Three DNA polymorphisms previously identified as markers for handgrip strength are associated with strength in weightlifters and muscle fiber hypertrophy

Running title: Genes for strength in weightlifters

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ABSTRACT

Muscle strength is a highly heritable trait. So far, 196 single nucleotide polymorphisms (SNPs) associated with handgrip strength have been identified in three genome-wide association studies. The aim of our study was to validate the association of 35 SNPs with strength of elite Russian weightlifters and replicate in Polish weightlifters. Genotyping was performed using micro-array analysis or real-time PCR. We found that the rs12055409 G-allele near *MLN* gene (P = 0.004), the rs4626333 G-allele near the *ZNF608* gene (P = 0.0338) and the rs2273555 A-allele in the *GBF1* gene (P = 0.0099)) were associated with greater competition results (total lifts in snatch and clean and jerk adjusted for sex and weight) in 53 elite Russian weightlifters. In the replication study of 76 sub-elite Polish weightlifters, rs4626333 GG homozygotes demonstrated greater competition results (P = 0.0155) and relative muscle mass (P = 0.046), adjusted for sex, weight and age, compared to carriers of the A-allele. In the following studies, we tested the hypotheses that these SNPs would be associated with skeletal muscle hypertrophy and handgrip strength. We found that the number of strength-associated alleles was positively associated with fast-twitch muscle fiber cross-sectional area in the independent cohort of 20 male power athletes (P = 0.021) and with handgrip strength in 87 physically active individuals (P = 0.015). In conclusion, by replicating previous findings in four independent studies we demonstrate that the rs12055409 G-, rs4626333 G-, and rs2273555 A-alleles are associated with higher levels of strength, muscle mass and muscle fiber size.

Key words: strength performance, muscle hypertrophy, DNA, polymorphism

INTRODUCTION

Muscle strength and power are highly heritable quantitative traits and critical in athletic events, such as sprinting, jumping, and weightlifting (17). Specifically, three studies have estimated 30 to 82% of heritability for strength-related phenotypes, such as muscle strength and handgrip strength (14,24,29). Considerable variation exists between people in the muscle strength response to resistance training, which may be partly explained by genetic variation (10,11). Overall, these findings provide strong evidence for muscular strength trait to be inheritable.

Several morphological (e.g. composition and cross-sectional area of muscle fibers) and neural (e.g. ability to maximally excite the motor neuron pool) adaptations are responsible for the muscle strength (9,13,22). Since the discovery of heritability of muscle strength there has been a growing interest to find genetic markers that influence muscle phenotypes, such as muscle strength, lean mass, composition and cross-sectional area of muscle fibers, testosterone levels and power athlete status (1,2,4,5,11,12,15,18,31,35,36). The most studied polymorphisms, which are associated with muscle strength and power athlete status, are located in *ACTN3* and *ACE* genes (19). Other muscle strength associated markers identified in candidate gene association studies include but not limited to *AGT*, *CCL2*, *CCR2*, *CNTF*, *FST*, *HIF1A*, *IGF1*, *IL6*, *MCT1*, *MSTN*, *NR3C1*, *PPARA*, *PPARG*, *PTK2* and *VDR* (2,3,11,18).

Using a genome-wide association study (GWAS) approach, 196 new DNA polymorphisms were associated with handgrip strength in three large GWASs. Specifically, the study conducted by Willems et al. of 195,180 white Europeans identified 16 single nucleotide polymorphisms (SNPs) near or within the genes involved in muscle structure and function, and associated them with handgrip strength (32). Matteini and co-workers examined associations of about 2.7 million SNPs in the GWAS with additional meta-analysis of individuals over 65 years old and reported 41 variants to be linked with handgrip strength (21). A more recent meta-analysis study by Tikkanen et al. (30) identified 139 loci associated with handgrip strength in a UK Biobank cohort. Additional association tests such as eQTL provided insight into a role of the identified markers in biological processes, e.g. some SNPs were seen to play role in brain function and the nervous system. These findings of gene variants involved in neuro-regulation supports the notion that muscle performance requires a well-functioning nervous system. Handgrip strength is a predictor of total muscle strength (33) and is associated with bone fracture risk (7), cardiac function (6) and risk of mortality (20,34). Thus, identification of genetic variants associated with strength is important to create resistance training programs for an aging population to prevent muscle strength decline (dynapenia) and further complications.

The aim of our study was to validate the association of 35 SNPs (of the 196 SNPs previously associated with handgrip strength) in a cohort of elite Russian weightlifters, and to replicate this in Polish weightlifters and other cohorts using functional analyses.

METHODS

Experimental Approach to the Problem

To identify genetic markers associated with strength in 53 elite Russian weightlifters, we performed a genotype-phenotype study using 35 SNPs previously discovered in non-athletic cohorts. The results were validated in 76 sub-elite Polish weightlifters. The functionality of significant SNPs was investigated in the muscle biopsy cohort of 20 male power athletes, in the body composition study of Polish weightlifters and in the handgrip strength study of 87 physically active men and women.

Subjects

Fifty-three elite weightlifters (22 females, 31 males) from the Russian cohort (all participants in Olympic Games or World / Europe Championships in 2008-2012, all negatively tested for doping by the WADA-accredited laboratories) and 76 sub-elite weightlifters (28 females, 48 males) from a Polish cohort (members of national junior team; no athletes tested were banned for taking any illegal substances) participated in the association study. In addition, 20 male sub-elite

Russian power athletes (9 sprinters, 3 weightlifters, and 8 powerlifters; regional competitors with at least four years of experience participating in their sports) and 87 physically active men (n=54) and women (n=33) were involved in the functional (muscle biopsy and handgrip strength, respectively) studies. The athletes were all Caucasians. Age, height and body mass of athletes from different groups are presented in Table 1.

The overall study was approved by the Ethics Committee of the Physiological Section of the Russian National Committee for Biological Ethics, and Ethics Committee of the Regional Medical Chamber in Szczecin (Approval number 09/KB/IV/2011). Written informed consent was obtained from each participant. The study complied with the guidelines set out in the Declaration of Helsinki and ethical standards in sport and exercise science research. The experimental procedures were conducted in accordance with the set of guiding principles for reporting the results of genetic association studies defined by the STrengthening the REporting of Genetic Association studies (STREGA) Statement.

Table 1. Anthropometric and performance variables in subjects from different groups

Characteristics	Group			
	Russian elite weightlifters	Polish sub-elite weightlifters	Russian sub-elite power athletes	Russian physically active people
Males	*n* = 31	*n* = 48	*n* = 20	*n* = 54
Age (years)	23.7±0.7	18.0±0.2	21.5±1.1	33.1±1.3
Height (cm)	179.0±1.6	173.9±1.2	179.7±1.2	179.5±0.8
Body mass (kg)	96.7±3.7	78.9±2.5	86.5±2.3	78.9±1.3
Competition scores (units)	241.1±2.8	168.9±2.6	-	-
Females	*n* = 22	*n* = 28	-	*n* = 33
Age (years)	22.5±0.8	17.6±0.2	-	28.4±1.5
Height (cm)	165.1±1.8	162.0±1.5	-	167.2±0.9
Body mass (kg)	69.4±2.5	65.1±3.1	-	57.8±0.9
Competition scores (units)	245.3±4.8	157.0±2.8	-	-

Procedures

Russian cohorts. Molecular genetic analysis in all Russian athletes was performed with DNA samples obtained from leukocytes (venous blood). Four ml of venous blood were collected in tubes containing EDTA (Vacuette EDTA tubes, Greiner Bio-One, Austria). Blood samples were transported to the laboratory at 4°C and DNA was extracted on the same day. DNA extraction and purification were performed using a commercial kit according to the manufacturer's instructions (Technoclon, Russia) and included chemical lysis, selective DNA binding on silica spin columns and ethanol washing. Extracted DNA quality was assessed by agarose gel electrophoresis at this step. HumanOmni1-Quad BeadChips (Illumina Inc, USA) were used for genotyping of 1,140,419 SNPs in 53 weightlifters and HumanOmniExpress BeadChips (Illumina Inc, USA) were used for genotyping of > 700,000 SNPs in participants of the Muscle Fiber (20 power athletes) and handgrip strength (87 physically active men and women) studies. The assay required 200 ng of DNA sample as input with a concentration of at least 50 ng/µl. Exact concentrations of DNA in each sample were measured using a Qubit Fluorometer (Invitrogen, USA). All further procedures were performed according to the instructions of Infinium HD Assay. Of the 196 SNPs for handgrip strength previously discovered via GWAS, 35 were included in the chips. We therefore performed a validation study to identify if these 35 DNA polymorphisms are also associated with strength (competition results) in weightlifters.

Polish cohort. Nylon swabs (Copan, Italy) were used to collect buccal cells donated by the subjects. DNA was extracted from the collected material using GenElute Mammalian

Genomic DNA Miniprep Kit (Sigma, Germany) according to the manufacturer's protocol. The purity and quantity of the DNA samples was determined by measuring their absorbance at 260 nm and 280 nm on the Eppendorf Biophotometer Plus (Eppendorf, Germany). To prevent multiple freezing and thawing, isolated DNA was aliquoted and stored at −20°C. All samples were genotyped in duplicate (by two separate researchers) in order for the results to be credible (eliminating false positives and false negatives) using an allelic discrimination assay on a CFX Connect Real-Time PCR System (Bio-Rad, Germany). Between the two researchers, the percentage of agreement in genotyping was 100%. To discriminate *ZNF608* rs4626333 alleles, TaqMan® Pre-Designed SNP Genotyping Assays were used (Applied Biosystems, USA) (assay ID: C__30716090_10) including primers and fluorescently labelled (FAM and VIC) MGB^{TM} probes together with the Brilliant III Ultra-Fast QPCR MasterMix (Agillent, USA) to detect alleles. Genotypes were assigned using all of the data from the study simultaneously. For the primary data analysis, CFX Maestro Software (Bio-Rad, Germany) was used.

Evaluation of muscle fiber composition and cross-sectional area

Samples of the vastus lateralis muscle of physically active participants were obtained with the Bergström needle biopsy procedure under local anesthesia with 1% lidocaine solution. After this procedure, serial cross-sections (7 μm) were obtained from frozen samples using an ultratom (Leica Microsystems, Germany). The sections were thaw-mounted on Polysine glass slides, kept for 15 min at RT and incubated with in PBS (3 × 5 min). Then the sections were incubated at RT in primary antibodies against slow or fast isoform of the myosin heavy chains (M8421, 1:5000; M4276; 1:600, respectively; Sigma-Aldrich, USA) for 1 h and incubated in PBS (3 × 5 min). After this, the sections were incubated at RT in secondary antibodies conjugated with FITC (F0257; 1:100; Sigma-Aldrich) for 1 h. The antibodies were removed and the sections were washed in PBS (3 × 5 min), placed in mounting media and covered with a cover slip. The image was captured using a fluorescent microscope Eclipse Ti-U (Nikon, Japan). All analyzed images contained > 100 fibers. The ratio of the number of stained fibers to the total fiber number was calculated. Fibers stained in serial sections with antibodies against slow and fast isoforms were considered as hybrid fibers. The cross-sectional area (CSA) of fast and slow fibers was evaluated using ImageJ software (NIH, USA).

Measurement of body composition

The body composition (relative muscle mass, i.e. muscle mass / body weight, %) in Polish weightlifters was measured in the fast state (for ≥ 8 hrs) with stand-on hand-to-foot 8-electrode body composition analyser Tanita MC-180MA (Tanita, Tokyo, Japan), according to manufacturer's instructions. Normal, athletic body type was selected for the manufacturer's inbuilt predictive algorithm. Standard positioning was used as described in the instruction manual in all measurements. In brief, participants were asked to stand with bare feet on the electrode panel and hold electrodes in both hands; arms were extended and hung down in a natural standing position with the electrodes in contact with thumb and palm during the measurements.

Strength measurement

Evaluation of strength in elite Russian weightlifters was assessed by their performance in snatch, and clean and jerk (best results in official competitions including Olympic Games, Europe and World Championships). The total weight lifted (in kg) is multiplied by the Wilks Coefficient (Coeff) to find the standard amount lifted normalised across all body weights.

$Coeff = \frac{500}{a+bx+cx^2+dx^3+ex^4+fx^5}$, where x is the body weight of the weightlifter in kilograms.

Values for males are: a = -216.0475144; b = 16.2606339; c =-0.002388645; d = -0.00113732; e = 7.01863E-06; f = -1.291E-08. Values for females are: a = 594.31747775582, b = -27.23842536447; c = 0.82112226871; d = -0.00930733913; e = 4.731582E-05; f = -9.054E-08.

Handgrip strength

The hand dynamometer (DK-140, Russia) was used for the handgrip strength testing of 87 physically active men and women. The strength of both the left and right hands was measured thrice each in a standing position (with the arm in complete extension without touching any part of the body with the dynamometer), and the best score of the dominant hand (kg) was used in the analysis.

Statistical Analyses

Statistical analyses were conducted using PLINK v1.90, R (version 3.4.3), and GraphPad InStat (GraphPad Software, Inc., USA) software. Differences in phenotype between different genotype groups were analysed using ANOVA (for three genotypes) or unpaired *t* tests (for two genotypes). Effect estimates (EE) were measured using R. Omega-Squared (ω^2) values were calculated as effect estimates for ANOVA using "omega_sq" function from the "sjstats" package. Cohen's D values were calculated as effect estimates for unpaired *t* tests using "cohensD" function from the "lsr" package. To perform the meta-analysis the Cochrane Review Manager (RevMan) version 5.3 was used. Random and fixed effect models were applied. The heterogeneity degree between the studies was assessed with the I^2 statistics. All data are presented as mean (standard deviation). *P* values < 0.05 were considered statistically significant. Due to the large number of SNPs investigated and, therefore, the large number of multiple comparisons, the Benjamini-Hochberg method was used where appropriate to control the false discovery rate (FDR).

RESULTS

Association studies in weightlifters

There were no differences in the standard amount lifted between Russian female and male weightlifters ($P > 0.05$), which allowed us to combine females and males of different body mass into one group. Using a panel of 35 strength-related SNPs (see Supplemental Digital Content 1, which demonstrates data of all 35 SNPs), we found that the associations of three SNPs were nominally ($P < 0.05$) replicated in the same direction in Russian weightlifters (Table 2). Specifically, weightlifters with rs12055409 G ($P = 0.0016$; $\omega^2 = 0.136$), rs4626333 G ($P = 0.047$; $\omega^2 = 0.042$) and rs2273555 A ($P = 0.0042$; $\omega^2 = 0.108$) alleles have shown significantly better results (additive model). Two of these markers (rs12055409 and rs2273555) have also passed Benjamini-Hochberg correction criteria for multiple testing (see Supplemental Digital Content 1). However, the rs4626333 was not excluded given that it was used in a validation study (Polish cohort).

In weightlifters, three SNPs (and rs4626333 in Polish athletes) met Hardy-Weinberg expectations ($P > 0.05$). There were no differences in allelic frequencies between males and females for these SNPs (rs12055409 G: 62.9 vs. 63.6%; rs4626333 G: 82.3 vs. 86.4%; rs2273555 A: 66.1 vs. 61.4%). In a separate analysis, we found that male weightlifters with rs12055409 G ($P = 0.025$) and rs4626333 G ($P = 0.011$) alleles have shown significantly better results (additive model), while in female weightlifters, carriers of these alleles only tended to have greater strength (*P* values from 0.07 to 0.3).

Next we performed a validation study in the Polish cohort of sub-elite weightlifters using only one available SNP for analysis (rs4626333). In accordance with Russian results, we found that carriers of the rs4626333 GG genotype had greater personal best results in weightlifting ($P = 0.0155$ for additive model; Cohen's D = 0.548) adjusted for their sex, weight and age than carriers of the A allele.

Table 2. Summary of the most significant SNPs replicated in Russian weightlifters (n=53) with their competition scores adjusted by Wilks formula.

SNP	Chr	Gene	Strength increasing allele (%)	Genotypes, their frequencies and strength (Wilks Score)			*P (ANOVA)*
rs12055409	6	Near *MLN*	G (63.2)	AA (18.9%) 225.5 (22.8)	AG (33.9%) 242.0 (25.2)	GG (47.2%) 249.1 (17.1)	0.004*
rs4626333	5	Near *ZNF608*	G (83.9)	AA (1.9%) 205.2	AG (28.3%) 235.1 (15.9)	GG (69.8%) 245.7 (23.9)	0.0338*
rs2273555	10	*GBF1*	A (64.2)	GG (13.2%) 223.1 (22.1)	AG (45.3%) 241.0 (22.5)	AA (41.5%) 248.9 (20.1)	0.0099*

Values are means (SD).
*$P < 0.05$, statistically significant

Functional studies

There were no differences in muscle fiber CSA between Russian sprinters and strength (weightlifters and powerlifters) athletes ($P > 0.05$), which allowed us to combine them for further analysis. In separate analyses, the positive association between rs12055409 G allele and CSA of fast-twitch muscle fibers was identified ($P = 0.0295$ for additive model; $\omega^2 = 0.187$). We also found that amongst Polish weightlifters, rs4626333 GG homozygotes had greater relative muscle mass ($P = 0.046$ for additive model; Cohen's D = 0.488) adjusted for their sex, weight and age than carriers of the A allele. We also have replicated the association between the rs2273555 A-allele in the *GBF1* gene and handgrip strength in both 54 physically active men ($P = 0.042$) and 33 women ($P = 0.035$) (adjusted for sex $P = 0.0026$ for additive model; $\omega^2 = 0.092$).

Meta-analysis

Using available data of strength-related phenotypes (i.e. competition results of Russian and Polish weightlifters, and handgrip strength of Russian physically active men and women) we performed a meta-analysis for three SNPs. The meta-analysis revealed two statistically significant markers, namely rs2273555 ($P = 6.34 \times 10^{-5}$; TE = 0.345; $I^2 = 0$) and rs4626333 ($P = 0.0024$; TE = 0.211; $I^2 = 0$) after correcting for multiple testing. The rs12055409 remained nominally significant ($P = 0.043$; TE = 0.174; $I^2 = 0.77$) and was not excluded from further polygenic analysis given its additional association with CSA of fast-twitch muscle fibers.

Polygenic analyses

We found that CSA of fast-twitch muscle fibers was significantly ($P = 0.021$) higher in athletes who carried 5-6 strength alleles (i.e. rs12055409 G, rs4626333 G and rs2273555 A) in comparison with carriers of 3 and 4 alleles (Table 3). In addition, physically active individuals (n=87) with 5-6 strength alleles had significantly (adjusted for sex $P = 0.015$) higher handgrip strength than individuals with 2-4 alleles.

Table 3. Cross-sectional area of muscle fibers in carriers of different number of strength-related alleles among Russian male power athletes (n=20).

Carriers of different numbers of strength alleles*	CSA of fast-twitch muscle fibers, μm^2	CSA of slow-twitch muscle fibres, μm^2
3 alleles (n=3)	6196 (934)	5602 (710)
4 alleles (n=9)	7049 (1244)	5732 (1081)
5-6 alleles (n=8)	10029 (3235)**	6904 (2458)

*strength-related alleles: rs12055409 G, rs4626333 G and rs2273555 A
**P=0.021 (ANOVA); values are means (SD).

DISCUSSION

In the current study we used a replication design to avoid finding false-positive results in the identification of strength-related markers in weightlifters. For this, we performed a genotype-phenotype study of 53 elite Russian weightlifters using 35 SNPs previously discovered as handgrip strength variants in non-athletic cohorts. For one SNP we also validated the obtained results in 76 sub-elite Polish weightlifters. The functionality of significant SNPs was further investigated in the muscle biopsy cohort of 20 male power athletes and in the handgrip strength study of 87 physically active men and women.

We have thus nominally ($P < 0.05$) confirmed the associations of three SNPs, namely rs12055409, rs4626333 and rs2273555, with strength in Russian weightlifters, and one of the SNPs (rs4626333) was also validated in Polish weightlifters. These findings were further supported by functional studies, where we found that polygenic scores composed of three SNPs were significantly associated with fast-twitch muscle fiber CSA and handgrip strength.

The rs12055409 SNP, previously identified as a handgrip strength marker in 334,925 individuals from the UK Biobank cohort (30), is located in the regulatory region next to the *MLN* gene. *MLN* encodes motilin, a polypeptide hormone which is secreted in small intestine and stimulates gastric motor activity. Sullivan et al. (28) have reported an increase in exercise-induced motilin secretion in endurance athletes. Interestingly, motilin is a peptide that promotes the secretion of growth hormone in a dose-related fashion in rat brain (26). According to the GTEx database, the rs12055409 G allele is associated with a high expression of the *IP6K3* gene in thyroid tissue (16). *IP6K3* encodes inositol hexakisphosphate kinase 3 which generates inositol pyrophosphates and regulate diverse cellular functions, including metabolism and body weight (23). Given that growth hormone has an anabolic effect on muscle mass, it can be speculated that the rs12055409 SNP in the regulatory region near the *MLN* gene might change the growth hormone concentration by altering its expression, thus giving athletes an advantage in strength. Indeed, we observed an association between the strength-increasing rs12055409 G allele and CSA of fast-twitch muscle fibers in power athletes.

The rs4626333 SNP, previously described as a handgrip strength marker in 27,581 older individuals of European descent (21), is a variant located in regulatory region next to the *ZNF608* gene, which encodes the transcription factor, paralog of the *ZNF609* gene. It has been shown that rs4836133 (which is not in linkage disequilibrium with rs4626333) is associated with body mass index in humans (27). Moreover, a study on pigs mentions *ZNF608* as a potential gene that impacts on absolute fat mass and in this manner might influence other traits as well (25). One might suppose that the association of rs4626333 with muscle strength is mediated via its muscle hypertrophic effect. Indeed, we found that rs4626333 GG homozygotes had greater relative muscle mass amongst Polish weightlifters.

The rs2273555 SNP, previously discovered as a handgrip strength marker in 195,180 white Europeans (32), is located inside intron of the *GBF1* gene. *GBF1* (golgi brefeldin A resistant guanine nucleotide exchange factor 1) encodes a protein that regulates the recruitment of proteins to membranes by mediating the GDP to GTP exchange and plays a role in vesicular trafficking by activating ADP ribosylation factor 1. The protein is expressed in skeletal muscles and is supposed to play a role in exercise-induced lipolysis in skeletal muscle (8).

In conclusion, by replicating previous findings in four independent studies we demonstrate that the rs12055409 G-, rs4626333 G-, and rs2273555 A-alleles are associated with higher levels of strength, lean mass and muscle fiber size, thus confirming previous associations of these polymorphisms with handgrip strength in different populations. Furthermore, we provide evidence that these genetic associations with strength are underpinned by greater lean mass and muscle fiber size in those with the “favourable” genetic variants, thus improving our

understanding of the genetic association with strength. Overall, this suggests that these particular gene variants predispose to greater muscle mass and strength and/or to a more favourable neuromuscular adaptation to chronic resistance training, primarily by influencing muscle fiber size.

PRACTICAL APPLICATIONS

Our results highlight the potential for the rs12055409, rs4626333 and rs2273555 polymorphisms to be used to indicate who is more likely to adapt more favourably to chronic resistance exercise, thus enabling resistance training to be prescribed on a more individual level, both for athletes and the general population.

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