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Nir Eynon[1], Alberto Jorge Alves[2], Yoav Meckel[1], Chen Yamin[1], Moshe Ayalon[3], Michael Sagiv[1], Moran Sagiv[1]

[1] Genetics and Molecular Biology Laboratory, Life Sciences Division, The Zinman College of Physical Education and Sport Sciences at the Wingate Institute, Israel.

[2] Research Centre in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto, Portugal.

[3] Biomechanics Laboratory, Life Sciences Division, The Zinman College of Physical Education and Sport Sciences at the Wingate Institute, Israel.

Corresponding Author:

Nir Eynon

Genetics and Molecular Biology Labratory, Life Sciences Division, The Zinman College of Physical Education and Sport Sciences at the Wingate Institute, Netanya 42902, Israel

Phones: Office + 972 9 8639368; Home + 972 4 6306334

Fax: + 972 9 8639365

E-Mail: eynon@wincol.ac.il

Running title: *HIF1A* Pro582Ser polymorphism and elite sprinters

Abstract

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that regulates gene expression in response to hypoxia, and has been associated with athletic performance. The aims of this study were 1) to determine the frequency distribution of *HIF1A* Pro582Ser (rs11549465) polymorphism among 155 Israeli athletes (sprinters and endurance athletes) and 240 healthy controls and 2) to analyze the influence of the interaction between *HIF1A* Pro582Ser and *ACTN3* R577X (rs1815739) genotypes on sprint performance. There were no differences across the *HIF1A* genotype and allele frequencies among endurance athletes, sprinters and controls. Similarly, no differences were found between the subgroups of top-level and national-level endurance athletes, or between top-level and national-level sprinters. Conversely, interaction effects were found between *HIF1A* Pro582Ser and *ACTN3* R577X polymorphisms and sprinters. The proportion of *HIF1A* Pro/Pro + *ACTN3* R/R genotypes was significantly higher in sprinters than in endurance athletes and healthy controls (P=0.002). In addition, the odds ratio for *HIF1A* Pro/Pro + *ACTN3* R/R genotype carriers being a sprinter was 2.25 (95% confidence interval 1.24-4.1), and for *HIF1A* Pro/Pro + *ACTN3* R/R genotype carriers being an endurance athlete was 0.5 (95% confidence interval 0.2-1.24). We conclude that *HIF1A* Pro582Ser polymorphism by itself is not critical in determining sprint performance. However, sprinter performance is determined by the interaction between the wild-type *HIF1A* Pro/Pro genotype and *ACTN3* RR genotype.

Introduction

Favorable genetic endowment together with environmental factors seem to be necessary for attaining the highest level of athletic performance. Although sprint performance is likely a polygenic trait, only a few single nucleotide polymorphisms (SNP's), namely ACTN3 R577X [1-4], ACE I/D [5-7] and more recently eNOS -786 T/C [8] and, IL-6 -174G/C ( ), were found to be associated with sprint performance.

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that is unique among mammalian transcription factors with respect to the demonstrated specificity and sensitivity of its induction by hypoxia [9]. HIF-1 is part of a family of heterodynamic basic helix-loop-helix (bHLH) proteins, which is composed of two subunits, HIF-1 α and HIF-1 β. Expression levels of the HIF-1α subunit is precisely regulated by cellular O_2 concentration such that levels of HIF-1α protein and HIF-1 DNA-binding activity increases exponentially as O_2 concentration decreases [10]. The α subunit of HIF1 also promotes cell survival and angiogenesis, and was suggested to influence glucose metabolism [11]. In connecting these data to muscle function, HIF-1 α mRNA and protein levels were found to be constitutively higher in type IIX muscle fibers, which have a high fatigability compared with the more oxidative type 1 muscle fibers, which have a low fatigability [12]

A functional SNP was identified in the *HIF1A* gene which encodes the α subunit of HIF1 protein, resulting in the replacement of proline (Pro) with serine (Ser) at amino-acid 582 [13]. The rare Ser582 allele, rather that the wild type Pro582 allele, was previously associated with increased transcription activity and stability of HIF1A protein. Ahmetov et al [14] have recently found that carriers of the Pro/Ser genotype had a significantly higher percentage of type IIX muscle fibers than homozygous for the Pro582 allele. Therefore, the Ser582 allele may increase the hypoxic resistance of cells, which would result in high glycolytic potentialities [15].

Since sprint performance requires a large amount of fast-twitch type IIX muscle fibers (Spencer MR & Gastin PB (2001). Energy system contribution during 200- to 1500-m running in highly trained athletes, it can be assumed that the proportion of the Ser582 allele will be higher among sprinters compared to endurance athletes and sedentary controls. Therefore, the purposes of the present study were 1) to compare the frequency distribution of the *HIF1A* Pro582Ser (rs11549465) polymorphism between athletes of sports with different demands (endurance vs. sprinters) as well as between athletes of competitive levels (elite-level vs. national-level), and 2) to test the influence of the interaction between

the *HIF1A* Pro582Ser and the *ACTN3* R577X (rs1815739) genotypes, since the *ACTN3* R577X polymorphism was previously reported to influence power-oriented top-level athletic performance in a broad variety of ethnic groups [1-4].

Methods

Subjects

The study followed recent recommendations for replicating genotype-phenotype association studies [16]. Owing to limitations, genotyping was not performed in two independent laboratories using different methodology. One hundred and fifty-five track and field athletes (119 men and 36 women, age=35.9$\pm$12.2 yrs) volunteered to participate in the study. Athletes were included in the study sample only if they had participated in national/international track and field championships. The control group consisted of 240 non-athletic healthy individuals who were randomly selected from the Israeli population. Controls were not engaged in physical activity on a regular basis. Athletes were divided into two groups: 1) An endurance-type group that included 74 long distance runners whose main events were the 10000m run and the marathon; 2) A sprint-type group that included 81 sprinters whose main event was the 100-200m dash. According to their individual best performances, athletes within each group were further divided into two subgroups: elite-level (those who had represented Israel in world track and field championships or in the Olympic Games) and national-level. All subjects, athletes and non-athletes, were Israeli Caucasians for $\geq$ 3 generations, with an equivalent ratio of mixed Jews coming from Arab countries (non-Ashkenazi) and Jews coming from Europe (Ashkenazi) (2:1). The study was approved by the Helsinki Committee, the formal ethics committee of the Hillel-Yaffe Medical Center, Hadera, Israel, according to the Declaration of Helsinki. A written informed consent was obtained from each participant.

Genotyping

Genomic DNA was extracted from peripheral EDTA treated anti-coagulated blood using a standard protocol. Genotyping of the *HIF1A* Pro582Ser (rs11549465) polymorphism was performed using polymerase chain reaction (PCR). The resulting PCR products were genotyped (in the Genetics and Molecular Biology Laboratory of the Zinman College of Physical Education and Sport Sciences at the Wingate Institute, Netanya, Israel) by restriction fragment length polymorphism (RFLP). Briefly, a 197-bp fragment of *HIF1A* Pro582Ser (C/T) was amplified by PCR using primers F 5'-GACTTTGAGTTTCACTTGTTT -3' and R 5'- ACTTGCGCTTTCAGGGCTTGCGGAACTGCTT -3'.

PCR was performed by first denaturation at 94°C for 5 min, 30 34 cycles of denaturation at 94°C for 30 60s, annealing at 62 55°C for 30 60 s, extension at 72°C for 3 1 min, and a final extension step of 7 10 min at 72°C. The amplified fragment subsequently underwent digestion by *Tsp451* (New England Biolabs, Beverly, MA, USA) in a condition recommended by the supplier. The digested products were then electrophoresed in a 3% agarose gel. This method yields a 197-bp fragment in the presence of the T (Ser) allele, and 154 and 43 bp in the presence of the C (Pro) allele.

The ACTN3 R577X polymorphism was genotyped according to a previously reported method [2]. To ensure proper internal control, for each genotype analysis we used positive and negative controls from different DNA aliquots that were previously genotyped by the same method, according to recent recommendations for replicating genotype-phenotype association studies [16]. The RFLP results were scored by two experienced and independent investigators who were blind to the subject data.

Data analysis

The SPSS statistical package, version 15.0, was used to perform all statistical evaluations (SPSS Inc., Chicago, IL, USA). Allele frequencies were determined by gene counting. A Pearson χ^2 test, Yates corrected χ^2 test, or Fischer exact test was used to confirm that the observed genotype frequencies were in Hardy-Weinberg equilibrium, and to compare the *HIF1A* Pro582Ser alleles and genotype frequencies between athletes and control subjects. One of these tests was also used to examine the interaction between the *HIF1A* Pro582Ser and the *ACTN3* R577X genotypes in relation to sprint performance, and in relation to the sprinter's level of performance. A logistic regression analysis was set in order to calculate the odds ratio for the interaction of both polymorphisms in sprint athletes and in control subjects. The level of significance was set at $P < 0.05$.

Results

The complete data on allele and genotype frequencies of the *HIF1A* Pro582Ser polymorphism are shown in Table 1. The genotype subtype did not differ by gender in the athletes group ($\chi^2 = 0.7$, d.f=2, P=0.71) or in the control group ($\chi^2 = 1.88$, d.f=2, P=0.39). Since the Israeli population includes Caucasians who are mixed non-Ashkenazi and Ashkenazi, we confirmed that there was an equivalent ratio of non-Ashkenazi and Ashkenazi descent in each group (2:1), and that there were no differences across *HIF1A* genotype between non-Ashkenazi and Ashkenazi descendants ($\chi^2 = 0.07$, d.f=2, P=0.9). *HIF1A* genotype distribution was in agreement with the Hardy-Weinberg equilibrium within the

endurance athletes (P=0.33), the sprinters (P=0.9), and the control group (P=0.64). Genotype distribution and allele frequencies were similar in the groups of endurance athletes, sprinters and controls (Table 1). Similarly, no statistical differences were found between the subgroups of top-level endurance athletes and national-level endurance athletes, or between top-level and national-level sprinters (Table 2). However, interaction effects were found between *HIF1A* Pro582Ser and *ACTN3* R577X polymorphisms and sprinters (see Table 3). *HIF1A* Pro/Pro + *ACTN3* R/R genotypes were more frequently found in the sprinters than in the control group (see Figure 1).

In the whole cohort of athletes, the odds ratio of *HIF1A* Pro/Pro + *ACTN3* R/R genotypes being a sprinter was 2.25 (95% confidence interval 1.24-4.1) and the *HIF1A* Pro/Pro + *ACTN3* R/R genotypes being an endurance athlete 0.5 (95% confidence interval 0.2-1.24).

Discussion

In the present study we investigated the association between *HIF1A* Pro582Ser polymorphism and elite athletic performance. The *HIF1A* gene was chosen as a genetic marker of athletic ability due to its proposed role in increasing transcription activity [13], promoting a shift of type I (oxidative) muscle fibers to type IIX (glycolitic) muscle fibers [14] and increasing hypoxic resistance of cells [15]. Our main findings were 1) genotype distribution and allele frequency within the *HIF1A* Pro582Ser polymorphism was similar in endurance athletes, sprinters and sedentary controls, and 2) *HIF1A* Pro/Pro + ACTN3 R/R genotypes were more frequently found in the sprinters than in the endurance athletes group and the control group. These findings suggest that the *HIF1A* Pro582Ser polymorphism by itself is not a factor in determining power/sprint performance. However, the wild-type *HIF1A* Pro/Pro genotype interacts with the *ACTN3* RR genotype, which was previously associated with power/sprint performance [1-4].

HIF-1 mediates increased glycolytic generation of ATP and possibly other intracellular metabolic adaptations to hypoxia [11]. Thus, one can expect that this protein will play a role in sprint events, since short distance sprint and power events rely largely on the anaerobic pathways, which are especially dependent on intramuscular stored CP, ATP and glycogen (Spencer & Gastin, 2001).

Reports regarding the interaction between the *HIF1A* Pro582Ser polymorphism and the *HIF1A* transcriptional activity are limited as well as inconsistent. The Ser582 allele was associated with higher transcription activity among head and neck squamous cell carcinoma (HNSCC) patients [15]. However,

another study suggested the opposite. An in vitro reporter gene transfection experiments confirmed that homozygous for the Ser582 allele have lower transcriptional activity than wild-type alleles at comparable expression levels [17]. Furthermore, the Ser582 allele by itself also had significantly decreased reporter gene activity at some of the concentrations, although results were less consistent than with the double mutant [17]. The cross-sectional comparison in this study revealed that a higher proportion of people showing a specific "preferred genotype" (e.g. *HIF1A* Pro/Pro + *ACTN3* R/R) were more likely to be sprinters, and thus it can be assumed that the power/sprint athletes will have higher transcription activity of the *HIF1A* gene which results in high glycolytic potentialities.

The results of the present study are not in agreement with the results of Ahmetov et al. [14], who suggested that the incidence of *HIF1A* Ser582 allele was significantly higher in weight-lifters than in controls. However, almost 29% of the sprinters in the present study harbored the Pro/Pro+ R/R genotype as opposed to only 8% and 15% in the endurance athlete group and the control group, respectively. This emphasizes the important role of the functional *ACTN3* R/X polymorphism in power/sprint performance, since it is well established that actinin-3 in these athletes is presented in higher amounts in the RR genotype than in other genotypes [4], and may be necessary for developing forceful contractions at high velocity [18].

The results of the present study also emphasize the assumption that many other yet-to-be-identified polymorphisms, which may not influence sports performance individually per se, could play an important role when combined with other variants. Furthermore, beyond genotype-phenotype associations, the effect of short, non-coding RNA molecules, namely MicroRNAs (miRNAs), on human muscle phenotypes remains to be determined. It is now believed that MicroRNAs regulate skeletal muscle post-transcriptional gene expression, and thus modulate important aspects of muscle function, including muscle contractility [19].

Our study was not without limitations. The group of elite athletes was relatively small, owing to the small number of available athletes. Nevertheless, it consisted of highly-selected endurance and sprint athletes having a unique phenotype. Also, genetic association studies must always be interpreted with caution. As with any statistical analysis, there is a non-trivial possibility of a false positive result [20].

In conclusion, the *HIF1A* Pro582Ser polymorphism is not associated with sprint performance. However, the *HIF1A* Pro/Pro + ACTN3 R/R genotypes were more frequently found in the sprinters than in the endurance athletes group and the control group. Further investigations are needed to clarify the possible

role of other polymorphisms and the combination of polymorphisms in determining athletic performance.

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Tables:

Table 1. Genotype and allele frequencies of *HIF1A* Pro582Ser polymorphism in all groups

		Genotype			**Allele frequencies**	
Athlete groups	n	Pro/Pro	Pro/Ser	Ser/Ser	Allele Pro	Allele Ser
Endurance	74	53 (72)	21(28)	0 (0)	127 (0.86)	21 (0.14)
Sprinters	81	59 (73)	20 (25)	2 (2)	138 (0.85)	24 (0.15)
Control	240	173 (72)	63 (26)	4 (2)	409 (0.85)	71 (0.15)

Data is absolute and relative values (within parentheses)

χ^2 = 1.85, d.f=2, P=0.76 for overall differences in genotype frequencies

χ^2 = 0.04, d.f=1, P=0.98 for overall differences in allele frequency

Table 2. The *HIF1A* Pro582Ser genotype and allele frequencies in sprinters and endurance athletes according to their level of competition.

			Genotype			**Allele frequencies**	
Athlete groups	Competitive level	n	Pro/Pro	Pro/Ser	Ser/Ser	Allele Pro	Allele Ser
Endurance	Top-level	20	16 (80)	4 (20)	0 (0)	36 (0.9)	4 (0.1)
	National level	54	37 (69)	17 (31)	0 (0)	91(0.84)	17 (0.16)
Sprinters	Top-level	26	20 (77)	6 (23)	0 (0)	46 (0.88)	6 (0.12)
	National level	55	39 (71)	14 (25)	2 (4)	92 (0.84)	18 (0.16)

Data is presented as absolute and relative values (within parentheses)

χ^2 = 1.07, d.f=2, P=0.58 for genotype frequencies in top-level vs. national level sprinters

χ^2 = 0.65, d.f=1, P=0.42 for allele frequency in top-level vs. national level sprinters

χ^2 = 0.95, d.f=2, P=0.33 for genotype frequencies in top-level vs. national level endurance athletes

χ^2 = 0.79, d.f=1, P=0.37 for allele frequency in top-level vs. national level endurance athletes

Table 3. Combined *ACTN3* R577X and *HIF1A* Pro582Ser polymorphisms genotype frequencies within the endurance athletes, the sprinters, and the control group.

ACTN3 Genotype	**HIF1A genotype**	**Endurance athletes (n=74)**	**Sprinters (n=81)**	**Controls (n=240)**
RR	Pro/Pro	6 (8.1)	23 (28.4)	36 (15)
RR	Pro/Ser	8 (10.8)	9 (11.1)	13 (5.4)
RX	Pro/Pro	30 (40.5)	23 (28.4)	104 (43.3)
RX	Pro/Ser	6 (8.1)	7 (8.6)	42 (17.5)
RX	Ser/Ser	0 (0)	2 (2.5)	3 (1.3)
XX	Pro/Pro	17 (23)	13 (16)	33 (13.8)
XX	Pro/Ser	7 (9.5)	4 (4.9)	8 (3.3)
XX	Ser/Ser	0 (0)	0 (0)	1 (4)

Data is presented as absolute and relative values (within parentheses)

χ^2 = 33.3, d.f=14, P=0.003 for overall combined genotype distribution

χ^2 = 12.4, d.f=2, P = 0.002 for RR+Pro/Pro

χ^2 = 2.3, d.f=2, P = 0.33 for RR+Pro/Ser

χ^2 = 3.4, d.f=2, P = 0.19 for RX+Pro/Pro

χ^2 = 4.9, d.f=2, P = 0.08 for RX+Pro/Ser

χ^2 = 2.5, d.f=2, P = 0.29 for RX+Ser/Ser

χ^2 = 2.6, d.f=2, P = 0.27 for XX+Pro/Pro

χ^2 =3.5, d.f=2, P = 0.17 for XX+Pro/Ser

χ^2 =3.5, d.f=2, P = 0.17 for XX+Pro/Ser

χ^2 =8.0, d.f=2, P = 0.02 for XX+Ser/Ser

Figure legends:

Figure 1. Genotype frequencies of the "optimal genotype" for power/sprint phenotypes within the sprinters, the endurance athletes, and the control group.

χ^2 = 12.5, d.f=2, P=0.002 for genotype frequencies differences in *ACTN3* RR+*HIF1A* Pro/Pro vs. other genotypes between endurance athletes, sprinters, and controls.

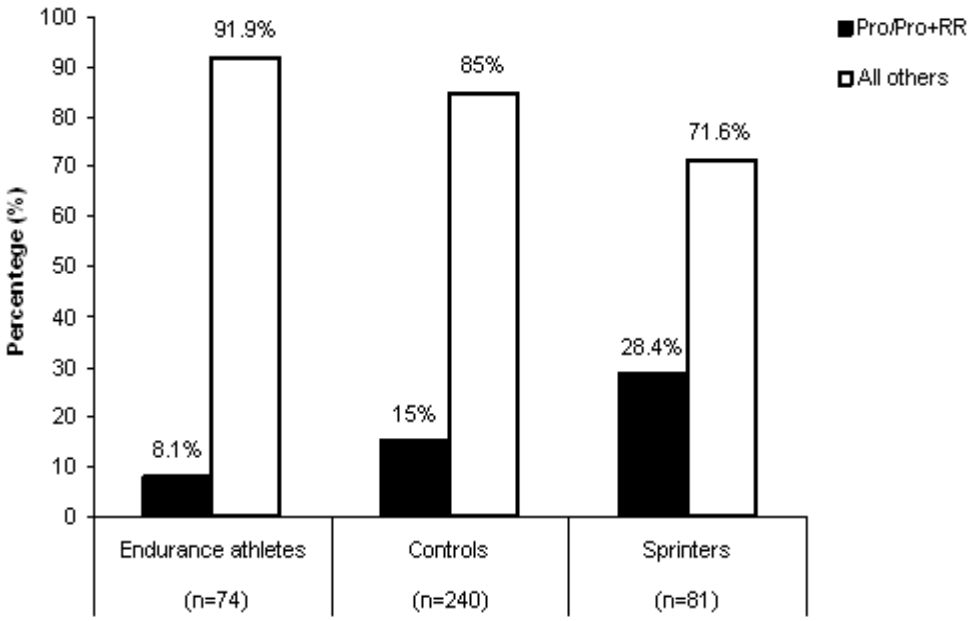

Figure 2. Genotype frequencies of the "optimal genotype" for power/sprint phenotypes stratified by gender within the sprinters group.

χ^2 = 0.18, d.f=1, P=0.78 for genotype frequencies differences in *ACTN3* RR+*HIF1A* Pro/Pro vs. other genotypes between male and female.