ACTN3 and TNF gene polymorphism association with C-reactive protein, uric acid, lactate and physical characteristics in young African cricket players

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The genotyping of α-actinin-3 (ACTN3) and tumor necrosis factor-α (TNF) genes of 31 Zulu South African males aged 20 to 27 years (14 cricketers and 17 controls) was performed by PCR amplification followed by restriction digestion. After ANOVA the associations with C-reactive protein (CRP), uric acid (UA), lactate (LA) and physical characteristics were examined using Chi² maximum likelihood test and Fisher’s exact test. ACTN3 genotype frequencies for the cohort were 90.3% RR and 9.7% RX. The XX genotype was absent. R allele at high frequency of 100% was associated with CRP <3.0 mg/L in cricketers (p=0.0001) and controls (p=0.0140). High R allele frequencies of 80 to 100% were associated in cricketers with lower uric acid (p=0.0001), lactate (p=0.0003) and with body mass index (BMI) and Fat mass (FM) (p=0.0001). TNF genotyping displayed 42% GG, 45% GA and 13% AA for the cohort. CRP <3.0 mg/L was associated (p=0.0001) with A allele frequency (40% cricketers and 18% controls). Associations (p=0.0001) of A allele frequency (9 to 22%) with BMI, Lean body mass (LBM) and FM were found. The genotyping of cricketers was done for the first time. The study demonstrates a strong ACTN3 R allele association with lower CRP, UA and LA levels. The findings of XX null genotype and TNF 308A allele association with CRP inflammatory marker provide evidence about the genotype distribution of previously unexamined homogeneous cohort of African athletes.

Key words: Tumor necrosis factor-α (TNF) genotype, α-actinin-3 (ACTN3) genotype, polymorphism, C-reactive protein, physical characteristics, Zulu South African cricket players.

INTRODUCTION

The human genome is assumed to have been subject to strong selective pressures in different geographical areas during various periods of the history of the humankind. The impact of these selective conditions favoured the reproductive fitness and should be considered with regard to the spread of mutations that promoted the function of genes responsible for physical performance and efficient energy extraction and utilisation during migrations of the tribes (Bertelli et al., 2008). Humans have 23 chromosomes containing about 25000 genes. The recent update of the human performance and fitness gene map includes about 214 genes (Bray et al., 2008).

One of the genes that has been of most interest is α-actinin - 3 (ACTN3) gene located at the long arm of chromosome 11(11q13-q14), encoding the expression of α-actinin3 protein in the fast glycolytic (type IIB) fibres responsible for the generation of rapid forceful contractions. This protein plays a structural role as a component of the Z-line of the sarcomere anchoring together actin filaments, maintaining muscle mechanical integrity (MacArthur and North, 2007). The cytosine – thymine (C-T) nucleotide transition in codon 577 of exon 16 leads to a stop-codon (R577X) resulting in α-actinin –
3 protein deficiency in muscle fibres (Druzhevskaya et al., 2008). The ACTN3 gene is presented in three possible genotypes (RR, RX and XX).

Recent publications provided a substantial body of evidence regarding the association of ACTN3 with athletic performance and certain physical characteristics which could be relevant for talent selection, for profiling the abilities of athletes to respond to training and to improve trainability (Yang et al., 2003; MacArthur and North, 2007; Gomez-Gallego et al., 2008; Norman et al., 2009). Elite weightlifters and competitive bodybuilders have shown an overrepresentation of RR genotype compared to controls. Some studies failed to support the relationship between ACTN3 R577X polymorphism and elite strength athletes and the association of XX genotype with endurance performance (Yang et al., 2005; Schneider and Rupert, 2009). On the other hand, the association of ACTN3 R577X polymorphism with power performance in athletes was reported (Moran et al., 2007; Druzhevskaya et al., 2008; Andonov et al., 2008; Lucia et al., 2010). However, later findings suggested that heterozygotes with RX and XX genotypes are not disadvantaged in elite sprint/power performance (Yang et al., 2007). Cohorts of sprint athletes of various origins have shown a very low frequency of XX genotype (Scott et al., 2010). The frequency of X allele was significantly lower in endurance-oriented athletes compared with the controls and none of the highly elite athletes had XX genotype (Ahmetov et al., 2010).

It was suggested that populations undergoing changes in climate and diet due to migrations may produce more free radicals (Bertelli et al., 2008) resulting in strong physical exertion-induced oxidative stress which in turn may affect signaling pathways in the muscles. Research on the association of ACTN3 and biomarkers of oxidative stress in various sports has been scarce (Bray et al., 2008). Taking into consideration that ACTN3 protein might be related to muscle signaling and metabolism (MacArthur and North, 2007) and the role of some endogenous antioxidants such as uric acid and lactate in scavenging free radicals (Djarova et al., 2009, 2010) it would be noteworthy to explore the possible relationship.

Tumour necrosis factor-α (TNF) gene is located on the short arm of chromosome 6 (6p21.3) encoding the tumour necrosis factor-α (TNF) protein, which stimulates the production of interleukin (IL-6) thereby inducing the hepatic production of C-reactive protein (CRP). CRP is a sensitive biomarker of the inflammatory status of the individual and exercise-induced oxidative stress (Mattusch et al., 2000; Pitsavos et al., 2003; Lakka et al., 2005; Murakami et al., 2009; Djarova et al., 2009a, 2010).

The substitution of guanine (G) to adenine (A) at position 308 located at the promoter region stimulates the rate of transcription of TNF gene. The TNF gene has three genotypes (GG, GA and AA). The TNF gene G308A polymorphism could affect downstream signaling of TNF-α protein and thus circulating CRP levels. To the best of our knowledge, only one paper establishing an association of TNF G308A polymorphism with CRP levels in response to exercise-training programme, has been published (Lakka et al., 2006), reporting that the AA genotype is associated with increased plasma CRP levels and body mass index (BMI) in African American men and women.

The aim of this study was to investigate ACTN3 R577X and TNF G308A polymorphism and the association with oxidative biomarkers – C-reactive protein (CRP), uric acid (UA), lactate (LA), and body mass index (BMI), lean body mass (LBM) and fat mass (FM) in African cricket players of Zulu origin.

MATERIALS AND METHODS

Experimental subjects

The cohort consisted of 31 Zulu South African males (14 cricketers age 22.85±0.65) from the University of Zululand cricket team and 17 students (age 22.64±0.66) as controls. All participants were volunteers and a written consent was obtained prior to the study. Experimental protocols were approved by the Ethic Committee of the Research Board of University of Zululand. Cricket players participated in regular 2 h training sessions 5 to 6 times weekly and played inter-universities matches over the weekend once or twice monthly. The students of the control group reported leisure physical activities such as playing volleyball, basketball or soccer for short periods not more than once or twice weekly.

Measurements of body mass index (BMI), fat percentage (Fat %), lean body mass (LBM) and fat mass (FM) were taken according to the procedures of the American College of Sports Medicine (Thompson et al., 2000).

Blood samples were collected at rest from the antecubital vein into vacutainers and analysed in the accredited Lancet laboratory at Bay Hospital, Richards Bay for determination of C-reactive protein (CRP), uric acid (UA) and lactate (LA) using Dimension Xpanda (Siemens, Germany) according to the South African standards of good laboratory practice. All participants were advised not to change their dietary habits and to refrain from physical exercise 24 h before blood sampling and were questioned about current and previous transient infections.

Genotyping

Blood spots were collected on FTA® classic cards according to the manufacturer’s instructions (Whatman International, UK). Samples were prepared by punching 1.2 cm discs from the cards and washing with FTA® purification reagent and TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) according to the manufacturer’s instructions. PCR was then performed directly from the dried disc. The detection of R577X polymorphism in ACTN3 gene was performed by a modified method of Mills et al. (2001). The primer sequences were ACTN3 (forward): 5′-CGTCTCCTGCTGGTAAAGTTG-3′ and ACTN3 (reverse): 5′-TGGTGACATATGTCGAGAGG-3′. The TNF G308A polymorphism was detected by a modification of the method of Wilson et al. (1992) using the primers TNF A1 (forward): 5′-AGGCAATAGGTAGTTGGAGGCAT-3′ and TNF A2 (reverse): 5′-TCCTCCCTGCTGGCTGAT-3′. PCR reactions were performed using the SensiMix™ dT kit according to the manufacturer’s instructions (Quantace, UK). The final reaction mixtures contained 1× SensiMix (with a final Mg²⁺ concentration of 3 mM) and 200 nM of each of the appropriate primer pair. 20 μl of
Table 1. ACTN3 genotype and allele frequency (%) in cricket players and controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype frequency (%) and numbers in brackets</th>
<th>Allele frequency (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>RR</td>
<td>RX</td>
</tr>
<tr>
<td>Cricket players (n=14)</td>
<td>85.7 (12)</td>
<td>14.3 (2)</td>
</tr>
<tr>
<td>Controls (n=17)</td>
<td>94.1 (16)</td>
<td>5.9 (1)</td>
</tr>
<tr>
<td>Total (31)</td>
<td>90.3 (28)</td>
<td>9.7 (3)</td>
</tr>
</tbody>
</table>

Table 2. C-reactive protein (CRP), uric acid (UA) and lactate (LA) blood levels at rest in cricket players and control group (mean ± SEM) and association tests.

<table>
<thead>
<tr>
<th>Biomarkers (mg/L)</th>
<th>Cricket players</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein</td>
<td>1.81 ± 0.37&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.81 ± 0.51&lt;sup&gt;*,b&lt;/sup&gt;,&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.31 ± 0.008&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.29 ± 0.007&lt;sup&gt;*&lt;/sup&gt; NS</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.55 ± 0.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.95 ± 0.11** NS</td>
</tr>
</tbody>
</table>

Student's t-test: *p<0.05 control group vs cricket players, **p<0.001 control group vs cricket players. Association tests: <sup>a</sup>p=0.0001 CRP <3 mg/L in cricketers – ACTN3 R allele frequency (100%), X allele (null), <sup>b</sup>p=0.0140 CRP <3 mg/L in controls – ACTN3 R allele frequency (100%), X allele (null), <sup>c</sup>p=0.0001 UA <0.30 mmol/L in cricketers – ACTN3 R allele frequency (80%), X allele (20%), <sup>d</sup>p=0.0001 UA <1.55 mmol/L in cricketers – ACTN3 R allele frequency (88%), X allele (12%), <sup>e</sup>p=0.0001 CRP <3 mg/L in cricketers and controls – TNF A allele frequency (40%), G allele (60%) in cricketers and A allele (18%), G allele (20%) in controls. NS – no significant association tests.

RESULTS

ACTN3 genotype and allele frequencies amongst cricket players and control group are shown in Table 1. No significant differences between two groups were noted. For the whole cohort, the ACTN3 gene the frequency of RR genotype was 90.3% and RX genotype was 9.7%. The XX genotype was absent (Figure 1). In this study, the R allele at extremely high frequency (100%) was associated with CRP levels (< 3.0 mg/L) in cricketers (p=0.0001) and controls (p=0.0140). R allele frequencies of 80 and 88% were associated in cricketers with lower UA (p=0.0001) and LA levels (p=0.0003) respectively (Table 2). A strong association of R allele frequency (100%) was found with BMI and FM in cricket players (p=0.0001) and controls (p=0.0007) (Table 3).

TFN genotype and allele frequencies are presented in Table 4. TFN genotyping displayed 42% GG, 45% GA and 13% AA frequency for the whole cohort, but no differences between both groups were established. CRP skewed; hence these variables were transformed for the Analysis of Variance (Unbalanced design). For the association tests, CRP levels were categorised as <3 mg/L (low) and >3 mg/L (high) according to Pearson et al. (2003). Other variables were categorised according to their median (M). After ANOVA, the association was examined using Chi<sup>2</sup> maximum likelihood test and Fisher’s exact test.

the appropriate mix was added to a single dried disc in a thin-walled 200 µL PCR tubes. All amplifications were performed in a Rotor-Gene 6000 (Corbett Research, Australia) using the following conditions: Activation step 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 90°C for 20 s, 72°C for 20 s. Restriction digests were performed in a total volume of 5 µL: 2.5 µL PCR product, 0.125 µL enzyme (1.25 U), and 1.875 µL water. Digests were performed for 16 h at 37°C. 1 µL 6x loading buffer was added to each digest and the whole sample loaded to a gel. ACTN3 amplicons were digested with Ddel and resolved in 2.5% (w/v) agarose 1x TBE gels. TNFα amplicons were digested with NcoI and resolved in 3% (w/v) agarose 1x TBE gels.

Statistical analysis

The Student’s t-test was used to analyse the statistical difference in the blood biomarkers and physical characteristics between the cricket players and the control group. The results are presented as mean ± SEM. Statistical significance was accepted at p<0.05. Statistical analysis for the genotype associations was done using GenStat Discovery Edition 3. The distribution of some variables was skewed; hence these variables were transformed for the Analysis of Variance (Unbalanced design). For the association tests, CRP levels were categorised as <3 mg/L (low) and >3 mg/L (high) according to Pearson et al. (2003). Other variables were categorised according to their median (M). After ANOVA, the association was examined using Chi<sup>2</sup> maximum likelihood test and Fisher’s exact test.
Table 3. Physical characteristics of cricket players and control group (mean±SEM) and association tests.

<table>
<thead>
<tr>
<th>Physical characteristics</th>
<th>Cricket players</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>68.68 ± 2.54</td>
<td>61.00 ± 1.61 **</td>
</tr>
<tr>
<td>Stature (cm)</td>
<td>175.08 ± 1.25</td>
<td>170.58 ± 0.33 **</td>
</tr>
<tr>
<td>Body mass index (BMI) (kg/m²)</td>
<td>22.40 ± 0.81 a,b,c</td>
<td>20.79 ± 0.36 ** b,e</td>
</tr>
<tr>
<td>Lean body mass (LBM) (kg)</td>
<td>61.81 ± 2.01 d</td>
<td>55.41 ± 1.49 ** b,e</td>
</tr>
<tr>
<td>Fat mass (FM) (kg)</td>
<td>6.87 ± 0.54 a,b,c</td>
<td>5.59 ± 0.22 ** b,e</td>
</tr>
<tr>
<td>Fat %</td>
<td>9.84 ± 0.39</td>
<td>9.13 ± 0.32 *</td>
</tr>
</tbody>
</table>

Student’s t-test: *p< 0.05 control group vs cricket players; **p<0.001 control group vs cricket players. Association tests for BMI, LBM and FM: a p=0.0001 BMI below 22.4 kg/m² and FM below 6.9 kg – ACTN3 R allele frequency (88%), X allele (12%) in cricketers; b p=0.0007 BMI below 22.4 kg/m², FM below 6.9 kg and LBM below 55.4 kg – ACTN3 R allele frequency (100%), X allele (null) in controls; c p=0.0202 BMI below 22.4 kg/m² and FM below 6.9 kg and TNF A allele frequency (9%), G allele (78%) in cricketers; d p=0.0001 LBM below 55.4 kg – TNF A allele frequency (16%), G allele (74%) in controls.

Table 4. TNF genotype and allele frequency (%) in cricket players and controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype frequency (%) and numbers in brackets</th>
<th>Allele frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
<td>GA</td>
</tr>
<tr>
<td>Cricket players (n=14)</td>
<td>35.7 (5)</td>
<td>57.1 (8)</td>
</tr>
<tr>
<td>Controls (n=17)</td>
<td>47 (8)</td>
<td>35.3 (6)</td>
</tr>
<tr>
<td>Total (31)</td>
<td>41.9 (13)</td>
<td>45.2 (14)</td>
</tr>
</tbody>
</table>

(<3.0 mg/L) was associated (p=0.0001) with A allele frequency of 40% for the cricket players and 18% for the control group and (Table 2) Associations (p=0.0001) of A allele frequencies from 9 to 22% with lower BMI, LBM and FM were observed (Table 3).

The biomarkers blood levels and the physical characteristics measurements are shown in Tables 2 and 4. C-reactive protein was significantly higher (p<0.001) in control group (5.81 mg/L) than in cricketers (1.81 mg/L). It is worth mentioning that Lancet Laboratories, South Africa, have set up CRP normal reference range of 0 to 8 mg/L, where the reference range of Ciba Laboratories in European Union are 0 to 5 mg/L. We found CRP levels of 6.0 mg/L only in one cricket player. CRP levels above 8.0 mg/L were observed in four controls. In both cases other hematological parameters of subclinical/clinical inflammation such as leucocytes and full blood count (FBC) were not observed. Uric acid was found slightly higher (p<0.05) and lactate lower (p<0.001) in cricket players compared to controls (Table 2). BMI, LBM and FM were significantly higher in cricketers than in control group (Table 3).

DISCUSSION

The finding of complete absence of XX genotype in our study was unexpected. XX null homozygosis was found in Nigerian athletes (Yang et al., 2007). Very low frequencies of XX genotype (1 to 2%) were observed in Kenyan, Jamaican and American sprinters (Scott et al., 2010). Low frequencies (11%) were reported in Ethiopian athletes (Yang et al., 2005). European studies reported 6 to 14% XX allele genotype in Russians power athletes (Druzhevskaia et al., 2008), 19% in Swedish healthy volunteers (Norman et al., 2009) and 20% in Bulgarian athletes (Andonov et al., 2008). In Spanish athletes with European origin, Australian Caucasians and Asians (Japanese and Javanese) XX genotype frequencies from 18 to 25% were established (Yang et al., 2007). Recently published data about the possible relationship between XX genotype and endurance performance remains controversial (Lucia et al., 2010; Paparini et al., 2007; MacArthur and North, 2007).

The RR genotype of ACTN3 gene is considered an advantage for power/sprint activities in track and field athletes (Wessner et al., 2009). The frequency of RR genotype found in elite Greek track and field athletes was 47.97% suggesting strong association with power performance (Papadimitriou et al., 2008). Furthermore, male athletes with ACTN3 577RR genotype showed better results in long-distance rowing than carriers of RX or XX genotype (Ahmetov et al., 2010). In this study, the percent distribution of RR genotype (85.7%) in cricket players is much higher compared to 47.9% found in 60 top level professional soccer players (Santiago et al., 2008). It appears that young cricketers of Zulu origin tend to display power/sprint genotype. This genotype is a potential basis for cricket-specific power and sprint development and trainability that are required for the...
successful performance in this sport. Maximal rapid force production is a very important characteristic in fast bowling and batting. Even fielders stationed in deeper positions have to throw the ball with great power (Noakes and Durant, 2000). The ability of cricketers to cope with repeated muscle contractions may require substantial muscle strength to reduce the extent of muscle damage (Noakes and Durant, 2000).

Our data about the stature, BMI and LBM in cricket players are very similar to the ones observed by Christie and King (2008) in 25 male South African cricketers (age 19.0± 1.1 years) recruited from a provincial cricket academy and Rhodes University. The results of our study also support the findings of higher stature and LBM in 102 Indian cricketers (age 18.3 ± 2.2 years) recruited from district and state level cricket teams of Amrisar, Punjab (Koley and Yadev, 2009). The percentage of body fat in the cricket players of University of Zululand is lower compared to the above-mentioned studies. The difference may be due to the diet and effects of the training programme. For high level of sporting performance in cricket higher stature (177 ± 7.5 cm), BMI (23.3 ± 1.9 kg/m²) and body fat of 13% have been suggested as an "ideal" body composition (Christie and King, 2008). In general, it is considered that height and LBM help cricketers to generate more force in the game (Koley and Yadev, 2009). Our finding carry practical applications in linking extensively used sports anthropometry with genotyping and talent selection.

The association of high R allele frequency with lower CRP, UA and LA biomarkers in cricket players is an important outcome of the present study. UA and LA at rest are important parameters for assessing overtraining and fatigue (Djarova et al., 2009, 2010). It is known that excessive free radical production during strenuous exercise could affect gene expression and regulation and act as secondary messengers in intracellular signaling cascades (Valko et al., 2007), having an impact on the circulating CRP, UA and LA levels (Djarova et al., 2009a, 2010). In cricket repeated eccentric muscle contractions occur during fast bowling, but also in repeated decelerations that occur when turning during batting and fielding may contribute to increased free radical production and exercise-induced oxidative stress inducing muscle damage (Noakes and Durant, 2000). On the other hand, low and physiological levels of free radicals are required for normal force production and adaptations in skeletal muscles (Power and Jackson, 2008). Further studies on large cohorts to confirm the association of R allele with above-mentioned blood markers, including markers for exercise-induced muscle damage are needed.

We found significantly lower CRP levels in cricket players submitted to 5 to 6 training sessions weekly compared to controls. This outcome is in agreement with reduced levels of CRP observed by Lakka et al. (2005, 2006) in response to exercise programme. It also supports our previous results of the CRP-decreasing effect of strenuous training in swimmers and middle and long distance runners (Djarova et al., 2009, 2009a, 2010).

Changes in circulating CRP levels are not only markers of the immune status and subclinical inflammation of individuals, but also indicators of the activation of signaling pathways including expression of TNF gene and cytokines production (Petersen and Petersen, 2005). Additional chemical pathology tests should be recommended for participants with higher CRP levels for future studies.

Investigating the TNF G-308A polymorphism association with CRP levels, Lakka et al. (2006) established genotype frequencies of 73.7% GG and 22.8% GA in 232 American males. In our study, TNF genotyping has shown more balanced distribution of 41.9% GG and 45.2% GA frequencies. In Black Americans, 3.5% AA frequency was found to be lower compared to 12.9% AA frequency in Zulu South Africans. Our findings of 64.8% G allele and 35.5% A allele frequencies associated with CRP levels below and above 3 mg/L are closer to the results of 75.6% G allele and 24.4% A allele frequencies reported by Lakka et al. (2006). The discrepancy between the two studies could be explained by the disparity in the sampling size.

In genetic studies of athletes in various sports, the sampling size may fluctuate significantly from 28 to more than 200 participants (Yang et al., 2007; Bray et al., 2008, Santiago et al., 2008; Gomez-Galego et al., 2008). According to Collins (2009) detailed genotyping and phenotyping, properly applied may improve the power to detect biological effects in a small cohort relative to a large cohort that is studied in less details.

The limitation of present study is the small sample size, but the high homogeneity of the cohort could be considered an advantage. The genotyping of cricketers was done for the first time.

In conclusion, this study demonstrates a strong ACTN3 R allele association with low CRP, UA and LA levels. The findings of ACTN3 XX null genotype and the association of TNF G308A polymorphism with CRP inflammatory marker provide evidence about the genotype distribution of previously unexamined cohort of Zulu South African athletes.

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