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Comparison of the genotypes and allele frequencies of ACTN3 of football players from different categories

Comparação dos genótipos e frequencia dos alelos de ACTN3 de jogadores de futebol de diferentes categorias

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Daniel Barbosa Coelho[1]
Eduardo Mendonça Pimenta[2]
Izinara Cruz Rosse[3]
Maria Raquel Santos Carvalho[3]
Gabriel Gonçalves Costa[1]
Emerson Silami-Garcia[4]

[1]Federal University of Ouro Preto
[2]University of Leon, Department of Biomedical Sciences, Spain
[3]Laboratory of Human and Medical Genetics, Department of General Biology – Institute of Biological Sciences – Federal University of Minas Gerais, Belo Horizonte, Minas Gerais
[4]School of Physical Education, Physiotherapy and Occupational Therapy, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais

RESUMO: O objetivo do presente estudo é identificar a freqüência dos diferentes genótipos do polimorfismo ACTN3 em jogadores de futebol brasileiros de diferentes níveis competitivos. Participaram do estudo 140 jogadores masculinos das categorias profissional (PRO) (N: 83, 23,3±4,5 anos), pré-infantil (U-14) (N:43, 14,5±0,4 anos) e amadora (AMA) (N:14, 22,2±3,43 anos). A genotipagem do gene ACTN3 foi feita através de PCR-RFLP com utilização da enzima de restrição DdeI e as comparações ocorreram entre as freqüências dos diferentes grupos genotípicos do ACTN3 que são o RR, RX e XX. Não foram encontradas diferenças significativas entre as frequências alélicas (p=0,769). Observou-se uma diminuição na freqüência do genótipo XX e um aumento na freqüência do genótipo RX nas categorias pré-infantil e profissional em relação à categoria amadora (p=0,03). O aumento da freqüência do genótipo RX e diminuição do genótipo XX na categoria profissional e pré-infantil em comparação a categoria amadora pode indicar uma seleção natural para o futebol de indivíduos de caráter misto (força/resistência) em detrimento dos mais fracos quando o nível de exigência enquanto treinamentos e competições é maior.

Palavras-chave: Futebol; Performance atlética; Expressão de gen; Seleção genética; Fibras Musculares, tipo II; Alfa-actinina-3 humana.

ABSTRACT: The purpose of this study is to identify the genotypes and allele frequency of the ACTN3 polymorphism in Brazilian football players at different competitive levels. The ACTN3 gene encodes α-actinin-3. A polymorphism in this gene (ACTN3 R577X) alters the expression of α-actinin-3 and is associated with performance of strength and speed as the predominant activities. The study included 140 male players in professional (PRO) (N: 83, 23.3 ± 4.5 years), Under-14 (U-14) (N: 43, 14.5 ± 0.4 years) and amateur (AMA) categories (N: 14, 22.2 ± 3.43 years). The ACTN3 gene was genotyped by PCR-RFLP using the restriction enzyme DdeI. The frequencies of the ACTN3 variants, RR, RX and XX, were compared among the study groups. No significant difference was found between allele frequencies (p = 0.77). Decreased frequency of the XX variant and increased frequency of the RX variant were observed in the U-14 and PRO categories when compared to AMA (p = 0.03). This finding suggests the existence of artificial selection of football players with mixed characteristics (strength and resistance). Weaker individuals would tend to be excluded as the demand during training and competition increases.

Key Words: Football; Athletic performance; Gene expression; Genetic selection; Muscle fibers, type II; Human alpha-actinin-3.

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Contato: Daniel Barbosa Coelho - danielcoelhoc@bol.com.br

Introduction

Football has a worldwide audience and can be played by people of both genders and all ages around the world[1]. During a football match, a player covers a distance of 8–12 km by movements that range from walking to running at maximum speed[2]. The aerobic energy pathway predominates during the long game, but exertions that lead to the final movements, such as sprints, jumps, heading, dribbling, and shots on goals, have anaerobic characteristics[3]. Due to these characteristics, football may be classified as an intermittent and high energy activity[4,5]. Therefore, football players should be aerobically well trained and possess significant strength and speed in order to perform at high levels[5,6].

Considering the difference of physical demand among different competitive levels, it has been observed that players of professional sports teams have a greater number of high intensity activities compared to amateur players[7] older categories have higher values aerobic capacity and anaerobic threshold[8]. These can interfere with the characteristics of the soccer practice considering the age and competitive level of the players.

Innate sports skill differences based on metabolic predominance (aerobic, anaerobic, or mixed) may have been selected during human evolution[9]. In athletes, a positive correlation has been observed between a higher competitive level and a greater contribution of the genes associated with the characteristics of a specific sport (strength or resistance)[9-11]. However, other studies did not find a correlation between physical fitness and the expression of genes associated with those skills[19-21].

The ACTN3 gene encodes the intrasarcomeric protein α-actinin-3. A biallelic polymorphism of this gene (ACTN3 R577X) alters the expression of α-actinin-3 and is associated with changes in the performance of activities that mainly require strength and speed[22]. This is due to the fact that α-actinin-3 is specific to type II fibers and plays a structural role in the arrangement and traction transfer between actin filaments and the Z-line[17,23,24].

Alpha-actinins (alpha-actinin-1, alpha-actinin-2, alpha-actinin-3, and alpha-actinin-4) belong to an ancient family of actinin-binding proteins in the Z-line of the sarcomere. Alpha-actinin-3 is the most specialized among the four alpha-actinins that are seen in mammals. This specialization is seen in humans, rather than in other species such as birds, is linked to human evolution, and is most likely a result of natural selection[25].

Different variants expressing α-actinin-3 (ACTN3-RR and RX) have been found to be associated or not associated, in different studies, with sport activities that mainly rely on strength and speed[28,31,32], such as football[33]. The variant that does not express α-actinin-3 (ACTN3-XX) mainly correlates with anaerobic activities[34-36]. Thus, one must consider that soccer practice may be influenced by competitive levels and genetic factors.

Considering the above the objective of this study was to identify the genotypic frequency of ACTN3 polymorphism in Brazilian football players of different competitive levels and categories.

Methods

This study was in compliance with all the standards established by the National Board of Health (Res. 196/96) involving research with human beings. All volunteers, or those responsible, signed the informed consent form after receiving clarifications about the procedures and possible risks. This project was analyzed and approved by the Research Ethics Committee of the Federal University of Minas Gerais (UFMG; ETIC 291/09).

In total, 140 male players were analyzed. The U-14 and professional (PRO) players belonged to the first division of Brazilian football clubs that maintained regular systematized trainings and conducted disputed competitions organized and/or recognized by the Brazilian Football Confederation (CBF). Apart from the competition periods, the players had at least 2 years of experience in competitive football and trained for an average of nine 2-h sessions over 6 days each week.

We evaluated a group of AMA athletes who were part of a university team and were not remunerated for their performance. The amount of training was less than

30% of the training that the PRO players included in this study underwent.

To define the cohort, physical evaluation of the participants was performed and provided measurements of body mass, height and skin folds. A previously calibrated digital scale (Filizola®) with a precision of 0.02 kg was used to measure the body mass (kg) of volunteers without clothes and footwear. Height (cm) was measured using a stadiometer (with a precision of 0.5 cm) that was connected to a scale (Filizola®).

Subscapular, subaxillary, suprailiac, and abdominal skin folds as well as triceps, biceps, chest, thighs, and legs were measured using a plicometer (Lange®), scaled in millimeters, according to a protocol described previously[36]. The sum of all folds (∑ folds) was used to estimate the percentage of fat.

To genotype the athletes, 2 blood samples were collected from each athlete using vacuum tubes containing 4 mL EDTA each (Vacuette®). Samples were collected through venipuncture performed by a researcher or a nurse who had been properly trained in phlebotomy. The tubes were stored at 4 °C until the extraction of genomic DNA.

Genomic DNA was extracted from the peripheral blood samples according to a protocol described in the literature that uses proteinase K followed by saline precipitation[39]. A DNA fragment containing exon 16 of the ACTN3 gene was amplified from the genomic DNA. The primers used were the following: forward, 5′-CTGTTGCCTGTGGTAAGTGGG-3′; reverse, 5′-TGGTCACAGTATGCAGGAGGG-3′, annealing in adjacent intronic sequences[40].

The PCR reaction was performed with approximately 100 ng genomic DNA as template in a final volume of 25 µL of a pH 8.4 solution containing 10 mM Tris, 50 mM KCl, 1.75 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM each dNTP (Invitrogen, Carlsbad, CA), 1 U *Taq* DNA polymerase (Phoneutria Biotechnology and Service, Belo Horizonte, MG, Brazil), and 1.0 µM each primer (Sinapse Biotecnologia, Sao Paulo, SP, Brazil). The amplification program consisted of an initial denaturation step of 5 min at 94 °C; followed by 30 cycles each of 1 min at 94 °C, 1 min at 64 °C and 1 min at 72 °C; and a final extension step of 5 min at 72 °C. R577X alleles (CGA and TGA codons) were distinguished by the presence (577X) or absence (577R) of a restriction site for the enzyme *Dde*I[40].

After PCR amplification, 1 µL PCR product was digested with 20 U DdeI enzyme in a final volume of 15 µL. The reactions were incubated overnight (O/N) at 37 °C. The digested fragments were separated by electrophoresis in a 8% polyacrylamide gel, which was then stained with silver nitrate solution.[41] The ACTN3 577R allele generated fragments of 205 and 86 base pairs (bp), and the ACTN3 577X allele generated fragments of 108.97 and 86 bp (Figure 1)[22].

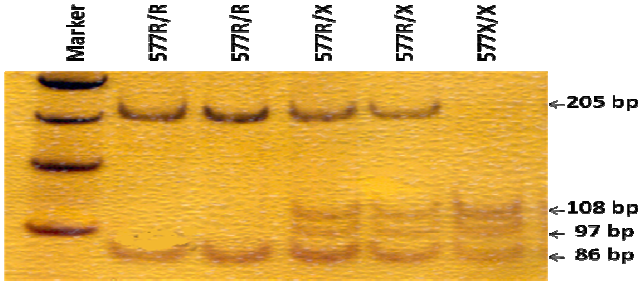

Figure 1. Polyacrylamide gel showing the restriction reaction. Individuals with 577R/R genotype present fragments of 205 and 86 bp; 577R/X genotype, 205, 108.97, and 86 bp; and 577X/X genotype 108. 97 and 86 bp. Marker: Ladder of 50

Statistical analysis

The Hardy-Weinberg equilibrium test was performed using GENEPOP v.4.0.10 software. The difference between genotypic and allelic frequencies between the different categories was assessed with the X^2 test using the Statistical Package for the Social Sciences for Windows®, version 11.0 software. A value of $p < 0.05$ was considered significant.

Results

Table 1 shows the characteristics of the volunteers in the different groups, classified according to their categories.

The genotypic and allelic frequencies observed for the ACTN3 locus in each category were; U-14 (58% RR, 30% RX, 12% XX, 73% X e 27% R), PRO (43% RR, 52% RX, 5% XX, 69% R and 31% X), Amateur (57%

RR, 21% RX, 21% XX, 68% R and 32% X). The allelic and genotypic frequencies were in Hardy-Weinberg equilibrium (p = 0.442).

Significant differences were not observed among the allelic frequencies (p = 0.77) (Figure 2). However, significant differences were observed among the genotype frequencies (p = 0.03) (Figure 3). We also observed reduced frequency of the XX and an increased frequency of the RX variant in U-14 and PRO categories when compared to AMA.

Table 1. Characteristics of the cohort: Height (cm), weight (kg), body composition (%G), and age of the following categories: Under-14 (U-14), professionals (PRO), and amateurs (AMA). Data presented in average and standard deviation

Category (N)	Age (years)	Body Mass (kg)	Stature (cm)	G (%)
U-14 (43)	14,23 ± 0,32	69,12 ± 7,53	178,21 ± 9,72	10,43 ± 3,51
PRO (83)	23,50 ± 3,64	74,25 ± 5,79	182,60 ± 8,55	8,20 ± 2,34
AMA (14)	22,31 ± 2,50	74,12 ± 3,43	180,33 ± 5,69	10,12 ± 3,48

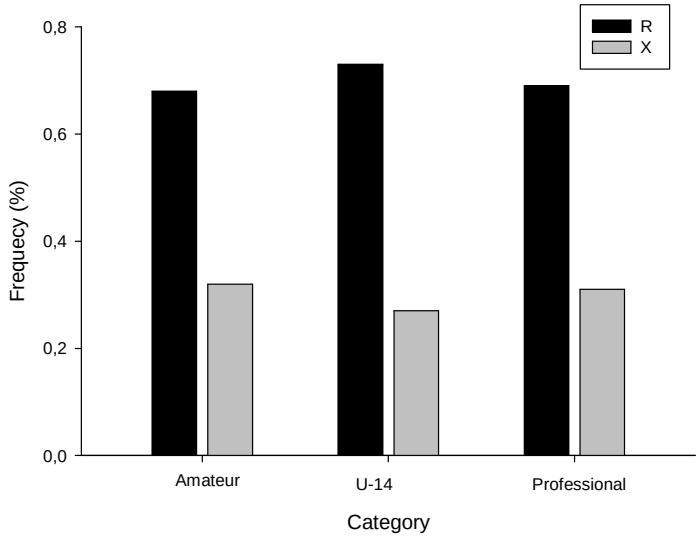

Figure 2. Allelic frequencies (%) of R and X in the different categories

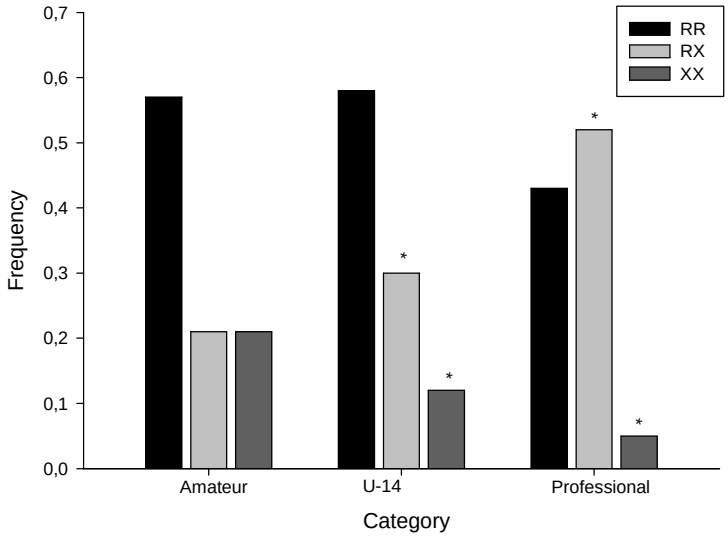

Figure 3. Genotype frequency of ACTN3- (RR, RX, and XX) for the amateur, Under-14 (U-14), and professional categories. * Difference in the amateur category (p < 0.05)

Discussion

The main finding of this study was the increase in ACTN3-RX and decrease in ACTN3-XX frequency in the U-14 and PRO categories compared to the frequencies for the AMA category. This finding suggests natural selection at the competitive levels of football.

Taking the characteristics of this sport into account, whereby strength and speed increasingly dictate the result of the game[42] and physical demands of the matches and trainings increase in higher categories, decreased frequency of ACTN3-XX and increased frequency of ACTN3-RX maybe would be expected in the PRO category when compared to U-14 category. However, despite the apparent visual observation of such differences, they were not statically confirmed. The increase in RX is consistent with the mixed character of this sport, in which both strength and endurance are important.

The difference between the categories can be due to factors such as the standard of match. It is argued that this fact may occur by artificial selection for the sport. What could justify this selection would be the difference in the standard of play between the different competitive levels. It has been identified that amateur players play fewer high-intensity activities during the games, but remain more time in each of these high-intensity stimulus. High levels of competitive players cover highest distances along the match, stay longer with the ball, carry a greater number of sprints and require less recovery time between sprints[7].

Even that was not statistically significant difference can be observed a higher frequency of the ACTN3 genotype-RX (Figure 3), which presents mixed (aerobic / anaerobic) character in the professional category. It has been observed that the anaerobic threshold of professional players is higher than younger players[8].

Some studies have found a correlation between ACTN3-RR frequency and the competitive levels of some athletes[9,13,15] or the performance levels, as classified by productivity[33]. Using the same observational methods, other studies did not find a correlation between the genotype frequency and performance[37,43].

Similar to the present study, Saunders et al.[37] did not find differences between the R or X allele frequency among higher competitive levels. However, Yang et al.[22] found an increased frequency of the R allele among higher competitive levels.

Santiago et al.[33] evaluated football players from different divisions of the Spanish Championship. These authors observed that RR and RX variants and the frequency of the R allele in football players were higher than in sedentary individuals and endurance athletes. The authors reported that the percentage of XX players in the first division was lower than that of sedentary individuals and endurance athletes.

Other studies transversally assessed ACTN3-R/X frequency in athletes practicing different sports and found different results. These studies observed a higher frequency of ACTN3-RR/RX genotype in athletes practicing sports that require a predominant use of strength and power[13,44] and of ACTN3-XX in sports characterized by aerobic resistance[17]. However, this relationship could not be established in all cases[20,21,29] because some authors used a small number of studies and some studied athletes with lower competitiveness[15,16,29].

Nevertheless, Scott et al.[45] found a relationship between the frequency of ACTN3-RR in American and Jamaican short-distance track Olympic finalists and the population of these countries. However, Yang et al.[19] did not observe the same correlation between African endurance athletes and the African populations. This indicates that a relationship between the populations of specific countries and the genetic ability for specific sports could not be established.

Our study did not include a control group due to methodology limitations, which may be considered a limitation of our study. However, if the genotype frequencies of athletes were similar to those of a control group composed of Brazilians, it could not be claimed that the Brazilian population possessed better football skills because of the environmental, technical, and tactical factors that influence football performance.

Interest among researchers in the influence of genetic profiles on sports performance has increased in the recent years[46,47], but it is essential that the quality of these studies increase[48].

ACTN3 is one of the most highly studied genes associated with sports performance. However, considering that 239 genes have been classified and found to be correlated with sports performance[46], combinations of multiple genes should be used to evaluate phenotypic expression.

Assessing the influence of genetic combinations in sports performance, Gómez-Gallego et al.[12] studied the expression of ACTN3 and ECA in 46 cyclists, relative to physical test performance, and compared them to a control group of the same size. The study observed that the genetic combination of ACTN3-RR/RX and ECA-DD were associated with higher strength and power compared with groups possessing intermediate combinations (ACTN3-RX/RR and ECA-II or ACTN3-XX and ECA-DD). ACTN3-XX and ECA-DD groups demonstrated higher values of aerobic capacity. Other studies did not find this association between performance and the same genetic combinations[18,20].

Ahmetov et al.[9] suggested an ideal genetic profile for studying aerobic and anaerobic athletes, such as rowers and football players. The authors evaluated five genes associated with sports performance and demonstrated a combination of a maximum of three of the genes that occurred among the better-positioned athletes. It can be concluded that the probability of occurrence of an ideal genotypic model is rare and that interaction with the environment is crucial. Other studies have also found an association between an increase in aerobic[10] and anaerobic performance[43] and an increase in gene expression.

The results emphasize the important probability that a greater number of genes and gene combinations are associated with performance in sports with predominance of specific physical activities. This reduces the probability that expression of just one gene may be responsible for a higher aerobic or anaerobic performance in these athletes.

Alpha-actinin-3 is a component of the sarcomeric Z-line[25], belonging to the family of actin binding proteins that are essential for the spatial maintenance of actin myofilaments, traction transfer between actin filaments, and the Z-line and for myofibrillar maintenance and repair[23,24,30]. The protein's specialization in humans is linked to human evolution and, most likely, to factors related to natural selection[25]. Besides the structural role and maintenance of the mechanical integrity of muscles, this protein is possibly associated with other functions, such as signaling and muscle metabolism[24,25,49].

Alpha-actinin has been associated with alteration of the contractile properties of type II fibers in the sarcomere, effects on the differentiation of the muscle fiber type, or hypertrophy via indirect interactions between alpha-actinin-3 and signaling proteins such as calcineurin, modification of the ability to resist and/or recover from exercise-induced muscle damage, and alterations in the metabolic profile of muscle fibers through modified interactions with metabolic enzymes such as fructose-1,6-bisphosphatase and phosphorylase[27,49]. None of these scenarios is mutually exclusive, and it is likely that the real mechanism is a combination of these different processes[25].

Studies using an animal model have demonstrated a more oxidative tendency of the skeletal muscles in a mouse knockout for ACTN-XX[26,35,50], with higher oxidative enzymatic activity, higher glycogen concentrations, increased relaxation times, and faster recovery from fatigue. These characteristics indicate a negative regulation of ACTN3-XX in sports requiring greater strength and power, although they suggest a positive influence on endurance activities. These aspects did not differ according to the gender but exacerbated with age[35].

Evaluation of the genetic profile of individuals may be important for directing individuals toward sports for which they may have more skills, assuming that the individuals are interested in the indicated sports. Such a capability could facilitate talent discovery. When the genetic profile of athletes is determined, adjustments in their training program or recovery periods during

competition might be performed based on such parameters[27]. Higher predisposition to muscle microtrauma has been described in ACTN3-XX individuals after eccentric training[30,49]. However, the effect was not confirmed in other studies[23], including an animal model[35].

With the tactical developments in football, some players that perform specific functions in the field may have different physical demands throughout the game. It is suggested that the defensive players as goalkeepers and fullbacks present higher values of anaerobic power[51], but Coelho et al[52] after evaluating the jumping ability and speed of 167 professional players did not identify the difference between tactical positions. However, considering the physical demands represented as time of work in areas of intensity of heart rate in the study of Coelho et al[53] were assessed 44 players over 29 an official competition games. In this study it was observed that the midfield players stay longer in areas of intermediate and aerobic intensity zones and the wing-backs work longer in anaerobic intensity zones nearest the maximum HR.

Considering that there is a difference between physical requirement between tactical positions[53] it may be that this factor interfered with natural selection of different genotypes of ACTN3 between footballers. However, in the present study this comparison was not performed by the small sample size for this analysis. However, one must consider this point a limitation of the study and a suggestion for further assessment. In addition, the main objective of the study was the comparison of genotype frequencies between the different competitive levels in football and not between tactical positions.

Another point that was not evaluated in this study was the relationship between genetic and physical distribution capabilities of the players. This also was not the main objective of the present study and the researchers did not have access to these data to physical assessment belonging to sports club. However this is something interesting research to be investigated in future research.

Access to high performance players to conduct such studies is difficult. By this fact the number of players evaluated is reduced. Yet, assuming you the total number of players per category in Brazil is 800 players, the sample is presented as an interesting sample.

A homozygous R577X gene (XX) has been reported to induce an alpha-actinin-3 deficiency in approximately 18% of the healthy European population; the frequencies vary in other populations[40]. A rate of approximately 11% was found in Ethiopians[19]. Deficiency of alpha-actin-3 is associated with reduced performance of fast muscle fibers in athletes and non-athletes. ACTN3 may be a selection factor for athletes but is not considered reliable selective factor yet. Even though ACTN3 expression is considered to be partially responsible for an athlete's success, environmental factors cannot be ignored[21,25,27].

The results showed increased RX frequency and decreased XX frequency in the PRO and U-14 categories, compared to those for the AMA category. This might suggest a natural selection for football in individuals with mixed characteristics (strength and resistance), to the detriment of weaker individuals when the demand level in training and competition is high. This statement should be taken with caution, because in collective sports, important cognitive factors influence technical and tactical performance. While a genetic profile may contribute to performance, it should not be the only factor considered.

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