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Modifier Genes and their effect on Duchenne Muscular Dystrophy

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Abstract

Purpose of Review—Recently, genetic pathways that modify the clinical severity of Duchenne Muscular Dystrophy have been identified. The pathways uncovered as modifiers are useful to predict prognosis and also elucidate molecular signatures that can be manipulated therapeutically.

Recent Findings—Modifiers have been identified using combinations of transcriptome and genome profiling. Osteopontin, encoded by the *SPP1* gene, was found using gene expression profiling. *LTBP4*, encoding latent transforming growth factor β binding protein 4 was initially discovered using a genomewide screen in mice and then validated in cohorts of Duchenne Muscular Dystrophy patients. These two pathways converge in that they both regulate TGF β . A third modifier, *Anxa6* that specifies annexin A6, is a calcium binding protein has been identified using mouse models, and regulates the injury pathway and sarcolemmal resealing.

Summary—Genetic modifiers can serve as biomarkers for outcomes in Duchenne Muscular Dystrophy. Modifiers can alter strength and ambulation in muscular dystrophy, and these same features can be used as endpoints used in clinical trials. Moreover, because genetic modifiers can influence outcomes, these genetic markers should be considered when stratifying results in muscular dystrophy.

Keywords

Muscular Dystrophy; TGF β ; osteopontin; latent TGF β binding

INTRODUCTION

Duchenne muscular dystrophy (DMD) and the allelic Becker Muscular Dystrophy are genetic disorders that affect one in 5600 males due to mutations in the gene encoding dystrophin [1,2]. Lack of dystrophin leads to the disruption of the dystrophin-glycoprotein complex and causes instability of the sarcolemma in muscle and the heart. As a result, there is ongoing degeneration and replacement of muscle with fibrosis and fat, loss of ambulation and eventual weakening of respiratory muscles and the heart. Currently treatment relies on

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Conflicts of Interest

A patent has been filed on the regulation of LTBP4 by one of the authors (EMM).

glucocorticoid steroids, typically prednisone or deflazacort, and use of such agents extends ambulation by 2 years [3]. Ongoing clinical trials in DMD are aimed at testing the utility of exon skipping, stop codon suppression, anti-inflammatory agents, anti-fibrotics, and alternative steroids. The discovery of modifiers and relevant pathways that alter the course of disease is necessary to help gain additional knowledge that could be used to develop potential therapeutics. Furthermore, modifiers can also have an immediate direct clinical impact. Genotyping modifier loci can identify individuals with specific phenotypes to develop personalized treatment. In this review, we will discuss the current modifiers that have been identified for DMD and their significance in pathogenesis.

Evidence for Genetic Modifiers in Muscular Dystrophy (MD)

Despite genetic homogeneity, DMD subjects express a range of phenotypic variability. The outcome in DMD is primarily determined by the primary *DMD* gene mutation; however, other factors also contribute. Environmental influences such as exercise and nutrition impart effect on outcome, but in recent years efforts have been directed at identifying genetic pathways that interact with the primary *DMD* gene mutation. A genetic modifier is a genetic locus that positively or negatively changes the phenotype of a primary disease causing mutation. In DMD, the large number of different mutations makes it challenging to dissect the effect of the primary *DMD* gene mutation compared to secondary genetic modifiers. Limb Girdle Muscular Dystrophy type 2C (LGMD 2C) arises from mutations in the *SGCG* gene, which encodes the dystrophin-associated protein γ -sarcoglycan, and in this disorder a single mutation is present in a large number of patients. Despite this genetic homogeneity, there is a range of outcomes including a broad range of time to loss of ambulation [4]. In MD, genetic modifiers may affect age of onset, affected muscle groups, disease progression, and severity. With recent advances in genomics and sequencing technology, it is becoming increasingly tangible to search for and identify potential modifiers of disease. Modifiers have been uncovered using gene expression and quantitative trait locus (QTL) mapping, as well as using candidate-driven approaches.

The TGF β pathway in muscle and muscle disease

The TGF β superfamily include more than 50 members that mediate cell growth, proliferation, apoptosis, differentiation, and control of extracellular matrix synthesis and degradation. TGF β 1, TGF β 2, and TGF β 3 constitute the primary TGF β subfamily; TGF β family members are secreted as a latent precursor that becomes activated before interaction with cell surface TGF β receptors [5,6] (Figure 1). Intracellular TGF β receptor binding elicits its effects through non-canonical and canonical (SMAD) signaling pathways that direct gene expression changes [7,8]. In non-muscle cells, TGF β has been shown to activate fibronectin independently of the Smad cascade through c-Jun N-Terminal kinases [9]. In muscle, multiple cell types express TGF β and TGF β receptors. TGF β acts on myofibers and satellite cells regulating response to injury, growth, differentiation and fibrosis.. Recently, there is evidence that enhanced canonical Wnt signaling triggers a fibrogenic signature in muscle satellite cells via the TGF β pathway by promoting TGF β 2 [10]. TGF β also directly promotes the expression of extracellular matrix (ECM) components from multiple cell types that remodel the matrix [11]. Myostatin, a muscle-specific member of the TGF β superfamily,

acts as a negative regulator of muscle growth and its inhibition also blocks fibrosis. A recent clinical trial with follistatin, an inhibitor of myostatin, suggests this is a safe means of potentially reducing fibrosis and improving muscle mass and function [12]. Therefore the TGF β family has multiple members that can influence DMD onset and progression.

DMD muscle has increased TGF β 1, and in non-dystrophic muscle TGF β 1 is elevated in response to exercise and injury [13–15]. The *mdx* mouse model of DMD similarly displays elevated TGF β 1 [16]. Inhibiting TGF β in *mdx* mice using losartan or neutralizing TGF β antibodies results in improved muscle function and fibrosis [17–19]. A meta-analysis of RNA microarray studies from many different types of degenerative muscle disease revealed a TGF β -centered network of 56 genes [20]. This TGF β signature was seen with normal muscle regeneration. However, the authors noted that in dystrophy where regeneration fails, the temporal specific expression pattern of these genes was disrupted. Loss of normal synchrony within this network, due to repetitive injury may contribute to the failed regeneration that characterizes DMD [20].

The intracellular pathways associated with increased TGF β are also targets for therapy in DMD. Reduction of Smad4, a co-Smad necessary for Smad2/3 signaling, improves cardiac function and skeletal muscle force but interestingly not fibrosis [21]. SMAD7 has been shown to act as a TGF β intracellular antagonist against myostatin, and mice null for SMAD7 have decreased muscle mass, force generation, and regeneration [22]. A dominant-negative mutation in *Tgfb2* expressed transgenically in muscle fibers improved muscle function in a mouse model of muscular dystrophy suggesting that hyper-TGF β signaling contributes to muscular dystrophy pathology [23]. Furthermore, antisense oligonucleotides directed against the TGF β Type I Receptor (ALK5) in the *mdx* mouse have been shown to reduce expression of genes related to fibrosis including *Colla1*, *Ctgf*, and *Serpine1* and, at the same time, increase expression of *Myog1*, a marker of regeneration [24]. Whether there is sufficient specificity to target these pathways preferentially in muscle as a therapy for muscle disease remains to be seen.

Osteopontin

Osteopontin, known as secreted phosphoprotein 1 (SPP1), is a secreted acidic glycoprotein that has roles in bone-remodeling, immune function, and muscle repair [25]. SPP1's promoter contains a Smad-binding region and is activated by TGF β family members [26]. TGF β has been shown to increase *SPP1* expression in myoblasts [25]. An intronic polymorphism, rs4522809, in *TGFBR2* is associated with *SPP1* expression levels [27,28]; this SNP is thought to be associated with regulating SPP1 levels in a steroid responsive manner. *mdx* mice lacking *SPP1* showed a significant reduction in TGF β , fibrosis, and inflammatory cell infiltrate [29]. *SPP1* is expressed in multiple cell types including fibroblasts and immune cells, and *SPP1* is increased after muscle injury [29]. Thus, SPP1 is positioned to play an important role in muscle disease and membrane repair although understanding the means by which it modifies DMD may be complicated by its broad expression pattern.

mRNA profiling of two separate DMD patient cohorts was used to identify *SPP1* as a genetic modifier of DMD. *SPP1* genotyping revealed a single nucleotide polymorphism (SNP) that correlated with both age of ambulation and grip strength. The rs28357094 SNP is found in the promoter of the *SPP1* gene, and the less common G allele was associated with earlier loss of ambulation and reduced grip strength [30]. The G allele was associated with reduced transcriptional activity of *SPP1* in a luciferase reporter; however, this effect may be mediated through the steroid responsiveness of this allele [31,32]. The *SPP1* genotype was also correlated with outcome in an Italian cohort of DMD patients. Patients with the G allele at rs28357094 performed less well on the North Star Ambulatory Assessment (NSAA) and 6-Minute Walk Test (6MWT) measures [33]. *SPP1* was confirmed as a modifier of age of ambulation in a separate cohort by Bello et al. but was found to have no significant effects in other studies [27,32,34,35]. *SPP1* is elevated in many MD subtypes indicating that it likely modifies multiple forms of muscle disease [36].

Identifying genetic modifiers faces the same challenges as measuring endpoints in DMD clinical trials. For example, 6MWT and measures of muscle strength are greatly influenced by volition and in children may not be so reliable. Loss of ambulation has been used in modifier studies, and may be among the more reliable measures, but may lack precision. In addition to measurements of phenotype, the rare nature of DMD limits the capacity to assemble large cohorts needed for these studies. All genetic cohort studies must be carefully considered for ethnicity and population stratification.

LTBP4

Latent TGF β Binding Protein 4 (*LTBP4*) has also been identified as a modifier and further reinforces that TGF β modifies MD. LTBP4 is a family of secreted glycoproteins that reside in the matrix. LTBP4s contain EGF-like and 8-cys repeats that are structurally similar to fibrillins. LTBP4s play a critical function in ECM remodeling by interacting with multiple matrix components in the ECM and can have a large impact in muscle degeneration diseases. LTBP1, LTBP2, and LTBP4 but not LTBP3 directly interact with latent TGF β [37]. LTBP4 stabilizes the TGFBR2, a TGF β receptor, to prevent its degradation suggesting the LTBP4 has additional mechanisms that promote TGF β [38]. In mice and human fibroblasts lacking LTBP4, protein but not mRNA levels of TGFBR1 and TGFBR2 are reduced [38]. LTBP4s also act as chaperones for latent TGF β ; LTBP4s are important for proper folding, secretion, ECM deposition, and ultimately the regulation of activating latent TGF β [39]. Proteolysis and a conformational change in LTBP4 are associated with the release of TGF β from the matrix allowing interaction with receptors [39]. *Ltbp4* was identified as a modifier in a mouse model of MD using an unbiased genomewide scan to identify genomic regions and variants associated with muscle disease [40]. Genomic sequencing revealed a 36bp insertion/deletion polymorphism that changes a proline-rich hinge region, resulting in more severe membrane damage and fibrosis. This size change in the hinge region is thought to change LTBP4's susceptibility to proteolysis and therefore release of latent TGF β [40]. Human LTBP4 protein has a shorter hinge region and is more susceptible to proteolysis than murine LTBP4. *mdx* mice expressing human LTBP4 also exhibit increased membrane damage and fibrosis as well as increased TGF β signaling

[41]. Genetic evidence supports that the *Ltbp4* polymorphism strongly correlates with membrane damage and fibrosis in all limb skeletal muscles [42].

In humans, *LTBP4* was also shown to correlate with outcome in human DMD cohorts [32,34,35]. Flanigan and colleagues examined two alleles in human *LTBP4* (Figure 2). The two alleles contain four non-synonymous single nucleotide polymorphisms (nsSNPs) in *LTBP4*. These four nsSNPs are in linkage disequilibrium and form two major amino acid haplotypes – IAAM and VTTT, so named for the amino acids specified by these nsSNPs. The IAAM haplotype significantly correlated with prolonged ambulation in both glucocorticoid-treated and non-treated patients. Treated patients homozygous for the IAAM haplotype had a mean age of ambulatory loss at 12.5 ± 3.3 years compared to 10.7 ± 2.1 years with other haplotypes. Non-steroid treated IAAM patients had a mean age of ambulatory loss at 11.2 ± 2.7 years compared to 9.8 ± 2.0 years with other haplotypes. Consistent with the idea that *LTBP4* modifies disease through TGF β , human fibroblasts with the IAAM haplotype have reduced TGF β signaling [35].

Two additional cohorts have been used to confirm the findings that *LTBP4* is a modifier of DMD. In one study, 265 Duchenne patients from European neuromuscular centers were analyzed and it was shown that the IAAM haplotype was significantly correlated to prolonged ambulation in both glucocorticoid-treated and non-treated patients [34]. Bello et al. studied the effects of *LTBP4* haplotype in another cohort of 340 DMD subjects from multiple continents. *LTBP4* genotyping for the protective IAAM haplotype trended towards association with age of ambulation. Once the cohort was stratified to focus on glucocorticoid-treated European or European-descent, a strong significant association with age of ambulation was seen [32]. Together, these data verify that *LTBP4* modifies ambulation in DMD.

Annexin A6

Anxa6 has also been identified as a modifier of MD [43]. Annexins bind phospholipids in a calcium-dependent manner and many different annexins are expressed in heart and skeletal muscle. Annexins are characterized by a variable amino-terminal domain that confers functional specificity, followed by four conserved calcium-binding annexin repeats. Annexin A6 has eight instead of four annexin repeats [44]. Annexins have been shown to function in calcium-mediated exocytosis and endocytosis, stabilization of the membrane, and membrane repair [45,46]. Annexins A1 and A2 directly interact with dysferlin, another muscular dystrophy-associated protein, in the process of membrane repair [47]. Furthermore, Annexins A1 and A2 correlate with disease severity in patients with limb girdle muscular dystrophy 2B [48]. In zebrafish, annexin A6 and other annexins localizes to the site of sarcolemma injury, where genetic evidence supports their role in repair [49].

In an intercross strategy using MD mice, *Anxa6* was found to be associated with abdominal muscle membrane damage and right ventricle heart mass [43]. Two synonymous SNPs were identified in *Anxa6* in exons 11 and 15, and these SNPs were associated with an alternative splice product that encodes low level production of a dominant-negative truncated annexin A6 protein (Figure 3). The truncated protein, which represents the first 265 of 673 total

amino acids, was shown to inhibit translocation of full length annexin A6 to the sites of membrane disruption. In the MD muscle, the presence of the truncated protein resulted in reduced full-length annexin A6 localization at the sarcolemma of muscle fibers with membrane disruption [43]. It is not known whether *Anxa6* also acts as a modifier of muscle disease in humans. However, recent data suggests that annexins may be useful as serum biomarkers, although this has yet to be applied to DMD [50].

Conclusions

A number of pathways have emerged from transcriptomic and genomic screens as pathways that modify the outcome of MD. Notably, muscle groups may exhibit differential responses to modifiers. In genetic screens, *Ltp4* was found to have a strong contribution to modifying pathological features in quadriceps, triceps, gastrocnemius/soleus and gluteus/hamstring, while *Anxa6* modified the abdominal muscles and the right ventricle. These observations do not preclude other muscle groups from these modifiers. *SPP1* appears to reflect a steroid response in muscle, which likely involves multiple cell types including inflammatory cells, while *LTBP4* modifies both membrane stability and fibrosis and may also be steroid sensitive. Further studies are needed to clarify the role of annexin A6 in human muscle. Each of these genes/proteins are pathways that can be exploited to improve sarcolemmal stability, repair, and to reduce fibrosis in MD.

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Key Points

- Single gene disorders like Duchenne Muscular dystrophy have a variable outcome, and part of this variability is mediated by genes that modify the outcome.
- *LTBP4*, the gene encoding the latent TGF β binding protein 4, was identified in mice as a modifier of muscular dystrophy and then shown in three independent studies to modify outcomes in human Duchenne Muscular Dystrophy.
- Elevated TGF β in Duchenne Muscular Dystrophy is pathogenic, and reducing TGF β signaling improves outcomes in animal models of muscular dystrophy.
- Annexin A6 regulates resealing of the sarcolemma and has been identified as a modifier of muscular dystrophy in mice.

Figure 1. TGFβ signaling in response to muscle injury leads to increased ECM remodeling

A. Following muscle injury, TGFβ signaling pathways are activated and lead to increased fibroblast proliferation and fibrotic programming. **B.** Proteolysis and conformational change of LTBP leads to activation of latent TGFβ signaling by releasing TGFβ from the extracellular matrix to interact with TGFβ receptors and further downstream Smad effectors to mediate target gene expression involving ECM remodeling.

Figure 2. LTBP4 haplotype modifies DMD severity

Non-synonymous single nucleotide polymorphisms across *LTBP4*, V194I, T787A, T820A, and T1140M, form the VTTT and IAAM *LTBP4* major haplotypes found in humans. Individuals homozygous for the IAAM haplotype are associated with prolonged ambulation when compared to all other haplotypes.

Figure 3. Annexin A6 localizes to site of membrane disruption in response to injury

A. Annexins are a family of proteins that bind phospholipids and assist in membrane repair. Annexins consist of a variable amino-terminal domain and four calcium-binding annexin repeats. Uniquely, Annexin A6 contains eight annexin repeats. Polymorphisms present in *Anxa6* mouse strains lead to expression of a truncated protein that is 265 amino acids instead of 673. **B.** In response to injury, annexins localize to the site of injury to facilitate membrane resealing and repair. The presence of the truncated version of annexin A6 lead to reduced localization of full-length annexin A6 and other associated annexins and impedes repair leading to increased membrane damage.