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ARTICLE

Comprehensive fine mapping of chr12q12-14 and follow-up replication identify activin receptor 1B (*ACVR1B*) as a muscle strength gene

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Muscle strength is important in functional activities of daily living and the prevention of common pathologies. We describe the two-staged fine mapping of a previously identified linkage peak for knee strength on chr12q12-14. First, 209 tagSNPs in/around 74 prioritized genes were genotyped in 500 Caucasian brothers from the Leuven Genes for Muscular Strength study (LGfMS). Combined linkage and family-based association analyses identified activin receptor 1B (*ACVR1B*) and inhibin β C (*INHBC*), part of the transforming growth factor β pathway regulating myostatin – a negative regulator of muscle mass – signaling, for follow-up. Second, 33 SNPs, selected in these genes based on their likelihood to functionally affect gene expression/function, were genotyped in an extended sample of 536 LGfMS siblings. Strong associations between *ACVR1B* genotypes and knee muscle strength (*P*-values up to 0.00002) were present. Of particular interest was the association with rs2854464, located in a putative miR-24-binding site, as miR-24 was implicated in the inhibition of skeletal muscle differentiation. Rs2854464 AA individuals were ~2% stronger than G-allele carriers. The strength increasing effect of the A-allele was also observed in an independent replication sample (*n*=266) selected from the Baltimore Longitudinal Study of Aging and a Flemish Policy Research Centre Sport, Physical Activity and Health study. However, no genotype-related difference in *ACVR1B* mRNA expression in quadriceps muscle was observed. In conclusion, we applied a two-stage fine mapping approach, and are the first to identify and partially replicate genetic variants in the *ACVR1B* gene that account for genetic variation in human muscle strength.

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INTRODUCTION

Because of its beneficial health effects, skeletal muscle strength is an important trait, even in populations that live in modern societies and no longer rely heavily on physical labor. Indeed, both in the elderly and the general population, muscle strength is inversely associated with all-cause mortality,[1] osteoporosis,[2] cardiovascular diseases,[3] metabolic syndrome prevalence[4] and other pathological conditions and chronic diseases.[5] Heritability estimates vary from 31 to 78% with large differences between muscle groups, contraction velocities or muscle lengths (reviewed in Peeters *et al*[6]). Apart from a genetic component, muscle strength is influenced by environmental factors such as nutrition, training or social status, and by the interaction between genes and environment, rendering it a complex multifactorial trait.

The 'Human gene map for performance and health-related fitness phenotypes'[7] indicates that only a limited number of genes has been implicated in muscle strength and even fewer associations have consistently been replicated. Genetic linkage studies regarding muscle strength are even scarcer. To date, only Tiainen *et al*[8] and our research group[9–14] reported results on genetic linkage for skeletal muscle strength characteristics. We were the first to perform linkage analyses on a unique collection of young male Caucasian siblings drawn from the Leuven Genes for Muscular Strength study (LGfMS).[9] Single and multipoint microsatellite marker-based linkage analyses revealed significant or suggestive linkage of chromosomal regions 12q12-14, 12q22-23 and 13q14.2 to knee muscle strength.[9,11] It can, however, not be excluded that genes other than the original myostatin pathway genes underlie these linkage findings and further fine mapping of the region surrounding these candidate genes is therefore warranted.

In this study, we further investigated the 12q12-14 region. We applied a two-staged genetic fine mapping approach, followed by a replication of the most promising associations and an evaluation of mRNA variation in relation to a *ACVR1B* SNP, in an independent

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study sample. We are the first to reveal evidence for variations in the *ACVR1B* gene to determine – at least in part – muscle strength properties. Our observations fit with available physiological data that demonstrate a potential role for ACVR1B in the regulation of muscle mass, as it is part of the transforming growth factor β (TGFβ) pathway regulating myostatin – a negative regulator of muscle mass – signaling.[15,16]

MATERIALS AND METHODS

Study design

A two-staged approach was designed to fine map the chr12q12-14 linkage peak. The most significant findings were then replicated in an independent study sample and the influence of a specific SNP on mRNA expression levels in quadriceps muscle tissue was tested. A schematic representation of the study design can be found in Figure 1.

Fine mapping stage 1. SNPs were selected under the 1-LOD confidence interval of the previously defined linkage peak,[9,11] using an empirical two-step strategy, in which candidate genes are prioritized using a bioinformatics approach, and the top genes are chosen for further SNP selection with a linkage disequilibrium (LD)-based method, as described in Supplementary file 1 (available online) and elsewhere.[17] A list of the candidate genes and corresponding polymorphisms is available in Supplementary file 2 (available online) (Supplementary Table S1).

Joint linkage and association analyses, and family-based association analyses tested the association of these polymorphisms with knee strength measurements.

Fine mapping stage 2. Candidate genes were ranked on the basis of (1) their significance in (a) the joint linkage and association approach, (b) the family-based association approach and (c) both methods; (2) their significance over different strength measurements; (3) the knowledge about their function in muscle tissue (known, putative, general function in different tissues or no known/unclear function). The highest ranked gene *ACVR1B* was selected for further follow-up. *INHBC* was also included because it is part of the same TGFβ signaling pathway as *ACVR1B,* even though it had a lower ranking. An extended sample was genotyped for the stage 1 SNPs, complemented with additional tagSNPs and polymorphisms likely to have functional consequences, such as exonic SNPs, SNPs in intron/exon boundaries (possibility to influence the splicing process), in (putative) transcription factor-binding sites or in highly conserved regions (Table 1). Assessment of potential functionality was based on queries from SNPselector,[18] SNPseek (http://snp.wustl.edu/cgi-bin/SNPseek/index.cgi) and publicly available genetic databases.

Replication. Genetic linkage and association analyses are prone to false-positive findings. Therefore, we applied a staged design, used complementary association methods and derived empirical *P*-values. Moreover, we replicated the associations with putatively functional polymorphisms, detected in the second stage of the fine mapping, in an independent sample.

Study samples

Two-staged fine mapping. Siblings analyzed in stage 1 and stage 2 of this study were selected from the LGfMS (748 men; 17–36 years), based on family size and DNA availability. The recruitment protocol and subject characteristics have been described elsewhere.[9,11] For stage 1, a total of 500 individuals were included (169 subjects overlap with the microsatellite-based linkage study[11]). For stage 2, a total of 536 subjects were included with an overlap of 464 subjects with stage 1. Subjects for whom genotyping failed in stage 1 were excluded from stage 2, and subjects for whom new DNA was collected between the two stages were included. Descriptive statistics of these samples can be found in Supplementary file 2 (Supplementary Table S2).

Replication sample. The replication sample consists of 193 Caucasian men (20–90 years) from the Baltimore Longitudinal Study of Aging (BLSA)[19–21] and 74 healthy Caucasian men (60–78 years) from a study conducted within the framework of the Flemish Policy Research Centre Sport, Physical Activity and Health (SPAH).[22,23]

The medical and ethical committee of the Katholieke Universiteit Leuven approved the LGfMS and SPAH studies. The BLSA experimental protocols were approved by the Institutional Review Boards for Human Subjects at Medstar Research Institute (Baltimore, MD, USA), Johns Hopkins Bayview Medical Center (Baltimore, MD, USA) and the University of Maryland (College Park, MD, USA). All subjects gave written informed consent before participating. Descriptive statistics of the replication subsamples can be found in Supplementary file 2 (Supplementary Tables S2 and S3).

Strength measurements

Isometric and dynamic strength of the knee extensors and flexors was measured using an isokinetic dynamometer as described in Supplementary file 1 and elsewhere (*LGfMS*,[9,11] *BLSA*[20,21] and *SPAH*[23]).

SNP genotyping

Detailed SNP genotyping methods can be found in Supplementary file 1. In short, DNA was extracted from whole blood (LGfMS, BLSA, SPAH) or from saliva (Oragene DNA Self-Collection Kits; Oragene, Ontario, Canada; LGfMS). Genotyping was performed on an Illumina Bead Array platform (Illumina Inc., San Diego, CA, USA; LGfMS stage 1), a Sequenom iPLEX Gold platform (Sequenom Inc., San Diego, CA, USA; LGfMS stage 2 and SPAH) or with Taqman allelic discrimination assays (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA; BLSA). Overall sample success rates, locus success rates and genotype call rates can be found in Supplementary file 1.

Quantification of ACVR1B mRNA expression

Muscle tissue (right vastus lateralis) was obtained from 16 healthy male volunteers (18–28 years) from an independent study conducted in our research center.[24] A detailed description of the mRNA extraction and real-time PCR

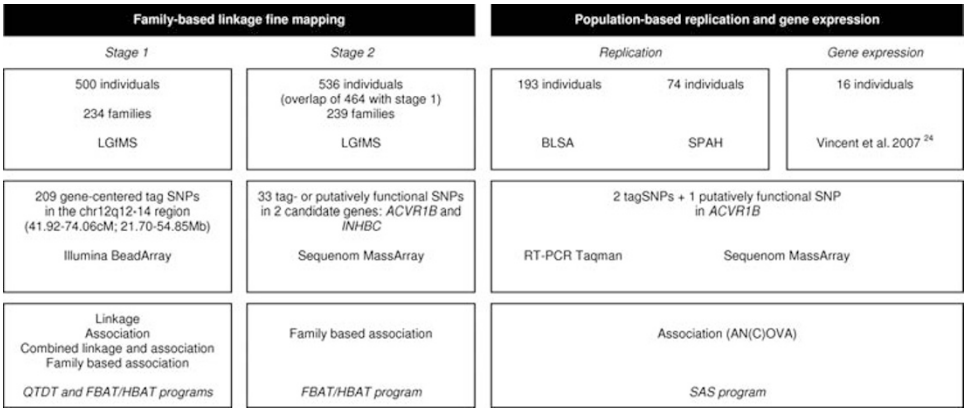

Figure 1 Study design. The different stages of the study are displayed according to their chronological order from left to right. Sample description, genotyping details and statistical analyses are presented from top to bottom. LGfMS, Leuven Genes for Muscular Strength study; BLSA, Baltimore Longitudinal Study of Aging; SPAH, study conducted within the framework of the Flemish Policy Research Centre Sport, Physical Activity and Health.

Table 1 Stage 2 genes and corresponding polymorphisms

SNP	*No.*	*Coordinate*	*Minor allele*	*Minor allele frequency*	*Putative function*
ACVR1B					
rs10431538	1	50605127	A	0.15	Located in transcription factor-binding site (TFBS)/conserved
rs706824[a]	2	50605518			Located in TFBS
rs1979921	3	50606344	T	0.08	Conserved
rs12230575[a]	4	50608313			Conserved
rs10783485	5	50622274	A	0.35	Tagging SNP, genotyped stage 1
rs746434	6	50628932	A	0.09	Tagging SNP, genotyped stage 1
rs808873[a]	7	50634449	C	0	Located in TFBS/conserved
rs11612312	8	50635355	A	0.22	Conserved
rs12809597[a]	9	50642590			Conserved
rs10783486[b]	10	50649053	A	0.27	Tagging SNP, genotyped stage 1
rs34488074[c]	11	50656482	C	0	Non-synonymous SNP (L/F)
rs2172603	12	50663079	A	0.02	Located in TFBS
rs12296553[a]	13	50663569			Located in TFBS
rs2641516[c]	14	50666953	C	0	Synonymous SNP (G/G)/conserved/located in TFBS
rs928906[a]	15	50666954			Located in TFBS/non-synonymous (V/L)
rs34050429[c]	16	50667165	A	0	Non-synonymous SNP (H/R)
rs2854464	17	50675158	G	0.27	Located in TFBS/miRNA-binding site
rs2714	18	50675551	A	0.10	Conserved
rs11169974	19	50676563	T	0.04	Located in TFBS
rs7955401[a]	20	50685018			Located in TFBS
INHBC					
rs7964492	1	56109852	C	0.25	Tagging SNP/conserved
rs12826906	2	56114925	G	0.006	5′ UTR
rs12831855[c]	3	56114969	T	0	Synonymous SNP (L/L)/conserved
rs12809642[c]	4	56115002	G	0	Synonymous SNP (A/A)
rs560048	5	56115396	T	0.45	Regulatory region intron 1
rs533975	6	56118251	A	0.45	Tagging SNP/conserved
rs2943693	7	56122232	A	0.22	Tagging SNP, genotyped stage 1
rs34137943[c]	8	56129647	T	0	Synonymous SNP (L/L)
rs2229357	9	56129978	A	0.26	Non-synonymous SNP (Q/R), genotyped stage 1
rs3741414	10	56130316	A	0.26	Tagging SNP, genotyped stage 1
rs34435057[c]	11	56130855	T	0	3′ UTR
rs11172223[c]	12	56132314	A	0	3′ conserved SNP
rs7136620[c]	13	56132489	A	0	3′ conserved SNP

The physical location of each SNP is based on NCBI Build 36. The putative function was derived from SNPselector and SNPseek output. All SNP genotype distributions are in Hardy–Weinberg equilibrium ($P>0.001$).
[a]Failed iPLEX assay.
[b]Failed iPLEX assay but also genotyped in stage 1.
[c]Monomorphic SNPs.

protocol for ACVR1B mRNA expression is available in Supplementary file 1 and elsewhere.[25]

Statistical analyses

Pedstats v0.6.4[26] was used to check pedigrees for Mendelian consistency and to test Hardy–Weinberg equilibrium (HWE). Unlikely genotypes were identified and discarded using the error checking option of Merlin v1.0.1.[27]

QTDT v2.5.1 software[28] was applied to compare a test for linkage between the SNPs and the strength measurements with a test for linkage while modeling association.[29] When association between a SNP and the studied trait is present, the linkage signal should become nonsignificant when association is added to the model. Merlin v1.0.1[27] was used to calculate IBD probabilities. Genetic distances were derived from physical distances (NCBI build 35) using Rutgers Map Interpolator based on Rutgers first-generation combined linkage-physical map (Rutgers map v.1).[30]

Haploview[31] was used to examine the LD structure across the region of interest and to calculate r^2 between all pairs of SNPs within a candidate gene.

Complementary to the combined linkage and association analysis, we conducted family-based association tests (FBAT v1.7.3) under the null hypothesis of 'linkage and no association'.[32,33] The haplotype version of this test (HBAT)[34] was used to obtain empirical *P*-values by means of the Monte-Carlo permutation procedure (10 000 permutations). An additive model was tested and trait offsets were specified as the sample mean of the trait. As only empirical *P*-values are reported, we should note that these were derived under the null hypothesis of 'no linkage and no association' since permutation tests are too computer-intensive under the alternative hypothesis. However, simulations show that tests under both null hypotheses render comparable results (data not shown).

SAS v9.1.3 (SAS Institute, Cary, NC, USA) was used to calculate descriptive statistics and to assess genotypic differences in muscle strength in the replication sample and in *ACVR1B* mRNA expression using analyses of (co)variance. Because the strength testing protocols between the replication samples differed (eg, in equipment, angle for the isometric test, measurement units), strength measurements were normalized to *Z*-scores (mean=0; standard deviation=1) per sample. Similar strength measurements were combined in the joined sample as described in Supplementary file 2, Supplementary Table S3. Because

of the large age range of the replication individuals, age was included as a covariate in the analyses.

Probability levels <0.05 are considered significant. Instead of correcting *P*-values for multiple testing, we relied on the use of complementary association analysis methods, calculation of empirical *P*-values and a staged design including replication in an independent sample to minimize the possibility of false-positive findings.

RESULTS

Fine mapping stage 1

In stage 1, candidate genes and SNPs in the 1-LOD region of the original chr12q12-14 linkage peak were selected using an empiric two-step approach. First, a bioinformatical prioritization approach was used to select 86 candidate genes out of the 454 genes in the region. Second, 181 tagging SNPs, determined on CEPH (Centre d'Etude du Polymorphisme Humain) genotypes downloaded from the Hapmap[35] website, were selected in 74 candidate genes. For the remaining 12 genes, no polymorphisms meeting our criteria (Illumina designability rank=1 (ie, high success rate of assay design); MAF>0.05) were present. These polymorphisms were supplemented with 28 SNPs in the gaps in-between the genes, to enhance power for linkage.[17] A list of the selected SNPs is available in Supplementary file 2 (Supplementary Table S1).

Of the 209 SNPs, 198 were successfully genotyped, of which 7 SNPs were discarded (2 were monomorphic and 5 departed from HWE at the $P<0.001$ level), yielding 191 SNPs for further analysis.

These SNPs were successfully genotyped in a sample of 499 young male siblings. Association between a SNP and the studied muscle strength characteristics was evaluated using a combined linkage and association approach.[29] Consistent drops in –log *P*-values after modeling a joint test for linkage and association – indicating the presence of association between SNP and trait – were present for rs10784948 in contactin 1 (*CNTN1*) and rs7960176 in YY1 associated factor 2 (*YAF2*; Figure 2, open arrow) and for rs10783485 and rs10783486 in activin receptor 1B (*ACVR1B*; Figure 2, black arrow).

In addition, family-based association analyses were performed. Figure 3 shows the empirical *P*-values for an additive model for the 191 SNPs. SNPs in five genes (*ACVR1B,* cyclin-dependent kinase 4 (*CDK4*), cullin-associated and neddylation-dissociated 1 (*CAND1*), inhibin β C (*INHBC*) and myosin IA (*MYO1A*)) were repeatedly highly suggestively associated ($P<0.01$ or $-\log P>2$) with different strength measurements.

Stage 2 candidate genes were ranked on the basis of significance in the different association tests, significance over different strength measurements and (putative) functionality in muscle tissue. *ACVR1B* got the highest ranking because of its high significance in both the combined linkage and association approach (Figure 2) and the family-based association analyses (Figure 3), and because the ACVR1B protein has a role in the molecular TGFβ pathway regulating the signaling of myostatin, a negative regulator of skeletal muscle mass.[15,16] Even though it ranked lower, INHBC was also included for follow-up because it is a part of the same superfamily of proteins as ACVR1B.

Fine mapping stage 2

In stage 2, 20 and 13 polymorphisms were selected within *ACVR1B* and *INHBC*, respectively, (Table 1 and Supplementary Figures S1 and S2; available in Supplementary file 2) based on their probability to influence *ACVR1B* and *INHBC* gene function or expression. Of these 33 SNPs, 7 failed during analyses (including rs10783486, a stage 1 tagSNP), and 10 were 100% homozygous. The resulting 16 informative SNPs were genotyped in 536 siblings. Genotype distributions for all successfully genotyped polymorphisms were in HWE ($P>0.001$).

ACVR1B. Family-based association results for the *ACVR1B* SNPs and knee muscle strength can be found in Table 2. For rs10783485 and rs746434 (two tagSNPs), the association results from stage 1 were confirmed in the extended sample (stage 2: N=536 *versus* stage 1: N=499; overlap of 464 individuals) with significant associations for most of the knee flexion strength and some of the extension strength parameters ($0.00002<P<0.039$). No additional subjects were genotyped for rs10783486, so stage 1 results are presented in Table 2. The conserved SNP rs11612312 and SNP rs2854464, located in a putative miRNA-binding site, both showed an association with the dynamic

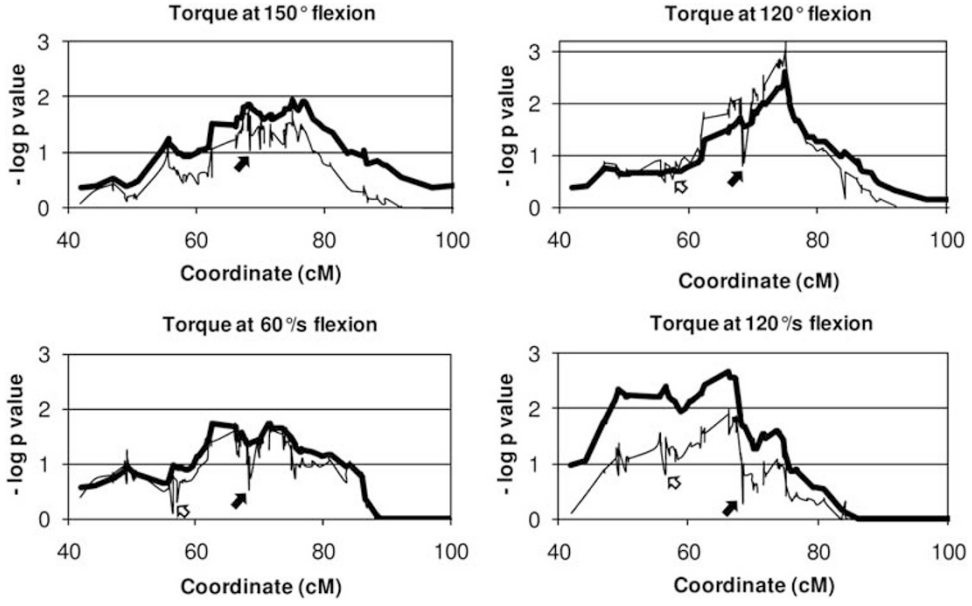

Figure 2 Combined linkage and association analyses on selected strength measurements. A drop in –log *P*-value for the combined linkage and association analysis (thin line) compared to the linkage analysis (thick line) indicates evidence for association. Associated SNPs rs10783485 and rs10783486 in *ACVR1B* are marked with a black arrow and associated SNPs rs10784948 (*CNTN1*) and rs7960176 (*YAF2*) are marked with an open arrow.

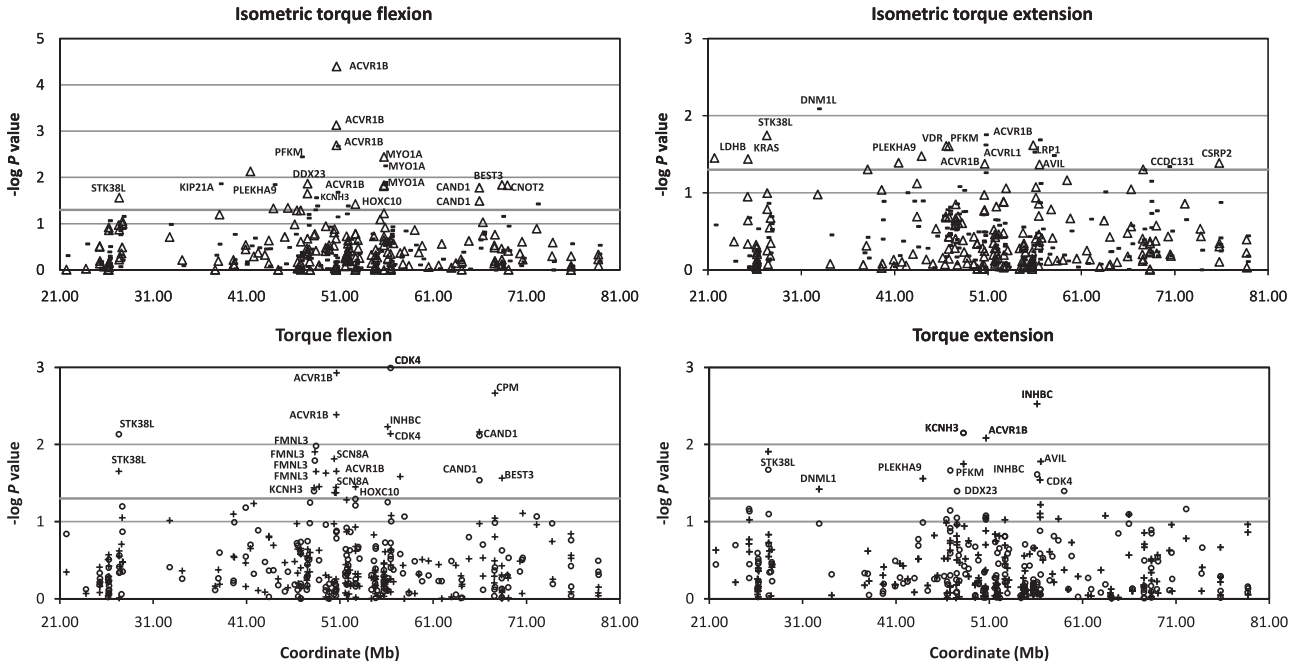

Figure 3 Family-based association results for selected strength measurements using single SNP markers. Empirical *P*-values from HBAT analyses were calculated after 10 000 permutations. Strength measurements at 150° (bold line), at 120° (triangle), at 60°/s (cross) and at 120°/s (circle) are shown. Most suggestive significant results ($P<0.05$, $-\log P\text{-value} > 1.3$) were marked with associated genes.

Table 2 Empirical *P*-values for association of *ACVR1B* polymorphisms and selected strength measurements

	Marker									
	rs10431538	*rs1979921*	*rs10783485*	*rs746434*	*rs11612312*	*rs10783486*	*rs2172603*	*rs2854464*	*rs2714*	*rs11169974*
Strength increasing allele			*C*	*G*	*T*	*G*		*A*		
Knee flexion										
Isometric at 150°, Nm	0.84	0.63	**0.028**	0.067	**0.034**	**0.0017**	0.50	0.083	0.94	0.95
Isometric at 120°, Nm	0.26	0.15	**0.0035**	**0.0038**	**0.00084**	**0.00002**	0.90	**0.0083**	0.67	0.82
Torque at 60°/s, Nm	0.15	0.39	**0.0069**	**0.023**	**0.0038**	**0.00066**	0.58	**0.029**	0.50	0.91
Torque at 120°/s, Nm	0.67	0.35	0.053	0.13	0.076	0.073	0.60	0.23	0.57	0.76
Knee extension										
Isometric at 150°, Nm	0.25	0.88	0.54	**0.025**	0.10	**0.034**	0.85	**0.045**	0.46	0.51
Isometric at 120°, Nm	0.84	0.76	0.30	0.19	0.052	**0.039**	0.88	**0.033**	0.37	0.88
Torque at 60°/s, Nm	0.34	0.098	0.10	0.18	**0.0076**	**0.0075**	0.43	**0.011**	0.89	0.68
Torque at 120°/s, Nm	0.52	0.47	0.12	0.057	0.13	0.074	0.29	0.14	0.88	0.92

Significant *P*-values are marked in bold.

knee flexion and extension measurements at 60°/s. Additionally, the polymorphisms show (a trend toward) significance for isometric strength.

INHBC. Only two of four selected tagSNPs showed limited levels of association with muscle strength (Table 3). Marker rs533975 was associated with torque flexion at 60°/s and 120°/s ($P<0.049$) and rs2943693 with torque flexion at 60°/s, and torque extension at both 60°/s and 120°/s ($P<0.021$). None of the other SNPs showed association with isometric or dynamic knee torque.

Replication analyses

We replicated the associations with putatively functional polymorphisms, detected in the second stage of the fine mapping, in men selected from two independent studies. Given the limited evidence for association for the *INHBC* gene, we focused the replication effort on rs10783485, rs10783486 and rs2854464 in *ACVR1B*.

All three polymorphisms were genotyped in 192 men from the BLSA study.[19] In the SPAH individuals[22] (n=74), only rs10783485 and rs2854464 were genotyped because of the technical difficulties. All genotype distributions were in HWE ($P>0.001$).

Replication analyses tested whether individuals homozygous for the strength increasing allele – as determined in stage 2 of the fine mapping – differed significantly from other individuals.

Because of testing protocol differences, strength measurements were normalized per sample before the analyses. Age was included as a covariate in the analyses because of the large age range of the individuals. Rs10783485 only showed significant association with moderate velocity torque of the knee extensors (GG: 0.074 ± 0.079

Table 3 Empirical *P*-values for association of *INHBC* polymorphisms and selected strength measurements

	Marker						
	rs7964492[a]	*rs12826906*	*rs560048*	*rs533975*[a]	*rs2943693*[a]	*rs2229357*	*rs3741414*[a]
Strength increasing allele			*T*	*A*	*A*		
Knee flexion							
Isometric at 150°, Nm	0.52	0.68	0.17	0.27	0.43	0.35	0.32
Isometric at 120°, Nm	0.60	0.63	0.40	0.49	0.57	0.40	0.39
Torque at 60°/s, Nm	0.70	0.14	0.068	**0.047**	**0.040**	0.72	0.61
Torque at 120°/s, Nm	0.70	0.86	0.056	**0.048**	0.11	0.73	0.76
Knee extension							
Isometric at 150°, Nm	0.095	0.68	0.092	0.066	0.57	0.25	0.22
Isometric at 120°, Nm	0.23	0.60	0.096	0.16	0.58	0.12	0.14
Torque at 60°/s, Nm	0.66	0.47	0.19	0.15	**0.0039**	0.43	0.49
Torque at 120°/s, Nm	0.50	0.64	0.41	0.23	**0.021**	0.39	0.42

Significant *P*-values are marked in bold; [a]Four selected tagging SNPs.

Table 4 Muscle strength characteristics according to rs2854464 genotype in the combined sample

	AA	*AG+GG*	
N	*81*	*64*	P-*value*
Knee flexion			
Isometric torque at 120°	0.14 ± 0.15	−0.19 ± 0.18	0.17
Slow velocity torque	0.021 ± 0.094	0.062 ± 0.11	0.77
Moderate velocity torque	−0.028 ± 0.096	−0.0070 ± 0.11	0.88
Knee extension			
Isometric torque at 140–150°	−0.015 ± 0.099	0.087 ± 0.11	0.50
Isometric torque at 120°	0.061 ± 0.096	0.0077 ± 0.11	0.72
Slow velocity torque	0.13 ± 0.080	−0.12 ± 0.086	**0.036**
Moderate velocity torque	0.16 ± 0.078	−0.17 ± 0.085	**0.0053**

All values are least square mean ± SEM from an analysis of covariance with age as covariate; significant *P*-values are marked in bold.

versus GT+TT: −1.15 ± 0.070; P=0.38). Individuals homozygous for the rs2854464 strength increasing A allele had significantly higher dynamic knee extensor strength than GG and AG individuals (all $P<0.036$; Table 4).

Quantification of mRNA expression

Effects at the mRNA level were tested only for rs2854464, located in a putative miR-24-binding site 985–1011 bp downstream of the stop codon of the *ACVR1B* gene, and inducing a higher strength in AA compared with AG+GG individuals (Tables 2–4).

We genotyped this SNP in a limited sample (N=16) from an independent study conducted in our research center from which muscle tissue biopsies were available.[24] Relative expression levels were 0.93 ± 0.18 and 0.95 ± 0.25 for AA homozygotes and G-allele carriers, respectively, and did not differ significantly (P=0.80).

DISCUSSION

We described the results of a two-staged gene-centered fine mapping approach applied on a previously identified linkage peak in the 12q12-14 region, and identified genetic variants in the activin receptor 1B (*ACVR1B*) gene to be associated with human muscle strength in a sample form the Leuven Genes for Muscular Strength study. A replication effort in a (size-limited) sample consisting of 266 men from two independent studies partly supports this association.

This is the first report that demonstrates a possible genetic link between knee strength and *ACVR1B*, which is a member of the TGFβ superfamily of proteins – as is myostatin[36] – and is known to be involved in the molecular pathway regulating myostatin and activin signaling. These signal pathways are triggered by the binding of the ligand to a type II receptor, the recruitment of a type I receptor and the formation of a heteromeric, active receptor complex. The type I receptor is the center of the signaling as it is essential for signal specificity as well as for signal propagation beyond the plasma membrane.[15,16]

Cross-linking studies demonstrated that activin receptor IIB (ACVR2B) is the primary type II receptor for myostatin.[37,38] Blocking the activity of this receptor in mice models leads to dramatic increases in muscle mass, comparable with those seen in myostatin knock out mice.[37,39] Additional evidence for a role of activin receptor signaling in determination of human muscle mass and strength comes from Walsh *et al*,[40] who showed a genetic association between ACVR2B and follistatin variants and skeletal muscle mass and strength. Moreover, resistance training could downregulate ACVR2B expression, suggesting a role for ACVR2B and the activin pathway in muscle response to strength training.[41,42]

Although it has been recognized that activin receptors are important in myostatin signaling, published studies have focused on the initial binding of myostatin to ACVR2B, whereas no studies reported on the subsequent steps of the signaling cascade. Cross-linking studies, however, showed that after binding of myostatin to ACVR2B, a type I receptor (ACVR1B (ALK4) or TGFβ receptor 1 (ALK5)) is recruited.[38]

Combined linkage and association, and family-based association results show that genetic variation in *ACVR1B* can influence human muscle strength. Of particular interest is the association between rs2854464 and muscle strength, with ∼2% lower knee strength for G-allele carriers compared to AA homozygotes in the LGfMS sample. Follow-up replication analyses in an independent but size-limited study sample also show an enhanced strength for AA homozygotes with regard to dynamic knee extensor strength.

The rs2854464 polymorphism is located in a putative miR-24-binding site in the 3′ untranslated region (UTR) of the *ACVR1B* mRNA. Wang *et al*[43] showed that miR-24 could decrease human ACVR1B expression at mRNA and protein levels. Published evidence

shows that genetic variation might influence organismal phenotypes[44,45] by perturbing miRNA-mediated gene regulation. Similarly, we hypothesize that the rs2854464 polymorphism affects muscle strength by interfering with miRNA binding.

Recently, Sun *et al*[46] showed that this miR-24 is involved in the inhibition of skeletal muscle differentiation by TGF*β*. Even though miR-24 is not a muscle-specific miRNA, they suggested that miR-24 might function during differentiation and homeostatic maintenance of cardiac and skeletal muscle tissue. We hypothesize that the effect of miR-24 on TGF*β* signaling is mediated through a miR-24-binding site in ACVR1B, one of the type I receptors important for TGF*β* as well as myostatin signaling.

It should be noted that the significances found for several of the selected *ACVR1B* SNPs are not completely independent findings, as these SNPs are in (high) LD with each other. The rs2854464 polymorphism is part of a haplotype block consisting of rs746434, rs11612312, rs10783486, rs2172603 and rs2854464 (determined using the four gamete rule in Haploview[31]). Haplotype analyses using HBAT software[34] confirm the single SNP analyses (data not shown). Although rs10783485 is not part of the same haplotype block, it does show considerable LD with SNPs in the haplotype block (r^2 range 0.15–0.44) and the observed significant results could therefore be related to rs2854464.

Even though a set of significant association results was observed for *INHBC* gene variants, these were not consistent over the different strength measurements. Therefore, these associations were not considered to be compelling evidence for a role of *INHBC* gene variants in determining muscle strength variation.

We find less significant results for associations at higher contraction velocities and different influences on knee flexors and extensors, suggesting a velocity and/or muscle group specific set of genes.[11] This is consistent with the observation that the importance of genetic factors decreases with increasing velocities in concentric torques due to genetic variation in contractile and elastic components, which contribute differently to strength production at different velocities.[47]

It could be argued that the currently applied two-stage strategy infers a bias toward genes with a known function in (the regulation of) muscle strength. Indeed, in both stages literature knowledge was used to prioritize genes for further follow-up. In addition, it should be noted that not all genetic variation in the linkage region was captured using our stage 1 SNP selection method because we only genotyped a limited set of SNPs with a MAF > 5%. It can therefore not be ruled out that genes and/or polymorphisms other than the ones we investigated are involved in the linkage signal. Moreover, genes for which no significant associations were found should not be considered negatively associated as not all SNPs within these genes were covered (eg, rare alleles). However, we were aware of these limitations from the beginning of our study and acknowledge that additional research will be necessary to determine which other genes/polymorphisms, in addition to *ACVR1B*, could be responsible for the linkage signal. These analyses should take into account that since the onset of this study a considerable amount of new information about human genetic variation has become available (eg, the 1000 genomes project; http://www.1000genomes.org).

In summary, we conducted a genetic fine mapping to identify variations in specific genes underlying a previously reported linkage peak for knee muscle strength on chr12q12-14. We are the first to describe, and replicate, a genetic link of ACVR1B, a receptor involved in the myostatin signaling pathway, to the determination of variation in muscle strength phenotypes. A SNP, rs2854644, located within a putative miR-24-binding site in the 3′ UTR of the *ACVR1B* gene, was found to be significantly associated with human knee muscle strength in three independent non-athletes samples from a wide age range.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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