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SHORT COMMUNICATION

DETECTION AND ANALYSIS OF POLYMORPHISM IN THE PROMOTER REGION OF EQUINE *PPARCG1A* GENE

D. Polasik[1*], B. Rogers[2], A. Bobrowska-Chwat[1] and R. Pikuła[3]

[1]Department of Genetics and Animal Breeding, West Pomeranian University of Technology, Szczecin, Poland,
[2]Performance Genetics LLC, USA,
[3]Laboratory of Horse Breeding and Animal-Assisted Therapy, West Pomeranian University of Technology, Szczecin Poland
Corresponding author e-mail: daniel.polasik@zut.edu.pl

ABSTRACT

The protein encoded by *PPARCG1a* gene is a key regulator of genes involved in energy metabolism. Numerous studies indicate that *PPARGC1a* gene may be a good marker for athletic performance in horses. Therefore the aim of this study was to detect polymorphisms in the promoter region of *PPARGC1a* gene and to evaluate its presence in horses belonging to nine different breeds (n = 268) as well as in elite and non-elite Thoroughbred racehorses with known racing outcomes (n = 276). By sequencing regulatory region of equine *PPARGC1a* gene one novel SNP (g.100.784.525 C>G) was detected which changes transcription factors binding sites. To genotype polymorphism PCR-RFLP method was applied. Frequency of alleles and genotypes showed considerable differentiation in analyzed breeds. *GG* genotype was found only in Standardbred, Polish Heavy Draft horses and Purebred Arabian (0.04-0.13). The distribution of *PPARGC1A* genotypes was in Hardy-Weinberg equilibrium except Polish Heavy Draft horses (P 0.05). Moreover disproportionate presence of the *GC* genotype in elite Thoroughbred racehorses over the *CC* genotype in the non-elite population (OR=1.69) was noticed. Similarly *GC* genotype was overrepresented in distance runners in relation to sprinters analyzing all Thoroughbreds (OR=2.00) as well as only elite horses (OR=1.31). While there are multiple genes involved in athletic performance, given the association of *PPARGC1A* to mitochondrial biogenesis and conversion slow-twitch type I muscle fibers, this novel SNP may explain adaption in aerobic metabolism. The relationship between genotypes and gene expression should be performed next to evaluate its functional role.

Key words: *PPARCG1a* gene, SNP, Thoroughbred, horses.

INTRODUCTION

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1) is a protein encoded by the *PPARGC1a* gene, which is involved in mitochondrial biogenesis, fatty acid oxidation, glucose utilization, thermogenesis, angiogenesis and muscle fiber-type conversion toward slow-twitch type I fibers (Ahmetov and Rogozkin, 2009). Different variants of this gene were associated with milk-fat composition, carcass and growth traits in cattle (Schennink *et al*. 2009, Shin and Chung, 2013, Li *et al*. 2014), muscle fiber characteristics and meat quality in pigs (Kim *et al*. 2012) and chicken skeletal muscle fiber types (Shu *et al*. 2014). *PPARGC1a* gene polymorphism in human was investigated only in relation to physical performance (Maciejowska *et al*. 2012, He *et al*. 2015). The *PPARGC1a* gene was proposed as a candidate for physical performance in the horses by Schröder *et al*. (2011).

The Equine *PPARGC1A* gene is localized on chromosome 3. (ECA3) and consists of 13 exons. The transcript length is 2814 bp, however the protein is – 796 aa (http://www.ensembl.org). Investigations of oxidative gene expression in equine skeletal muscle showed significant (P<0.05) difference in *PPARGC1A* transcripts levels 4h after exercise. Moreover velocity at maximum heart rate (VHR-$_{max}$) and peak post-exercise plasma lactate concentration ($[LA]T_1$) were also associated with *PPARGC1A* expression (P<0.05) (Eivers *et al*. 2010). A further study showed mRNA expression changes in genes encoding transcriptional coactivators of PGC-1 and genes that function upstream and downstream of PGC-1 in known metabolic pathways such as angiogenesis, mitochondrial respiration, glucose transport, insulin signaling and transcriptional regulation (Eivers *et al*. 2012). Because of its proven role in the skeletal muscle response to exercise *PPARGC1A* gene should be considered as a good candidate gene responsible for athletic performance in equine breeds.

Therefore, the aim of this study was to detect polymorphism in the regulatory region of *PPARGC1a* gene which may affect its expression and to analyze it in different horse breeds as well as in Thoroughbred Racehorses with known racing outcomes and race distances.

MATERIALS AND METHODS

Blood samples were collected from 268 horses representing 9 breeds: Purebred Arabian (n = 16), Holstein Breed (n = 33), Małopolski Horse (n = 30), Wielkopolski Horse (n = 36), Standardbred (n = 50), Deutsche Reitpony (n = 20), Polish Heavy Draft (n = 33) Polish Konik (n = 33), Hutsul (n = 17). DNA was isolated by use MasterPure™ DNA Purification Kit for Blood Version II (Epicentre, USA).

Additional investigations were carried out on 276 Thoroughbred racehorses of known racing outcomes with 190 determined to be elite racehorses (average earnings per start US$ 212,154) and 86 determined to be non-elite (average earnings per start US$ 4,192). Additionally, the race distance of these horses was taken into consideration – sprinters (n = 123; average win distance 1277m) and distance runners (n = 153; average win distance 1737m). All horses started at least 5 times, had won a race and had no known performance related injury or disease. In case of Thoroughbred horses DNA was isolated from hair roots by using the QIAamp DNA Mini Kit (Qiagen, Netherlands) as per manufacturers instructions.

The following PCR primers were designed to span 5'upstream region (426 bp), exon 1 (168 bp) and part of intron 1 (118 bp) of equine *PPARGC1a* gene: *forward* 5'-AGCTGGAATCCACTTGGAGA-3', *reverse* 5'-GGGCTACTTTTCTCGCTCCT-3'. PCR reactions were performed in a final volume of 15µl containing 80-100ng of DNA, 2xPCR Master Mix (A&A Biotechnology, Poland), 10pmol of each primer and PCR grade water. The PCR cycling profile was as follows: 94°C for 5min, 32 cycles of 94°C for 45s, 55°C for 45s, 72°C for 45s and 72°C for 5min. Polymorphism detection in 9 studied breeds was performed by direct sequencing in both directions, using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA). Screening for the detected polymorphism was completed by PCR-RFLP method with primers described previously. Obtained amplicons were digested with restriction enzyme *Bsa*HI (GR CGYC) (Thermo Scientific, USA). Restriction fragments were separated in 2% agarose gels stained with Midori Green (Nippon Genetics, Japan).

For population statistics following parameters were calculated using Power Marker (ver. 3.25) software (Liu and Muse, 2005): genotypes and alleles frequency, expected heterozygosity (He) and Hardy-Weinberg equilibrium (^{2}). Transcription factors binding sites were analyzed by use TFSEARCH (ver. 1.3) software and TRANSFAC database (Heinemeyer *et al*. 1998).

RESULTS AND DISCUSSION

By sequencing the equine *PPARGC1A* gene fragment, one novel single nucleotide polymorphism (SNP) was found in position -219 bp counting from the ATG codon in exon 1 (g.100.784.525 C>G). Sequence polymorphism is submitted in Genbank with the accession number JX948085. Analysis of transcription factors binding sites showed that the *G* allele introduces site for v-ErbA and AML-1a transcription factors that are abolished by the *C* allele.

Detected SNP was genotyped in 9 equine breeds and Thoroughbred racehorses by PCR-RFLP based on the following restriction fragments lengths: *C* allele – 327, 202, 183 bp, *G* allele – 529, 183 bp. Genotypes and alleles frequency with other population statistics are given in Table 1.

Analysis of genotypes frequency showed that *CC* genotype appeared with highest frequency in all horse breeds (0.53-1.00) except Purebred Arabian (0.27). *GG* genotype was present only in Standardbred, Polish Heavy Draft and Purebred Arabian horses (0.04-0.13). The distribution of *PPARGC1A* genotypes deviated from Hardy-Weinberg equilibrium only in Polish Heavy Draft horses (P 0.05). Highest observed and expected heterozygosity was noticed for Purebred Arabian (0.60, 0.49 respectively) whereas lowest in Polish Konik (0.06, 0.06). In Hutsul horses only *CC* genotype was observed. Analysis of alleles frequency indicated that *C* allele occurred more often (0.57-1.00) in relation to *G* allele, which highest frequency was observed in Purebred Arabian (0.43).

Analysis of Thoroughbreds showed that *GC* genotype was overrepresented (0.23) in elite racehorses when compared to non-elite (0.15) (OR=1.69; p=0.129). In case of race distance in all Thoroughbreds statistically significant differences were found between distance runners and sprinters (OR=2.00; p=0.029). *GC* genotype appeared with higher frequency in distance runners (0.26) in relation to sprinters (0.15). When comparing elite sprinters against elite distance runners the similar tendency was observed (OR=1.31; p=0.522). Due to small number of none-elite distance horses these subgroups were not compared.

Many studies have focused on searching for polymorphisms in equine genes among different breeds and utility types; myostatin (*MSTN*), alpha amylases (*AMY1*, *AMY2*) and actin alpha 1 (*ACTA1*) genes may serve as good examples (Baron *et al*. 2012, Coizet *et al*. 2014, Polasik and Pikuła, 2014).

In our study we observed presence of *GG* genotype only in three breeds. While it is present in other closely related breeds to the Thoroughbred, in the Arabian and Standardbred, there was an absence of the *GG* genotype in the racehorses samples studied. This may be due to the low average competing distance range of

the horses sampled (1507m), so it is possible that the *GG* variant exists in outlier sub-populations of the Thoroughbred breed including but not limited to National Hunt performers who regularly run distances of 4000m and beyond which are similar distances to those that the Arabian breed compete at with the latter breed having the highest frequency of the GG genotype.

Presence of *GG* genotype in Polish Heavy Draft may be explained by high strength and endurance of this breed.

Previous studies in Thoroughbreds have found polymorphisms within exercise relevant genes associated with elite racetrack performance. Gu *et al.* (2010) studied polymorphism in candidate genes in relation to racing performance in Thoroughbred horses including the *PPARGC1A* gene. Analyzed horses in that study were divided into elite (n=150) and non elite (n=80) in a similar fashion to our study. An investigated polymorphism (A>G) was detected in noncoding region of *PPARGC1A* gene – intron 10 however statistical analysis showed that it was not associated with elite racing performance (OR = 0.884, p = 0.621) and therefore not considered for further analysis. In that study 3 SNPs in creatine kinase, muscle (*CKM*), pyruvate dehydrogenase lipoamide kinase isozyme 4 (*PDK4*) and cytochrome c oxidase, subunit 4, isoform 2 (*COX4I2*) genotype frequency distributions were significantly (P<0.05) different between elite and non-elite thoroughbred racehorses. A subsequent study by Pereira *et al.* (2015), in Quarter Horses, a breed noted for their sprinting speed and high percentage of type II muscle fiber, indicated that the SNPs in *PDK4* and *COX4I2* showed no significant associations with a Quarter Horse speed index. Pereira *et al.* suggested that the alleles of the *PDK4* and *COX4I2* genes are probably associated with beneficial adaptations in aerobic metabolism and therefore play secondary roles in sprint racing performance in Quarter Horses, which is mainly anaerobic.

Given the association of peroxisome proliferator-activated receptor gamma coactivator 1-alpha to mitochondrial biogenesis and conversion slow-twitch type I muscle fibers, it is reasonable to suggest that *GG* genotype, more readily associated with distance breeds in this study, may also explain adaptions in aerobic metabolism and similar to the other SNPs in the study by Pereira *et al* (2015) be not present in breeds generally associated with sprinting.

This novel polymorphism in the regulatory part of *PPARCG1a* gene is one of many polymorphisms found in equine genome. While it showed variability in different breeds and an association with elite racehorse performance, the relationship between genotypes and gene expression should be performed next to evaluate its functional role. Moreover investigations on a larger Thoroughbred racehorse population including those excelling over longer distance ranges could confirm obtained results and allow to application of *PPARCG1a* gene as a marker for racing performance.

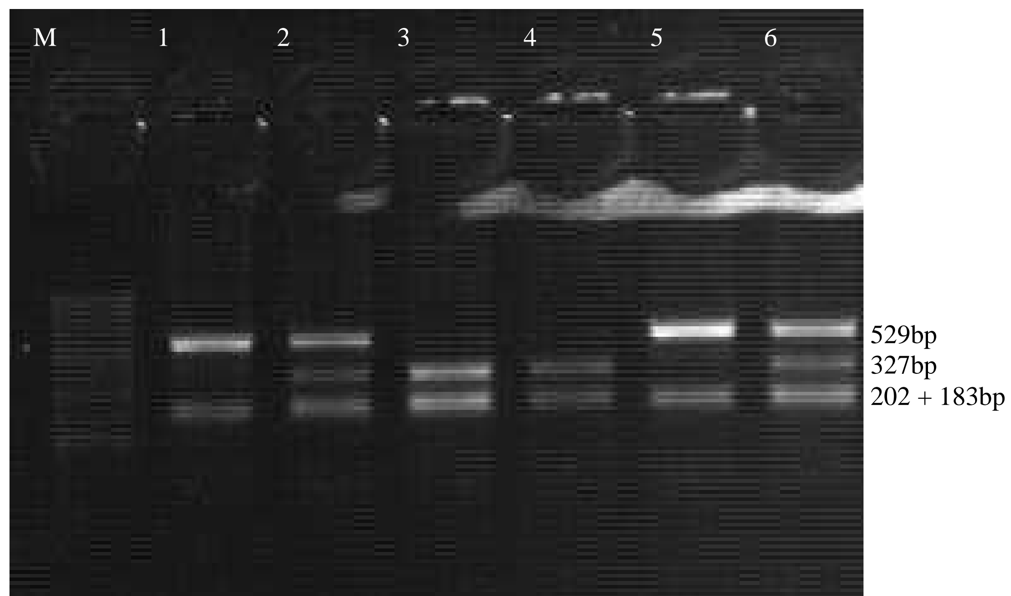

Figure 1. Restriction fragments obtained by digestion of the equine *PPARCG1a* gene amplicons by *Bsa*HI enzyme. Lane M – 100-1000bp ladder (A&A Biotechnology, Poland), lanes 2 & 6 – *GG* genotype, lanes 3 & 7 – *GC* genotype, lanes 4 & 5 – *CC* genotype.

Table 1. Population statistics in analyzed horses based on *PPARGC1A* gene polymorphism.

Breed	He	^{2}	p	Genotype frequency			Allele frequency	
				GG	*GC*	*CC*	*G*	*C*
Purebred Arabian	0.49	0.74	0.39	0.13	0.60	0.27	0.43	0.57
Holstein Breed	0.24	0.86	0.35	-	0.28	0.72	0.14	0.86
Malopolski Horse	0.26	0.98	0.32	-	0.31	0.69	0.16	0.84
Wielkopolski Horse	0.20	0.58	0.44	-	0.23	0.77	0.11	0.89
Standardbred	0.38	0.80	0.37	0.04	0.43	0.53	0.26	0.74
Deutsche Reitpony	0.14	0.14	0.70	-	0.16	0.84	0.14	0.86
Polish Cold-blooded	0.24	4.00	0.04	0.06	0.16	0.78	0.14	0.86
Polish Konik	0.06	0.03	0.85	-	0.06	0.94	0.03	0.97
Hutsul	-	-	-	-	-	1.00	-	1.00
Thoroughbred	0.19	3.66	0.06	-	0.21	0.79	0.10	0.90

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