

## **ACTN3 polymorphism affects thigh muscle area**

### **Abstract**

Muscle mass is an important factor influencing the activity of daily living in older adults. We aimed to investigate whether  $\alpha$ -actinin-3 (ACTN3) gene R577X polymorphism affects muscle mass in older Japanese women. A total of 109 women (mean  $\pm$  SD, 64.1  $\pm$  6.0 years) were genotyped for the R/X variant of ACTN3. Mid-thigh muscle cross-sectional area (CSA) was assessed using MRI and compared using analysis of covariance models adjusted for body weight. In addition, physical activity and protein intake were measured as the living environmental factors affecting muscle mass. The ACTN3 R577X genotype distributions of the subjects were 19, 63 and 27 for the RR, RX, and XX genotypes, respectively. No difference in physical activity and protein intake were observed among the genotypes. The XX genotype showed lower thigh muscle CSA compared with RR&RX genotype (mean  $\pm$  SEM; XX: 69.1  $\pm$  1.8 cm<sup>2</sup>, RR&RX: 73.6  $\pm$  1.1 cm<sup>2</sup>;  $P < 0.05$ ). The results of the present study suggest that ACTN3 R577X polymorphism influences muscle mass in older Japanese women.

## Introduction

The actin-binding protein,  $\alpha$ -actinin is an important protein related to muscle contraction and is a structural component of the Z-lines in skeletal muscle [19, 24]. The  $\alpha$ -actinin isoform,  $\alpha$ -actinin-3 (ACTN3) is observed only in type II fibers [25, 26]. One billion people are homozygous recessive for  $\alpha$ -actinin-3 (ACTN3) gene (Chr. 11q13.2) R577X polymorphism (rs1815739), which causes complete deficiency of ACTN3 (XX genotype). However, it is not clear how ACTN3 deficiency affects muscle mass.

Muscle mass is affected by both genetic and environmental factors. A study of postmenopausal women twins showed that the heritability of lean body mass is 56% [6]. In addition, living environmental factors such as physical activity and protein intake have been identified as important components in improving and maintaining muscle mass [8, 16]. Both genetic and living environmental factors need to be assessed for preventing age-related loss of skeletal muscle mass (sarcopenia). Previous studies have shown that the *ACTN3* R577X polymorphism is related to muscle strength [9, 30], power [10], running ability [22], and athletic performance [27, 33] in Caucasians. *Actn3*

knockout (KO) mice used as a model for the human XX genotype showed lower lean body mass than wild-type mice [18], suggesting that *ACTN3* R577X polymorphism affects muscle mass. Although a few studies have shown that there is no such linkage in Caucasians [9, 10 and 30], they did not consider physical activity and protein intake.

Sarcopenia, which leads to reduced muscle strength and reduced activities of daily living (ADL) [16], affects the lower body with greater frequency and severity than the upper body [15], ultimately resulting in reduced walking ability and an increased risk of falling [16]. This is an important social issue in Japan because 37.7% of the cases where patients are frail and bedridden involve sarcopenia. Therefore, prevention of sarcopenia is expected to improve the quality of life of older adults. Sarcopenia, which causes selective atrophy of type II fibers and disarrangement of Z-lines, affects older people more than young people [11]. *ACTN3* deficiency in type II fibers might affect sarcopenia. This study is based on the hypothesis that older people with *ACTN3* deficiency have a lower muscle mass than those without it. We investigate the influence of the *ACTN3* R577X polymorphism on thigh muscle cross-sectional area (CSA) in

older Japanese women, and examine physical activity and protein intake as living environmental factors affecting muscle mass.

## **Methods**

### **Subjects**

This cross-sectional study examined 109 healthy Japanese postmenopausal women aged 50–78 years (means  $\pm$  SD, 64.1  $\pm$  6.0 years). The subjects were non-smokers who did not have significant cardiovascular, metabolic or musculoskeletal disorders. None of the women had taken antidepressant, hypolipidemic or hormonal drugs. The study was approved by the Ethical Committees of the Institute of Health and Sport Sciences and the Institute of Clinical Medicine of the University of Tsukuba. The study conformed to the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from all subjects before their inclusion in the study.

### **Genotyping**

The genotype of the *ACTN3* R577X polymorphism was identified as described previously [23]. Genomic DNA was prepared from EDTA-whole blood samples

using a kit (QIAamp DNA Blood mini kit; Qiagen, Tokyo, Japan). Genotyping for the *ACTN3* R577X polymorphism was determined by real-time polymerase chain reaction assays using TaqMan probes (Pre-Designed SNP Genotyping assays; Applied Biosystems, Tokyo, Japan) and a sequence detector (ABI Prism 7700; Applied Biosystems, Foster City, CA). The oligonucleotide sequences were as follows:

*ACTN3* forward: 5'-ACGATCAGTTCAAGGCAACACT-3'

*ACTN3* reverse: 5'-ACCCTGGATGCCCATGATG-3'

*ACTN3/R* probe: 5'-TCGCTCTCGGTCAGC-3'

*ACTN3/X* probe: 5'-CGCTCTCAGTCAGC-3'

### **Thigh muscle CSA**

Thigh muscle CSA was determined by magnetic resonance imaging (MRI) using modified techniques as reported previously [5]. Each subject's right foot was visualised by MRI (0.25 Tesla, AIRIS Mate; Hitachi Medical, Tokyo, Japan). All the cross-sectional MRI scans were obtained using a conventional T1-weighted

spin-echo sequence (28 ms echo time, 360 ms repetition time), with a 10 mm slice thickness, a 20 mm intersection interval, a 240 mm field of view, 256 × 180 matrix, 90° flip angle and 4 signals on an average. The MRI images were then analysed by manual planimetry using a hand-writable liquid crystal display (Cintiq 17SX; WACOM, Tokyo, Japan) and image analysis software (NIH Image ver. 1.62; National Institute of Health, USA) on a personal computer (Macintosh Power PC; Apple, Tokyo, Japan). The DICOM image data from MRI were converted into 652 × 652 pixels JPEG picture images. The MRI scans were assessed in a blind manner, i.e. the identity of the subject was not revealed. The cross-sectional MRI corresponding to the area between the greater trochanter and medial condyle of the femur was analysed. The regions of thigh muscle CSA included quadriceps femoris, hamstrings and adductor magnus muscles. Thigh muscle CSA was measured from the mean value of three traces.

### **Measurement of daily physical activity**

Daily physical activity was measured using a uniaxial accelerometer (Life-Corder; Suzuken, Nagoya, Japan) as described in a previous study [14]. All subjects wore the accelerometer on their waist continuously for 14 days, except

during sleeping and bathing. The total physical energy expenditure (kcal) was calculated from the energy measured by the accelerometer combined with a questionnaire based on the Compendium of Physical Activity [4], which was administered to all subjects.

### **Measurements of blood components**

The blood components were measured as described in a previous study [14]. Subjects were instructed to abstain from vigorous exercise and fast on the day prior to the measurements. Plasma glucose concentrations and fasting serum concentrations of cholesterol, triglycerides and insulin were determined using standard enzymatic techniques. The homeostasis model for assessing insulin resistance (HOMA-IR) was calculated using the formula  $\text{fasting plasma insulin } (\mu\text{U/ml}) \times \text{fasting plasma glucose (mg/dl)}/405$  [21].

### **Measurement of dietary intake**

A nutritionist assessed each subject's daily nutritional intake of carbohydrates, fat, protein and calories by conducting a 3-day survey of food intake.

## Statistical analysis

Statistical analyses were performed using SPSS Base System 14.0 J for Windows (SPSS Japan, Tokyo, Japan) and R (Ver. 2.6.0) [2]. The *ACTN3* R577X genotype distribution was evaluated for conformity with the Hardy-Weinberg equilibrium using the  $\chi^2$  test with one degree of freedom. Differences in physical characteristics between XX, RR and RX genotypes were tested using analysis of variance (ANOVA), and between the XX and RR&RX genotypes using unpaired *t*-tests. Thigh muscle CSA was compared between the genotype groups using an analysis of covariance (ANCOVA) model with age, height, body weight, physical activity, and protein intake as covariates. Linear regression analysis, including likelihood ratio tests between full (genotype and body weight) and constrained (only body weight) models, were performed to estimate the degree of variance in thigh muscle CSA attributable to the *ACTN3* genotype. Values are expressed as mean  $\pm$  SEM;  $P < 0.05$  is considered to be statistically significant.

## Results

Table 1 shows the *ACTN3* R577X genotype distributions of the subject

population. The genotype frequencies are in Hardy-Weinberg equilibrium ( $\chi^2$  value = 2.87, df = 1,  $P = 0.09$ ). No significant differences were found for age, height, body weight, body mass index (BMI), physical activity, daily nutritional intake, blood parameters or HOMA-IR among the *ACTN3* R577X genotypes (RR vs. RX vs. XX using ANOVA), or among cases with and without ACTN3 (RR&RX vs. XX using *t*-test) (Table 2).

As described in a previous study [9], we assessed all factors that were considered to be covariates that influence thigh muscle CSA. Body weight was found to have a significant influence, while age, height, physical activity and protein intake did not. We therefore statistically adjusted thigh muscle CSA with body weight as a covariate. This showed that the thigh muscle CSA of the subjects was significantly lower in the XX genotype than in the RR&RX genotype (Table 2). Table 3 shows that the *ACTN3* R577X genotypes accounted for 3.3% of the variability in thigh muscle CSA.

## Discussion

The results of this study show that there is a relationship between thigh muscle

CSA and the *ACTN3* R577X polymorphism in older Japanese women. The XX genotype with *ACTN3* deficiency shows a smaller muscle CSA than the RR&RX genotype in which *ACTN3* is present. These differences appear to be influenced more by genetic factors than by living environmental factors such as daily physical activity and nutritional intake.

This study suggests the possibility that *ACTN3* R577X polymorphism influences thigh muscle size, although the underlying mechanisms are not known. It has been reported that *Actn3* KO mice simulating human XX genotype have lower lean mass than wild-type mice [18]. In addition, Walsh et al. recently reported that the absence of *ACTN3* affects fat-free mass in women but not in men [31]. However, CSA and the muscle fiber area of the quadriceps muscle in younger people shows no differences among the *ACTN3* R577X genotypes [30]. We assume that *ACTN3* R577X genotypes affect the acuteness of sarcopenia. A recent study showed that this polymorphism is significantly associated with the age-related changes in the human phenotype [28]. That is, it influences the phenotypes of older adults but not young people. Future studies should investigate the extent to which the *ACTN3* R577X polymorphism affects the

alteration in muscle mass with aging and the mechanisms by which ACTN3 deficiency influences muscle mass.

ACTN3 is expressed only in type II fibers, particularly type II b fibers. A deficiency of ACTN3 therefore changes the characteristics of type II b fibers. For example, *Actn3* KO mice simulating the human XX genotype have lower lean mass than wild-type mice because KO mice have a smaller type II b fiber diameter [18]. In humans, the XX genotype has fewer type II x (also called type II b/x) fibers than the RR genotype [30]. Because type II fibers are related to sarcopenia and ACTN3, the XX genotype may show a decrease in type II b fibers with aging. Further investigation of changes in characteristics of ACTN3-deficient muscle fiber characteristics with aging is needed.

ACTN3 deficiency may affect the muscle size through the survival motor neuron 1 (SMN1) pathway in skeletal muscles. Recently, the human *SMN1* gene, which is responsible for spinal muscle atrophy (SMA), was reported to interact with  $\alpha$ -actinin in Z-lines [29]. In fact, SMA causes remarkably more atrophy in the trunk and lower body than in the upper body, and frequently, selective atrophy of

type II fibers [7]. These features resemble sarcopenia. It is therefore possible that the interaction of ACTN3 with SMN1 causes selective atrophy of type II fibers in sarcopenia observed in older adults. We are also interested in the calcineurine pathway discussed by Vincent et al. [30]. These molecular changes observed in cases of sarcopenia with ACTN3 deficiency should be investigated further in future.

ACTN3 anchors actin filaments to the Z-disk and incorporates the contractile properties of actomyosin. However, 22–25% of Asian people have an ACTN3-deficient XX genotype [3]. The results presented here (24.7%) are in agreement with this number. In addition, the subjects' characteristics are representative of the mean height and body weight of the general population of middle-aged and older Japanese women [1]; the mean BMI was categorised as normal weight. Moreover, the subjects had typical physical activity and daily nutritional intake, along with normal blood parameters. They had no history of cardiovascular, metabolic or musculoskeletal disorders. Therefore, the subjects were regarded as healthy, making the present findings applicable to general population.

We found that the variability attributable to the *ACTN3* R577X genotype was 3.3% for thigh muscle CSA in older Japanese women. The result is similar to Clarkson's observation that the *ACTN3* R577X genotype explained 2.2% of the baseline arm muscle strength in young women. Recently, three studies have investigated the relationship between height and gene polymorphism indicating 12-27 SNP explained 2.0-3.7 % of human height [13, 17, 32]. Therefore, one SNP contribution is not likely to have a high impact on morphology.

There are some limitations to the current investigation. Although, this study indicates that the XX genotype results in a significantly smaller thigh muscle CSA compared to the RR&RX genotype, the study was performed on a small sample size. In addition, thigh muscle size was not evaluated as muscle volume. However, for lower limbs, the middle muscle CSA is commonly used to indicate the muscle size of the thigh [5, 20]. Previous studies have indicated that thigh muscle CSA measured from a single MR image provides a good indication of muscle volume [20]. Another limitation was that this study only considered older women. Previous studies have reported that the *ACTN3* R577X

polymorphism influences fat-free mass [31], muscle strength [9], and athletic performance [32] in women but not men. However, sarcopenia affects both sexes. Therefore, future studies should include Japanese men.

In conclusion, the interaction of genetic and living environmental factors affects the human muscle phenotype. The subjects examined in this study were typical middle-aged or older Japanese women who were healthy and of normal weight. The genotype groups examined showed many similar characteristics; however, the XX genotype showed markedly lower thigh muscle CSA than the RR&RX genotype. The results show that *ACTN3* R577X polymorphism influences the size of the thigh muscles of older women. Our data suggests that *ACTN3* deficiency might affect the muscle mass in older adults.

The *ACTN3* R577X polymorphism is an important consideration for future studies aiming to determine how muscle mass can be preserved in older people.

### **Conflict of interest**

We have no financial, consultant, institutional and other relationships that might lead to bias or a conflict of interest.

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**Table 1** *ACTN3* R577X genotype distributions of the subject population

|                           | <b>Genotype</b> |            |            | <b>Allele<br/>Frequency</b> |          |
|---------------------------|-----------------|------------|------------|-----------------------------|----------|
|                           | <b>RR</b>       | <b>RX</b>  | <b>XX</b>  | <b>R</b>                    | <b>X</b> |
| All subjects<br>(n = 109) | 19 (17.4%)      | 63 (57.8%) | 27 (24.8%) | 0.463                       | 0.537    |

**Table 2** Characteristics of the subject by *ACTN3* R577X genotype

|                                       | RR<br>(n = 19) | RX<br>(n = 63) | XX<br>(n = 27) | RR&RX<br>(n = 82) | <i>P</i> Value<br>RR vs. RX<br>vs. XX | <i>P</i> Value<br>RR&RX vs.<br>XX |
|---------------------------------------|----------------|----------------|----------------|-------------------|---------------------------------------|-----------------------------------|
| Age (years old)                       | 64.1 (1.3)     | 63.8 (0.8)     | 64.9 (1.1)     | 63.9 (0.7)        | 0.76                                  | 0.47                              |
| Height (cm)                           | 151.2 (1.5)    | 151.2 (0.7)    | 150.3 (0.9)    | 151.2 (0.7)       | 0.76                                  | 0.46                              |
| Body Weight (kg)                      | 52.5 (1.7)     | 53.0 (0.8)     | 53.3 (1.2)     | 52.9 (0.7)        | 0.93                                  | 0.79                              |
| BMI (kg/m <sup>2</sup> )              | 22.9 (0.6)     | 23.2 (0.3)     | 23.6 (0.5)     | 23.1 (0.3)        | 0.71                                  | 0.45                              |
| Thigh muscle CSA (cm <sup>2</sup> ) † | 75.2 (2.2)     | 73.0 (1.2)     | 69.1 (1.8)     | 73.6 (1.1)        | 0.07                                  | 0.04                              |
| Physical Activity (kcal/day)          | 178.9 (15.4)   | 178.2 (10.6)   | 163.2 (16.6)   | 178.4 (8.9)       | 0.71                                  | 0.41                              |
| Daily Nutritional Intake              |                |                |                |                   |                                       |                                   |
| Total caloric Intake (kcal/day)       | 2013 (74)      | 2057 (59)      | 2047 (76)      | 2047 (48)         | 0.92                                  | 1.00                              |
| Carbohydrate Intake (g/day)           | 303.9 (12.7)   | 306.3 (11)     | 308.3 (14.4)   | 305.8 (8.9)       | 0.98                                  | 0.89                              |
| Fat Intake (g/day)                    | 52.9 (3.5)     | 53.8 (2.1)     | 53.5 (3.0)     | 53.6 (1.8)        | 0.98                                  | 0.99                              |
| Protein Intake (g/day)                | 79.4 (3.5)     | 81.6 (2.1)     | 78.9 (2.7)     | 81.1 (1.8)        | 0.73                                  | 0.54                              |
| Blood parameters                      |                |                |                |                   |                                       |                                   |
| Total Cho (mg/dl)                     | 218.2 (7.8)    | 217.5 (4.4)    | 224.7 (6.7)    | 217.7 (3.8)       | 0.66                                  | 0.36                              |
| LDL-Cho (mg/dl)                       | 128.1 (6.9)    | 132.5 (3.6)    | 139.1 (5.8)    | 131.5 (3.2)       | 0.43                                  | 0.24                              |
| HDL-Cho (mg/dl)                       | 59.4 (3.0)     | 60.7 (1.5)     | 60.6 (2.9)     | 60.4 (1.3)        | 0.92                                  | 0.93                              |
| Triglycerides (mg/dl)                 | 109.4 (17.2)   | 101.7 (6.1)    | 98.8 (7.5)     | 103.5 (6.1)       | 0.78                                  | 0.68                              |
| Insulin (μU/ml)                       | 5.3 (0.6)      | 6.8 (0.7)      | 9.3 (3.4)      | 6.5 (0.5)         | 0.35                                  | 0.42                              |
| Glucose (mg/dl)                       | 97.1 (1.7)     | 96.7 (1.0)     | 105.1 (5.7)    | 96.8 (0.8)        | 0.07                                  | 0.16                              |
| HOMA-IR                               | 1.29 (0.17)    | 1.69 (0.20)    | 2.37 (0.80)    | 1.59 (0.16)       | 0.28                                  | 0.35                              |

Values are mean (SEM). †Adjusted for body weight by ANCOVA. BMI, Body mass index; CSA, Cross-sectional area; Cho, Cholesterol; LDL, low density lipoprotein; HDL, High density lipoprotein; HOMA-IR, Homeostasis model assessment of insulin resistance.

**Table 3** Variability in thigh muscle cross-sectional area of subjects attributable to *ACTN3* R577X genotype.

| Measurement      | r <sup>2</sup> Full model | r <sup>2</sup> Constrained Model | Variability Attributable to Genotype Effect | Likelihood Ratio Test <i>P</i> Value <sup>†</sup> |
|------------------|---------------------------|----------------------------------|---------------------------------------------|---------------------------------------------------|
| Thigh muscle CSA | 0.219                     | 0.186                            | 3.3%                                        | 0.037                                             |

<sup>†</sup>Likelihood ratio test comparing full model (with genotype and body weight) to constrained model (body weight only). CSA, Cross-sectional area.