Analysis of the Nebulin-Related Anchoring Protein Gene (N-RAP) SNP Polymorphism *(C/T)* in Slovak Warmblood Horse by PCR-RFLP Method

Anna Trakovická, Michal Gábor, Martina Miluchová, Tomáš Minarovič, Danka Štastná

Slovak University of Agriculture in Nitra, 949 76-Nitra, Tr. A Hlinka, 2, Slovakia

Abstract

The show-jumping is a important breeding goal in many breeds of warmblood horses. By using of genome-wide association studies (GWAS) were discovered a several genes which could be identified as candidate genes for physical performance in show jumping of horses. The one of this genes is the nebulin-related anchoring protein gene (NRAP) localized on ECA1. The aim of this work was genotyping the Slovak warmblood horse for SNP mutation (rs68545483) in NRAP gene and analysis of genotype structure in population of Slovak warmblood horses. The SNP polymorphism C/T of the N-RAP gene was studied in a group of 40 animals. Genomic DNA was isolated from samples of blood by using salting-out method. The SNP C/T was analyzed by PCR-RFLP method with restriction endonuclease *Bse*GI. The allele T was detected by two restriction fragments 93 bp and 40 bp and the allele C with 133 bp fragment. In the analyzed population of Slovak warmblood horses were detected the following frequency of alleles and genotypes for the SNP polymorphisms C/T of the N-RAP gene. Frequencies of allele T and allele C were 0.6125 and 0.3875 and frequencies of genotypes were 0.15 (genotype CC), 0.475 (genotype CT) and 0.375 (genotype TT).

Keywords: N-RAP gene, PCR-RFLP, show-jumping, Slovak warmblood horse

1. Introduction

Worldwide, horses play an important role within human cultures. Since domestication, the focus of breeding has been to improve the horses' usefulness to man [1]. The equine breeding selection has been developed by applying quantitative genetic methods for calculating the heritability of the complex traits such as performance in racing, show jumping or sport competitions [2]. Due to the long generation interval, genetic improvement in horses needs longer time-spans to be realised, so the application of genetic markers in selection schemes to improve physical performance appears highly desirable. The rapid development in the equine molecular genetics that has evolved around the second assembled genome sequence of the horse (EquCab2.0), the prospects of dissecting the genetic components of multigenic traits such as performance and conformation have increased dramatically [1]. With the rapid progress in equine genetics, new applications in early performance evaluation became available. Using a new SNP chip (Equine SNP50), which includes 54,602 SNP markers distributed among the whole equine genome, other genome-wide scan projects are ongoing to detect SNP markers of show jumping and endurance race ability [1,2]. The last update of the gene list validated by experimental studies revealed 214 autosomal genes and quantitative trait loci and 7 others on the X chromosome [3]. Schröder et al. [1] detected by using of genome-wide association studies (GWAS) a several functional candidate genes within the QTLs on

*Corresponding author: Trakovická, Tel: +421641-4285, Email: anna.trakovicka@uniag.sk

ECA 1, 8, 9 and 26 as well further candidate genes within the putative QTLs. The one of potentialy candidate genes from the putative QTLs for performance in show-jumping, which is an economical important breeding goal in warmblood horses is the nebulin-related anchoring protein gene (N-RAP) localized on ECA1. The N-RAP is a striated muscle-specific scaffolding protein involved in myofibril assembly [4-7]. The structural proteins found in mature myofibrils, some proteins exhibit a transient association with developing myofibrils. These include NRAP, which colocalizes with the earliest myofibril precursors as well as being transiently associated with newly formed mature myofibrils [8-10]. The N-RAP are hypothesized to promote key steps in myofibril assembly [6,9].

The aim of this study was to detect SNP *rs68545483* in population of Slovak warmblood horses, which are using for show-jumping.

2. Materials and methods

In this study were collected blood samples from 40 of Slovak warmblood horses. Genomic DNA was isolated by using commercial kit Nucleospin Blood (Macherey Nagel).

PCR: The PCR amplification of specific 133 bp fragment DNA included SNP *rs68545483* of the N-RAP gene which is characterize substitution of C/T. For PCR amplification were used specific primers designed by software BatchPrimer3 v1.0 [11]. The sequences of primers were: forward primer 5´CCTTTGACAGTTCGTACATCCAG - 3´ and reverse primer 5´ -ATGAAAGTCC CCCACTATCTTCTC - 3´. The reaction mixture in the total volume 25 µl containing 50 ng DNA, 1 U Taq polymerase (Fermentas), 1 x PCR buffer $(NH4)_2SO_4$, 3 mM $MgCl_2$, 200 µM dNTP, 5 pM of each primer. The PCR reaction was optimized in the gradient thermocycler C1000^{TM} (Biorad, USA). The following amplification parameters were applied: 95^{o}C for 3 minutes followed by 30 cycles: 95°C for 10 seconds, 60°C for 30 seconds, 72°C for 30 seconds. The reaction was completed by the final synthesis: 72°C for 5 minutes.

RFLP: The PCR products were digested by restriction endonuclease FastDigest *Bse*GI (Fermentas). The digestion was performed with 10 µl of PCR product mixed with 1 µl of the restriction enzyme, 2 µl of 10 x FastDigest buffer in total volume 20 µl. The run conditions were 5 minutes at 37^{o}C. Digested fragments were visualized by electrophoresis on 2.5 % agarose gel (Invitrogen) containing GelRed dye (Biotium) at 180 V in 1 x sodium borate buffer [12] for 15 minutes and the gel was analyzed by UV transilluminator and photographed with an documentation system Olympus C 7070.

3. Results and discussion

The digestion of 133 bp PCR product with restriction endonuclease *Fok*I (Fermentas) differentiated alleles C and T for SNP (rs68545483) of the N-RAP gene. The *Fok*I digestion of the PCR products produced one fragment for allele C (133 bp) and two fragments for allele T (40 bp, 93 bp). The PCR-RFLP method was used for genotyping of 40 horses of Slovak warmblood. In this group of animals were detected the all genotypes (CC, CT, TT) for single nucleotide polymorphism C/T (rs68545483) (Figure 1).

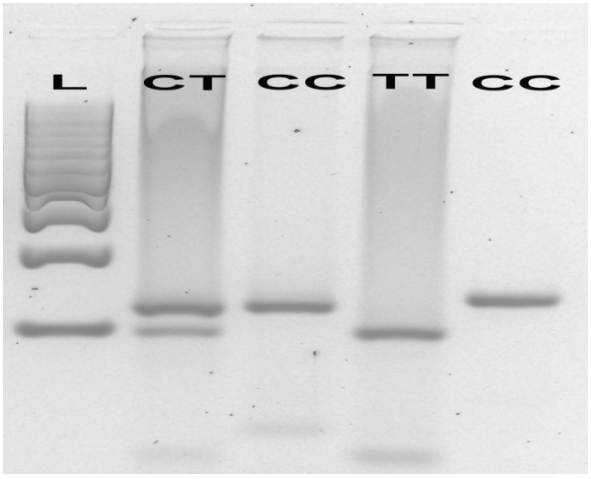

Figure 1. Representatively results of PCR-RFLP analysis by restriction enzyme *Bse*GI on 2.5% agarose gel
L–ladder 100 bp (Fermentas), CT-genotype (133 bp, 93 bp, 40 bp), CC–genotype (133 bp), TT–genotype (93 bp, 40 bp).

In population of Slovak warmblood horses we detected the presence of all genotypes as homozygote genotypes CC–6 horses, heterozygote genotypes CT–19 horses and homozygote genotypes TT–15 horses. The frequencies of genotypes and alleles are presented in Table 1.

Table 1. Genotype and allele frequencies for SNP rs68545483

BREED	n	GENOTYPES FREQUENCY			ALLELES FREQUENCY	
		CC	CT	TT	C	T
Slovak warmblood horse	40	0.15	0.475	0.375	0.3875	0.6125

The frequencies of minor allele C and major allele T for population of 40 Slovak warmblood horses were 0.3875 and 0.6125. Schröder et al. [1] detected in population of 115 Hanoverian warmblood stallions the same SNP (rs68545493) with frequence of the minor allele 0.29. Their study confirmed a highly additive effect with value $P<0.01$ and significant dominance effect of this SNP (rs68545493) for show-jumping in the investigated stallion population. The results of the genome-wide association (GWAS) in the study of Schröder et al. [1] suggest that genes involved in muscle structure, development and metabolism are crucial for elite show jumping performance. The importance of he N-RAP gene and its product a nebulin-related anchoring protein for myofibril assembly were decribed in the other studies [5-9]. Hill et al. [12] found a sequence polymorphism in the *MSTN* gene that is strongly associated with best race distance among elite racehorses. Schröder et al. [1] was not found the MSTN gene within a QTL or putative QTL for show-jumping in Hanoverian warmblood horses. The GWAS realized by Schröder et al. [1] detected a several QTL regions with the functional candidate genes and the others candidate genes localized in the putative QTL regions which can be used for selection in show-jumping. Our results of PCR-RFLP analysis the confirmed the presence of both allele C and T for SNP rs68545483, which is in neighboring with the N-RAP gene localize in the putative QTL region for show-jumping. The association study between genotypes CC, CT, TT (SNP rs68545483) and show-jumping performance will be the object of the study in near future.

4. Conclusions

It may be concluded that the population of Slovak warmblood horses is a polymorphic for SNP rs68545483 which is situated in region of the putative QTL for the show-jumping performance and is neighboring with the N-RAP gene. It was detected the homozygous genotype CC (0.15), the heterozygous genotype CT (0.475) and the homozygous genotype TT (0.375). The results proved the predominance of allele T (0.6125) before the allele C (0.3875) in population of Slovak warmblood horses.

Acknowledgements

This work has been supported by the Slovak Research and Development Agency under the contract No. LPP-0220-09.

References

1. Schröder, W., Klostermann, A., Stock, F.K., Distl, O., A genome wide association study for quantitative trait loci of show-jumping in Hanoverian warmblood horses, Animal Genetics, 2011. doi: 10.1111/j.1365-2052.2011.02265.x
2. Barrey, E., Review: Genetics and genomics in equine exercise physiology: an overview of the new applications of molecular biology as positive and negative markers of performance and health, Equine vet. J., 2010, doi: 10.1111/j.2042-3306.2010.00299.x
3. Bray, M.S., Hagberg, J.M., Pérusse, L., Rankinen, T., Roth, S.M., Wolfarth, B., Bouchard, C., The human gene map for performance and health-related fitness phenotypes: the 2006-2007 update. Med. Sci. Sports Exerc., 2009, 41, 35-73
4. Luo, G., Zhang, J.Q., Nguyen, T.P., Herrera, A.H., Paterson, B., Horowits, R., Complete cDNA sequence and tissue localization of N-RAP, a novel nebulin-related protein of striated muscle, Cell Motil.Cytoskel., 1997, 38, 75-90
5. Carroll, S.L., Herrera, A.H., Horowits, R.**,** Targeting and functional role of N-RAP, a nebulin-

related LIM protein, during myofibril assembly in cultured chick cardiomyocytes, J. Cell Sci., 2001, 114, 4229-4238.
6. Carroll, S., Lu, S., Herrera, A. H., Horowits, R., N-RAP scaffolds I-ZI assembly during myofibrillogenesis in cultured chick cardiomyocytes, J Cell Sci., 2004, 117, 105–114
7. Dhume, A., Lu, S., Horowits, R., Targeted disruption of N-RAP gene function by RNA interference: A role for N-RAP in myofibril organization, Cell Motil Cytoskeleton, 2006, 63, 493–511
8. Carroll, S.L., Horowits, R., Myofibrillogenesis and formation of cell contacts mediate the localization of N-RAP in cultured chick cardiomyocytes, Cell Motil Cytoskeleton, 2000, 47, 63–76
9. Lu, S., Carroll, S.L., Herrera A.H., Ozanne, B., Horowits, R., New NRAP-binding partners *a*-actinin, filamin and Krp1 detected by yeast two-hybrid screening: Implications for myofibril assembly, J Cell Sci., 2003, 116, 2169–2178
10. Lu, S., Borst, D.E., Horowits, R., N-RAP expression during mouse heart development. Dev Dyn, 2005, 233, 201–212.
11. Frank, M.Y., Naxin, H., Yong, Q.G., Ming-cheng, L., Yaqin, M., Dave, H., Gerard, R.L., Jan, D. and Olin D, A. BatchPrimer3: a high throughput web application for PCR and sequencing primer designi. BMC Bioinformatics, 2008, doi:10.1186/1471-2105-9-253
12. Hill, E.W., Gu, J., Eivers, S.S., Fonseca, R.G., McGivney, B.A., Govindarajan, P., Orr, N., Katz, L.M., MacHugh, D.A., Sequence polymorphism in MSTN predicts sprinting ability and racing stamina in Thoroughbred horses. PLoS ONE, 2010, 5, e8645.