ACTN3 POLYMORPHISM PREVALENCE IN GENERAL POPULATION OF SIX BALKAN COUNTRIES

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Konakli H., Z. Konakli, S. Dogan, D. Marjanovic, S. Dogan (2017): *ACTN3 polymorphism prevalence in general population of six Balkan countries*. - Genetika, Vol 49, No.3, 959-968.
The aim of this study was to investigate the prevalence of RR, RX and XX genotypes of rs1815739 single nucleotide polymorphism of *ACTN3* gene in general population of six Balkan countries. This SNP is currently thought to give genetic predisposition for advantageous sport performance, with its R allele positively correlating with better performance in strength/power sports, and X allele being present in higher frequency in elite athletes in endurance disciplines. A total of 483 individuals (281 males and 202 females) from seven populations from six countries was tested. Samples were collected by buccal swabbing method, DNA was isolated according to salting-out protocol and genotyping was performed using PCR and RFLP analysis. Obtained results suggest that RR and RX genotypes (43.7% and 44.1%, respectively) are overrepresented when compared to XX genotype (12.2%). The XX genotype in the study samples is present in lower frequency when compared to the global (16-18%) and European (18%) average. This study reports the first population data on genotype prevalence in sports-related genes for this part of Europe and is the beginning of research interests that intend to investigate genetic predispositions of elite athletes from this region competing internationally in different disciplines.
        Key words: ACTN3, endurance disciplines, rs1815739, strength/power disciplines

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INTRODUCTION

The human α-actinin muscle isoform 3 gene (*ACTN3*) encodes for an α-actinin-binding protein whose expression is limited to the fast-twitching skeletal muscle fibers and that helps to anchor the actin filaments and is involved in coordination of muscle fiber contraction (KIKUCHI and NAKAZATO, 2015). The gene itself is located on the long arm of chromosome 11 with an exact chromosomal locus 11q13.2 (BAUMERT *et al.,* 2016; KENT *et al.,* 2002). As all other actinin genes, *ACTN3* is a member of actin-binding protein family. It is the first structural gene encoding skeletal muscle proteins that has been connected to genetic predisposition to improve sports performance back in 2003 by Yang and colleagues (KIKUCHI and NAKAZATO, 2015; YANG *et al.,* 2003).

ACTN3 single nucleotide polymorphism (SNP) rs1815739 is a C>T transition that converts arginine residue at position 577 to a premature stop codon (non-sense mutation) usually abbreviated as R577X (BAUMERT *et al.,* 2016). Homozygosity for the premature stop codon is labelled as XX genotype and results in complete deficiency of α-actinin-3 protein in fast-twitch skeletal muscle fibers, but without any pathogenic or disease-causing phenotype related to this mutation (BROOS *et al.,* 2016). This genotype reduces strength, muscle mass and fast-twitch fiber diameter, but is increasing metabolic efficiency of the skeletal muscle at the same time (FRIEDLANDER *et al.,* 2013). The approximate abundance of XX homozygotic individuals in the global human population has been reported to be 16% (BROOS *et al.,* 2016) and 18% (FRIEDLANDER *et al.,* 2013; MILLS *et al.,* 2001), thus making X a minor allele. In Europe, the abundance of XX genotype is 18%, while this frequency reaches 25-29% in Japan and is as low as 1% in Africa (KIKUCHI and NAKAZATO, 2015; MAFFULLI *et al.,* 2013). Previous research suggests that the high frequency of the 557X allele in some human populations could be a result of selective pressure for increasing metabolic efficiency, which could in turn enhance the capability for endurance running. This phenomenon could favor the development of novel kinds of human activities that require high endurance, such as persistence hunting (AMORIM *et al.,* 2015).

As it is usually referred to as "a speed gene" (BROOS *et al.,* 2016), *ACTN3* polymorphisms have mostly been investigated within elite athletes, while the general population studies of this gene are rather scarce in the current literature. The major goal of *ACTN3* genotyping studies is to determine whether there is a correlation between the strength/power elite athletes' success and the increased incidence of R allele in the same population, as well as whether endurance performance sports are practiced by individuals with X allele of the same polymorphism (BAUMERT *et al.,* 2016; KIKUCHI and NAKAZATO, 2015). However, without genotype data for general population of a specific country or geographic region, it is impossible to assess enrichment in R/X alleles in professional athletes.

The goal of the present study is to investigate the frequency of RR, RX and XX genotypes in the general population of six Balkan countries (Albania, Bosnia and Herzegovina, Croatia, FYR Macedonia, Montenegro and Serbia) and to test the hypothesis that RR and RX genotypes are more prevalent, while X is a minor allele in general population as it arose from a non-sense mutation. In addition, the goal of the study was to check if geographically close populations can be differentiated on the basis of R/X alleles of *ACTN3* gene and if it is possible to cluster them based on the genetic distance measures. Finally, this study represents the first data on sports-related SNP variants of *ACTN3* gene in this part of Europe and the first report of

upcoming research efforts that intend to decipher the relationship between genetic variants and predisposition to success in sports in the Balkan populations.

MATERIALS AND METHODS

Study populations and sample collection

In order to investigate the distribution of *ACTN3* gene polymorphisms in seven populations from the Balkan Peninsula, a total of 483 samples from unrelated individuals were collected as follows: 64 samples from Albania (45 males, 19 females), 96 samples from Bosnia and Herzegovina (60 males, 36 females), 99 samples from Croatia (39 males, 60 females), 33 samples from Albanians inhabiting FYR Macedonia (22 males, 11 females), 50 samples from Macedonian population living in FYR Macedonia (25 males, 25 females), 60 samples from Montenegro (31 males, 29 females), and 81 samples from Serbia (59 males, 22 females).

The samples were collected using oral buccal swabbing method. Prior to sample collection, all study participants signed an informed consent. The approval for conducting the study was obtained from the Ethical Committee of International Burch University, Sarajevo, Bosnia and Herzegovina prior to sample collection process.

DNA isolation

Buccal swab DNA samples were stored in paper envelopes at 4°C until further analyzed. DNA isolation was performed by salting-out method (MILLER *et al.,* 1988) in the Research laboratory at the Genetics and Bioengineering Department, International Burch University.

PCR amplification

ACTN3 polymorphism rs1815739 was genotyped via PCR. The amplification of exon 16 of *ACTN3* gene was performed using primers proposed by MILLS *et al.* (2001) in a total reaction volume of 5 μl and using the same concentrations of chemical components as suggested by the authors of the original publication (MILLS *et al.,* 2001). The sequence of the forward primer was 5'-CTGTTGCCTGTGGTAAGTGGG-3', while the reverse primer was 5'-TGGTCACAGTATGCAGGAGGG-3'. The reaction was carried out under the following conditions: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 59.8°C for 30 s and extension at 72°C for 30 s. The final extension was performed at 72°C for 10 minutes.

RFLP analysis

Restriction fragment length polymorphism (RFLP) analysis of the resulting PCR products was performed using *DdeI* restriction enzyme and CutSmart Buffer (New England Biolabs, Ipswich, MA, USA) at 37°C for 45 minutes (MILLS *et al.,* 2001). RFLP result analysis was performed on 4% agarose gel that was run in two phases. In the first phase, the gel was run for 10 minutes at 30 V and 20 mA, while in the second phase, it was run for additional 90 minutes at 90 V and 60 mA. DNA fragments were stained using SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA) and the results were checked and photographed using Molecular Imager GelDoc XR System (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical and phylogenetic analyses

In order to test for the possible deviations from the Hardy-Weinberg equilibrium in the study populations, Pearson's chi-squared (χ^2) test with a significance level of $p=0.05$ has been used. The analysis has been performed using SPSS software package, version 20 (IBM Corporation, Armonk, NY, USA). Phylogenetic tree was constructed using POPTREE2 software (TAKEZAKI *et al.,* 2009). Heat map based on Pearson's correlation coefficient was created using Hierarchical Clustering Explorer (HCE) version 3.5 (SEO *et al.,* 2006).

RESULTS AND DISCUSSION

In the present study, 483 individuals (281 males and 202 females) from six countries of the Balkans (Albania, Bosnia and Herzegovina, Croatia, FYR Macedonia, Montenegro and Serbia) were genotyped for the single nucleotide polymorphism rs1815739 on *ACTN3* gene. A tested individual had one of three possible genotypes: RR, RX or XX.

The results of the study in terms of the abundance of obtained genotypes as well as compliance with Hardy-Weinberg equilibrium are given in Table 1.

Table 1. The results of the current study presenting the frequency of ACTN3 rs1815739 polymorphism genotypes in seven populations from six countries, along with gender distribution of the detected genotypes.

	Albania	B&H	Croatia	FYR Macedonia (Albanian)	FYR Macedonia (Macedonian)	Montenegro	Serbia	Total
N	64	96	99	33	50	60	81	483
RR, N (%)	29 (45.3)	44 (45.8)	39(39.4)	14(45.5)	10(38)	39(65)	26 (32.1)	214 (43.7)
RX, N (%)	27 (42.2)	35 (36.5)	47 (47.5)	14(45.5)	28(56)	17(28.3)	44 (54.3)	213 (44.1)
XX, N (%)	8 (12.5)	17 (17.7)	13 (13.1)	3(9.1)	3(6)	4(6.7)	11 (13.6)	59 (12.2)
HWE *p* value	**0.66**	**0.04**	**0.84**	**0.78**	**0.08**	**0.27**	**0.26**	**0.64**
Males	45	60	39	22	25	31	59	281
RR, N (%)	16 (35.6)	26 (43.3)	15 (38.5)	9(40.9)	13(52.0)	18(58.1)	18 (30.5)	115 (40.9)
RX, N (%)	22 (48.9)	21 (35.0)	17 (43.6)	10(45.5)	10(40.0)	10(32.3)	32 (54.2)	122 (43.4)
XX, N (%)	7 (15.6)	13 (21.7)	7 (17.9)	3(13.6)	2(8.0)	3(9.7)	9 (15.3)	44 (15.7)
Females	19	36	60	11	25	29	22	202
RR, N (%)	13 (68.4)	18 (50.0)	24 (40.0)	6(54.5)	6(24.0)	21(72.4)	8 (36.4)	96 (47.5)
RX, N (%)	5 (26.3)	14 (38.9)	30 (50.0)	5(45.5)	18(72.0)	7(24.1)	12 (54.5)	91 (45.0)
XX, N (%)	1 (5.3)	4 (11.1)	6 (10.0)	0 (0.0)	1 (4.0)	1 (3.4)	2 (9.1)	15 (7.4)

General results for seven populations show that only Bosnian-Herzegovinian population does not have polymorphism distribution in agreement with Hardy-Weinberg equilibrium with *p* value of 0.04, while the remaining six studied groups are in compliance with expectations. This observation is the consequence of the fact that genotype XX is by far the most abundant in B&H population, reaching the regional peak value of 17.7%. On the other hand, heterozygous genotype accounts for around one-third of the analyzed individuals from Bosnia and Herzegovina, thus making this population an exception to the Hardy-Weinberg equilibrium. When the results for all seven populations are analyzed, it is observable that XX genotype is highly underrepresented, accounting for only 12.2% of all tested individuals with relatively even distribution through populations which makes this genotype less abundant in the Balkans when compared to the global average of 16-18% (BROOS *et al.,* 2016; FRIEDLANDER *et al.,* 2013) and European average of 18% (KIKUCHI and NAKAZATO, 2015). In general, RR and RX genotypes seem to have almost the same distribution, accounting for 43.7% and 44.1%, respectively. However, the frequencies of these two genotypes differ in individual populations, as in Bosnian-Herzegovinian and Montenegrin populations homozygous RR genotype is more prevalent, while heterozygous genotype is more prevalent in Croatian, Macedonian and Serbian populations. Finally, Albanian populations from Albania and FYR Macedonia both have relatively equal distribution of RR and RX variants.

When separate results for male and female participants are analyzed, a certain level of gender influence is observable across the populations. Generally, XX genotype is twice as prevalent in males as in females (15.7% vs. 7.4%). Also, RR genotype is the most abundant in females, while heterozygous genotype is prevalent in males, when joint results for all tested individuals are considered. When observed separately, gender seems to have the strongest influence on the overall results in Albanian populations from Albania and FYR Macedonia, as XX genotype is present in one female in the population from Albania, while it was not detected in Albanian females from FYR Macedonia (Table 1).

In order to compare the results among study populations, a neighbor-joining phylogenetic tree and a heat map have been constructed (Figures 1 and 2). As expected, the phylogenetic tree based on genetic distance is clustering study populations according to the ratio of RR to RX genotypes, as XX genotype has similarly low abundance in all seven of them. In that manner, Serbian, Croatian and Macedonian (from FYR Macedonia) populations cluster together in one subclade of the tree, while the remaining four populations appear to be more distant (Figure 1). These results are partially reproduced in a heat map created using Pearson's correlation coefficient, in which the observed clustering is following the one observed in the tree with an exception of Bosnian-Herzegovinian and Albanian population from FYR Macedonia (Figure 2). The difference is justified by the low genetic distance between the populations and the fact that algorithmic approach might influence differential clustering of closely related populations. In addition, the probability of higher impact of XX genotype frequency in the latter method has caused minor changes in the heat map appearance when compared to the N-J tree. Genetically close clustering of the study populations has been expected, mainly due to geographical proximity and shared history of the studied groups. The region of the Balkan Peninsula has been inhabited since the Paleolithic period with the first known human civilization in this part of Europe being Illyrians. In the 6th and 7th century, the region has been conquered by the Roman Empire, which led to the immigration of the Latin-speaking population. The Ottoman Empire that was ruling and inhabiting the area for around 500 years has also left an important

genetic imprint on the modern Balkan populations. Other minor influences include Gothic and Slavic migration routes through this area. Apart from migrations, the genetic diversity of the Balkan populations has been affected by many geographic, ecological, religious and cultural factors (HADŽISELIMOVIĆ, 1984; MALCOLM, 1996; MARJANOVIĆ et al., 2008).

The comparison of worldwide populations on the basis of allele and genotype frequency of *ACTN3* polymorphism was not possible, as previous studies from different populations have been examining different study groups, that is, non-athletes, national athletes, elite athletes, as well as competitors in both strength/power and endurance disciplines, thus making comparison efforts meaningless.
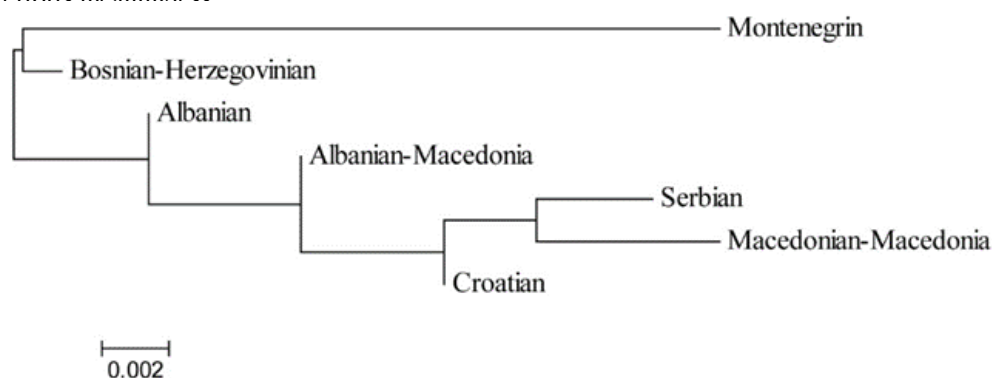

Figure 1. Rectangular N-J phylogenetic tree showing the similarities and differences in *ACTN3* polymorphism genotype distribution throughout seven populations.
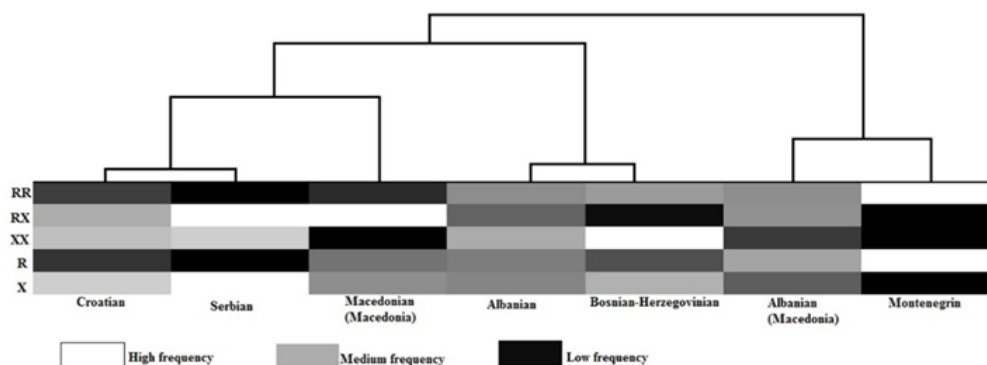

Figure 2. Pearson's correlation coefficient-based clustering of Balkan populations based on *ACTN3* rs1815739 polymorphism. RR genotype is present in a relatively low frequency in Croatian, Serbian and Macedonian populations and medium in B&H and two Albanian populations (from Albania and FYR Macedonia). On the other hand, RX genotype is observed in high frequency in Serbian and Macedonian, and in low frequency in B&H and Montenegrin populations.

Since a great majority of *ACTN3*-related studies is performed on elite sportspersons with the goal of determining the impact of SNP genotype on genetic predisposition for either strength/power or endurance sports, it is interesting to assess the results obtained in those studies. The studies have been done on different sport disciplines and on athletes of different ethnic backgrounds, making it possible to assess the importance of *ACTN3* genotypes on the performance of athletes of different backgrounds. While some studies yield expected results in terms of R allele positively correlating with an extraordinary success in strength/power disciplines and X with endurance disciplines (CHIU *et al.,* 2011; MASSIDDA *et al.,* 2009; PAPADIMITRIOU *et al.,* 2016; SHANG *et al.,* 2010), other studies are giving completely opposite results (for example, a study by MAGI *et al.,* 2016, in which Estonian cross-country skiers were enriched in R allele, although they are performing in an endurance discipline) or did not find any correlation at all (GINEVICIENE *et al.,* 2016; GREALY *et al.,* 2013; MANY *et al.,* 2016; MASSIDDA *et al.,* 2015; YVERT *et al.,* 2016). The present study is serving as groundwork with general population data for seven populations from the Balkan countries to which genotype frequencies from elite sportspersons can be compared in order to assess the importance of the study *ACTN3* polymorphism alleles in predisposition for advantageous sport performance.

CONCLUSIONS AND FUTURE PERSPECTIVES

In the present study, the frequency of RR, RX and XX genotypes of rs1815739 SNP of *ACTN3* gene has been assessed in six Balkan countries for the first time. According to the obtained results, XX genotype is underrepresented in all seven populations when compared to European average (12.2% vs. 18%, respectively). RR and RX genotypes have similar distributions through all study populations.

ACTN3 gene polymorphism is mainly a focus of recent research interest because of possible connection between its R allele and advantageous performance in strength/power sports and X allele with predisposition for endurance disciplines. However, in order to judge its importance in elite athletes, it is first necessary to obtain population data from randomly chosen non-athletes to be able to determine if there is an over- or underrepresentation of a certain allele or genotype. Even with general population data, study design is still of crucial value when examining the impact of *ACTN3* polymorphism. While population size is not of the highest importance, what is important to consider is gender, racial and ethnic background of the athletes in question, the exact discipline within either strength/power or endurance group and the performance level of athletes, that is, whether they are competing at national, international or elite level (such as world championships in their respective disciplines or the Olympic Games). It is expected that any of these variables can affect the results of an *ACTN3*-related study, as original research articles are sometimes giving opposing results on genotype effect on sport performance.

As for the Balkan populations, further research is to be undertaken on other genes that are expected to be influencing sport performance, most prominently *ACE*, as well as to collect data from national and elite athletes from different disciplines and compare them to the general population to assess whether *ACTN3* alleles and genotypes really are influential, as well as to what extent and in which manner in this part of Europe.

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RASPROSTRANJENOST *ACTN3* POLIMORFIZMA U POPULACIJAMA ŠEST ZEMALJA ZAPADNOG BALKANA

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Izvod

Cilj ove studije je istraživanje rasprostranjenosti genotipova RR, RX i XX rs1815739 SNP-a (single nucleotide polymorphism) gena *ACTN3* u populacijama šest zemalja Balkana. Trenutno se smatra da ovaj SNP daje genetičku predispoziciju za izvanredne sportske izvedbe. Alel R ovog SNP-a ima pozitivnu korelaciju sa boljom izvedbom u disciplinama koje zahtevaju snagu i/ili jačinu, dok alel X ima veću učestalost kod vrhunskih sportaša u disciplinama koje traže izdržljivost. Ukupno 483 osobe (281 muškarac i 202 žene), pripadnici sedam populacija iz šest zemalja, su testirani. Uzorci su prikupljeni koristeći ćelije bukalne sluznice, DNK je izolovana metodom isoljavanja, dok je genotipizacija vršena upotrebom PCR-a i RFLP metode. Dobijeni rezultati pokazuju da su genotipovi RR i RX (43,7%, odnosno 44,1%) mnogo zastupljeniji nego XX genotip (12,2%). XX genotip je u zemljama Zapadnog Balkana zastupljen ispod svetskog (16-18%) i evropskog (18%) proseka. Ova studija nudi prve populacijske podatke o zastupljenosti genotipova gena povezanog sa sportom u ovom delu Evrope i predstavlja početak istraživanja koje ima za cilj otkrivanje genetskih predispozicija vrhunskih sportaša iz Balkana koji se takmiče na međunarodnom nivou u različitim disciplinama.