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Genetic association between *ACTN3* polymorphism and risk of non-acute ankle sprain

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ABSTRACT. In this study, we investigated the association between *ACTN3* R577X polymorphism and non-acute ankle sprain by measuring the allele frequency and genotype distribution of *ACTN3* in a Chinese Han population. We recruited 100 patients with non-acute ankle sprain and 100 healthy controls with no history of ankle injuries. Mass spectrometric analysis of single nucleotide polymorphism was used to analyze the genotype and allele frequencies of *ACTN3*. Results showed that the genotype frequency of RR in patients was 12.0%, which was significantly lower than that of the controls (24.0%) (OR = 1.7; 95%CI = 1.5-2.7; P = 0.001). The frequency distribution of the R allele in patients and controls were 68.5 and 56.7%, respectively (P = 0.002). Moreover, frequency of the RR genotype exhibited a downward linear trend with increased incidences of ankle sprain. Our results suggest that *ACTN3* R577X polymorphism is associated with non-acute ankle sprain in the Chinese Han population.

Key words: *ACTN3*; Gene; Polymorphism; Non-acute ankle sprain

INTRODUCTION

Ankle sprain, which occurs mostly during middle age, is one of the most common musculoskeletal joint injuries. Ankle sprain is categorized as either acute or non-acute ankle sprain (NAAS) based on the duration of the injury. Acute ankle sprain (AAS) lasts less than one week, and is the result of physical trauma. NAAS is not caused by injury, and is usually due to muscle weakness or capsular ligament laxity; duration of NAAS is often longer than 2 weeks. NAAS can easily relapse, leading to recurrent ankle sprains, which seriously affects the patient's quality of life. Recent clinical trials show that a variety of internal and external factors are associated with NAAS (Müller et al., 2015).

Recent studies demonstrated that chronic injury of tendons and ligaments around the ankle is associated with genetic susceptibility (Kaux et al., 2016). Mutations in genes that encode the extracellular matrix proteins in the tendons and ligaments surrounding the ankle were also associated with NAAS (Shang et al., 2015; van Dijk et al., 2016). Moreover, increasing number of studies have found that *ACTN3* R577X polymorphisms can have an effect on athletic abilities in humans. *ACTN3*, with a length of 16.4 kbp, is located on human chromosome 11, which contains 21 exons and 20 introns. *ACTN3* is expressed only in fast twitch fibers encoding α-actinin-3. It has been shown that RR overexpression is significantly higher in strong athletes and sprinters as compared to that in the general population (Schimke et al., 2015). Furthermore, a large number of cross-sectional studies confirmed that the R allele is positively correlated with muscle contraction velocity (Loiselle et al., 2016), suggesting that overexpression of the R allele could benefit skeletal muscle function and strength, especially during high-speed muscle contraction (Rodeo et al., 2015; Sutton et al., 2016). Currently, the relationship between *ACTN3* R577X polymorphism and incidence of NAAS has not yet been reported. This study was designed to investigate the association between *ACTN3* R577X polymorphism and NAAS by measuring allele frequency and genotype distribution of *ACTN3* in the Chinese Han population.

MATERIAL AND METHODS

Subjects

Between December 2015 and March 2016, 100 NAAS patients (44 men and 56 women), diagnosed by a senior orthopedic physician, were recruited into the study from the Luoyang Orthopedic Hospital of Henan Province. The mean age of the patients was 27.7 ± 14.7 years (range: 23-37), and the average duration of NAAS was 12 ± 5.7 months (range: 0.7 to 1.5 years). In addition, 100 healthy volunteers who underwent physical examinations (43 men and 57 women) were included as controls. The mean age of control subjects was 25.7 ± 5.5 years (range: 20-35). No significant differences were found in age, gender, and general health between the two groups ($P > 0.05$).

This study has been pre-approved by the Ethics Committee of the Luoyang Orthopedic Hospital of Henan Province. Written informed consent was given by all the subjects prior to their enrollment in this study.

Reagents and Instruments

DNA extraction kit was purchased from R&D Systems (Minneapolis, MN, USA). The

ABI 7300 PCR instrument, SNP assay reagents and TaqMan Genotyping Master Mix were purchased from Applied Biosystems (Waltham, MA, USA).

Method

Blood collection

Peripheral blood (5 mL) was collected from each subject using EDTA as the anticoagulant. Blood samples were centrifuged, and the supernatants were collected and stored at -80°C for later use.

Primer design and synthesis

The Assay Designer 4.2 software was used to design primers. Primers were synthesized by the Sichuan University Huaxi Medical Center. Primer sequences were as follows: forward 5'-TGA CAG CGC ACG ATC AGT TCA-3', reverse 5'-GAT GTA GGG ATT GGT GGA GCA-3'.

ACTN3 genotyping

ACTN3 genotyping was performed by the Sichuan University Huaxi Medical Center and Beijing Genomics Technology Co., Ltd. The Sequenom's MassARRAY system was used to perform the assay. The PCR mixture contained the following: 34.15 μL ddH_2O, 2.5 μL 20X buffer, 5.0 μL $MgCl_2$, 0.5 μL dAGC, 0.5 μL dTTP, 0.55 μL Taq polymerase, 0.3 μL forward primer, 0.6 μL reverse primer, 0.3 μL Fam probe, 0.6 μL JOE probe, and 5.0 μL template DNA. The PCR reaction protocol was as follows: denaturation at 37°C for 2 min, followed by 40 cycles at 94°C for 15 s and at 58°C for 60 s.

NAAS grading

Currently, no uniform standard for NAAS grading is available. However, standards from the American Academy of Orthopaedic Surgeons (AAOS) is widely used (Chapman, 1975). AAOS standard does not distinguish between ASS and NAAS. Grade 1 NAAS mainly refers to over-stretched or weakened anterior talofibular ligament (ATFL), which shows clinical manifestations such as pain, mild edema, lack of congestion, and limited ankle mobility. Grade 1 NAAS patients can still bear weight. Grade 2 NAAS refers to full tear of the ATFL and partial tear of the calcaneofibular ligament (CFL), which leads to pain, moderate edema, mild to moderate congestion, and limited ankle mobility. Grade 2 NAAS patients cannot fully bear weight. Finally, grade 3 NAAS refers to full tear of the ATFL and the CFL, which leads to extreme pain, swelling, and moderate to severe congestion. As a result, grade 3 NAAS patients cannot bear any weight.

Statistical analysis

The IBM SPSS 17.0 software was used for statistical analysis. Counting data are reported as means ± SD, and were analyzed by the c^2 test. The Student *t*-tests were used to compare the groups. Correlation was analyzed by Spearman rank correlation test. $P < 0.05$ was considered statistically significant.

RESULTS

General information of subjects

No significant differences were found between the NASS patients and controls in terms of age, gender, height, weight, family history of NAAS, and general health (P > 0.05) (Table 1).

Table 1. General information of subjects.

Groups	N	Age (year)	Male [N (%)]	Height (cm)	Weight (kg)	NAAS family history [N (%)]
NAAS	100	27.7 ± 14.7	44 (44%)	165.5 ± 5.5	62.3 ± 11.5	9 (9.00%)
Control	100	25.7 ± 11.5	43 (43%)	167.5 ± 4.7	65.2 ± 11.3	7 (7.00%)

Genotype and allele frequency distribution of *ACTN3* in NAAS patients and controls

Results showed that genotype frequencies of RR, RX, and XX in the *ACTN3* gene were 39.8, 43.4, and 16.8%, respectively. All genotype frequencies were in line with Hardy-Weinberg equilibrium (χ^2 = 3.209; d.f. = 1, P = 0.07). Genotyping analysis showed that the frequency distributions of RR, RX, and XX in the NAAS and control groups were 12.0, 39.0, and 49.0% *vs* 24.0, 36.0, and 40.0%, respectively. Allele frequencies of R and X in the NAAS group were 68.5 and 31.5% respectively; allele frequencies of R and X in the control groups were 56.7 and 43.3%, respectively. In addition, the genotype frequency of RR in the NAAS group was significantly lower than that of the control group (12.0 *vs* 24.0%; OR = 1.7; 95%CI = 1.5-2.7; P = 0.001) (Table 2).

Table 2. Genotype and allele frequency distribution of *ACTN3* in NAAS patients and controls.

	NAAS (N = 100)	Control (N = 100)	P value	OR (95%CI)
Genotype				
RR	12	24	0.001	1.7 (1.5-2.7)
RX	39	36	0.197	0.8 (0.624-1.356)
XX	49	40	0.356	0.8 (0.454-1.135)
Allele				
R	31.5	42	0.002	1.5 (1.0-2.1)
X	68.5	58	0.451	1.249 (0.934-1.145)

ACTN3 RR genotype and NAAS grading

Results showed that frequencies of RR in control subjects, grade I NAAS, grade II NAAS, and grade III NAAS were 26.7, 21.9, 17.9, and 11.5%, respectively. As compared to that of the controls, the *ACTN3* RR genotype in NAAS exhibited a downward linear trend with increasing NAAS grading (r = -0.756, P < 0.05) (Figure 1).

DISCUSSION

NAAS is one of the most common musculoskeletal joint injuries, and is usually caused by repeatedly delayed healing of AAS. Studies have shown that approximately 40% of AAS would eventually become NAAS. NAAS does not have a history of trauma; it is usually caused by muscle weakness or capsular ligament laxity. Its duration is longer than 2 weeks. NAAS can easily relapse, leading to recurrent ankle sprain.

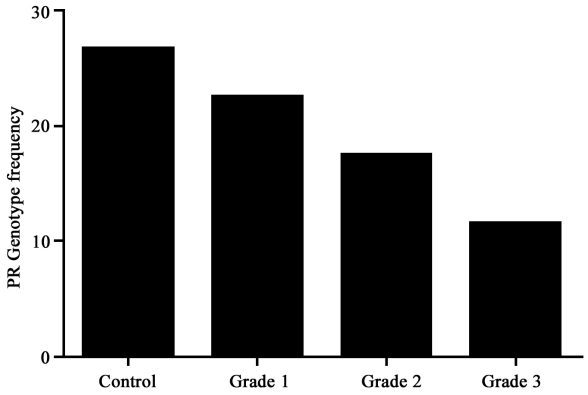

Figure 1. *ACTN3* RR genotype and NAAS grading.

The *ACTN3* gene is located on human chromosome 11. It encodes α-actinin-3, and is positively correlated with muscle strength and athletic ability. The correlation between *ACTN3* and NAAS still remains unclear.

The current study is the first study that investigates the correlation between *ACTN3* polymorphism and NAAS in the Chinese Han population. Results showed that there were significant differences in *ACTN3* genotype and allele frequencies between NAAS patients and controls. In addition, the RR genotype frequency in NAAS patients was significantly lower as compared to that of the controls, suggesting that high-risk NAAS populations carry a greater proportion of the RR genotype.

To date, few studies have examined the genetic factors in ankle tendon and ligament injury (Lieberthal et al., 2015; Saggini et al., 2015). Lieberthal et al. (2015) reported that genetic factors contribute to a portion of tendon and ligament injuries; however, no specific genes were identified. Many recent studies have confirmed that *COL1A1* (Bible and Mir, 2015), *COL5A1* (Gallamini et al., 2015), *COL12A1* (Gaut and Duprez, 2016), and *TNC* (Cody et al., 2015) are associated with tendon and ligament injuries. *COL1A1*, *COL5A1*, *COL12A1*, and *TNC* encode collagen I (Salamanna et al., 2015), collagen V (Cody et al., 2015), collagen XII (Harvey et al., 2015), and troponin (Niemi and Majamaa, 2005), a glycoprotein widely present in a large number of tendon tissues, which can withstand high tension and pressure. Studies have shown that as compared to the general population, the genotype frequency of XX was lower in the athletic population. In contrast, the genotype frequency of RR was found to be higher in the athletic population. Papadimitriou et al. (2008) found that *ACTN3* plays an important role in fast-twitch muscle fibers, suggesting that the R allele bestows beneficial effects on skeletal muscle functions, particularly on high-speed muscle shrinkage. Other studies have also shown similar results in Finnish sprinters (XX genotype frequency: 0 *vs* 9.2%) (Roth et al., 2008), Greek track and field athletes (RR genotype frequency: 50 *vs* 26%) (Brossi et al., 2015), and Spanish professional soccer players (RR genotype frequency: 48 *vs* 29%) (Verdiyeva et al., 2015).

Using a mouse knockout model, Maffulli et al. (2015) described the relationship between muscle power and the *ACTN3* gene. They found that the muscle contraction strength of *ACTN3* knockout mice was weaker than that of wild-type mice. Chan et al. (2008) reported that *ACTN3* knockout mice showed a slow muscle contractile response, indicating that a lack of *ACTN3* results in transformation of fast-twitch muscle fibers to slow-twitch muscle fibers.

Quinlan et al. (2010) showed that *ACTN3* knockout mice have higher level of muscle glycogen as compared to that of control mice, but the activity of glycogen phosphorylase was lowered by 50%. This was one of the basic explanations of the association between *ACTN3* and athletic ability.

Various clinical studies demonstrated that the pathogenesis and mechanism of NAAS are very complicated, and genetic factors play very important roles in the prognosis of the disease. Studies have shown that individuals with poor physical fitness have higher probabilities of being affected by NAAS as compared to those with good physical fitness. The cause for fragile ATFL is fatigue. Exercises that increase leg muscle strength, flexibility, as well as ankle joint flexibility, are effective means of preventing NAAS. Strong muscle strength can help to stabilize the ankle joint during front, left, and axial movements. Expression of *ACTN3*, which promotes muscle strength and reaction, can improve an individual's reaction time and processing speed. Neuromuscular unresponsiveness increases ankle injury, thereby increasing the incidence of NAAS. This may explain the correlation between *ACTN3* genetic polymorphisms and NAAS in the Chinese Han population. Therefore, participating in exercises that strengthen calf muscles can effectively prevent ankle injuries and NAAS.

In summary, we showed that *ACTN3* R577X polymorphism was correlated with incidence and severity of NAAS in the Chinese Han population. *ACTN3* gene polymorphism can be used as an independent risk predictor for NAAS in the Chinese Han population.

Conflicts of interest

The authors declare no conflict of interest.

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