere component, a titin (Powers et al., 2016).

The titin, encoded by a *TTN* gene, is the largest known protein which constitutes a third most abundant myofilament in human muscles (after myosin and actin). It performs a wide range of important functions in muscle tissue for example provides scaffold for sarcomere assembly and organization during muscle development, maintains structural integrity of sarcomere during contraction, generates passive tension, as well as serves as a sensory and signaling mediator (Labeit et al., 1997; Chauveau et al., 2014; Ge et al., 2019). However, the mentioned above properties can vary significantly according to titin isoform expression. This heterogeneity may be a favorable or unfavorable factor for sport performance and may be associated with different post-training changes in human muscles (Stebbings et al., 2017).

A literature search revealed that the *TTN* gene is very promising genetic marker for sport performance which may underlie differences in the potential to be an elite athlete. However, analysis of the whole *TTN* gene are very difficult, due to its large size and complex structure. Recently, with development of technology for rapid DNA sequencing becoming widely available, the examination of the *TTN* gene structure and the identification of the individual polymorphisms that contribute to human health and athletic performance have been possible. So far, more than 120 *TTN* coding sequence variants associated with human phenotype have been reported (Chauveau et al., 2014). However, the sequencing of the *TTN* gene in various populations, as well as diversity of isoforms and reference sequences used, stress difficulties in assessing the importance and association with the phenotype of the *TTN* sequence

changes. Thus, the role of the *TTN* variants potential modifying effect on parameters linked to elite athlete status is almost unknown.

The main aim of the present study is to review current progress in the understanding of the titin protein and the *TTN* gene variants role in formation of skeletal and cardiac muscle properties. We discuss function and structure of this myofibril's protein, the gene, and the isoforms, as well as molecular mechanisms, disease-causing mutations, associated phenotypes, and their implications for human health, physical performance, adaptive changes of muscles in response to training, and injury risk.

Function and structure of titin protein

The titin (also known as a connectin) has been described in late 70s (Maruyama, 1976) as a main sarcomere component. It is the largest known protein (3-4 MDa) that extends from the Z-disk to the M-line (Granzier & Labeit, 2005, 2006; Labeit & Kolmerer, 1995). In the structure of the titin, four main structurally and functionally distinct regions can be identified: an amino-terminal Z-disc region, a middle I-band and A-band regions, and the carboxy-terminal part spanning the M-line. The protein structure is very repetitive, with many sequences' domain repeats (Ge et al., 2019; Chauveau et al., 2014).

The N-terminal part acts as anchor, is embedded in the Z-disc binding to proteins such as α-actinin while the titin myofilaments from adjacent sarcomeres overlap within the Z-discs (Gregorio et al., 1998). In fact, this region functions as a mechanical stretch sensor machinery part (Knöll et al., 2002) since the Z-disc, like other regions of the titin, is able to bind to the main sarcomere components (Gautel & Djinović-Carugo, 2016; Linke, 2018; Seto et al., 2011). It contains multiple Ig-like domains, large interdomain sections (is), and two to seven (depending on the muscle type) alternatively-spliced Z-repeats (Zr1 - Zr7) (Chauveau et al., 2014).

The I-band region spans between 0.8 and 1.5 MDa of the titin myofilament, contains highly repetitive domains that functions as a molecular spring, providing elasticity of the protein and maintain the Z- and M-line connections during elongation and contraction of muscles (Granzier & Irving, 1995; Chauveau et al., 2014; Linke, 2018; Wu et al., 2000). The I-band region is formed by: Ig-like domains; three unique sequences named novex-1, -2, and -3; a N2A (skeletal- and cardiac- specific) and a N2B (cardiac-specific) segments; a PEVK region (rich in proline, glutamic acid, valine, and lysine residues); and another Ig-like domains (Bang et al., 2001; Helmes et al., 1999; Labeit & Kolmerer, 1995; Chauveau et al., 2014). The PEVK domain is largely responsible for a spatially hierarchical arrangement of local elasticity: the N-terminal part is the most stiff and the C-terminal part is the most flexible (Nagy et al., 2005).

The A-band region is connected with the myosin in so-called "I/A zone" which is important for the thick filament termination, while the A-band "C zone" is involved in interaction with myosin-binding protein C (Freiburg & Gautel, 1996) and modification of the thick filament length (Tonino et al., 2017). It contains of Ig-like and fibronectin type III (Fn3) domains super-repeat segments distributed in two types of structures (a seven-domain stretch occurring six times and a eleven-domain segment repeated eleven times) (Labeit & Kolmerer, 1995; Tskhovrebova & Trinick, 2004; Chauveau et al., 2014).

The C-terminal part of the titin is localized within the sarcomere M-band and is involved in multiple signaling pathways (Centner et al., 2001; Gotthardt et al., 2003; Peng et al., 2007; Witt et al., 2005b, 2005a). The M-line extremity includes at the periphery a unique serine-threonine kinase domain (TK), which modulates the titin expression and turnover, as well as is negatively regulated by intramolecular interactions with autoinhibitory tail. Additionally, molecular analysis suggests that TK acts as a mechanical strain sensor during muscle activity (Chauveau et al., 2014). The titin myofilaments from opposite half-sarcomeres completely overlap within the M-line region, that has scaffolding role and possibly conveys mechanosensitivity (Zacharchenko et al., 2015). Such overlapping ends connect to the other sarcomere components, which form a contiguous system along myofibrils (Obermann et al., 1996).

Titin gene and isoforms

The titin protein is encoded by the *TTN* gene, which is located on the long arm of human chromosome 2 (locus 2q31.2). It consists of 365 exons of which 363 exons are coding and transcribes a mRNA over 100 kb long (Bang et al., 2001). Due to alternative splicing of the gene, there are multiple transcript variants, what cause variability in the I-band, the M-line, and the Z-disc regions of the titin, and consequently the isoforms with different spring compositions (Freiburg et al., 2000). To date, seven human titin isoforms have been described. The skeletal muscle titin isoforms tend to be longer than the cardiac isoforms in adult tissues (Granzier & Irving, 1995). It is worth to notice that the titin length is associated with variability in titin's elastic properties and in titin-based passive tension in skeletal muscles: the longer isoforms are more elastic, while the shorter isoforms with fewer exons spliced in are more rigid (Linke et al., 1998; Wang et al., 1991). This is connected with postnatal differences in the PEVK region localized in the I-band part, that lead to the shortening of the myofibril, thus increasing titin-based passive stiffness and possibly improving a motor control during the muscle development (Freiburg et al., 2000; Ottenheijm & Granzier, 2010).

The main skeletal muscle N2A isoform is the longest known isoform of the titin, expressing 312 exons, but lacking the exon 49, which encoding the N2B

domain (Bang et al., 2001; Freiburg et al., 2000). The skeletal-specific N2A isoforms have the longest PEVK region when comparted to the cardiac isoforms (Freiburg et al., 2000; Labeit & Kolmerer, 1995). Depending on skeletal muscle types (Prado et al., 2005) and developmental stage (Ottenheijm et al., 2009), numerous different length N2A titin isoforms are generated as a result the alternative splicing processes. The single titin isoform of determined length is expressed in a specific type of skeletal muscles, what may be considered as a modulation mechanism which allows skeletal muscles to adapt the sarcomere's passive stiffness (Prado et al., 2005; Ottenheijm et al., 2009).

In contrast, the cardiac-specific N2B isoform, produced form 191 exons (specifically: exons 49/50 to exon 219), lacks expression of both the N2A exon and some PEVK-encoding regions (Bang et al., 2001; Freiburg et al., 2000; Lahmers et al., 2004). The other major cardiac isoform, N2BA, is encoded by up to 313 exons, specifically: exon 49 (coding for the N2B element) and exons 102–109 (coding for the N2A element). It contains both the N2A and the N2B exon-encoded domains, the PEVK region, and additional the Ig domains. Due to alternative splicing, both the cardiac-specific isoforms may also differ in length of the Ig and the PEVK domains, what causes variability in myocardial stiffness (Cazorla et al., 2001; Freiburg et al., 2000; Wu et al., 2000). The isoforms are co-expressed with expression ratios N2BA:N2B of approximately 50:50 (range from 30:70 to 40:60) (Neagoe et al., 2002, 2003).

Properties of the different titin isoforms are not only dependent on the alternative splicing. The titin molecules undergo the posttranslational modifications which modify its elasticity and passive stiffness of the muscles. Phosphorylation and oxidation changes in the I-band part was associated with failing hearts, thus they are thought to cause pathological myocardial stiffening in patients with systolic or diastolic heart failure (Beckendorf & Linke, 2015; Hamdani et al., 2017). The analyses have confirmed that phosphorylation of the N2B segment lowers titin stiffness (Hamdani et al., 2013; Krüger et al., 2009; Yamasaki et al., 2002), whereas phosphorylation of the PEVK region rises it (Hidalgo et al., 2009). It is worth to notice, the titin expression pattern is shifted toward an increased proportion of the more elastic N2BA isoforms than the stiffer N2B isoforms in failing hearts (Makarenko et al., 2004; Nagueh et al., 2004; Neagoe et al., 2002). Consequently, it decreases sarcomeric stiffness, which could be a compensatory mechanism preventing from the increased myocytes stiffness resulted from changed titin phosphorylation often noted in failing hearts (Linke, 2018).

TTN gene mutations and skeletal muscle diseases

Considering the wide range of important functions which the titin performed in muscle tissue, the *TTN* gene variants are associated with human conditions concern the numerous skeletal and/or cardiac muscle diseases. One of these disorders is muscular dystrophy (MD) with genetic background resulting in increasing weakness and atrophy of skeletal muscle mass. Studies of MD patients showed that mutations of the *TTN* gene causing the titin protein changes are linked to specific disease types from this group – such muscle disorders are named as titin myopathy or titinopathy. The disease severity and affected muscles are different depending on the *TTN* mutations localization and type. To date, about forty *TTN* mutations have been associated with four skeletal muscle disorders with no cardiac involvement: tibial muscular dystrophy (TMD), limb girdle muscular dystrophy (LGMD), hereditary myopathy with early respiratory failure (HMERF), and centronuclear myopathy (CNM), as well as in few patients with uncharacterized muscle diseases (Vasli et al., 2012; Evila et al., 2014).

The first described human titinopathy was TMD, an autosomal-dominant late-onset distal myopathy, that begins after the age of 30 and involves the leg muscles (Udd et al., 1993). It is caused by missense mutations or insertion-deletion found in exon 363 encoding the titin M-band (Hackman et al., 2008), mutations in exon 340 for the A-band (Evilä et al., 2017), as well as missense mutations in the A-band and frameshift mutations in the *TTN* (Evilä et al., 2014). Next disease, LGMD, presents in the first two decades of life and affects predominantly hip and shoulder muscles (Hackmac et al., 2008). It is caused by a nonsense mutation in the last exon of the *TTN* gene (Pénisson-Besnier et al., 2010), as well as mutation designed as 107788T>C (W35930R) (Zheng et al., 2016). HMERF, also named Edström myopathy, is an autosomal-dominant disease, which is a slowly progressive myopathy that usually stars in the third to fifth decades of life. The common symptoms are gait disturbance linking to distal leg weakness or nocturnal respiratory symptoms due to weakness of respiratory muscles. The weakness finally generalizes and involves both proximal and distal muscles. At first, HMERF was initially reported only in Swedish families, however, later it has been identified in families of different origins. To date, all described mutations causing HMERF are localized in the *TTN* exon 344, encoding the 119th Fn3 domain in the titin A-band and considered quite rare. Recently, HMERF in several newer North European families have been associated with mutation c.95134T>C p.C31712R (Palmio et al., 2019). In contrast, CNM, named after the presence of numerous muscle fibers with centrally localized nuclei, is a congenital myopathy that usually presents in infancy or childhood with amyotrophy and weakness involving cranial, axial, proximal, and distal muscles. A recent study identified 12 autosomal-recessive mutations of the *TTN* gene: ten truncating and two in-frame insertions/deletions, all present at the compound heterozygous state in the affected patients (Chauveau et al., 2014).

TTN gene mutations and cardiomyopathies

In cardiac muscle the titin protein also has a multifunctional role, especially in proper sarcomeric assembly, thus the *TTN* gene mutations would also have consequences for physiological cardiac function. The detailed molecular studies indicated that targeted mutations in the *TTN* gene causing deletion of the titin M-band particles in cardiomyocytes impair the integrity of the titin C-terminal region, which is critical for myosin filament assembly, the M-line formation and maturation of the Z-disk (Gotthardt et al., 2003; Weinert et al., 2006). The results of these analyses revealed that the *TTN* homozygous mutations have much more severe consequences, such as complete absence of sarcomeric organization, when compared to heterozygous carriers in which functional sarcomeres are still found, although with altered structure that may impair a normal cardiac function in adulthood (Carmignac et al., 2007; Gerull et al., 2002, 2006; Satoh et al., 1999). Till now many mutations in the *TTN* gene have been associated with four cardiomyopathies: dilated cardiomyopathy (DCM) (Carmignac et al., 2007; Gerull et al., 2002, 2006; Herman et al., 2012; Itoh-Satoh et al., 2002; Matsumoto et al., 2005; Yoskovitz et al., 2012), hypertrophic cardiomyopathy (HCM) (Arimura et al., 2009; Herman et al., 2012; Matsumoto et al., 2005; Satoh et al., 1999), arrhythmogenic right ventricular cardiomyopathy (ARVC) (Taylor et al., 2011), and restrictive cardiomyopathy (RCM) (Peled et al., 2014).

Specifically, the Val54Met and Ala743Val missense substitutions (located in the *TTN* region encoding of the titin Z-disc segment; that have been showed to decrease the binding affinity of the titin to the α-actinin and T-cap/telethonin, respectively), mutation Gln4053ter (located in the *TTN* region encoding the N2B region of titin), mutation Leu4855Phe, change designed as Ser19628IlefsX1, insertion mutation (in exon 326) or Trp930Arg mutation (located in the Z-disk–I-band transition zone) as well as deletion (in exon 335; causing a frameshift, resulting in a premature stop codon following the addition of ten novel amino acid residues; designed as Glu28386LysfsX10) were associated with familial DCM (Gerull et al., 2002, 2006; Itoh-Satoh et al., 2002; Roncarati et al., 2013; Yoskovitz et al., 2012). The *TTN* mutations have been reported in HCM – the first case of the *TTN* mutation in the establishment of HCM was the Arg740Leu replacement, showing an increased binding affinity of the titin to the α-actinin by about 40% (Satoh et al., 1999). Also, Ser3799Tyr mutation (located in the gene part encoding the N2B region), that increased the binding affinity of the titin to a four and a half LIM domains protein 2 (FHL2), was indicated as important in HCM (Matsumoto et al., 2005). Moreover, two new *TTN* mutations were found in the N2A domain, which increased the binding of the titin to the Z-disk protein, were described in HCM patients (Arimura et al., 2009). The *TTN* mutations, such as Thr2896IIe (located in the *TTN* gene region encoding Ig10 domain of the titin responsible for development of passive tension), may also be the precursor of ARVC (Taylor et al., 2011). Moreover, the Tyr7621Cys (in the *TTN* exon 266 encoding the A-/I-band junction region) substitution was implicated in the onset of RCM (Peled et al., 2014).

Association between *TTN* gene polymorphisms and physical performance

Although the *TTN* mutations have been associated with a wide range of cardiac and skeletal muscle diseases, their relationship with physical performance is almost unknown. At first, a genome-wide linkage scan for endurance training-induced changes in submaximal exercise stroke volume (ΔSV50) in the HERITAGE Family Study including 483 white participants showed two chromosomal regions (2q31-q32 and 10p11.2) with an evidence of association among studied population. After reviewed the chromosome linkage area in detail, Rankinen et al. (2003) suggested that the *TTN* gene appears to be very promising genetic marker for human variation in ΔSV50 in the sedentary state and its response to training program. They also pointed that the titin is a biological candidate for the adaptation of cardiac function to endurance training (Rankinen et al., 2003).

To date, the best-known sport associated *TTN* mutation is a missense C>T transition (rs10497520), which results in the lysine codon transforming into a glutamic acid codon (causing Lys1201Glu change) in the I-band region of the N2A isoform, what may contribute to the variability within the titin isoforms expression in cardiac and skeletal muscle. The combining RNA profiling with single-gene DNA marker association analysis gave an evidence that the polymorphism is associated with the differences in the training response of maximal oxygen consumption (VO2max) in studied population (Timmons et al., 2010). Next, Stebbings et al. (2017) investigated the association between the C>T polymorphism and muscle fascicle length in recreationally active men and marathon personal best time in male marathon runners (the sample comprised 278 Caucasian men). The obtained results revealed that the T allele is linked to the shorter skeletal muscle fascicle length, what require less energy to produce a given force and conveys an advantage for marathon running performance in habitually trained men. It was suggested that changed the *TTN* gene splacing, due to the presence of the T allele, may affect the expression of the N2A isoforms, what may explain the mentioned results. In theory, individuals with the CC genotype with

longer skeletal muscle fascicles would have a rightward shift in their length-tension relationship and greater optimal joint angles for maximal torque production. Such the shift has been associated with a decrease in injury occurrence, as a longer optimal skeletal muscle length would provide that less of the muscle's functional range would be along the more unstable descending limb of the length-tension curve (Brughelli & Cronin, 2007). For this reason, knowledge of the *TTN* genotype in populations at higher injury risk, such as physically active people, would help to predict the consequences of performed exercises (Stebbings et al., 2017).

The importance of the *TTN* gene for muscle performance and injury risk was confirmed by Vera et al. (2019), who tried to determine the prevalence of 60 gene variants linked to connective tissue disorders (CTDs) that included hypermobility spectrum disorder (HSD) as a phenotype in a 51 professional male and female ballet dancers. They showed that CTD-associated genetic variants, particularly in the *TTN* gene, were highly prevalent in the study group. Due to the key role of the titin in elasticity of muscle tissue, the authors suggested that the *TTN* gene variants in the population allow the dancers greater elasticity in their muscles (and thus greater range of motion) through variations in the titin isoforms allowing more elasticity of the titin myofilaments. Additionally, the study found an inverse association between variants in the morphology of muscle, including the *TTN*, and morphology of skeleton gene groups and patient-reported outcome scores regarding the foot, ankle, and hip. These findings imply that participants with the gene variants experience increased pain than those without these variants (Vera et al., 2019). These conclusions are consistent with other studies that have demonstrated a relationship between hypermobility and chronic or severe pain (Celletti et al., 2013).

Conclusion

All the above-mentioned studies have showed that the various *TTN* gene mutations (including insertion/deletion changes leading to production of the shortered titin isoforms unable to range between the M-line and the Z-disk and missense mutations causing normal-sized titin isoforms but poorly attached to the Z-disk) may result in originating the titin myofilaments with changed elastic properties and, in consequence, sarcomeres with impaired contractile characteristic. Taken together, the studies highlighted the association between the *TTN* gene variants and formation of the essential skeletal and cardiac muscle properties such as stiffens and strength with regard to sport skills and injury risk (Maciejewska-Skrendo et al., 2020). Thus the knowledge of regarding biochemical, physiological, and genetical factors that affect muscle performance are key to achieve success in sports. In the future, the information of the *TTN* genotype in physically active people such as athletes would help to predict the consequences of performed exercises, thus making training programs much more efficient (possibility of accurate prediction of the training results) and safer (early prevention of injuries). In conclusion, we propose to include the *TTN* gene in the group of genetic marker for sport performance which may underlie differences in the potential to be an elite athlete. However, much more various studies are needed to establish the relationship between the *TTN* variants and their implications for human health, physical performance, adaptive changes of muscles in response to training, as well as injury risk.

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