Original Articles

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ACTN3 R577X Genotype Is Associated with ACTN3 Protein Expression Levels and Myosin Heavy Chain Composition in Japanese College-Level Male Sprinters

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Objective: Alpha-actinin (ACTN) 3 R577X polymorphisms have three genotypes: RR, RX and XX. Only RR and RX genotypes express ACTN3 protein in type II fibers. The purpose of this study was to clarify whether there are differences in ACTN3 protein expression levels and myosin heavy chain (MyHC) composition between RR and RX genotypes in Japanese college-level male sprinters.

Materials and Methods: Forty-three Japanese college-level male sprinters participated in this study. Subjects were genotyped for ACTN3 R577X using a real-time polymerase chain reaction assay. Furthermore, muscle biopsies from the vastus lateralis muscle were obtained from a subset of subjects who had R allele and gave their consent (4 RR and 9 RX). ACTN3 protein expression levels were assessed by western blotting. MyHC composition was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Results: There was no difference in 100-m sprint performance among RR, RX and XX genotypes in all 43 subjects. In 13 biopsy-sampled subjects, there was also no difference in 100-m sprint performance between RR and RX and ACTN3 protein expression levels tended to be higher in RR genotype than in RX genotype. Although there were no differences in the proportion of type I and II MyHC isoforms between both genotypes, RR genotype had a significantly higher proportion of type IIx MyHC isoform and a significantly lower proportion of type IIa MyHC isoform than RX genotype.

Conclusions: ACTN3 protein expression levels and the proportion of type IIx MyHC isoform are higher in RR genotype compared with RX genotype in Japanese college-level male sprinters.

Key words: ACTN3 R577X genotype, ACTN3 protein, muscle fiber composition, human skeletal muscle, sprinters

Introduction

In mammalian skeletal muscle, two alpha-actinin (ACTN) isoforms, ACTN2 and ACTN3, are major components of the Z-lines, where they form a lattice structure that anchors the actin-containing thin filaments and stabilize the contractile apparatus[1)-3)]. ACTN2 protein is expressed in both type I (slow twitch) and II (fast twitch) skeletal muscle fibers, whereas ACTN3 protein is restricted to type II fibers in human skeletal muscle[4)]. In general, it is thought that the expression of ACTN3 protein in type II fibers contributes to the generation of greater muscular strength and power. ACTN3 protein is encoded by ACTN3 gene, and ACTN3 gene R577X polymorphisms have three genotypes

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(RR, RX and XX). RR and RX genotypes express ACTN3 protein in type II fibers, whereas XX genotype results in a complete deficit of ACTN3 protein[5]. Therefore, it may be important for sub-elite and elite sprinters to have R allele. Indeed, it has been reported that the frequencies of RR and RX genotypes are higher than that of XX genotype in sprinters and there is no XX genotype in top-level sprinters[6]. However, the differences between RR and RX genotypes in sub-elite and elite sprinters have not been studied very well, although sprint performance of RR and RX genotypes with ACTN3 protein has been compared with that of XX genotype without ACTN3 protein. In addition, there has been no study comparing ACTN3 protein expression levels between RR and RX genotypes in sprinters, although it was reported that ACTN3 protein expression levels were higher in healthy male adults with RR genotype than in those with RX genotype[7].

It is generally accepted that muscle fiber composition influences sprint performance[8]. Ahmetov *et al.*[9] stated that the ACTN3 gene R577X polymorphism may be one of the contributing gene variations in the determination of muscle fiber composition and showed an association between ACTN3 R577X genotypes and average percentage of slow twitch fibers in physically active subjects including different racing distance speed skaters. We have also shown that RR+RX genotypes have a higher proportion of type IIx myosin heavy chain (MyHC) isoform than XX genotype in young men[10]. In contrast, Norman *et al.* found no differences in muscle fiber composition among three genotypes in healthy young men and women[11]. Clearly, there is a lack of consistent findings regarding the relationship between ACTN3 R577X genotype and muscle fiber composition, and specifically whether muscle fiber composition in sprinters differs between RR and RX genotypes.

The purpose of this study was to clarify whether there are differences in ACTN3 protein expression levels and MyHC composition between RR and RX genotypes in Japanese college-level male sprinters.

Materials and Methods

1. Subjects

Forty-three Japanese college-level male sprinters (100-m personal best time: 10.91 ± 0.31 s) participated in this study. All subjects trained for 2-3 h, five times a week. All subjects gave their written consent to participate in this study after being fully informed of the study protocol, procedures, and risks. This study was approved by the Ethics Committee of the Graduate School of Health and Sports Science, Juntendo University (approval number: GHSS 25-53) and was conducted in accordance with the principles of the Declaration of Helsinki.

2. Analysis of ACTN3 R577X genotypes

Blood samples were extracted from all subjects to identify their ACTN3 R577X genotype (rs1815739) using the Custom TaqMan SNP Genotyping Assay (Applied Biosystems, Tokyo, Japan). For the ACTN3 R577X polymorphisms, the sequences of the primers and probes were 5'-CACGATCAGTT CAAGGCAACA-3' (forward primer), 5'-CCCTGGATGCCCATGATG-3' (reverse primer), VIC-5'-CTGACCGAGAGCGA-3' (probe for the R allele), and FAM-5'-AGGCTGACTGAGAGC-3' (probe for the X allele), as previously described[12]. Amplification and detection were performed using a 7300 real-time polymerase chain reaction (PCR) system with TaqMan PCR Master Mix (Applied Biosystems, Tokyo, Japan). The thermal cycle protocol consisted of 2 min at 50℃ and 10 min at 95℃, followed by 15 s of denaturation at 95℃ and 60 s of annealing/extension at 60℃ for 40 cycles in a total reaction mixture of 25 μl. All reactions were set up manually, and allelic discrimination was determined in duplicate.

3. Muscle biopsy

Muscle biopsies were obtained from the dominant vastus lateralis muscle of 4 RR and 9 RX subjects who had given their consent for muscle biochemical analyses. Using a disposable biopsy instrument (14-gauge, Max Core; C. R. Bard, Covington, GA, USA), 5-15 mg of muscle was obtained three times under local anesthesia with 1% lidocaine solution. After removal, the muscle samples were frozen immediately in liquid nitrogen and stored at -80℃ in preparation for western blotting and MyHC composition analyses.

4. Sample preparation

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), muscle samples

were homogenized in × 15 ice-cold buffer [50 mM HEPES pH 7.4, 4 mM EGTA, 10 mM EDTA, 50 mM β-glycerophosphate, 25 mM sodium fluoride, 5 mM sodium orthovanadate, 0.1% Triton X-100, Protease Inhibitor Cocktail Complete EDTA-free (Roche, Penzberg, Germany) and PhosSTOP (Roche, Penzberg, Germany)] and centrifuged at 12,000 × *g* for 15 min at 4℃, as described by Kakigi *et al*[13]. After discarding the supernatant, the insoluble pellets were resuspended in ×15 1% SDS buffer (20 mM HEPES, 1% SDS, 250 mM sodium chloride (NaCl)), the samples were centrifuged at 15,000 × *g* for 5 min at 4℃. The supernatant was collected, and the total protein concentration was determined using a BCA™ Protein Assay Kit (Thermo Scientific Inc., USA) with bovine serum albumin (BSA) as a standard. For ACTN3 protein and MyHC analyses, the supernatant was mixed with an equal volume of 2 × sample buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 50% glycerol, 0.01% bromophenol-blue, 10% 2-mercaptoethanol] using the same protein concentration in each sample and then boiled at 95℃ for 5 min. The samples were stored at -80℃ prior to western blot and MyHC composition analyses.

5. SDS-PAGE and western blot analysis for ACTN3 protein

The same amount of protein (1.05 μg) was loaded into each well of an SDS-PAGE gel (4% stacking gel, 10% separating gel) and then run at 150 V for 1 h. After separation, the proteins were transferred to PVDF membranes at 100 V for 1 h. The membranes were then blocked for 1 h using 5% skim milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.4) supplemented with 0.1% Tween-20 (T-TBS). After that, the membranes were incubated with primary antibody specific to ACTN3 (1:20000) in T-TBS containing 5% skim milk. The membranes were subsequently washed with T-TBS and incubated with an anti-rabbit HRP conjugated secondary antibody (1:10000, HRP-linked anti-rabbit IgG: Cell Signaling) in 5% skim milk in T-TBS for 30 min. After repeated washing, each protein band was visualized using a chemiluminescence reagent (ECL Prime Western blotting Detection System, GE Healthcare UK, Buckinghamshire, UK) and images were captured using an image analysis apparatus (Light Capture; ATTO, Bunkyo-ku, Japan). Each band was evaluated using computer software (CS Analyzer 3.0, ATTO). For each protein, all optical density values were normalized using actin (A3853, Sigma-Aldrich, USA) as a loading control. The protein expression levels are shown in arbitrary units (AU). All incubations were performed at room temperature. The antibody against ACTN3 was kindly provided as a gift from research group[14]. Standard curves were constructed for each protein analysis.

6. Determining MyHC composition

The MyHC composition was determined using glycerol—SDS-PAGE according to the modified method described by Sugiura and Murakami[15]. Briefly, 1.75 μg of samples was applied to the glycerol—SDS-PAGE gel (stacking gel: 4% acrylamide, 34.7% glycerol, 125 mM Tris-HCl pH 6.8; separating gel: 8% acrylamide, 33.3% glycerol, 375 mM Tris-HCl pH 8.3). Electrophoresis was started at 60 V with the stacking gel at 8℃. The voltage was set at 150 V and the gel was run for 18 h at 8℃ at which point the tracking dye had completely entered the separating gel. After separation, each gel was stained with Coomassie Brilliant Blue (Biosafe G250; Bio-Rad Laboratories) and then rinsed with distilled water. To determine the relative percentage of each MyHC isoform, each gel was scanned using a calibrated densitometer (GS800; Bio-Rad Laboratories) and the MyHC composition was determined using ImageJ (NIH) software.

7. Statistical analyses

All data are presented as mean ± standard deviation (SD). The 100 m best times for the RR, RX, and XX genotypes for all subjects were analyzed by one-way analysis of variance (ANOVA). Student's t-tests were used to determine differences in the limited samples between RR and RX genotypes for all parameters. Statistical significance was set at $p < 0.05$. All analyses were performed using SPSS ver. 23.0 statistical software (SPSS, Chicago, IL, USA). Since XX genotype subjects have no detectable ACTN3 protein, they were excluded from biochemical analyses.

Table-1 Genotype frequency and 100-m best time among ACTN3 R577X genotypes

	RR	RX	XX
Number	12 (4)	25 (13)	6
Distribution (%)	27.9	58.1	14.0
100-m best time (s)	10.83 ± 0.29 (11.13 ± 0.27)	10.92 ± 0.31 (10.95 ± 0.39)	10.99 ± 0.31

Values are mean ± SD.
The data in parentheses indicate muscle biopsy-sampled subjects.

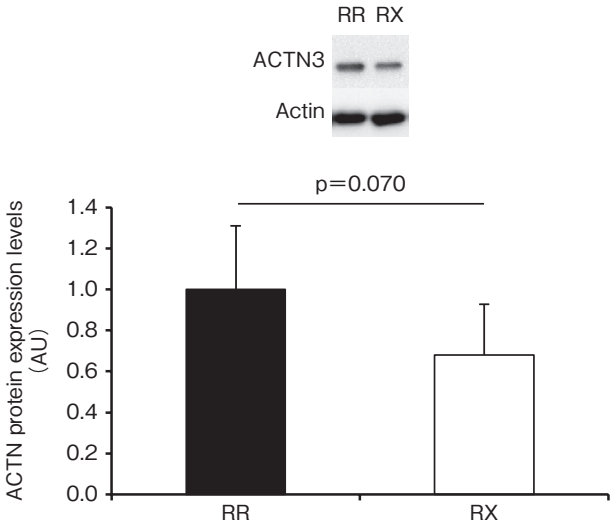

Figure-1 Alpha-actinin (ACTN) 3 protein expression levels in subjects with RR and RX genotypes
The upper panel shows an image of a typical western blot and the lower panel shows a quantitation of 13 subjects.
RR (black bar); RX (white bar). Values are mean ± SD.

Results

1. ACTN3 R577X distribution and sprint performance

Table-1 shows ACTN3 R577X genotype distribution (RR, RX, and XX) for all 43 with biopsy-sampled 13 subjects and their corresponding sprint performance times. Genotyping of the all subjects showed that 12 (27.9%) were RR, 25 (58.1%) were RX, and 6 (14.0%) were XX. There were no statistically significant differences in average 100-m sprint performance times among three genotypes in all subjects and between RR and RX genotypes in biopsy-sampled subjects.

2. ACTN3 protein expression levels and MyHC composition

Figure-1 shows ACTN3 protein expression levels of RR and RX genotypes in the subset of subjects. ACTN3 protein expression levels tended to be higher in RR genotype than in RX genotype (RR vs. RX; 1.00 ± 0.31 vs. 0.68 ± 0.25, $p = 0.070$).

Table-2 MyHC composition in RR and RX genotypes

	RR (n = 4)	RX (n = 9)
Type I (%)	27.1 ± 2.7	26.3 ± 6.0
Type IIa (%)	40.5 ± 6.2*	50.7 ± 6.0
Type IIx (%)	32.4 ± 5.1**	23.0 ± 4.6

Values are mean ± SD.
*$p < 0.05$, vs. RX genotype
**$p < 0.01$, vs. RX genotype

Table-2 shows the relative content of all MyHC isoforms between RR and RX genotypes. No significant differences were found in the proportion of type I and II MyHC isoforms between RR and RX genotypes (RR vs. RX: type I; 27.1 ± 2.7% vs. 26.3 ± 6.0%, type II; 72.9 ± 2.7% vs. 73.7 ± 6.0%). However, RR genotype had a significantly higher proportion of type IIx MyHC isoform and a significantly lower proportion of type IIa MyHC isoform than RX genotype (RR vs. RX; type IIx: 32.4 ± 5.1% vs. 23.0 ± 4.6%, $p < 0.01$, type IIa: 40.5 ± 6.2% vs. 50.7 ± 6.0%, $p < 0.05$).

Discussion

In the present study, we obtained the first evidence demonstrating a relationship between ACTN3 R577X genotype, ACTN3 protein expression levels, and MyHC composition in Japanese college-level male sprinters. The results indicate that ACTN3 protein expression levels and the proportion of type IIx MyHC isoform are higher in RR genotype compared with RX genotype. However, there is no difference in their sprint performances between RR and RX genotypes.

We recruited 43 college-level male sprinters for this study. The average 100-m best time for all and a subset of biopsy-sampled subjects were 10.91 ± 0.31 s (n = 43) and 11.00 ± 0.36 s (n = 13). The

frequencies of ACTN3 R577X genotype in the present study were 27.9% RR, 58.1% RX, and 14.0% XX. It is reported that ACTN3 R577X genotype frequencies were 20.3% RR, 53.3% RX, and 26.3% XX in Japanese controls[16]. On the other hand, it has been reported that the frequencies of RR and RX genotypes were higher and there were few or no XX genotype in top-level sprinters[6) 16)]. Thus, it is not unusual for a certain number of XX genotype to exist in the present study. Mikami *et al.*[16] demonstrated that sprinters with RR and RX genotypes were significantly faster in 100-m sprints than those with XX genotype (RR and RX vs. XX; 10.42 ± 0.05 s vs. 10.64 ± 0.09 s). In contrast, there are no differences in muscular performance among three genotypes in healthy men[11) 17)]. These results suggest that genetic polymorphisms may influence sprint performance in high-level sprinters. Therefore, in the present study of college-level sprinters, it is not surprising to find no significant difference in sprint performance among ACTN3 R577X genotypes and the genotype might not be critical in determining sprint performance in Japanese college-level male sprinters.

This study is the first to show that ACTN3 protein expression levels tended to be higher in RR genotype than in RX genotype in well-trained male sprinters. It is important to evaluate muscle samples of subjects who perform sprint training continuously. Since the result of the present study is consistent with a previous report[7)], we predict that ACTN3 protein expression levels may be affected by ACTN3 R577X genotype regardless of the subject's status (trained or non-trained).

Given that the expression of ACTN3 protein is limited to type II fibers[2) 4)], it has been suggested that ACTN3 protein expression levels are associated with muscle fiber composition. North and Beggs[2)] showed by immunohistochemistry that ACTN3 protein was in 100% of type IIb/x fibers and 50% of type IIa fibers in human skeletal muscle. In addition, Vincent *et al.*[18)] have demonstrated that in healthy young RR genotype men, the staining intensity of ACTN3 protein in type IIx fibers was higher than in type IIa fibers. These results suggest that the proportion of type IIx fibers influences ACTN3 protein expression levels. Indeed, our results showed higher ACTN3 protein expression levels and a significantly higher proportion of type IIx MyHC isoform in RR genotype compared with RX genotype. Therefore, we suggest that ACTN3 protein expression levels may also be influenced by muscle fiber composition.

From the above, ACTN3 R577X genotype might affect muscle fiber composition. Previous studies have reported that the frequency of RR genotype was increased in international-level sprint/power-oriented athletes compared with that in national-level sprint/power-oriented athletes[6) 16) 19) 20)]. Since top-level sprint/power-oriented athletes require a higher proportion of type IIx fibers for rapid muscle contraction, RR genotype may possibly have an advantage over RX genotype. Thus, these results suggest that ACTN3 R577X genotype influences muscle fiber composition and that RR genotype seems to confer an advantage to sprint/power-oriented athletes. However, effects of ACTN3 R577X genotype on sprint performance is not clear in college-level sprinters in this study. Therefore, while genetic factors may influence sprint performance to some extent, daily training and sprint skills seem to be more important for sprint performance at the college-level.

There are several limitations in our study including sample size, sample recruitment, and the evaluation of skeletal muscle performance. First, we were unable to examine biopsy samples for all subjects, resulting in the small sample size. In addition, although ACTN3 protein expression levels in RR genotype tended to be higher than those in RX genotype, the difference was not statistically significant, possibly due to the small sample size. Therefore, a larger sample size is needed to confirm this association. Second, we did not recruit control subjects in this study. A previous report[21)] demonstrated that resistance training induces a skeletal muscle fiber type transformation (type IIx to IIa). In this study, we were unable to detect the effects of sprint training on ACTN3 protein expression levels. Lastly, while it is thought that the expression of ACTN3 protein in type II fibers is related to the generation of greater muscular strength and power, this remains to be determined. Further studies are therefore required to examine the effects of ACTN3 protein expression levels on muscular strength and power.

Conclusions

It is concluded that ACTN3 protein expression levels and the proportion of type IIx MyHC isoform are higher in RR genotype compared with RX genotype in Japanese college-level male sprinters.

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Conflict of interests

The authors declare no conflict of interests regarding the publication of this article.

References

1) Beggs AH, Byers TJ, Knoll JH, Boyce FM, Bruns GA, Kunkel LM: Cloning and characterization of two human skeletal muscle alpha-actinin genes located on chromosomes 1 and 11. J Biol Chem, 1992; 267: 9281-9288.
2) North KN, Beggs AH: Deficiency of a skeletal muscle isoform of alpha-actinin (alpha-actinin-3) in merosin-positive congenital muscular dystrophy. Neuromuscul Disord, 1996; 6: 229-235.
3) Luther PK, Squire JM: Muscle Z-band ultrastructure: titin Z-repeats and Z-band periodicities do not match. J Mol Biol, 2002; 319: 1157-1164.
4) Mills M, Yang N, Weinberger R, *et al*: Differential expression of the actin-binding proteins, alpha-actinin-2 and -3, in different species: implications for the evolution of functional redundancy. Hum Mol Genet, 2001; 10: 1335-1346.
5) North KN, Yang N, Wattanasirichaigoon D, Mills M, Easteal S, Beggs AH: A common nonsense mutation results in alpha-actinin-3 deficiency in the general population. Nat Genet, 1999; 21: 353-354.
6) Niemi AK, Majamaa K: Mitochondrial DNA and ACTN3 genotypes in Finnish elite endurance and sprint athletes. Eur J Hum Genet, 2005; 13: 965-969.
7) Hogarth MW, Garton FC, Houweling PJ, *et al*: Analysis of the ACTN3 heterozygous genotype suggests that α-actinin-3 controls sarcomeric composition and muscle function in a dose-dependent fashion. Hum Mol Genet, 2016; 25: 866-877.
8) Costill DL, Daniels J, Evans W, Fink W, Krahenbuhl G, Saltin B: Skeletal muscle enzymes and fiber composition in male and female track athletes. J Appl Physiol, 1976; 40: 149-154.
9) Ahmetov II, Druzhevskaya AM, Lyubaeva EV, Popov DV, Vinogradova OL, Williams AG: The dependence of preferred competitive racing distance on muscle fibre type composition and ACTN3 genotype in speed skaters. Exp Physiol, 2011; 96: 1302-1310.
10) Kumagai H, Tobina T, Ichinoseki-Sekine N, *et al*: Role of selected polymorphisms in determining muscle fiber composition in Japanese men and women. J Appl Physiol (1985), 2018; 124: 1377-1384.
11) Norman B, Esbjornsson M, Rundqvist H, Osterlund T, von Walden F, Tesch PA: Strength, power, fiber types, and mRNA expression in trained men and women with different ACTN3 R577X genotypes. J Appl Physiol, 2009; 106: 959-965.
12) Paparini A, Ripani M, Giordano GD, Santoni D, Pigozzi F, Romano-Spica V: ACTN3 genotyping by real-time PCR in the Italian population and athletes. Med Sci Sports Exerc, 2007; 39: 810-815.
13) Kakigi R, Naito H, Ogura Y, *et al*: Heat stress enhances mTOR signaling after resistance exercise in human skeletal muscle. J Physiol Sci, 2001; 61: 131-140.
14) Chan Y, Tong HQ, Beggs AH, Kunkel LM: Human skeletal muscle-specific alpha-actinin-2 and -3 isoforms form homodimers and heterodimers in vitro and in vivo. Biochem Biophys Res Commun, 1998; 248: 134-139.
15) Sugiura T, Murakami N: Separation of myosin heavy chain isoforms in rat skeletal muscles by gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Biomed Res, 1990; 11: 87-91.
16) Mikami E, Fuku N, Murakami H, *et al*: ACTN3 R577X Genotype is associated with sprinting in elite Japanese athletes. Int J Sports Med, 2014; 35: 172-177.
17) Hanson ED, Ludlow AT, Sheaff AK, *et al*: ACTN3 genotype does not influence muscle power. Int J Sports Med, 2010; 31: 834-838.
18) Vincent B, De Bock K, Ramaekers M, *et al*: ACTN3 (R577X) genotype is associated with fiber type distribution. Physiol Genomics, 2007; 32: 58-63.
19) Eynon N, Duarte JA, Oliveria J, *et al*: ACTN3 R577X polymorphism and Israeli top-level athletes. Int J Sports Med, 2009; 30: 695-698.
20) Kikuchi N, Miyamoto-Mikami E, Murakami, *et al*: ACTN3 R577X genotype and athletic performance in a large cohort of Japanese athletes. Eur J Sport Sci, 2015; 16: 694-701.
21) Kraemer WJ, Patton JF, Gordon S, *et al*: Compatibility of high-intensity strength and endurance training on hormonal and skeletal muscle adaptations. J Appl Physiol, 1995; 78: 976-989.