Linkage of myostatin pathway genes with knee strength in humans

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Abstract

Skeletal muscle mass and muscle strength have been identified as major somatic components of physical fitness and are also determining factors of health-related fitness (6), e.g., for coping with stressful events, and for evaluating success in many sports (8). Since muscle mass, especially cross-sectional areas (Fig. 1), is a major determinant of work efficiency, an increased interest in muscular mass and strength has emerged during the last decades, with both increases in myofiber size (hypertrophy) and myofiber number (hyperplasia) (20), and mutations in the GDF8 gene in cattle result in the double-muscling phenotype (11, 19). The negative regulation of myostatin on postnatal muscle growth is confirmed by a study where a myostatin blocker, in vivo, increased muscle size and strength in mice suffering from Duchenne muscular dystrophy (5). In addition, systemically administered myostatin inhibitors in adult mice show a postnatal positive effect on both muscle and fat loss (38).

However, whether myostatin regulates skeletal muscle mass in humans in the same way as in nonhuman species is unclear. A study on HIV-infected men demonstrated a strong association between muscle wasting and increased levels of myostatin in serum and muscle tissue (10). Ferrrell et al. (8) identified five missense substitutions in the coding sequence of human myostatin, but only two of them were polymorphic (K153R, A55T). In their sample of Caucasian and African-American subjects, no significant association was found with differential muscle mass response to strength training. Yet, one allele (R153) was overrepresented in the nonresponder group, suggesting that this allele may play a role in other muscle phenotypes (8). Since muscle mass, especially cross-sectional areas of the muscle, and muscle strength are two strongly related characteristics, more muscle mass would implicate (in part) more strength. Indeed, two studies, both on women, suggest an association between strength (28) or gain in strength after a training program (13) with human myostatin variant K153R.

Here, briefly we provide a description of our selection of the candidate genes. As economic implications of myostatin on muscle growth for the cattle-breeding industry are obvious, many studies on animals have been performed and have unraveled the physiological pathway (16, 18, 26, 30, 31), although not all interactions with other muscle regulatory proteins are known. Myostatin pathway genes can directly or indirectly interact at each stage of the muscle development (Fig. 1). Mesoderm precursor cells transform into myoblasts under control of Myf5 and MyoD. The phosphorylation status of retinoblastoma (Rb) regulates the cell cycle (DNA synthesis) and therefore also the proliferation of myoblasts. After
proliferation, committed myoblasts differentiate and fuse into mature myotubes under control of Myf6 and myogenin. Thomas et al. (31) propose the following model for myostatin (GDF8) in regulating muscle mass (Fig. 1A): myostatin signaling results in an upregulation of p21 (or Cdkn1a), which is an inhibitor of cyclin-dependent kinase 2 (Cdk2). This causes a hypophosphorylation of Rb and a cell cycle arrest (G1) in proliferating myoblasts. Thus myoblast number and, hence, fiber number is regulated by GDF8. In addition, titin-cap (Tcap) can interact with myostatin and decreases the secretion of active myostatin, suggesting a possible regulatory effect on muscle development (22).

Myostatin can also inhibit differentiation (Fig. 1B) by up-regulation of MADH3 (or Smad3) that binds MyoD. This interaction represses MyoD transcriptional activity. As a result, several regulatory factors are downregulated (myogenin, p21, Myf5, Rb), which results in improper cell cycle withdrawal and inhibition of myoblast differentiation (3, 16, 26). Figure 1 further shows a schematic representation of the main (temporal) interactions of Igf1 on both proliferation and differentiation (3, 9, 25, 27), and in Table 1 the candidate genes depicted for this linkage study are given.

Since the allele frequencies of the polymorphisms in human myostatin are very low, association studies become difficult. Linkage analysis could counter this problem by using markers in the vicinity of the gene of interest, rather than nonsynonymous sequence polymorphisms in association analyses. However, so far no linkage study on muscle strength or mass has

### Table 1. Gene and marker positioning

<table>
<thead>
<tr>
<th>Gene</th>
<th>Marker</th>
<th>Physical Position, bp</th>
<th>Genetic Position, cM</th>
<th>No. of Alleles</th>
<th>Heterozygosity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYOG</td>
<td>D1S2683</td>
<td>200.735.504–200.738.298</td>
<td>220.7</td>
<td>7</td>
<td>76.2</td>
</tr>
<tr>
<td>GDF8</td>
<td>D2S118</td>
<td>189.114.675–189.800.894</td>
<td>189.800.716–189.800.894</td>
<td>6</td>
<td>78.6</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>D6S1051</td>
<td>36.648.597–36.657.219</td>
<td>36.635.107–36.635.335</td>
<td>4</td>
<td>63.7</td>
</tr>
<tr>
<td>MYOD1</td>
<td>D11S4138</td>
<td>18.862.381–18.864.900</td>
<td>18.876.970–18.877.174</td>
<td>6</td>
<td>73.9</td>
</tr>
<tr>
<td>MYF5</td>
<td>D12S326</td>
<td>78.316.207–78.316.459</td>
<td>81.085.949–81.088.595</td>
<td>13</td>
<td>67.6</td>
</tr>
<tr>
<td>MYF6</td>
<td>D12S708</td>
<td>81.076.594–81.078.399</td>
<td>82.180.214–82.180.382</td>
<td>13</td>
<td>67.6</td>
</tr>
<tr>
<td>RB1</td>
<td>D13S153</td>
<td>46.877.857–47.055.986</td>
<td>46.890.784–46.890.918</td>
<td>9</td>
<td>83.2</td>
</tr>
<tr>
<td>MADH3</td>
<td>D1S988</td>
<td>63.151.204–63.276.844</td>
<td>63.119.350–63.119.623</td>
<td>7</td>
<td>52.6</td>
</tr>
<tr>
<td>TCAP</td>
<td>D17S3293</td>
<td>32.329.857–32.330.140</td>
<td>32.330.98–32.330.140</td>
<td>7</td>
<td>87.4</td>
</tr>
</tbody>
</table>

Physical map positions are according to the UCSC Genome Bioinformatics web site. Genetic map positions are according to Marshfield (http://research.marshfieldclinic.org/genetics/) and Genethon (http://www.ceph.fr/ceph-genethon-map.html). MYOG, myogenin; GDF8, myostatin; CDKN1A, p21; MYF5, myogenic factor 5; MYF6, myogenic factor 6; IGF1, insulin-like growth factor 1; RB1, retinoblastoma; MADH3, Smad3; TCAP, titin-cap.
been performed. This literature overview above shows that myostatin has an important functional role in muscle mass of mice and cattle, that myostatin blockers have positive effects on muscle growth, and that linkage studies on muscularity in humans are lacking. Therefore, this linkage study is, to our knowledge, the first to explore the potential role of candidate genes in the myostatin pathway as QTLs for muscle strength and estimated muscle cross-sectional area in young male sib-pairs.

MATERIALS AND METHODS

Subjects

We randomly selected 329 male Caucasian sibs from 146 families from the Leuven Genes for Muscular Strength study (LGfMS). Of these families, 115 included sib-pairs, 25 trios, and 6 quads, which resulted in a maximum of 204 pairwise comparisons. These young male adult (17–36 yr) and healthy participants from the LGfMS study, ascertained from the general population, were volunteers, and no special selection based on sport, exercise, or physical activity was made, to avoid a selected physically active sample. In the LGfMS study, 23.9% of the subjects were not active in any sport, whereas 43% were active in two or more sports. Most of them (63.7%) were familiarized with the testing procedure (velocity, range of motion). Peak torque over the complete range of motion (0–90°) of knee extension and flexion was measured at 60°/s (3 repetitions), at 120°/s (25 repetitions), and at 240°/s (5 repetitions). During these contractions, torques at specific angles was also recorded. Following the force-strength relationship of a muscle, optimal strength is generated at longer muscle length, i.e., at an angle of 60° for knee extension (musculus quadriceps) and 30° for knee flexion (hamstring). Subjects were verbally encouraged to perform at their maximum effort, and visual feedback of their performance was presented after each test. For optimal comparison between subjects of different body size, “muscle quality” was calculated as the ratio of knee torque over muscle and bone cross-sectional area of the midhigh (N·m/cm²) and used in further linkage analysis.

Laboratory Methods

Genomic DNA was prepared from EDTA whole blood by the salting out method (21). Microsatellite markers (di- and tetranucleotide) were selected from the build 31 STS map (http://www.ncbi.nlm.nih.gov/genome/sts). Criteria for good markers were: location to the candidate gene (within 1 cM region), heterozygosity (>65%), and uniqueness of the primers. Positioning of the markers to the genes and heterozygosity is given in Table 1.

PCR conditions. Five PCR protocols were used to amplify the 11 markers of the 10 candidate genes. A marker was selected in or near the uniqueness of the primers. Positioning of the markers to the genes and heterozygosity is given in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Marker</th>
<th>Primer Volume, µl</th>
<th>Primer sequence (5′ → 3′)</th>
<th>Temp</th>
<th>MgCl₂</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR A</td>
<td>GDF8</td>
<td>D2S118</td>
<td>F: CAGGACAGGGTCCAAATCCGAG</td>
<td>R: ATTAAGGAAGATGAAACCTCT</td>
<td>60°C</td>
<td>2 mM</td>
</tr>
<tr>
<td></td>
<td>MYOD1</td>
<td>D1S4138</td>
<td>1</td>
<td>F: TGGTGACGCGGTGAAAGGAAATC</td>
<td>1</td>
<td>R: CAAGGAGGTTAATAGGGTCCA</td>
</tr>
<tr>
<td></td>
<td>CDKN1A</td>
<td>D6S1051</td>
<td>1.5</td>
<td>F: CAGAGAACGACATTTACAGGACG</td>
<td>1.5</td>
<td>R: GTATATTTTGCCGCAATGAGC</td>
</tr>
<tr>
<td>PCR B</td>
<td>MYF5/MYF6</td>
<td>D1S1708</td>
<td>0.5</td>
<td>F: GGGAACTTATGCGAAGCTGAGGA</td>
<td>0.5</td>
<td>R: GACTTAGTGCTGCAAGGGGTCTCC</td>
</tr>
<tr>
<td></td>
<td>RB1</td>
<td>D1S153</td>
<td>1</td>
<td>F: CATTTGTGTAGTCTATACAGCG</td>
<td>1</td>
<td>R: CAGGAGTGAAAGTCTAAGCC</td>
</tr>
<tr>
<td></td>
<td>IGF1</td>
<td>D1S1030</td>
<td>1</td>
<td>F: TCCCACTTTGACCTAGTTGAGG</td>
<td>1</td>
<td>R: AGGTTTAAATTGCCTACAGGGG</td>
</tr>
<tr>
<td>PCR C</td>
<td>TCAP</td>
<td>D17S1814</td>
<td>0.8</td>
<td>F: TCCCAATGAGGGTGATGAT</td>
<td>0.8</td>
<td>R: CGGAGGACACGGAAGCTGAG</td>
</tr>
<tr>
<td></td>
<td>MYOG</td>
<td>D15S2683</td>
<td>1</td>
<td>F: TGGCGTTGTCTGCTAAGGCGAG</td>
<td>1</td>
<td>R: GGCTGACAGGAAATCCTGGA</td>
</tr>
<tr>
<td></td>
<td>MYF5/MYF6</td>
<td>D12S326</td>
<td>1.5</td>
<td>F: CCCGACGCTGATGTTGAGTGA</td>
<td>1.5</td>
<td>R: GGGCTGAGGTGAGATGACAA</td>
</tr>
<tr>
<td>PCR D</td>
<td>TCAP</td>
<td>D17S1293</td>
<td>1</td>
<td>F: TGGAGGCTTGGAGGTTCCTTCT</td>
<td>1</td>
<td>R: CTTGAGGCTGGAGGAGTTTGAG</td>
</tr>
<tr>
<td>PCR E</td>
<td>MADDH3</td>
<td>D15S988</td>
<td>1</td>
<td>F: ATGAAAAACATGCTGCTGAGATTACG</td>
<td>1</td>
<td>R: CTTGCTGCTGCTGCTGCGAC</td>
</tr>
</tbody>
</table>

Further details of PCR reaction mix are outlined in the MATERIALS AND METHODS. F, forward primer; R, reverse primer.
(PCR-D and PCR-E) PCRs together with the primer sequences, gene and marker names. The specifications of the total PCR reaction mix (15 μl) were as follows: 1.5 μl PCR buffer [200 mM Tris·HCl (pH 8.4) and 500 mM KCl; Invitrogen], 0.5 μl dNTPs, 10 mM each (Amersham Biosciences), and 0.1 μl of Taq DNA polymerase (5 U/μl; Invitrogen), 1 μl genomic DNA, 0.6 μl of 2 mM MgCl₂ (or 0.675 μl of 2.25 mM MgCl₂ in PCR-D), and primers (see Table 2 for details). Sterile water was added to this mixture to end up with 15 μl mix. Amplification in Biometra T1 thermal cyclers started with an initial denaturing of 5 min at 94°C and ended with a final extension of 7 min at 72°C. PCR-specific annealing temperatures are given in Table 2.

Genotyping. PCR products were diluted 5–10 times before being pooled. Labeled fragments were size-resolved by capillary electrophoresis in the ABI3100 Genetic Analyzer, and size calling was performed by the GeneScan software (ver. 3.7, Applied Biosystems). Two independent, experienced researchers scored the genotypes, and a third independent expert was consulted to verify discrepant results.

Statistical Analysis

Heritability estimations. Upper-limit heritabilities of the traits (h²) were estimated by the variance components analysis procedure (VC) in QTDT (1). This estimate also includes common environmental variation in addition to the additive genetic component, because these two factors cannot be separated with sib-pairs only. Hence, it is called the upper-limit heritability.

Linkage analyses. After a pedigree check that revealed no genotyping errors, single-point linkage analysis was performed using Merlin software ver. 0.9.11 (2). Genotypic and phenotypic information was analyzed with two different model-free linkage methods. The first is a linear regression for quantitative traits (“qtl” option in Merlin; Ref. 2) that uses the framework of Whitemore and Halpern (37) for calculating a mean deviate for each founder allele, and an LOD score is defined by the Kong and Cox (15) statistic (QTL). The second is the VC method that tests a model with unique environment (E) and additive genes (A) against a model with A, E, and a quantitative trait locus (Q). Test statistic is a χ² with 1 df for comparison of the −2ln likelihoods of the two models. LOD scores are reported as (χ²/df). Allele frequencies and heterozygosity of the markers were estimated by allele counting procedures, and a Hardy-Weinberg equilibrium test was performed in Merlin. Suggestive evidence for linkage was defined by P < 0.01.

RESULTS

Descriptive Statistics

Table 3 shows the subject characteristics (means and SD) and the estimated upper-limit heritabilities (h²). The subjects had normal body mass and height; the body mass index (BMI; 22.6 ± 2.7 kg/m²) indicates that they were, on average, not overweight. Strength-contraction velocity relationships were observed in both extension (60°/s, 205.3 ± 53.5 N·m; 120°/s, 167.3 ± 27.4 N·m; 240°/s, 115.8 ± 20.7 N·m) and flexion (60°/s, 125.9 ± 22.9 N·m; 120°/s, 109.3 ± 19.1 N·m; 240°/s, 81.6 ± 16.4 N·m). This represents a decline in torque from 60°/s to 240°/s of 43.6% and 35.2% for extension and flexion, respectively.

As expected, the upper-limit heritability estimate of stature was high (92%), and genetic determination of body mass and BMI were somewhat lower (both 81%). Fat-free mass and other musculature variables (12) showed h² estimates between 70% and 99%, but lower h² values were observed for adiposity indicators (50–70%). In this sample, all strength phenotypes were under strong familial control (64% < h² < 75%).

Linkage Analyses

Heterozygosity of all markers was on average 72.5%. Genotype frequencies were tested for Hardy-Weinberg equilibrium and revealed no significant deviation from this equilibrium.

Single-point linkage analysis was performed between markers in or near the candidate genes from the myostatin pathway with muscle strength and mass phenotypes. Three markers showed consistent LOD scores on different strength phenotypes and are reported in Table 4. However, the two analysis methods (QTL and VC) did not always confirm each other. Marker D2S118 (GDF8, 2q32.2) had the strongest evidence for linkage with LOD scores between 2.63 and 1.24 (0.0002 < P < 0.008), except for extension at 120°/s and at 240°/s (P > 0.04). For QTL and VC, similar P values were observed in most cases, but large differences exist for extension at 60°/s (P = 0.0002 vs. P = 0.008) and flexion at 240°/s (0.007 vs. 0.2). D6S1051 (CDKN1A or p21, 6p21.2) and D11S4138 (MYO1D, 13q14.2) showed relative consistent linkage results with the VC method for most strength phenotypes (0.01 < P < 0.004), but the QTL method often failed to result in P values that were similar to the VC method. In addition, the number of significant LOD scores decreased with increasing velocity for the three loci. Moreover, only one significant LOD score (P = 0.007) was found with the highest velocity (flexion at 240°/s), i.e., with marker D2S118. The markers of the other candidate genes did not show a pattern of linkage with muscularity or knee strength. Suggestive linkage with estimated muscle cross-sectional area was only found with the marker for myostatin: MBA of the midthigh and muscle cross-sectional area of the quadriceps showed borderline significance (P = 0.01) with D2S118 applying the VC method.

DISCUSSION

This study is the first to explore the role of the myostatin pathway in explaining interindividual variation in estimated muscle cross-sectional area and strength using linkage analysis in a sample of young healthy male siblings. Choosing a physiological pathway has several advantages: 1) many genes in the cascade are covered if GDF8 is not the primary controlling gene, 2) a set of genes in that pathway can be ruled out in case of negative findings, 3) there is direct physiological evidence of its function, and 4) the importance of this pathway.
in explaining the variance in estimated muscle cross-sectional area and strength can be determined.

The maximum LOD score (QTL) of 2.63 ($P = 0.0002$) was observed for the ratio (Nm/cm$^2$) of knee extension at 60°/s with marker D2S118, ∼680 kb near the myostatin gene (GDF8). However, the VC analysis revealed a lower LOD score (1.24), suggesting difference in power with QTL, although other phenotypes showed in general comparable LOD scores (Table 4). The pattern of LOD scores for this marker was consistent over the different muscle groups (musculus quadriceps and hamstrings for extension and flexion, respectively) and velocities with exception of knee flexion at 120°/s and 240°/s. On chromosome 6, marker D6S1051 was chosen close to CDKN1A (13 kb), and marker D11S4138 on chromosome 11 is located 14.6 kb near MYOD1, and the same pattern, although less strong, emerges with LOD scores ranging between 1.17 and 1.87 ($P < 0.002$). Together with some borderline significant LOD scores ($0.05 < P < 0.01$) (Table 4), these data can be interpreted as an indication of linkage with these loci but not as strong evidence.

The myostatin pathway was chosen for its possible effects on muscle mass. Surprisingly, linkage was mainly found with strength phenotypes, but only marginal evidence was present for its effects on estimated muscle cross-sectional area. Two muscle cross-sectional areas had LOD scores that just reached $P = 0.01$ with D2S118 (myostatin) (Table 4). However, a recently revisited Haseman and Elston method, also implemented in Merlin (“merlin-regress” option) (29) showed confirmation of suggestive linkage with estimated muscle cross-sectional area (LOD 1.12–1.18). Unfortunately, this study lacks more accurate MRI- or CT scan-based measures of muscle mass. Maybe the anthropometric estimates of muscle mass cannot differentiate enough between levels of muscularity and therefore fail to detect higher allele sharing between sibs with similar muscle mass. However, previous studies have shown a good correlation between CT scan measures of muscle mass and estimates of muscle mass by anthropometric equations (32). Nevertheless, caution should be taken when interpreting results of estimated muscle cross-sectional area as muscle mass indicators.

Whether earlier reported polymorphisms in the myostatin gene A55T (exon 1) and BanII K153R, TaqI E164K, and BsrNI P198A (all in exon 2) (8) are responsible for the weak signal as found in this linkage study is unclear. In an earlier study we found no variation in the A55T, E164K, and P198A genotypes of 57 strength athletes and 57 control individuals (34). Only one individual was heterozygous for the K153R polymorphism in both strength athletes and control group. Because of the very low predicted allele frequencies of the rare alleles in these polymorphisms, subjects were not genotyped for these sequence variants in the present linkage analysis.

When phenotypes are compared over the different markers, the slow contraction velocities tend to be more linked to the three candidate regions (GDF8, CDKN1A, and MYOD1) than the higher velocities. Since the myostatin mechanism described earlier in animals mainly affects muscle mass, and since muscle mass is more correlated with isometric strength, it is not surprising that we found more suggestive linkage signals with the slower velocities. Another explanation is given by Carlson et al. (7) and Wehling et al. (36). They studied the effects of modified muscle use (unloading) on myostatin expression in different fiber types and found higher concentration of myostatin mRNA in fast-twitch muscle than in slow-twitch muscle. This, probably more prominent, inhibitory role of myostatin in fast-twitch muscle could be caused by fiberspecific, posttranslational modifications in myostatin. But it is not clear why the two other candidate genes are also more linked to strength phenotypes at lower contraction speeds.

The linkage pattern between the three loci and maximal knee muscle strength becomes more apparent when torque is considered in a specific angle during the three different movement speeds. The angles were chosen in which optimal strength could be generated following the force-length relationship (30° for flexion and 60° for extension). These torque measurements represent the same biological trait as the concentric peak torque values, and if similar linkage results are found with these traits, power is added to the interpretation of (suggestive) linkage. Indeed, the LOD scores of the angle-specific torques replicate the peak torque results (data not shown).
As could be expected, not all examined traits gave a signal for linkage despite the fact of biological relatedness. Also, some genes encoding proteins that play an important role in myogenesis (e.g., myogenin, IGF1) did not show signs of linkage to explain variation in muscle mass or strength. A number of reasons could explain the discrepancies in these findings.

First, one must consider the modest effect that any single gene would be expected to have on muscle mass or strength. These phenotypes are complex multifactorial traits that likely cannot be explained by a single gene, and, in addition, environmental factors contribute significantly to the observed variability. Moreover, one might assume that estimated muscle cross-sectional area and strength are also partly determined by gene-gene interactions as well as gene-environment interactions, which make it harder to determine a single gene effect.

Second, single-point rather than the more powerful multipoint linkage analysis was performed because this was an explorative study where the primary goal was to screen the myostatin pathway for its potential in explaining interindividual differences in estimated muscle cross-sectional area and strength. Third, the power of this study was limited. With optimal ascertainment of muscle cross-sectional area and strength, which make it harder to determine a single gene effect.

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Because of the exploratory nature of this study, patterns of (suggestive) linkage over different phenotypes are more interesting than individual LOD scores. The patterns seen in the region of 3 of 10 candidate genes indicate that this was a successful approach and strongly suggest that these loci (GDF8, CDKN1A, and MYOD1) may explain part of the interindividual variance of knee strength.

In conclusion, this study was the first explorative linkage study in humans to see whether the myostatin pathway might explain interindividual differences in estimated muscle cross-sectional area and knee strength. The present findings suggest that the chromosomal regions 2q32.2, 6p21.2, and 13q14.2 might harbor potential QTLs for skeletal muscle strength with GDF8, CDKN1A, and MYOD1 as good candidate genes. Since 3 of 10 candidate genes revealed suggestive single-point linkage on a limited sample size, it indicates that the myostatin pathway plays a role in human variation of muscle strength. Further studies on a larger sample and using a more dense marker saturation of these candidate regions (allowing multipoint linkage analysis) are required to confirm these results.

Acknowledgments

We thank all siblings of the LGIMS study for their maximum efforts and cooperation.

The following web sites for electronic databases are relevant to this paper: National Center for Biotechnology Information UniSTS web site http://www.ncbi.nlm.nih.gov/genome/stcs; the Genetic Power Calculator web site (http://statgen.iop.kcl.ac.uk/gpc/gplink.html); and UCSC Genome Bioinformatics web site (http://genome.ucsc.edu/).

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