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The *ACTN3* Gene is a Potential Biomarker for the Risk of Non-Contact Sports Injury in Female Athletes

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Abstract

Sports injuries can become serious impairments for all athletes. Most notably, female athletes are at higher risk than men for sports injury, for example, anterior cruciate ligament (ACL) disorder. However, there is currently no genetic marker to determine if a female athlete harbors a predisposition for muscle trauma. Hence, we performed single nucleotide polymorphism genotyping of the α-actinin-3 (*ACTN3*), angiotensin-converting enzyme (*ACE*), and uncoupling proteins (*UCP1, UCP2,* and *UCP3*) in 99 young female athletes who had been injured during a sports activity, and we compared the occurrence of muscle traumas with the genotypes using the chi-square test. For the *ACTN3* 577R allele, the subjects who had non-contact muscle injury had a marked increase in frequency (p-value=0.0015; odds ratio=2.52). The significant increase in non-contact muscle injury related to *ACTN3* 577R alleles suggests that ACTN3 is likely to be involved in muscle strain and that non-contact muscle injury might occur due to the presence of this allele. It is crucially important for young female athletes to understand their risk for injury, as they might be able to modify their training program to avoid injury, depending on their specific genetic markers.

Keywords: Single nucleotide polymorphism; ACTN3 genotype; Sports injury; Female athletes

Introduction

The incidence of sports injuries, such as injury to muscles, ligaments, and tendons and fractures, remains high in young athletes. Despite the introduction of some successful prevention programs [1-4], ACL injury continues to be the single largest problem in orthopedic sports medicine, with the incidence of non-contact ACL injuries being much higher in female athletes in various sports [2]. As non-contact muscle injury remains a significant problem in young female athletes, procedures to improve the prevention and management of muscle injuries are required.

There are few biomarkers for sports trauma; however, there are many known biomarkers related to athletic or muscle performance, some of which are described below [5-9]. ACTN3 is highly synthesized in and is a major component of the skeletal muscle Z-disk in fast-twitch skeletal muscle fibers [5]. Homozygosity for a common nonsense polymorphism (rs1815739) in the *ACTN3* gene results in complete ACTN3 deficiency in an estimated 16% of the global population [6] and is associated with variations in human muscle performance. The human *ACE* gene exhibits a polymorphism based on the presence (insertion; I) or absence (deletion; D) of a 287-bp Alu-repeat element within intron 16 of the *ACE* gene [7]. The *ACE*-I allele is associated with an increase in Type I skeletal muscle fibers [8]. Mitochondrial-targeted UCPs are members of the mitochondrial anion-carrier superfamily, with three known mammalian forms (UCP1–3). Until recently, it was unclear whether UCP polymorphisms were involved in muscle performance; however, one report identified a common genetic variation at the UCP2/3 gene locus (rs659366 and rs1800849, respectively) associated with training-related improvements in delta efficiency, an index of skeletal muscle performance [9]. Our study investigated the relationship between sports injury and genetic markers in athletes through SNP analysis.

Material and methods

Participants

This study utilized a sports injury data survey and the genetic variants for 99 students from the Department of Health and Sports Sciences, Mukogawa Women's University. The participants were all female, future professional athletes, and their sports were as follows: 37, touch football; 28, softball; 25, basketball; and 9, badminton. The age range of the participants was 18-22 (average, 19.7), and other pertinent information has been provided as Supplementary Information. The study protocol was approved by the institutional review board of Mukogawa Women's University, and written informed consent was obtained from each participant. After providing informed consent, each participant filled out the questionnaire described in the "Data survey for sports injury" sub-section. Simultaneously, DNA was collected when the participants supplied a few drops of saliva on water-soluble paper (Mishima Dissolve Paper, 60 MDP; Nippon Paper Papylia Co., Ltd.), according to a previously described protocol [10].

Genotyping

We performed SNP genotyping for the *ACTN3, UCP1* (rs1800592), *UCP2,* and *UCP3* genes using TaqMan assay (TaqMan®SNP Genotyping Assays; Life Technologies, Carlsbad, CA). PCR analyses

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were carried out utilizing the ABI Prism 7300 Real Time PCR System (Life Technologies) with a 20 μL total volume reaction mixture using the DNA swab (1.2 mm diameter water-soluble paper with dried saliva) as a template [10], 6.6 μL or 7.1 μL of distilled water, 2 μL of 2× PCR buffer that was provided with the KOD FX neo DNA polymerase kit (TOYOBO, Japan), 0.4 μL of 50× ROX reference dye, 10 μL of Thunderbird ™ Probe qPCR Mix (TOYOBO), and 1 μL or 0.5 μL of 20× or 40× Probe Primer mixture, combined additively. The PCR conditions were the following: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

ACE gene polymorphism tests were performed using PCR with primers flanking the polymorphic region: the forward primer was 5'-CTGGAGACCACTCCCATCCTTTCT-3' and the reverse primer was 5'-GATGTGGCCATCACATTCGTCAGAT-3'. PCR was carried out using the Applied Biosystems Veriti® 96-Well Thermal Cycler (Life Technologies) with a total reaction mixture volume of 15 μL, containing the water-soluble paper with saliva as a template, 3.7 μL of distilled water, 7.5 μL of 2× PCR buffer for KOD FX neo, 1.5 μL of 2 mM dNTPs, 10 μM of each primer for *ACE*, and 0.3 U of KOD FX neo DNA polymerase (TOYOBO). The PCR conditions for ACE amplification were the following: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 68°C for 30 s. The resulting fragments were analyzed using the MultiNA Microchip Electrophoresis System MCE-202 (Shimadzu, Japan).

Data survey for sports injury

To collect data pertaining to sports injury, a questionnaire was developed and filled out by each participant. The questionnaire asked whether the participant had suffered severe traumatic injuries during sports activity in the past. For patients who had a history of injuries, we also asked questions regarding the following aspects: the affected body part, type of injury, cause of the injury, and duration of the injury. The injury classifications were based on the "Daily Report on Injuries and Illnesses in International Olympic Committee", which is used by the medical staff of the National Olympic Committees (see Supplementary Information) [11].

Statistical analysis

All of the statistical analyses were performed using JMP software for Macintosh, version 10.0.2 (SAS Institute Inc., Cary, NC, USA). The Hardy-Weinberg equilibrium was assessed by Peason's chi-square analysis. And odds ratio for verification of the relationship between sports injury and genetic markers in athletes through SNP analysis. A p-value threshold of <0.05 for chi-square analysis was considered to be a significant correlation. The 95% confidence interval (95% CI) of the odds ratio was calculated to confirm the significant association between genotypes and injuries.

Results

Using a case study design, we assessed the injuries of athletes along with 5 SNPs in biological candidate genes that have been associated previously with sports performance or muscle activity (*ACTN3, ACE, UCP1, UCP2,* and *UCP3*). "Injury" was considered any "non-contact injury" in this study, and we removed "contact" injuries from the participants' injury history, because most contact injuries were not associated with muscle performance. For the same reason, contusion and inflammation, such as arthritis and fasciitis, were also removed. Finally, muscle strain and/or ligament ruptures, which consist of sprains and ACL ruptures, were independently compared with the 5 SNPs. Consequently, only muscle strain was significantly associated with the *ACTN3* allele frequency and *ACE* genotypes (Table 1 and Table s1).

Gene	Genotype	Injured		Non-injured			p-value
		23	%	76	%		
ACTN3		RR	9	39.1	17	22.3	0.1944
		RX	9	39.1	30	39.5	
		XX	5	21.8	29	38.2	
		RR	9	39.2	17	22.4	0.1095
		RX+XX	14	60.8	59	77.6	
		RR+RX	18	78.3	47	61.8	0.1463
		XX	5	21.7	29	38.2	
	Allele	R	27	58.7	64	42.1	0.0479*
		X	19	41.3	88	57.9	
ACE		DD	8	34.8	7	9.2	0.0038*
		ID	5	21.7	39	51.3	
		II	10	43.5	30	39.5	
		DD	8	34.8	7	9.2	0.0027**
		II+ID	15	65.2	69	90.8	
	Allele	D	21	45.6	53	34.9	0.1853
		I	25	54.4	99	65.1	
UCP1		A/A	7	30.4	24	31.6	0.9505
		A/G	12	52.2	37	48.7	
		G/G	4	17.4	15	19.7	
		A/A+A/G	19	82.6	61	80.3	0.8024
		G/G	4	17.4	15	19.7	
	Allele	A	26	56.5	85	55.9	0.9427
		G	20	43.5	67	44.1	
UCP2		G/G	5	21.7	22	28.9	0.7204
		G/A	11	47.9	36	47.4	
		A/A	7	30.4	18	23.7	
		G/ G+G/A	16	69.6	58	76.3	0.5197
		A/A	7	30.4	18	23.7	
	Allele	G	21	45.6	80	52.6	0.4067
		A	25	54.4	72	47.4	
UCP3		C/C	1	4.3	11	14.5	0.2721
		C/T	8	34.8	31	40.8	
		T/T	14	60.9	34	44.7	
		C/C	1	4.3	11	14.5	0.1923
		C/T+T/T	22	95.7	65	85.5	
	Allele	C	10	21.7	53	34.9	0.0939
		T	36	78.3	99	65.1	

*: P-value < 0.05, **: P-value < 0.01

Table 1: Allele distribution associated with muscle strain of injured and non-injured athletes.

The results of the comparative analysis indicated that the combination of sports injuries, i.e., muscle strain and ligament rupture, was significantly correlated with the *ACTN3* genotype (Table 2) but not with the other genetic variants. Notably, compared to the findings for each *ACTN3* genotype, the percentage of *ACTN3* 577XX athletes who did not experience sports injuries was significantly greater than the percentage with the *ACTN3* 577RR genotype (p-value=0.0133; Table 2 and Figure s1a). Furthermore, *ACTN3* 577R allele frequency was higher than 577X frequency in athletes who experienced muscle injuries (p-value=0.0015; Table 2 and Figure s1b). This association was very significant with an odds ratio of 2.52 (95% CI, 1.42-4.47), and it suggested that the *ACTN3* genotype contributed to muscle injury susceptibility.

Gene		Genotype	Injured		Non-injured		p-value
			50	%	49	%	
ACTN3		RR	18	35.3	8	16.7	0.0133*
		RX	22	43.1	17	35.4	
		XX	11	21.6	23	47.9	
		RR	18	35.3	8	16.7	0.0353*
		RX+XX	33	64.7	40	83.3	
		RR+RX	40	78.4	25	52.1	0.0058**
		XX	11	21.6	23	47.9	
	Allele	R	58	57.0	33	34.7	0.0015**
		X	44	43.0	63	65.3	
ACE		DD	11	21.6	4	8.3	0.1855
		ID	21	41.1	23	47.9	
		II	19	37.3	21	43.8	
		DD	11	21.6	4	8.3	0.0664
		II+ID	40	78.4	44	91.7	
	Allele	D	43	42.2	31	32.3	0.1516
		I	59	57.8	65	67.7	
UCP1		A/A	17	33.3	14	29.2	0.8725
		A/G	25	49.0	24	50.0	
		G/G	9	17.7	10	20.8	
		A/A+A/G	42	82.3	38	79.2	0.6874
		G/G	9	17.7	10	20.8	
	Allele	A	59	57.8	52	54.2	0.6024
		G	43	42.2	44	45.8	
UCP2		G/G	14	27.5	13	27.1	0.5659
		G/A	22	43.1	25	52.1	
		A/A	15	29.4	10	20.8	
		G/G+G/A	36	70.6	38	79.2	0.3262
		A/A	15	29.4	10	20.8	
	Allele	G	50	49.0	51	53.1	0.5636
		A	52	51.0	45	46.9	
UCP3		C/C	7	13.7	5	10.4	0.6679
		C/T	18	35.3	21	43.8	
		T/T	26	51.0	22	45.8	
		C/C	7	13.7	5	10.4	0.6142
		C/T+T/T	44	86.3	43	89.6	
	Allele	C	32	31.4	31	32.3	0.8896
		T	70	68.6	65	67.7	
*: P-value < 0.05, **: P-value < 0.01							

Table 2: Allele distribution associated with a combination of sports injuries (muscle strain+ligament rupture) of injured and non-injured athletes.

Discussion

For young female athletes, it is important to determine whether they have a risk of suffering from severe traumatic injuries. This risk is especially important for ACL injuries, since women are at an increased risk and experience a slower recovery compared to men [12-16]. If women were able to know their genetic predisposition for developing sports injury prior to training, then they may be able to develop specific training programs and avoid injury. However, until now, no biomarkers had been identified to assess this risk associated with genotype.

Here, we found a relationship between the injury history during sports activity and genetic variants in young female athletes. The frequency of the *ACTN3* 577R allele in the contribution to a combination of muscle strain and ligament rupture was remarkably higher than that to ligament or muscle strain alone (Table 2 and Figure s1). This result is the first demonstration that *ACTN3* genotype is likely to be concerned with injury, since *ACTN3* is associated with the performance of human muscle structure until now [6]. Furthermore, other reports have shown that 577X allele frequency is associated with bone fracture [17,18]. In this study, *ACTN3* 577X allele frequency was higher than 577R allele's in fractured subjects, but the result was not significant (p-value=0.3475, data not shown). This could be due to the fact that bone mineral density (BMD), a cause of osteoporosis, remains high in young populations. Since the previous findings suggested pleiotropic effects of ACTN3 for bone and muscle [17,18], our results suggested that ligament was also affected with ACTN3 protein.

Muscle strain appeared to be dependent on the *ACE* genotype, as seen in the significant differences among the genotypes (Table 1); moreover, the significance seemed to be derived from the number of ID hetero-type, non-injured participants. Therefore, we examined that allele frequency. Because the *ACE*-I allele existed at a higher frequency than the *ACE*-D allele in both injured and non-injured participants, our data did not clearly indicate whether the *ACE* gene was genetically correlated with this injury.

Prospectively, young female athletes might be able to modify their training program as described in reviews [19,20] based on their genetic predisposition. Further studies are required to verify the genetic mechanisms responsible for muscle injuries. In addition, future studies will include genetic association testing of male athletes. We are also interested in the frequency of *ACTN3* polymorphism in male athletes, also because there are gender differences in sports injuries such as ACL [2]. Once this association is determined, genetic variance should be utilized as a marker for injury risk and also for clinical use.

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