Association analysis of *ACE* and *ACTN3* in Elite Caucasian and East Asian Swimmers

Guan Wang[1], Eri Mikami[2,3], Li-Ling Chiu[4], Alessandra de Perini[5], Michael Deason[1], Noriyuki Fuku[6], Motohiko Miyachi[7], Koji Kaneoka[8], Haruka Murakami[7], Masashi Tanaka[6], Ling-Ling Hsieh[9], Sandy S. Hsieh[10], Daniela Caporossi[5], Fabio Pigozzi[5], Alan Hilley[11], Rob Lee[1], Stuart D.R. Galloway[12], Jason Gulbin[13], Viktor A. Rogozkin[14], Ildus I. Ahmetov[14,15], Nan Yang[16], Kathryn N. North[17], Saraslanidis Ploutarhos[18], Hugh E. Montgomery[19], Mark E.S. Bailey[20], Yannis P. Pitsiladis[1].

Affiliations: [1] Institute of Cardiovascular and Medical Sciences, [11] School of Medicine and [20] School of Life Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK. [2] Graduate School of Sport Sciences, and [8]Faculty of Sport Sciences, Waseda University, Saitama, Japan. [3]Japan Society for the Promotion of Science, Tokyo, Japan. [4]Department of Nutrition and Health Sciences, Chang Gung University of Science and Technology, Tao-Yuan, Taiwan. [5]Department of Health Sciences, University of Rome "Foro Italico" - IUSM, Rome, Italy. [6]Department of Genomics for Longevity and Health, Tokyo Metropolitan Institute of Gerontology (TMIG), Tokyo, Japan. [7]Department of Health Promotion and Exercise, National Institute of Health Nutrition, Tokyo, Japan. [9]Department of Public Health, Chang Gung University, Tao-Yuan, Taiwan. [10]Graduate Institute of Exercise and Sport Science, National Taiwan Normal University, Taipei, Taiwan. [12]School of Sport, University of Stirling, Stirling, UK. [13]Strategy and Innovation, Australian Institute of Sport, Canberra, Australia. [14]Sports Genetics Laboratory, St Petersburg Research Institute of Physical Culture, St Petersburg, Russian Federation. [15]Laboratory of Molecular Genetics, Kazan State Medical University, Kazan, Russian Federation. [16]Suzhou Academy, Xi'An Jiaotong University, Xi'An, China. [17]The Children's Hospital at Westmead, Sydney,

Australia. [18]*Department of Physical Education and Sport Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece.* [19]*The UCL Institute for Human Health and Performance, University College London, London, UK.*

Running title: ACTN3 and ACE genotypes in elite swimmers

Corresponding Author: Dr Yannis P Pitsiladis

Institute of Cardiovascular and Medical Sciences

College of Medicine, Veterinary and Life Sciences

University of Glasgow

Glasgow

G12 8QQ

United Kingdom

Phone: +44 (0) 141 330 3858

E-mail:yannis.pitsiladis@glasgow.ac.uk

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Abstract

Purpose: Polymorphic variation in the angiotensin-converting enzyme (*ACE*) and α-actinin-3 (*ACTN3*) genes has been reported to be associated with endurance and/or power-related human performance. Our aim was to investigate whether polymorphisms in *ACE* and *ACTN3* are associated with elite swimmer status in Caucasian and East Asian populations. **Methods:** One hundred and ninety-three elite Caucasian swimmers from European, Commonwealth, Russian and American cohorts (short and middle distance, SMD ≤ 400 m, n = 125; long distance, LD > 400 m, n = 68) and 326 elite Japanese and Taiwanese swimmers (short distance, SD ≤ 100 m, n = 160; middle distance, MD: 200 – 400 m, n = 166) were genotyped for *ACE* I/D and *ACTN3* R577X. Genetic associations were evaluated by logistic regression. **Results:** *ACE* I/D was associated with swimmer status in Caucasians, with the D-allele being overrepresented in SMD swimmers under both additive and I-allele-dominant models (permutation adjusted $p = 0.003$ and $p = 0.0005$, respectively). *ACE* I/D was also associated with swimmer status in East Asians. In this group, the I-allele was overrepresented in the SD swimmer group (permutation adjusted $p = 0.036$ and $p = 0.008$ under the additive and the D-allele-dominant models, respectively). *ACTN3* R577X was not significantly associated with swimmer status in either Caucasians or East Asians. **Conclusions**: *ACE* I/D associations were observed in these elite swimmer cohorts, with different risk alleles responsible for the associations in swimmers of different ethnicities. The functional *ACTN3* R577X polymorphism did not show any significant association with elite swimmer status despite numerous previous reports of associations with 'power/sprint' performance in other sports.

Key words: ACE/ACTN3 polymorphisms; elite swimmer status; case-control association study

Introduction

Paragraph Number 1 Genetic contributions to performance at the elite level in swimming have received little attention (8,35). We were interested in identifying genes that predispose to high performance in swimming and in investigating whether such genes act across multiple populations. We thus set out to do a candidate gene association study of elite swimmers in both Caucasian and East Asian populations.

Paragraph Number 2 Two candidate genes were selected for the study: those encoding angiotensin converting enzyme (*ACE)* and α-actinin-3 (*ACTN3).* Variants in both genes have been reported to be associated with elite athletic performance and with normal, quantitative physical performance traits in the general population. Angiotensin converting enzyme plays a critical role in circulatory homeostasis as a component of the circulating renin-angiotensin system (RAS), catalysing the conversion of angiotensin I to the vasoconstrictor angiotensin II and the degradation of the vasodilator bradykinin. However, local (tissue or cellular) RAS in a variety of tissues subserve diverse roles, including the regulation of inflammation, cell growth and aspects of metabolism (25). A 287bp *Alu* repeat insertion/deletion (I/D) polymorphism (rs4340) in intron 16 is associated with circulating and tissue ACE levels, with higher ACE activity being associated with the D (deletion) variant in both Caucasians (26) and East Asians (36). In contrast, in populations of sub-Saharan African descent, the I/D polymorphism is associated with ACE activity to a considerably lower extent, reflecting the linkage disequilibrium (LD) structure across the gene and the fact that the I/D variant is not thought to be the functional variant affecting ACE activity (40).

Paragraph Number 3 *ACE* I/D is associated with a variety of exercise-related phenotypes, including sporting performance (20), fatigue resistance in response to physical training, the cardiac growth response, differences in muscle efficiency and strength, hypoxic ventilatory drive and skeletal muscle fiber distribution (reviewed in (25)). A number of studies have examined the relationship between this polymorphism and elite athlete status. In Caucasian populations, the I-allele has previously been reported to be associated with enhanced elite endurance performance in long-distance runners and rowers and with enhanced performance at high altitude (25), all activities requiring endurance capabilities; the D-allele, on the other hand, has been reported to be associated with strength/power sports, such as sprinting (22) and swimming events of ≤ 400 m (8,35). Despite the consistency of such findings, data from populations of East Asian descent have revealed conflicting results, the D-allele being associated with elite Japanese long distance runner status (33) and the I-allele with elite Korean power-oriented athlete status (14).

Paragraph Number 4 The *ACTN3* gene encodes α-actinin-3, an actin-binding protein with a structural role at the sarcomeric Z-line in glycolytic (type II, fast-twitch) muscle fibers and an increasingly evident role in the regulation of muscle metabolism (reviewed in (5)). A common nonsense polymorphism, p.R577X, exists in many human populations. The 577X-allele is a protein-null allele, from which no ACTN3 is produced, so that XX homozygotes do not express ACTN3 at all in their muscles (5). In the knockout mouse, it is clear that ACTN3 deficiency alters skeletal muscle function (5). The 577X-allele is found worldwide but at widely differing frequencies in different populations (5). Associations have been reported between R577X and physical performance both in elite athletes and in the general population, with the 577R-allele being associated with increased sprint performance (21,37). The 577XX null genotype has been reported to be found at a reduced frequency in elite Australian

Caucasian and Finnish sprinters and other sprint/power athletes (23,37). The 577X-allele is found at very low frequency in sub-Saharan Africa (5) and, in line with this, associations with sprint or power athletic status in Nigerians, Jamaicans and African-Americans (29,38), or with endurance athletic status in East Africans (38), have not been found.

Paragraph Number 5 We sought to explore further the associations of *ACE* and *ACTN3* genotype with elite swimmer status and to investigate whether such associations differed by swimming event distance or by ethnicity, focusing on Caucasian and East Asian populations. We have thus conducted analyses using a case-control approach in world-class elite athletes, such as world record holders, world champions and Olympians, including world-class competitive swimmers at all distances ranging from 50 m to 25 km; this overall cohort of swimmers is considerably larger than any previously assembled.

Methods

Subjects

Paragraph Number 6 Two elite swimmer cohorts, comprising Caucasian and East Asian subjects, respectively, were studied with the approval of the respective local ethics committees (the Sports Studies Ethics Committee (SSEC) at the University of Stirling, Scotland; the Institutional Review Board of Tokyo Metropolitan Institute of Gerontology, National Institute of Health and Nutrition, Japan; and the Institutional Review Board of Chang Gung Memorial Hospital, Taiwan). Written informed consent was obtained from all subjects. Parental consent was sought for subjects under 16 years of age in both cohorts.

Paragraph Number 7 *Caucasians*. A total of 193 elite Caucasian swimmers from European, Commonwealth, Russian and American cohorts were sampled during swimming competitions during 2005 and 2006 and categorized as short and middle distance (SMD ≤ 400 m, n = 125) or long distance swimmers (LD > 400 m, n = 68) (Table 1). Several Russian swimmers in this cohort (n = 21) were elite competitors at very long distances (5 – 25 km). Distances of 400 m and below have been used in previous studies to define swimmers excelling at power-dominated swimming events (35). Competitive swimmers are generally unable to excel (i.e. win world-class competitions) in events in both short distance and longer-distance categories. All swimmers were of world-class status or highly competitive in international competitions. Controls for this group comprised individuals of known genotype from the general population reported in previous studies (*ACE*-C: n = 1248, (35); *ACTN3*-C: n = 1694, (1,17,27,28,37)).

Paragraph Number 8 *East Asians*. Elite Japanese (n = 158) and Taiwanese (n = 168) swimmers were recruited and classified as short distance (SD ≤ 100 m, n = 160) and middle distance (MD: 200 – 400 m, n = 166) swimmers (Table 1). All had either participated in international competitions such as the Olympics, World Championships and Asian Games, or were nationally competitive swimmers. Controls for this group came from two sources - Japanese controls were recruited for this study from the general population in Tokyo and its environs (n = 649); Taiwanese controls were a randomly selected subset (n = 603) of a larger cohort (n = 3000) recruited from the general Taiwanese population, as previously described (15). All controls were healthy adults of both genders and were not professionally connected with athletics/sport. Japanese and Taiwanese subgroups were combined in the analysis as a single control group except in models testing ethnicity x genotype interactions.

DNA collection/extraction/quantification

Paragraph Number 9 *Caucasians*. Subjects were asked not to consume food or drink for at least 30 minutes, after which time buccal cell samples were taken. Buccal cell samples were collected by a trained individual by firmly rubbing a brush (Medical Packaging Corporation, Camarillo, CA, USA) against the inside of each subject's cheek for approximately 15 seconds. The head of the brush was cut into a screw cap tube containing cell lysis solution (0.1 M Tris-HCl pH 8.0, 0.1 M EDTA; 1% SDS) and stored at -20 °C. DNA was extracted using the QIAamp® DNA Mini kit (QIAgen, Hilden, Germany) according to the instructions of the manufacturer with minor adjustments. Following extraction, DNA samples were quantified using a Nanodrop® ND-8000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Subsequently, the DNA was diluted with Qiagen buffer AE to a working concentration of 3 $ng \cdot \mu l^{-1}$ and stored at 4 °C in Rigid Thin Wall 96 x 0.2 ml Skirted Microplates (Starlabs UK Ltd, Buckinghamshire, UK) during the genotyping analysis.

Paragraph Number 10 *East Asians*. For the Japanese swimmers and controls, genomic DNA was isolated from either 7 ml venous blood or 2 ml saliva using QIAamp® DNA Blood Mini Kit (QIAgen, Hilden, Germany) or Oragene® DNA Self-Collection Kit (DNA Genotek Inc., Ottawa, Ontario, Canada). DNA was then quantified using either a Nanodrop or a GeneQuant Pro (Amersham Biosciences, Amersham, UK) spectrophotometer. For the Taiwanese swimmers and controls, 5 ml venous blood was collected into heparinized tubes (Vacutainer) and centrifuged within 24 hours and buffy coat cells stored at -70 °C until extraction of genomic DNA as previously described (12).

Genotyping

Paragraph Number 11 *Taqman single nucleotide polymorphism (SNP) genotyping method.* Genotypes were determined using Taqman® assays (Applied Biosystems, Warrington, UK; Applied Biosystems, CA, USA). For the Caucasian swimmers and for the Japanese swimmers and controls, genotypes were obtained at *ACE* SNP rs4341 (ABI assay ID: C__29403047_10) and at *ACTN3* p.R577X (rs1815739; ABI assay ID: C____590093_1_). rs4341 is known to be in perfect LD with I/D (rs4340) in Caucasian and Asian populations (10,32). For Caucasian swimmers, amplifications were carried out in 20 μl reactions containing 10 μl universal master mix, 1.0 μl ABI assay mix (20 ×), 6 μl distilled water and 9 ng genomic DNA. For Japanese subjects, amplifications were carried out in 5 μl reactions containing 2.5 μl Taqman® GTXpress™ master mix, 0.125 μl ABI assay mix (40 ×), 1.375 μl distilled water and 10 ng genomic DNA. Amplifications were carried out using StepOnePlus™ Real Time PCR (Applied Biosystems, CA, USA). Genotypes were called from end-point reads using StepOne™ Software v2.1. *ACE* I/D genotypes were calculated from rs4341 genotypes as follows: rs4341 G/G was called as D/D; C/G was called as I/D; C/C was called as I/I.

Paragraph Number 12 *Allele discriminatory PCR method.* Genotyping of *ACE* I/D (rs4340) in Taiwanese swimmers and controls was performed using a standard gel-based allelic discrimination assay method as previously described (3). The PCR primers for *ACE* gene were Forward: 5′-CTGGAGACCACTCCCATCCTTTCT-3′ and Reverse: 5′-GATGTGGCCATCACATTCGTCAGAT-3’. The PCR was performed in a 25 μl reaction using a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany). The PCR constituents were 100 ng genomic DNA, 3.5 mm $MgCl_2$, 200 μM dNTPs, 1 unit of Taq

polymerase and 400 nM of each primer in 1× PCR buffer for 35 cycles under the following conditions: 95 °C for 1 min, 58 °C for 30 s, and 72 °C for 40 s. PCR products were electrophoresed through an 8% polyacrylamide gel, stained with ethidium bromide and photographed under UV light. The I- and D-alleles yielded fragments of approx. 480 bp and 190 bp, respectively. Because amplification of the I-allele can be suppressed in ID heterozygotes, resulting in allelic dropout and miscalling of heterozygotes as DD homozygotes, all samples classified as DD genotype were subjected to a second PCR using an I-allele-specific primer pair: Forward: 5′-TGGGACCACAGCGCCCGCCACTAC-3′ and Reverse: 5′-TCGCCAGCCCTCCCATGCCCATAA-3′ (24) (using 30 PCR cycles of 1 min at 95 °C, 40 s at 67 °C, and 2 min at 72 °C). Products were detected by 6% polyacrylamide gel electrophoresis. A 335 bp fragment indicated the presence of the I-allele, and samples positive for both this 335 bp fragment and the 190 bp fragment in the first PCR were called as ID heterozygotes.

Paragraph Number 13 *PCR-RFLP Genotyping*. Genotyping of Taiwanese swimmers and controls at *ACTN3* R577X (rs1815739) was carried out after PCR amplification across the polymorphic site and restriction digestion, as previously described (7).

Statistical analysis

Paragraph Number 14 Genotype and allele frequencies were calculated for both *ACE* and *ACTN3* polymorphisms and Hardy-Weinberg equilibrium (HWE) assessed using a χ^2 test. Multinomial logistic regression was used to analyse genotypic associations with case/control status. Three outcome states were used in the models. The regional samples were analysed separately. For Caucasians, the outcome states were SMD swimmer, LD swimmer and

control. For East Asians, the outcome states were SD swimmer, MD swimmer and control. Associations of genotype with outcome were modeled using three genetic models - additive allelic effects and two models assuming complete dominance of each allele in turn. A permutation test tool (http://rosalind.infj.ulst.ac.uk/Software.html#PTest (6)) was employed to generate association test *p*-values adjusted for multiple testing while maintaining an experiment-wide type I error rate of 0.05. For each calculation, 99,999 permutations were computed. Genetic model-adjusted *p*-values were also calculated using MAX3, an efficiency robust trend test implemented in the R Package Rassoc (39), reporting empirical *p*-values calculated using the 'boot' method. Additionally, the effect of ethnicity within the East Asian cohorts was evaluated by including a genotype x ethnicity interaction term in the multinomial logistic regression models and assessing significance using a likelihood ratio test. Analyses were carried out using IBM® SPSS® Statistics 19 software (SPSS, Inc., Chicago, USA) and R (R Foundation for Statistical Computing, Vienna, Austria).

Results

Paragraph Number 15 In the Caucasian cohort, genotype data were available for 191 cases (swimmers) and 1248 controls for *ACE* and 193 cases and 1694 controls for *ACTN3*. For East Asians, data were available for 326 cases and 1244 controls for *ACE* and 326 cases and 1252 controls for *ACTN3*. Both polymorphisms were in HWE in both cases and controls for both Caucasian and East Asian cohorts (See Tables, Supplemental Digital Content 1 and 2, which illustrate *ACE* and *ACTN3* genotype and allele frequencies in elite Caucasian and East Asian swimmer cohorts, respectively). Allele frequencies for *ACE* I/D (as measured using rs4341 in Caucasian swimmers and Japanese subjects) in the control populations were as expected, with the I-allele being at relatively higher frequency in East Asians (14,33). Allele

frequencies for *ACTN3* showed only small differences between the regional subgroups and were in line with expectation (1,17,21,27,28,37). The *ACTN3* data in Caucasian controls were obtained from 5 separate studies (1,17,27,28,37). There were no differences in allele frequencies or genotype distributions between these studies ($\chi^2 = 6.03$, $p = 0.64$; See Table, Supplemental Digital Content 3, which describes *ACTN3* genotype and allele frequencies in Caucasian controls from the five published reports).

Paragraph Number 16 *ACE* I/D genotype was associated with elite swimmer status in Caucasians. The multinomial logistic regression models were significant (Table 2; $p = 0.017$ for the additive model and $p = 0.005$ for the I-allele-dominant model). This association was mediated by effects in SMD swimmers (Figure 1, Table 2), with the largest effect size observed for the I-allele-dominant model (D-allele homozygotes vs. I-allele carriers: odds ratio = 1.90; logistic regression $p = 0.001$; adjusted permutation test $p = 0.0005$), with the D-allele being over-represented in the swimmers. We thus conclude that the D-allele is associated, in recessive fashion, with elite SMD swimmer status in Caucasians. The genetic model-adjusted *p*-value remained strongly significant (MAX3 test statistic = 3.37; $p = 0.0017$). No significant association was found between the *ACE* I/D polymorphism and Caucasian LD swimmer status (Figure 1, Table 2).

Paragraph Number 17 Before deciding how to treat the East Asian sample in the association analyses, multinomial logistic regression models were evaluated for genotype by ethnicity interactions (i.e. the models were Outcome = genotype + ethnicity[Japanese/Taiwanese] + (genotype x ethnicity) + error) to determine whether effects on outcome differed between the two sub-cohorts. In models evaluated for both *ACE* and *ACTN3* under all three genetic models (additive and both dominant models), the interaction

term was not significant ($p \geq 0.11$; See Table, Supplemental Digital Content 4, which demonstrates the results of likelihood ratio tests for the effect of the interaction term on the overall model in the elite East Asian swim cohort). As a result, the Japanese and Taiwanese subgroups were treated as a single East Asian cohort in all subsequent analyses.

Paragraph Number 18 In East Asian SD swimmers, *ACE* I/D genotype was also associated with swimmer status (Figure 2, Table 2). Under the D-allele-dominant model, the multinomial logistic regression model was significant (Table 2; $p = 0.032$), with the additive model test also approaching significance ($p = 0.097$). This association was mediated by effects in SD swimmers (I-allele homozygotes vs D-allele carriers: odds ratio = 1.53; logistic regression $p = 0.012$; adjusted permutation test $p = 0.008$), with the I-allele being over-represented in the swimmers. Thus we conclude that the I-allele predisposes, in recessive fashion, to elite SD swimmer status in East Asians. The genetic model-adjusted *p*-value remained significant (MAX3 test statistic = 2.53; $p = 0.025$). No significant association was found between the *ACE* I/D polymorphism and East Asian MD swimmer status (Figure 2, Table 2).

Paragraph Number 19 For *ACTN3* R577X, no statistically significant associations were observed in either regional subgroup for any of the swim distance subgroups (Table 2; also see Table and Figures, Supplemental Digital Content 2, which demonstrates *ACTN3* genotype and allele frequencies in elite Caucasian and East Asian swim cohorts and Supplemental Digital Content 5 and 6, which report the genotype frequency distribution of *ACTN3* in elite Caucasian and East Asian swimmers and controls, respectively). The multinomial logistic regression model for East Asian swimmers approached significance ($p = 0.07$) under the

additive and 577X-allele-dominant models, with most of the effect coming from the SD swimmers (p = 0.022) in whom the 577R-allele would be the performance-enhancing allele.

Discussion

Paragraph Number 20 Our results show that the *ACE* I/D polymorphism is associated with elite swimmer status in both Caucasians and East Asians. The association is not seen in the longer distance events in each group, but only in SMD swimmers in Caucasians and only in SD swimmers in East Asians. *ACTN3* p.R577X genotype was not significantly associated with swimmer status in these samples.

Paragraph Number 21 The findings for *ACE* I/D need to be interpreted in the context of population differences in I/D allele frequency. The allele frequencies observed were as expected for the populations used here (13). The lower minor allele frequency in East Asians reduces power to detect associations somewhat but did not prevent an association being detected, at least in the shorter distance swimmers. Despite the association in Caucasians being observed in swimmers of combined SD and MD designation (the SMD swimmer subgroup), there was no tendency for genotypes of East Asian MD swimmers to differ from controls in the same direction as in the significantly associated SD swimmers. Limited power is unlikely to explain this lack of trend and the possibility should therefore be entertained that the populations differ in the extent to which *ACE* I/D affects swimmers at different distances.

Paragraph Number 22 In terms of direction of effect, the observation that the D-allele was associated with SMD swimmer status in Caucasians while the I-allele was associated with SD swimmer status in East Asians is particularly notable. Previous studies, though using smaller

samples, have reported associations between the D-allele and elite SMD swimming status (8,35). The direction of effect in East Asians is consistent with previous reports if *ACE* affects other endurance/power-related sports in the same way as it does swimming - the D-allele has been reported to be associated with endurance performance in elite Japanese marathon runners (33), whereas the I-allele has been reported to be associated with elite power athlete status in Koreans (14). No associations with longer distance events were observed in our study but it is not always the case that complementary associations must be observed in opposing phenotypes and whether in fact these genotype effects operate across the entire phenotypic distribution in the whole population is not known.

Paragraph Number 23 While associations of opposite direction in different ethnic groups can be a result of type I error, there are several other possible explanations consistent with real association. Firstly, it may be that, although the causative variant(s) are identical in Caucasians and East Asians, the *ACE* haplotype networks found in Caucasians and East Asians are sufficiently different in the environs of these variants that different I/D alleles are on the predisposing haplotype more of the time in each group. Secondly, it may be that there are different causative variants in Caucasians and East Asians, with I- and D-alleles being on different haplotypes with respect to these more of the time in each regional subgroup. While the idea that common polymorphisms show association with phenotypes because of so-called 'synthetic associations' - where a number of different, individually rare causative alleles are all captured by a single tagging variant - is popular at present (34), there is remarkably little evidence for associations between a single complex phenotype and different predisposing alleles in different populations (9). In addition, the relatively simple haplotype structure around I/D and relatively deep haplotype branching pattern (40), suggesting haplotype divergence predating the separation of the Caucasian and East Asian populations

approximately 30,000-50,000 years ago, would argue against this explanation here. A third possible explanation is that ACE affects the relevant phenotypes differently in Caucasians and East Asians as a result of other changes in physiology appearing since the two population subgroups diverged. Thus, for example, higher ACE activity may predispose to short distance swimming performance in one population and lower ACE activity may have the same effect in the other population.

Paragraph Number 24 The failure of previous studies to observe associations between *ACE* I/D and power-related performance in sub-Saharan African and African American/Jamaican samples (29), or indeed with endurance-related performance (4,30), is easier to explain. The I/D polymorphism is not thought to be the causative site influencing serum ACE activity, which is thought to be located between intron 18 and the 3' UTR (40), with potential additional functional sites located in the 5' region of the gene (19,40). The haplotype structure in Caucasians and East Asians means that I/D is in very strong LD with at least one of these functional sites, almost certainly as a result of the out-of-Africa bottleneck. In Africa, however, there is much greater haplotype diversity across *ACE* and the LD structure means that I/D is not strongly associated with serum ACE activity (40); this is likely to be a large part of the explanation for the lack of association with sporting performance in African populations. An alternative explanation, however, may be that serum ACE activity is not the important factor influencing associations with performance and that local actions of ACE within skeletal or cardiac muscle that influence blood flow or other determinants of muscle performance over the life course or during performance tasks, for example, are more important (20,25). Other commonly genotyped *ACE* variants may capture the effects of functional variants on such local ACE actions more effectively than the I/D polymorphism does.

Paragraph Number 25 The lack of clear association between *ACTN3* genotype and swimmer status is interesting in light of previous studies. In Caucasians, multiple studies have reported the *ACTN3* 577X-allele to be under-represented in elite sprint/power event athletes (reviewed in (18)). Few studies have focused on this polymorphism in East Asian elite athletes (7,31). Of the two ethnic groups studied here, the East Asians came closest to showing an association, this effect being in the same direction as in previous studies (with the 577R-allele being modestly over-represented in SD swimmers). Although ACTN3 deficiency has a modest effect on muscle fiber distribution (11), its impact on ability to perform in elite power events may have just as much to do with the role of ACTN3 in muscle metabolism rather than in relation to structure or fibre-type distribution *per se* (5,21). It may be that none of these roles subserved by ACTN3 are of particular importance in swimming, or it may be that the aspect of power performance affected by the polymorphism is under-engaged in swimming relative to other sports, possibly because of the relatively lower stress put on muscles supported in water and lack of eccentric contractions (16). It may also be that swimming performance has a much greater component of technique than other power events. Lastly, there is the possibility that type II error accounts for the fact that we do not see an association here. Large studies with power to detect significant associations at genome-wide level have not yet been conducted. Although a meta-analysis of associations between *ACTN3* and sprint/power athlete status has been published and does find evidence for a real association (2), many studies, mainly with small sample sizes, have failed to observe any association between *ACTN3* variants and sporting performance.

Paragraph Number 26 The limitations of the study reported here relate primarily to this issue of sample size. Although our study was carried out using the largest elite swimmer sample yet assembled, it is still a relatively modest sample size for a genetic association

study. Future efforts should be focused on collecting a sample large enough to allow testing of many more candidate genes using next generation sequencing approaches to find additional putative functional variants, or perhaps analysis at the genome-wide level and this may entail using a cohort that lies towards the top of the swimming performance distribution but that is less elite than the present sample. Further studies could also focus on whether the associations reported here are observed in trained/competent swimmers from the general population, as effects observed in the extremes of the distribution may or may not reflect physiological processes operating across the entire population distribution. It would also be interesting to know whether such associations are observed in cohorts of swimmers adhering to different training regimes/intensities, whether history of injury affects the association, or whether the range of event distances in which the effect is observed is wide or narrow. Our findings should be interpreted with caution until confirmed by additional, larger studies of these kinds but are interesting nonetheless.

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Conflict of Interest

Paragraph number 28 There are no conflicts of interest. The results of the present study do not constitute endorsement by ACSM.

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Figure 1. Genotype frequency distribution for *ACE* I/D in elite Caucasian swimmers and controls.

Figure 2. Genotype frequency distribution for *ACE* I/D in elite East Asian swimmers and controls.

List of Supplemental Digital Content (to the end of the MS)

- Supplemental Digital Content 1. Table that illustrates *ACE* genotype and allele frequencies in elite Caucasian and East Asian swim cohorts. doc
- Supplemental Digital Content 2. Table that demonstrates *ACTN3* genotype and allele frequencies in elite Caucasian and East Asian swim cohorts. doc
- Supplemental Digital Content 3. Table that shows *ACTN3* genotype and allele frequencies in Caucasian controls from five published studies. doc
- Supplemental Digital Content 4. Table that shows the results of likelihood ratio tests for the effect of genotype x ethnicity interaction on the overall model in elite East Asian swim cohort. doc
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- Supplemental Digital Content 6. Figure that reports the genotype frequency distribution of *ACTN3* in elite East Asian swimmers and controls. tiff

Table 1. Total numbers of Caucasian and East Asian swimmers recruited in this study.

Cohort	Event	Male	Female	Total
Caucasians	SMD (≤ 400 m)	71	54	125
	LD (> 400 m)	40	28	68
East Asians	SD (≤100 m)	95	65	160
	MD (200 – 400 m)	101	65	166

Table 2. Multinomial logistic regression analysis of associations between *ACE* and *ACTN3* polymorphisms and elite Caucasian and East Asian swimmers

Gene	Cohort	Group	Risk allele[#]	Additive Model				Dominant Model[†]				
				Model *p*	O.R.[$] (95% C.I.)	pairwise *p*[¶]	PT adjusted *p*[§]	Dom. allele	Model *p*	O.R.[$] (95% C.I.)	pairwise *p*[¶]	PT adjusted *p*[§]
ACE	Caucasians	SMD	D	0.017	1.46 (1.12 - 1.90)	0.005	0.003	I	0.005	1.90 (1.30 –2.78)	0.001	0.0005
		LD	(D)		1.04 (0.74 - 1.47)	0.82	N/A	I		1.12 (0.65 - 1.93)	0.70	N/A
	East Asians	SD	I	0.097	1.32 (1.02 - 1.72)	0.034	0.036	D	0.032	1.53 (1.10 - 2.13)	0.012	0.008
		MD	(I)		1.06 (0.83 - 1.35)	0.67	N/A	D		0.94 (0.68 – 1.30)	0.69	N/A
ACTN3	Caucasians	SMD	(X)	0.27	1.12 (0.86 – 1.44)	0.41	N/A	X	0.12	1.20 (0.80 – 1.80)	0.37	N/A
		LD	(R)		0.78 (0.55 – 1.12)	0.18	N/A	X		0.63 (0.39 – 1.03)	0.065	N/A
	East Asians	SD	R	0.071	1.32 (1.04 – 1.68)	0.022	N/A	X	0.072	1.51 (1.06 – 2.14)	0.022	N/A
		MD	(R)		1.03 (0.82 – 1.30)	0.80	N/A	X		1.14 (0.79 - 1.63)	0.49	N/A

risk allele is designated as the allele whose frequency is higher in the relevant swimmer group than in controls; it has no meaning where tests reveal no significant association - see parentheses

$ O.R. - odds ratio; 95% C.I. - 95% confidence interval

¶ *p*-value for the estimate of β for the effect of genotype on the pertinent pairwise outcome comparison (e.g. SMD vs Control) in the multiple logistic regression

§ adjusted *p*-value for the permutation test (PT) to control for multiple testing

† for each cohort, the model *p* value is given for the dominant model (as indicated in the 'Dom. allele' column) with the lowest *p* value; odds ratios under each model are reported for the designated risk allele for *ACE*, and for the *ACTN3* 577X-allele in Caucasians and the 577R-allele in East Asians

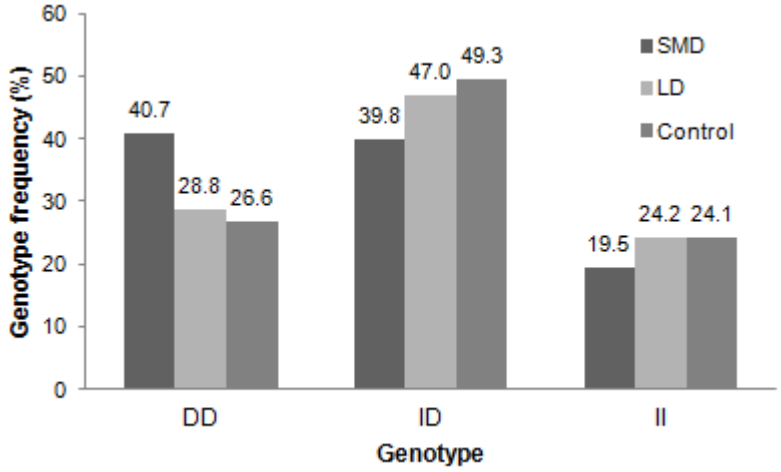


Figure 1. Genotype frequency distribution for *ACE* I/D in elite Caucasian swimmers and controls.

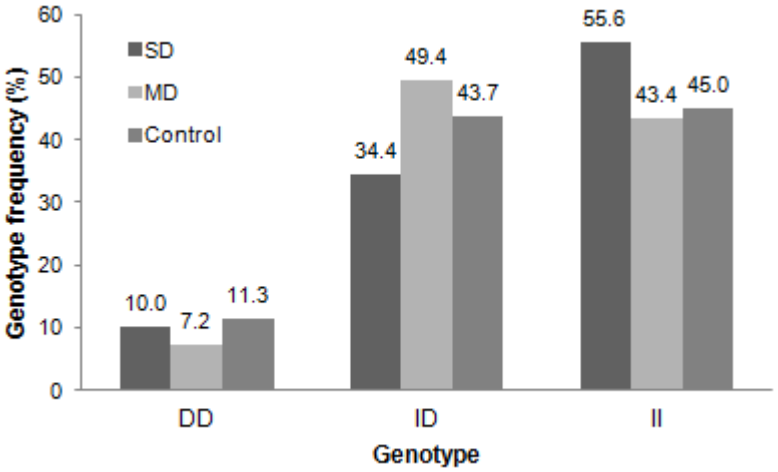


Figure 2. Genotype frequency distribution for *ACE* I/D in elite East Asian swimmers and controls.

Supplemental Digital Content 1. Observed *ACE* genotypes and allele frequencies in Caucasians and East Asians.

		Caucasian cohort			East Asian cohort		
Groups		**SMD**	**LD**	**Controls** [a]	**SD**	**MD**	**Controls**
Observed Genotype Counts, n (%)	**D/D**	51 (40.7)	19 (28.8)	332 (26.6)	16 (10)	12 (7.2)	140 (11.3)
	I/D	50 (39.8)	31 (47)	615 (49.3)	55 (34.4)	82 (49.4)	544 (43.7)
	I/I	24 (19.5)	16 (24.2)	301 (24.1)	89 (55.6)	72 (43.4)	560 (45)
Total		125	66	1248	160	166	1244
Allele Frequency	**D**	0.61	0.52	0.51	0.27	0.32	0.33
	I	0.39	0.48	0.49	0.73	0.68	0.67
HWE *P*-value		0.07	0.63	0.63	0.096	0.079	0.65

a. *ACE* controls were drawn from a previous published study (35)

Supplemental Digital Content 2. Observed *ACTN3* genotypes and allele frequencies in Caucasians and East Asians.

		Caucasian cohort			East Asian cohort		
Groups		**SMD**	**LD**	**Controls** [a]	**SD**	**MD**	**Controls**
Observed Genotype Counts, n (%)	**R/R**	35 (28)	29 (42.6)	540 (31.9)	55 (34.4)	47 (28.3)	323 (25.8)
	R/X	65 (52)	27 (39.7)	840 (49.6)	76 (47.5)	79 (47.6)	640 (51.1)
	X/X	25 (20)	12 (17.6)	314 (18.5)	29 (18.1)	40 (24.1)	289 (23.1)
Total		125	68	1694	160	166	1252
Allele Frequency	**R**	0.54	0.625	0.57	0.58	0.52	0.51
	X	0.46	0.375	0.43	0.42	0.48	0.49
HWE *P*-value		0.60	0.21	0.69	0.76	0.55	0.41

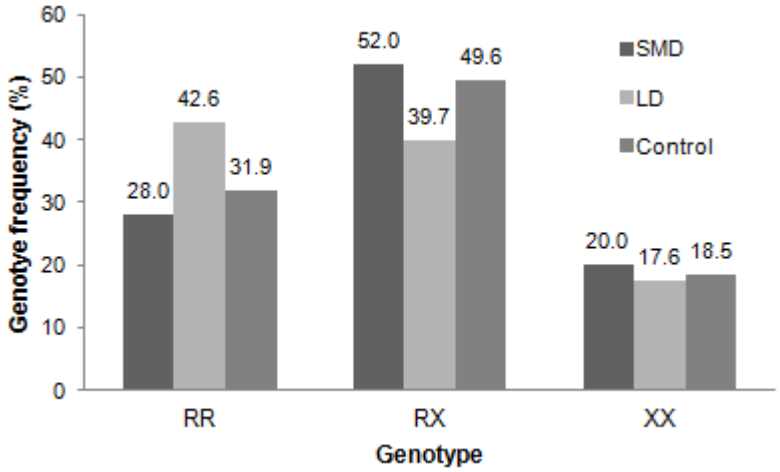
a. The total controls combined from five published *ACTN3* Caucasian controls (see Supplementary Digital Content 3)

Supplemental Digital Content 3. Observed *ACTN3* genotypes and allele frequencies in Caucasian controls drawn from 5 published studies.

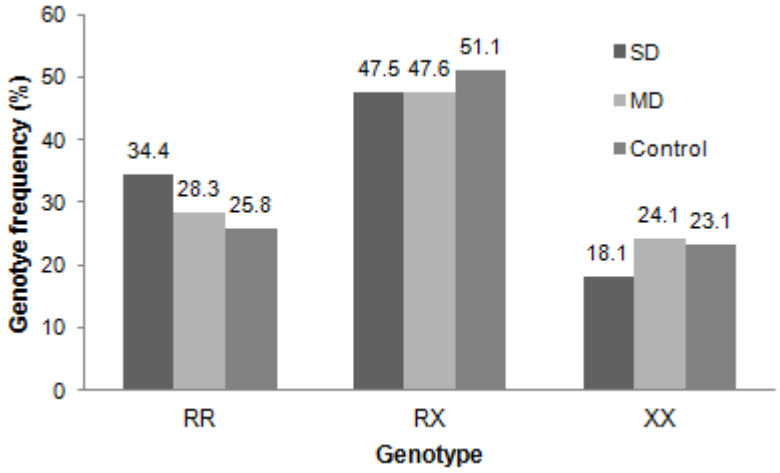
		ACTN3 Caucasian controls				
Studies		**Yang et al. 2003**	**Lucia et al. 2006**	**Roth et al. 2008**	**Santiago et al. 2010**	**Ahmetov et al. 2010**
Observed Genotypes Counts, n (%)	**R/R**	130 (29.8)	35 (28.5)	218 (32.6)	90 (31.8)	67 (36.4)
	R/X	226 (51.8)	66 (53.7)	317 (47.5)	141 (49.8)	90 (48.9)
	X/X	80 (18.3)	22 (17.9)	133 (19.9)	52 (18.4)	27 (14.7)
Total		436	123	668	283	184
Allele Frequency	**R**	0.56	0.55	0.56	0.57	0.61
	X	0.44	0.45	0.44	0.43	0.39
HWE *P*-value		0.29	0.34	0.36	0.80	0.72
Chi-squared *P*-value		0.64 (Chi-squared statistic = 6.03)				

Supplemental Digital Content 4. The likelihood ratio tests for examining the effect of genotype x ethnicity interaction on the overall model in East Asian cohort.

	Likelihood Ratio Test		
	Chi-square	**d.f.**	**Significance *p***
ACE			
Intercept	.000	0	.
I-ADD	.000	0	.
Ethnicity	3.34	2	0.19
I-ADD x Ethnicity	**0.89**	**2**	**0.64**
Intercept	.000	0	.
D-DOM	.000	0	.
Ethnicity	6.91	2	0.032
D-DOM x Ethnicity	**1.74**	**2**	**0.42**
Intercept	.000	0	.
I-DOM	.000	0	.
Ethnicity	1.54	2	0.46
I-DOM x Ethnicity	**0.17**	**2**	**0.92**
ACTN3			
Intercept	.000	0	.
R-ADD	.000	0	.
Ethnicity	7.67	2	0.022
R-ADD x Ethnicity	**2.30**	**2**	**0.32**
Intercept	.000	0	.
R-DOM	.000	0	.
Ethnicity	9.71	2	0.008
R-DOM x Ethnicity	**4.34**	**2**	**0.11**
Intercept	.000	0	.
X-DOM	.000	0	.
Ethnicity	7.38	2	0.025
X-DOM x Ethnicity	**0.18**	**2**	**0.92**



Supplemental Digital Content 5. Genotype frequency distribution for *ACTN3* R577X in elite Caucasian swimmers and controls.



Supplemental Digital Content 6. Genotype frequency distribution for *ACTN3* R577X in elite East Asian swimmers and controls.