

ACTN3 Genotyping by Real-Time PCR in the Italian Population and Athletes

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ABSTRACT

PAPARINI, A., M. RIPANI, G. D. GIORDANO, D. SANTONI, F. PIGOZZI, and V. ROMANO-SPICA. *ACTN3* Genotyping by Real-Time PCR in the Italian Population and Athletes. *Med. Sci. Sports Exerc.*, Vol. 39, No. 5, pp. 810–815, 2007. **Purpose:** Development of two novel sets of primers and probes to detect R577X and Q523R polymorphisms of the α -actinin-3 (*ACTN3*) gene by real-time PCR. We report the allelic frequencies observed in Italian individuals from the general population and athletes. Athletic performance is influenced by training, environmental factors, and genetic predisposition. Actn3 belongs to a family of actin-binding proteins and is supposed to influence sport performance. **Methods:** Primer-probe set design and protocol optimization for real-time PCR genotyping of R577X and Q523R polymorphisms. The assay was verified using a traditional PCR-RFLP approach and applied on an Italian population sample (102 male subjects and 42 athletes). **Results:** Haplotype distribution confirmed the presence of linkage disequilibrium between the polymorphisms, both in the Italian general population and athletes (respectively: $\chi^2 = 54.4$, $P \leq 0.001$ and $\chi^2 = 24.5$, $P \leq 0.001$). Within the general population, a large percentage of homozygous subjects (21.6%) was deficient for Actn3. No significant differences were observed in athletes. The concordance between PCR-RFLP and real-time PCR results was 100 and 93% for polymorphisms Q523R and R577X, respectively. **Conclusion:** Real-time PCR represents an effective approach for typing *ACTN3* alleles. Allelic frequencies in the Italian population are consistent with those seen in other studies on Caucasians. **Key Words:** ATHLETIC PERFORMANCE, GENETIC EPIDEMIOLOGY, R577X, Q523R, GENETIC PREDISPOSITION, SPORT

Physical performance is thought to be influenced by training, environmental factors, and genetic predisposition, in accordance with a complex multifactorial, polygenic model (14,29). Our growing insight into the genetic basis of variation in movement and athletic skills is well testified by the extent of the human gene map for performance and health-related fitness phenotype. The map now includes 140 autosomal gene entries and quantitative trait loci, plus four on the X chromosome, and 16 mitochondrial genes (29). A recent study on Ethiopian elite runners highlighted the role of nuclear polymorphisms, compared with mitochondrial ones, in the generation of successful running performance (24). Several genes are supposed to play a role in sport performance, including angiotensin-converting enzyme (*ACE*), muscle-specific creatine kinase (*CKMM*), α -fibrinogen (*FGA*), uncoupling protein 3 (*UCP3*), α -actinin-3 (*ACTN3*), α -2A-adrenoceptor (*ADRA2A*), endothelial PAS domain

protein 1 (*EPAS1*), β -2-adrenoceptor (*ADRB2*), and peroxisome proliferator-activated receptor- α (*PPARA*) (1,7–9, 11,14,19,30,31).

Alpha-actinins (α -Actn) are a family of actin-binding proteins with multiple roles in different cell types. They belong to the same protein group of dystrophins and spectrins and have been highly conserved during evolution (3,28). Homologous proteins have been identified in several different taxa, such as protists, invertebrates, birds, and mammals (13). In the latter class of organisms, six protein products are associated with either muscle or nonmuscle tissues. Thus, α -Actn isoforms are classified as nonmuscle cytoskeletal (calcium sensitive) or as muscle sarcomeric (calcium insensitive) (2). The various isoforms are encoded by four genes (*ACTN1*, *ACTN2*, *ACTN3*, and *ACTN4*) and are differentially expressed (15).

Human sarcomeric actinins are α -Actn2 and α -Actn3 and are found, respectively, in all types of fibers or in type II only. Muscle isoforms are considered functionally redundant because of their close localization within the sarcomere and the absence of pathogenic effects associated with *ACTN3* deficiencies (15,22,32). Deficiency is caused by a C→T transversion in exon 16 of the *ACTN3* gene (11q13–q14); this converts an arginine residue to a premature stop codon at residue 577. The single-nucleotide polymorphism (SNP) R577X is found at position 1747. The homozygous condition for the stop codon occurs in about 18 and 16% of the European and world populations, respectively (15,17).

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The physiological roles of sarcomeric α -Actn are not yet fully understood. Measurement of several indices of functional capacity has revealed no association between ACTN3 absence and the deleterious effect of aging on muscle output in 577XX homozygous female subjects (22). Conversely, fascinating observations have been elicited from studies on professional athletes (4,31). The presence of α -Actn3 seems to have a beneficial effect on muscular function, in generating forceful contractions at high velocity. The observation of an unexpectedly high number of R577X heterozygotes in sprint athletes, and an unexpectedly low number in endurance athletes, has suggested a genotype effect in female athletes but not in males (31). A second polymorphism, Q523R, has been described for the ACTN3 gene and has been reported to be in linkage disequilibrium with R577X (17). The SNP consists of an A→G transition at position 1586 that changes a glutamine to an arginine.

Although several papers have analyzed ACTN3 genotypes in a broad variety of ethnic groups (4,12,16, 22,31,32), no report has focused specifically on both R577X and Q523R in southern-European populations. Comprehensive epidemiological data are available on R577X, but there is only limited information regarding Q523R (17). Real-time PCR is considered a great improvement over gel-based methods (6,10). This technique has been applied previously for genotyping one of the ACTN3 polymorphisms (R577X) (4,5). Here, we describe the use of novel real-time PCR primer-probe sets for the allelic discrimination of polymorphic sites of ACTN3. Allelic frequencies of R577X and Q523R were evaluated in samples of healthy Italian males from the general population and in elite male athletes.

METHODS

The sample included 144 Caucasian males who were either from the general population ($N = 102$) or from a selected group of athletes ($N = 42$). The athlete sample population included Italian rowers who were participating in pre-Olympics training. Rowing has been considered an endurance sport specialty previously, in the original investigation by Yang et al. (31). Individuals from the general population were unrelated healthy subjects from randomly distributed areas of northern, central, and southern Italy. The donors were recruited from a population-based sample of Italian subjects of Italian ancestry and had been identified on a volunteer basis through local outpatient and university departments. Samples belong to a DNA repository representative of Italian regions that was previously used to measure allelic frequencies in the general population (20). The use of subjects was approved by the local ethics committee. After written informed consent had been obtained from the subjects, whole genomic DNA was extracted either from whole-blood samples or from oral swabs. Genomic DNA was isolated by SDS/proteinase K digestion, followed by organic

solvent extraction (21). Preparations were quantified spectrophotometrically, normalized, and checked for purity and integrity by agarose gel electrophoresis. Primers and probes were designed by Primer Express 2.0 Oligonucleotide Design Software (Applied Biosystems). The sequence of the ACTN3 gene was obtained from GenBank (accession number: M86407). For the ACTN3 Q523R polymorphisms, primers and probes were ACCTCTAGCGGATGGA-GAAGCT (forward primer), CCGCCGGGCAAACCTC (reverse primer), Vic-ACCATTGACCGGCTG (probe for R allele), and Fam-CATTGACCAGCTGCAA (probe for Q allele). For the ACTN3 R577X polymorphism, primers and probes were CACGATCAGTTCAAGGCAACA (forward primer), CCCTGGATGCCCATGATG (reverse primer), Vic-CTGACCGAGAGCGA (probe for R allele), and Fam-AGGCTGACTGAGAGC (probe for X allele). Allelic discrimination was performed by an Applied Biosystems Sequence Detection System 7000, using TaqMan PCR Master Mix (Applied Biosystems), TaqMan MGB probes (Applied Biosystems) (250 nM each), and primers (900 nM each). The thermal cycle protocol involved 10 min at 95°C and 15 s at 95°C, plus 60 s at 60°C, for 40 cycles, in 25- μ L reaction volume. For PCR-RFLP analysis, 0.2 units of Taq DNA polymerase (Promega) were used. Amplification was carried out in 96-well plates, in 25 μ L. Conditions included 5 min at 94°C, followed by 25 cycles with 30 s at 94°C, 30 s at 58°C, 60 s at 72°C, and a final extension step at 72°C for 7 min, with a primer concentration of 1 μ M each. DNA samples from all the homozygous individuals, carrying

TABLE 1. Genotyping of ACTN3 gene polymorphisms.

Polymorphism	Group (N)	Genotype	No. with Genotype (%)	Allele Frequency	
				Q	R
ACTN3Q523R	Male athletes (42)	523 RR	8 (19.05)	0.51	0.49
		523 QQ	9 (21.43)		
		523 RQ	25 (59.52)		
	Male subjects (102)	523 RR	23 (22.55)	0.54	0.46
		523 QQ	31 (30.39)		
		523 RQ	48 (47.06)		
ACTN3R577X	Male athletes (42)	577 RR	9 (21.43)	0.51	0.49
		577 XX	8 (19.05)		
		577 RX	25 (59.52)		
	Male subjects (102)	577 RR	32 (31.37)	0.55	0.45
		577 XX	22 (21.57)		
		577 RX	48 (47.06)		

An Italian population sample of male subjects and athletes was genotyped by real-time PCR using fluorescent probes, designed for the R577X and Q523R polymorphisms of the alpha-actinin-3 gene.

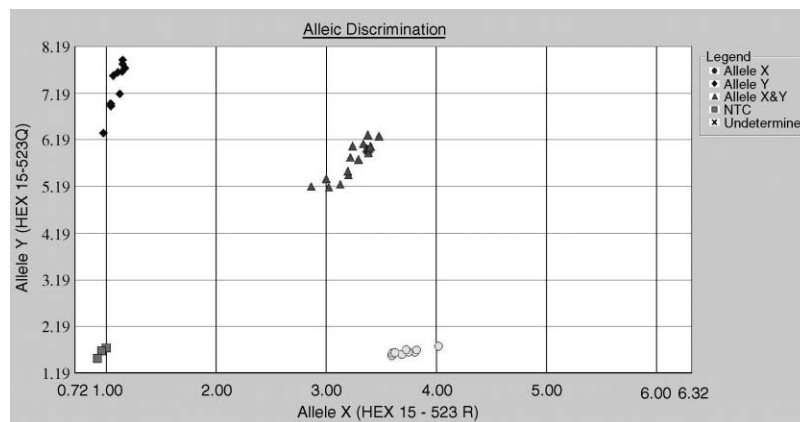


FIGURE 1—Example of Q523R allelic discrimination of the *ACTN3* gene by real-time PCR. Extracted DNA was amplified in the presence of two allele-specific fluorescent probes. A genotype was assigned to each subject on the basis of the relative fluorescence (shown on the x- and y-axes) associated with each probe. NTC, no template control.

mutant alleles, and previously identified by real-time PCR, were also analyzed by PCR-RFLP. Additional digestions of unpurified PCR products, from previously identified heterozygotes and wild-type homozygotes (523QQ and 577RR), were also included as controls. After a 3-h incubation with enzymes *MspI* and *DdeI*, digestion products were run on a 4% Nusieve electrophoresis agarose gel (Cambrex) (15). Expected fragments were 37 bp and 27 bp for allele 523R incubated with *MspI*, and 36 bp and 32 bp for allele 577X incubated with *DdeI*. Alleles 523Q and 577R could be revealed by full-length amplicons of 64 bp and 68 bp, respectively. Statistical analysis was carried out to determine allelic frequencies and to test for Hardy-Weinberg equilibrium. PHASE software (v. 2) was used to infer haplotype distributions, starting from genotypic data, and to study linkage disequilibrium (25,26). To calculate the approximate direct costs of the genotyping methods, we averaged prices from different manufacturers, and the following estimates were considered: one primer (€90 for 80 nmol), 96-well plate (€150 for 25), adhesive covers (€215 for 100), PCR master mix (€100 for 200 reactions), TaqMan PCR master mix (€530 for 400 reactions), *MspI* (€50 for 2000 u), *DdeI* (€100 for 1000 u), agarose (€450 for 500g), and DNA molecular weight marker (€160 for 50 lanes). Estimated electrophoresis time may vary from 3 to 12 h depending on the numbers of wells that can be simultaneously loaded on the gel.

RESULTS

Q523R and R577X polymorphisms of the *ACTN3* gene were successfully analyzed by the real-time PCR approach. Observed genotypic distributions are reported in Table 1. Amplification conditions were optimized to maximize allelic discrimination and to enhance the effectiveness of the primer-probe sets. The discrimination of the emission signals generated by the allele-specific fluorescent probes was clear and unambiguous (Fig. 1). Genotypes inferred by

real-time PCR were also confirmed by PCR-RFLP analysis. Coherent restriction profiles were obtained and visualized by agarose gel electrophoresis (Fig. 2) (15). We found 100% concordance between Q523R genotypes, detected by the novel real-time PCR assay, compared with the previously described enzyme assay (15). Concordance was slightly lower (93%) for R577X because of inconsistent results related to partial digestion or presence of spurious amplicons. Conversely, the real-time PCR always provided very reliable data.

Although reaction setup and amplification time were basically the same for the TaqMan assay and the traditional PCR (1 h 30 min vs 1 h 45 min), the entire PCR-RFLP protocol was considerably longer and much more laborious (6 h 45 min to 17 h 45 min vs 1 h 50 min) because of the additional restriction and electrophoresis steps. By shortening the restriction incubation to 1 h, we managed to shorten the PCR-RFLP protocol. However, although real-time PCR allowed the simultaneous analysis of up to 90 samples (plus controls), the electrophoresis-based genotyping method was necessarily constrained by the number of samples that could be loaded on a gel (Table 2).

In the sample from the general population, observed allelic frequencies were 0.45 and 0.46 for 577X and 523R,



FIGURE 2—Genotyping by restriction fragment-length polymorphism (RFLP) analysis. Amplification products previously typed by real-time PCR were digested with allele-specific enzymes and were loaded on a high-resolution 4% agarose gel. *DdeI* and *MspI* cleave alleles 577X and 523R, respectively. RFLP confirmed the allelic discrimination obtained by the real-time PCR assay. 1) Molecular weight standard; 2) 577XX; 3) 577XX *DdeI*-digested; 4) 577RX; 5) 577RX *DdeI*-digested; 6) 577RR; 7) 577RR *DdeI*-digested; 8) negative control; 9) negative control *DdeI*-digested; 10) 523RR; 11) 523RR *MspI*-digested; 12) 523RQ; 13) 523RQ *MspI*-digested; 14) 523QQ; 15) 523QQ *MspI*-digested; 16) negative control; 17) negative control *MspI*-digested.

TABLE 2. Comparison of real-time PCR and PCR-RFLP protocols used to genotype *ACTN3*: estimated costs and required time.

Method	Step	Time	Consumables (Cost/100 rxn)
Real-time PCR	PCR setup	30 min	96-well plate (6); adhesive covers (2); PCR mix (130); primer pair (3); probe pair (60)
	Amplification	1 h	
Total PCR-RFLP	Analysis	20 min	201
	3	1 h 50 min	
	PCR setup	45 min	
Total			148
	Amplification	1 h	
	Restriction setup	1 h	
	Incubation	1 to 3 h	
	Electrophoresis	3 to 12 h	
Total	5	6 h 45 min to 17 h 45 min	148

Considering direct costs, real-time PCR is a more expensive technique, but it has the advantage of being less time- and labor consuming.

Costs are given in euros and are calculated for a reaction volume of 25 μ L. The direct cost for 100 reactions is indicated for each item. Rxn, reaction; PCR, polymerase chain reaction; RFLP, restriction fragment-length polymorphism.

respectively. The frequency of mutant alleles in the sample of endurance athletes was 0.49 for both polymorphisms. Occurrence of 577XX homozygous null mutants was comparable in the general population and the athletes: 21.57 vs 19.05%, respectively. The distribution of athletes' genotypes seemed slightly enriched in heterozygotes ($P > 0.1$) for both SNP. Q523R and R577X polymorphisms are in Hardy–Weinberg equilibrium in the general population (respectively: $\chi = 0.29$, $P > 0.5$ and $\chi = 0.25$, $P > 0.5$) and among the athletes (respectively: $\chi = 1.53$, $P > 0.2$ and $\chi = 1.53$, $P > 0.2$).

We observed strong evidence for linkage disequilibrium occurring between the polymorphic loci Q523R and R577X, in accordance with previous studies (17). Inferred haplotype distributions were used to calculate expected allelic and genotypic frequencies within the two groups. Comparison between obtained and expected distributions confirmed a highly significant linkage disequilibrium between Q523R and R577X alleles in the Italian general population and also in the athletes (respectively: $\chi = 54.38$, $P \leq 0.001$ and $\chi = 24.54$, $P \leq 0.001$). No significant differences in the estimated haplotype distributions were observed between the general population and the athletes ($\chi = 1.323$, $P < 1$). The distribution of double homozygotes also did not change significantly. A combination of mutant alleles does not seem to be associated with pathologic effects. In particular, among subjects who were homozygous for at least one of the loci, we detected the 523R/577R haplotype in four healthy subjects and in one athlete. Healthy heterozygotes and homozygotes carrying haplotypes with a single mutation 523RQ/577XX, 523QQ/

577RX, 523QQ/577XX, and 523RR/577RR were also observed. This findings seem to confirm the absence of possible pathogenic effects associated with particular haplotypes.

DISCUSSION

Athletic performance can be considered a multifactorial, polygenic trait (14,29). Efficient human movement, like many other complex traits, is deeply influenced by environmental and behavioral factors, including training or diet, and also by genetic endowment (14,29). This latter issue may underpin what is referred to as individual sport aptitude. Recent studies suggest that α -Actn3 may influence muscle functionality and individual excellence in sport. *ACTN3* is a fast-twitch-specific isoform that is expressed only in type II myofibers. No significant differences in the R577X allele frequency between men and women have been described in the general population (4,31). However, gender-specific relationships have been reported between *ACTN3* genotype and athletic or muscle performance (4,12,14,16,31). Protein deficiency may represent an advantageous condition for female endurance athletes, whereas for sprint and power sports, the presence of Actn3 in the muscular tissue seems beneficial for both genders. To account for these observations, it has been hypothesized that androgens can reduce *ACTN3* effects (31). Several other factors may also influence athletic performance and modulate *ACTN3* effects, including mitochondrial oxidative phosphorylation genotypes (16).

In this study, a real-time PCR-based approach was developed to assess genotypic distribution of Q523R and R577X *ACTN3* polymorphisms, and this approach was applied to an Italian population sample. A fluorescent-based primer-probe set for R577X genotyping had previously been validated, and capillary electrophoresis was used to detect fluorescently labeled digestion fragments after *ACTN3* amplification (4,5,12). However, we have found no previous reports describing the application of TaqMan assays to SNP Q523R. This is the first study reporting frequencies of both SNP in a southern-European population. The presence of a Hardy–Weinberg equilibrium and the allelic frequencies observed in our group of Italians are in accordance with the results from previous studies on Caucasians (4,12,15,17). A similar R577X allele frequency has been found previously in Japanese subjects, whereas major differences have been detected in other populations (27). Interestingly, our findings confirm the absence of an association between *ACTN3* polymorphisms and endurance performance at the top level in males (12,31). As has been found previously in other populations, we detected phenotypically healthy subjects carrying the 523R/577R haplotype, with strong evidence of linkage disequilibrium (17). Apart from a small Bantu sample ($P < 0.05$), linkage disequilibrium for *ACTN3* polymorphisms was found in several ethnic groups (17). It has been

speculated that allele 577X is derived from a single mutational event and that a protective role against the possible deleterious effects determined by the mutant allele 523R is unlikely (17).

TaqMan assay results were confirmed by PCR-RFLP analysis. This method is a traditional, validated technique that is widely used to differentiate genotypes through the electrophoretic pattern obtained after amplicon restriction. In real-time PCR, the amount of product accumulating during amplification is monitored during the course of the reaction by recording fluorescence levels (10). TaqMan assay is based on fluorescence released after DNA polymerase 5'-3' nuclease activity on labeled probes. This procedure allows robust, high-throughput data acquisition and is fully automated. Its main disadvantages are elevated costs for reagents, instrumentation, and software. The supply of consumables for the amplification step, such as gloves, multiwell plates, and pipette tips, were basically comparable between PCR-RFLP and the TaqMan assay. PCR-RFLP is cheaper but more labor intensive, and the processing time is drastically delayed by the bottleneck of gel electrophoresis. Hazards and inconvenience due to the manipulation of toxic substances such as ethidium bromide are avoided by real-time PCR (10).

Although PCR-RFLP analysis of Q523R showed full concordance with real-time PCR, the R577X confirmation was slightly divergent in a minority (7%) of samples because of lack of amplification, low yield, or spurious bands. Likely, the R577X PCR-RFLP protocol was affected mainly by the quality of the DNA template and the oligonucleotide design, which was not designed for PCR-RFLP but, rather, was optimized to perform as a whole set together with the TaqMan probe.

Rapid, effective tests to identify the possible influences of individual genetic endowment on sport performance are highly desirable (18,23). Future advances will likely provide insights into the role of genetics on athletic aptitude, response to training, or injury recovery. However, the benefits of genetic testing on athletes require accurate evaluation of the ethical, legal, and social implications in addition to any technical issues.

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