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***LTBP4* genotype predicts age of ambulatory loss in Duchenne Muscular Dystrophy**

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Abstract

Objective—Duchenne Muscular Dystrophy (DMD) displays a clinical range that is not fully explained by the primary *DMD* mutations. *Ltbp4*, encoding latent transforming growth factor- β binding protein 4, was previously discovered in a genomewide scan as a modifier of murine muscular dystrophy. We sought to determine whether *LTBP4* genotype influenced DMD severity in a large patient cohort.

Methods—We analyzed nonsynonymous SNPs from human *LTBP4* in 254 nonambulatory subjects with known *DMD* mutations. These SNPs, V194I, T787A, T820A, and T1140M, form “VTTT” and “IAAM” *LTBP4* haplotypes.

Results—Individuals homozygous for the IAAM *LTBP4* haplotype remained ambulatory significantly longer than those heterozygous or homozygous for the VTTT haplotype. Glucocorticoid-treated patients who were IAAM homozygotes lost ambulation at 12.5 ± 3.3 years compared to 10.7 ± 2.1 years for treated VTTT heterozygotes or homozygotes. IAAM fibroblasts exposed to TGF β displayed reduced phospho-SMAD signaling compared to VTTT fibroblasts, consistent with *LTBP4*'s role as regulator of TGF β .

Interpretation—*LTBP4* haplotype influences age at loss of ambulation, and should be considered in the management of DMD patients.

Keywords

muscular dystrophy; genetic modifier; TGF β

A spectrum of dystrophin mutations causes Duchenne muscular dystrophy (DMD). The extreme genomic size of the *DMD* gene (2.2 Mb) leads to kilobase size deletions or duplications in approximately 70% of DMD patients, and the remaining 30% arise from point mutations that introduce premature stop codons or disrupt splicing¹. *DMD* mutations typically completely ablate dystrophin protein expression, while the milder Becker muscular dystrophy (BMD) mutations are most often in-frame deletions that produce truncated, but partially functional dystrophin. DMD patients are clinically defined by loss of ambulation before the age of 12, whereas BMD patients often remain ambulatory beyond age 15, a distinction that generally correlates with the degree of protein expression. Those who lose ambulation between 12 and 15 years are described as intermediate muscular dystrophy (IMD). Many DMD boys now ambulate beyond the age of 12 reflecting their treatment with prednisone or deflazacort, which remain the only medications that alter the natural history of DMD, prolonging ambulation for up to 1 to more than 3 years²⁻⁵.

Despite the complete absence of dystrophin, the DMD phenotype is associated with a range in rates of progression, supporting that genetic or environmental modifiers help determine

clinical course. Animal models, particularly mice, can be used to map genetic modifiers since inbred strains and standardized environmental conditions reduce variation. Like DMD patients, muscular dystrophy patients with a founder mutation in the dystrophin associated protein, γ -sarcoglycan, (*SGCG*) lose ambulation over a wide age range, consistent with the presence of genetic modifiers^{6, 7}. The *Sgcg* null mouse was used in a genome-wide screen for modifiers of muscular dystrophy and identified the *Ltbp4* gene encoding the latent TGF β binding protein (LTBP) 4 as a modifier of muscular dystrophy⁸. Given the shared pathological mechanisms between loss of dystrophin and its associated proteins, the sarcoglycans, we reasoned that *LTBP4* may modify DMD. LTBP4, a member of the fibrillin superfamily, binds to transforming growth factor (TGF)- β in the extracellular matrix where it regulates TGF β activity⁹.

We now examined human *LTBP4* polymorphisms in 254 nonambulatory patients from the United Dystrophinopathy Project cohort. This United Dystrophinopathy cohort includes 900 DMD patients, most who still ambulate, identified from seven sites representing by far the largest uniformly characterized DMD population. We assessed the effect of *LTBP4* genotype on the age at ambulatory loss, since it reflects disease progression, is not influenced by ascertainment bias, and is a milestone for which the month or age is recalled by most patients and/or families. A protective haplotype in *LTBP4* was identified.

Results

LTBP4 genotype and ambulation in DMD

To examine the age of ambulatory loss, we included all patients from the United Dystrophinopathy Cohort with a *DMD* mutation who had lost ambulation before age 20. All had confirmed dystrophin mutations¹⁰. Truncating mutations that interrupt the dystrophin reading frame positively predict a DMD phenotype in only 87% of patients and non-truncating mutations predict BMD in 63%¹⁰. To take an unbiased approach, we included all subjects who lost ambulation before age 20 who had a confirmed *DMD* mutation. 254 subjects met these inclusion criteria; 244 (96%) were DMD patients based on the diagnosis of the expert neuromuscular physician at the treating site.¹⁰ Also meeting these entry criteria were eight IMD patients who lost ambulation between age 12 and 15, and two BMD patients who lost ambulation after age 15 but before age 20.

Three *LTBP4* SNPs, rs2303729, rs1131620 and rs10880 were genotyped (Figure 1). A fourth nsSNP, rs1051303 (T820A), is located less than 200 bp from rs1131620 (Figure 1B). Because of this close proximity between rs1051303 and rs1131620, the genotype of rs1131620 was inferred. There is strong linkage disequilibrium across the entire *LTBP4* locus so that all four nsSNPs are most often co-inherited. In the 254 patients selected for study, the frequencies of the referent allele at each *LTBP4* nsSNP were 57.5% (rs2303729), 60.1% (rs1131620) and 65.3% (rs10880) matching the known frequency of these SNPs in the general population (Table S1).

Age of ambulatory loss was correlated with genotype for each of the *LTBP4* nsSNPs (Figure 2A). rs2303729 encodes V194I altering the matrix-binding domain of LTBP4. Homozygous individuals (AA, Ile:Ile) had prolonged ambulation ($p=0.01$) compared to those who were

heterozygous (AG, Ile:Val) or homozygous (GG, Val:Val). A similar association was seen for rs1131620 encoding T787A within the 8th EGF motif; DMD subjects homozygous for GG (Ala:Ala) allele had delayed wheelchair use compared to those with AG (Ala:Thr) or AA (Thr:Thr) at this position ($p=0.006$). The third nsSNP rs10880 (T1140M) alters a residue near the specialized “8-cysteine” repeat directly implicated in TGF β binding¹¹. Homozygous individuals (TT, Met:Met) allele had prolonged ambulation ($p=0.001$). We removed the eight patients clinically defined as IMD and the two patients clinically defined as BMD (Table S2), and the rs10880 association with age of ambulatory loss remained significant ($p=0.02$). For each of the three SNPs, delayed ambulatory loss was seen with homozygosity of the minor *LTBP4* allele. Heterozygosity or homozygosity of the referent *LTBP4* allele was similar to each other in time to loss of ambulation.

Given the extent of linkage disequilibrium observed for these three nsSNPs (Figure 1B), we estimated *LTBP4* protein haplotype frequencies in the 254 patients and evaluated these haplotypes for association with ambulatory loss (Table 1). The observed frequencies of *LTBP4* protein haplotypes were: VTTT = 0.53, IAAM = 0.30, IAAT = 0.06, ITTT = 0.05 and 0.06 for pooled rare haplotypes, in close agreement with reference frequencies from individuals of European ancestry in the 1000 Genomes database¹² (Figure 1B and Table S3). For age of ambulatory loss using a recessive model, the global haplotype score statistic was 12.26, and with 2f, yielded a P value from the χ^2 distribution of 0.002, while the haplotype-specific p value for the IAAM haplotype was 6×10^{-4} (Table 1).

The DMD genotype does not account for differences in age of ambulatory loss

To evaluate the effect of the primary *DMD* mutation, *DMD* mutations were separated into two classes: truncating *DMD* mutations and “other” mutations, which include in-frame deletions/duplications, predicted stop codons that could possibly generate residual in-frame dystrophin via exon skipping¹³, and small non-frameshifting mutations (Tables S4 and S5). The *LTBP4* IAAM haplotype remained significantly associated with ambulatory loss when considering only those with truncating *DMD* mutations (Table S6). When considering *DMD* mutation class as a covariate, the IAAM *LTBP4* haplotype remained highly correlated with delayed ambulatory loss (4.9×10^{-4} , Table S6). The *DMD* mutation class did not vary in the group of patients with the protective IAAM *LTBP4* haplotype, as the frequency of truncating versus “other” mutations was similar between those with the CC, CT and TT alleles of rs10880 (Figure 2B). These data are consistent with the minor alleles of common *LTBP4* nonsynonymous SNPs, encoding the isoleucine, alanine, alanine, and methionine or IAAM haplotype (including the rs1051303 T820A variant, Figure 1B), acting in a recessive fashion to delay loss of ambulation caused by muscular dystrophy. Correspondingly, the valine, threonine, threonine, threonine, or VTTT haplotype, acts dominantly as a risk allele in muscular dystrophy.

LTBP4 genotype effect with glucocorticoid treatment in DMD

Glucocorticoids promote prolonged ambulation in DMD^{2, 3} and are now more commonly used by younger ambulatory DMD patients. In the DMD subjects for whom glucocorticoid therapy prior to ambulatory loss could be established ($n = 137$) compared to those who were steroid naïve ($n=102$), treatment was highly correlated with prolonged ambulation ($p=0.001$,

Table S7). The *LTBP4* IAAM haplotype-specific association (Table 1) remained significant in the steroid treated ($p = 0.004$) and trended in the steroid naïve groups ($p = 0.06$), and remained significant when both steroid treatment and mutation classification were used as covariates ($p = 8.9 \times 10^{-4}$, Table S7). Six of the seven oldest individuals to lose ambulation were homozygous for the IAAM haplotype and five were among the glucocorticoid-treated group. There were two sets of brothers discordant for their *LTBP4* haplotypes, where one brother was homozygous for the IAAM haplotype (Figure 3A). In each pair, the brother with the protective IAAM allele walked longer (2.5 or 4.5 years longer, respectively) than the brother without the IAAM allele.

Figure 3B shows that the homozygous *LTBP4* IAAM haplotype associates with prolonged ambulation in both glucocorticoid-treated and naïve patients. In treated patients, the mean age of ambulatory loss for the homozygous IAAM haplotype is 12.5 ± 3.3 years compared to 10.7 ± 2.1 years for other haplotype pairs, while in steroid-naïve patients the mean age for the homozygous IAAM haplotype is 11.2 ± 2.7 compared to 9.8 ± 2.0 years for the other haplotype pairs. Both the rs10880 genotype and glucocorticoid treatment remained significant when tested as covariates in a Cox proportional hazards model ($p=0.001$ and $p=0.001$, respectively), and yielded hazard ratios of 0.52 (rs10880 genotype, 95% CI 0.34 to 0.78) and 0.65 (glucocorticoid treatment, 95% CI 0.50 to 0.84). The full model had a -2 log likelihood = 22.4 ($p=1 \times 10^{-5}$), indicating that both *LTBP4* genotype and glucocorticoid treatment contributed to prolonged ambulation observed within this cohort.

Common variation in *LTBP4*

We re-sequenced the coding region of *LTBP4* from 20 unrelated (ten Caucasian, ten African American, 40 chromosomes) and queried the 1000 Genomes database (Table S2, Figure S1)¹² finding no evidence for additional nonsynonymous coding variation except for rs1051303 (A820T). The alleles for rs2303729, rs1131620, rs1051303 and rs10880 are common (>30% minor allele frequency) in all 1000 Genomes populations of European, West African, East Asian and admixed American ancestry (Table S2, Figure S2 and S3), with VTTT and IAAM haplotypes observed as the most frequent haplotypes in each of these populations. In West African populations, the rs10880 Met allele is observed at higher frequencies associated with ITTM and VTMM haplotypes (Figure S3). Thus, we infer the major protective *LTBP4* haplotype IAAM and the risk haplotype VTTT are common protein isoforms. Polyphen-2 (PP2)¹⁴ predicts that the rs10880 Thr1140Met substitution is possibly damaging, while the other variants are predicted to be benign. This residue is also highly conserved (Table S8).

Fibroblasts with homozygous “IAAM” *LTBP4* have reduced TGF β signaling

LTBP4 directly binds TGF β in the extracellular matrix where it sequesters TGF β , regulating TGF β availability to cell surface receptors^{11, 15}. TGF β receptor binding triggers SMAD activation seen as phosphorylated SMAD2/3. We examined fibroblasts of each *LTBP4* genotype under confluent conditions where *LTBP4* protein was equally expressed (S4). These cultured fibroblasts were treated with latent TGF β to test whether IAAM fibroblasts differentially sequester TGF β compared to VTTT fibroblasts^{8, 16}. In this assay, decreased *LTBP4* binding of TGF β is expected to increase TGF β signaling. Fibroblasts homozygous

for the protective IAAM *LTBP4* protein variant had reduced pSMAD2/3, consistent with enhanced sequestration of TGF β , while fibroblasts homozygous for VTTT *LTBP4* protein had increased pSMAD2/3 (Figure 4). Fibroblasts heterozygous for IAAM/VTTT showed an intermediate level of pSMAD2/3. Fibroblasts with IAAM/T, which are homozygous for the IAA nsSNPs, but heterozygous for rs10880 M/T nsSNP, trended towards being significantly different from VTTT fibroblasts (S4). The level of *LTBP4* protein did not differ for each of the *LTBP4* genotypes and exposure to TGF β did not alter *LTBP4* levels (S5). Although these fibroblasts differ at many genetic loci in addition to the *LTBP4* gene, these data are consistent with a role for *LTBP4* nsSNPs as modifiers of TGF β signaling.

Discussion

We identified a common *LTBP4* haplotype that correlates with prolonged ambulation in DMD patients. The entire United Dystrophinopathy Project cohort includes more than 1100 subjects with dystrophin mutations, including 908 with a DMD phenotype. Because our cohort includes many younger DMD participants, only around 28% of current subjects have progressed to ambulatory loss. The 254 subjects included in this study represent the largest cohort of non-ambulant DMD patients yet assembled for evaluation of genetic modifiers. Determining modifier status in a homogeneous genetic disease such as DMD is fundamentally distinct from examining genetic contribution in common, genetically heterogeneous diseases, which requires much larger populations. This study was aided not only by narrowly defined cohort, where the allelic spectrum of *DMD* mutations was defined, but also by the animal data that demonstrated the potency of the *LTBP4* modifier. *LTBP4* emerged from a genomewide scan in the animal model as the only locus for limb skeletal muscles with a LOD score $>24^{17}$. The animal model used in the genomewide scan and DMD share a common molecular pathology, namely a disrupted dystrophin protein complex.

Treatment with glucocorticoid steroids is recommended for ambulatory DMD boys since it prolongs ambulation^{2, 3}. The effect size of the *LTBP4* genotype is on par with corticosteroid treatment, the only effective drug in use for DMD. Both *LTBP4* genotype and corticosteroid treatment yielded equivalent hazard ratios of 0.5 as estimated from a Cox proportional model. How steroid use prolongs ambulation is not fully known but likely mechanisms may include anti-inflammation, anti-fibrosis, or direct promotion of muscle strength. *LTBP4* genotype remained predictive of improved ambulation even for those DMD subjects who use glucocorticoids, and the magnitude of effect suggests that glucocorticoid treated subjects homozygous for the protective allele have the most improved ambulation. Although the *LTBP4* association remained significant in our cohort after removing 8 patients clinically defined as IMD, it is noteworthy that 6 of the 8 IMD patients were homozygous for the protective *LTBP4* IAAM haplotype, and all 8 IMD patients were treated with glucocorticoids. Previously considered “outliers”, these DMD individuals who remain ambulatory far longer than would be expected can now be explained by the contribution of both a genetic modifier and glucocorticoid treatment.

Elevated TGF β has been noted in DMD^{18, 19}. Genetic or drug-mediated reduction of TGF β signaling in animal models shows that reducing TGF β signaling improves muscle function

and regeneration²⁰⁻²². *LTBP4* IAAM may differentially regulate TGF β signaling by altered TGF β binding or release. *SPPI*, encoding osteopontin was previously reported to modify DMD, and the *SPPI* promoter is responsive to TGF β ^{23, 24}. Although we did not replicate the *SPPI* association (Table S9), the prior study and the current study independently identify the TGF β pathway as a modifier of DMD.

LTBP4 nsSNPs should be contemplated for DMD prognosis, especially when considering steroid treatment. *LTBP4* genotype in DMD may also influence interpretation of clinical trial data, since ambulation is a key endpoint for DMD. The *LTBP4* genotype as a regulator of TGF β signaling may also influence other diseases. For example, *LTBP4* SNPs have been associated with exercise capacity in chronic obstructive pulmonary disease, and progression in colorectal cancer and aortic aneurysms suggesting that the *LTBP4* IAAM haplotype may portend a broad protective effect beyond muscle disease²⁵⁻²⁷. *LTBP4* with its link to multiple human diseases suggests an important therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

LTBP4 protein and position of amino acid substitutions induced by single nucleotide polymorphisms (SNPs). A) SNPs rs2303729, rs1131620, rs1051303 and rs10880 alter LTBP4; the amino acid substitutions are shown in red. The amino terminus of LTBP4 binds to the extracellular matrix (ECM). The protein is composed of epidermal growth factor (EGF)-like repeats (light blue boxes). B) *LTBP4* protein haplotypes and frequencies estimated from 381 individuals found in the 1000 Genomes interim Phase I data from populations with European ancestry. The rs1051303 nsSNP alters the protein at amino acid 820 and is in perfect linkage disequilibrium ($r^2 = 1$) with rs1131620. The pairwise linkage disequilibrium (r^2) between the other three nsSNPs in our DMD cohort versus 1000 Genomes was: rs2303729:rs1131620 (0.67 vs. 0.68), rs2303729:rs10880 (0.50 vs. 0.45), and rs1131620:rs10880 (0.62 vs. 0.65).

Figure 2. *LTBP4* genotypes are associated with age of loss of ambulation in patients with Duchenne muscular dystrophy. A) Patients who have lost ambulation by 20 yrs of age are classified by their nonsynonymous *LTBP4* genotype. For each genotypic class, the survival curve to age of loss of ambulation and the Wald test asymptotic p-value (recessive) using a linear model applied to age of ambulatory loss as a quantitative trait is shown. B) *DMD* mutation class does not differ for those with the protective *LTBP4* allele. Distribution of age at ambulatory loss grouped by *LTBP4* rs10880 genotype (individuals per genotype; Thr:Thr = 109, Thr:Met = 114, Met:Met = 31), with dystrophin mutation class indicated by color: green = truncating mutations, gold = other mutations (see Tables S3 and S4 for definitions of truncating *DMD* mutation versus other *DMD* mutation classes). Detailed dystrophin mutation nomenclature is shown for patients A through J from the upper ambulatory range in the rs10880 Thr:Met and Met:Met groupings.

Figure 3.

The *LTBP4* rs10880 minor homozygote (TT = Met:Met) and IAAM/IAAM diplotype is associated with extended ambulation in glucocorticoid-treated Duchenne muscular dystrophy patients. A) Age of ambulatory loss classified by rs10880 genotype and color-coded for patients by their steroid treatment. Two pairs of brothers discordant for their rs10880 genotypes are shown connected by the dotted lines. B) Survival curves for DMD patients with the *LTBP4* IAAM/IAAM versus other haplotype pairs, with steroid treated versus naïve individuals plotted separately. The IAAM haplotype consists of SNPs: rs2303729 (I=Ile), rs1131620 (A=Ala), rs1051303 (A=Ala) and rs10880 (M=Met) respectively. There were 16 IAAM homozygotes in the steroid treated group and 10 IAAM homozygotes in the naïve group.

Figure 4. Fibroblasts homozygous for the IAAM or VT TT LTBP4 alleles or heterozygous (IAAM/VT TT) were cultured and latent TGFβ was added. LTBP4 protein binds and sequesters latent TGFβ, and it is predicted that IAAM binds less latent TGFβ in the matrix leading to reduced TGFβ signaling, seen as phosphorylated SMAD (pSMAD). IAAM fibroblasts had less pSMAD/total SMAD at baseline and at two different doses of latent TGFβ (p =0.02, ANOVA with repeated measures).

Haplotype analysis of nonsynonymous *LTBP4* variants associated with age of ambulatory loss for DMD patients.

Table 1

All (n=254) Global $P^d = 0.002$			Steroid treated (n=137) Global $P^d = 0.013$			Steroid naïve (n=102) Global $P^d = 0.12$			
Haplotype ^b	freq	score	p-val ^c	freq	score	p-val ^c	freq	score	p-val ^c
VTTT	0.53	-1.51	0.1	0.52	-1.04	0.3	0.52	-1.12	0.3
IAAM	0.31	3.43	6×10^{-4}	0.32	2.92	0.004	0.29	1.91	0.06

^aThe haplo.stats package was used to test for association between haplotypes and age of ambulatory loss as a quantitative trait using a recessive model.

^bThese haplotypes consist of SNPS: rs2303729, rs1131620, rs10880 respectively.

^cp value from the χ^2 , df=1, distribution of the haplotype-specific score.