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Research Article
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Identification of Horse *B3GNT5* gene expression and genetic variation

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Abstract

Horses are raised for various fields such as horse racing, and it was breed mainly for physical ability. Horses are suitable models for exercise research. In skeletal muscle of horses, many genes are expressed after exercise. Previous studies, we found differentially expressed genes (DEGs) related to exercise in horses. The transcription factor (TF) binding site of the 5'-regulatory region of the *B3GNT5* was predicted through PROMO. In addition, the genotypic frequency of nsSNP (rs69214296) and the Hardy-Weinberg equilibrium test were performed using 98 Thoroughbred race horses. We conducted a study to identification gene function using gene expression analysis and bioinformatic analysis for horse UDP-GlcNAc:BetaGal Beta-1,3-N-Acetylglucosaminyl transferase 5 (*B3GNT5*). The expression of *B3GNT5* was increased about 3.37 times after exercise compared with before exercise. Six transcription factors were predicted at the upstream 600 bp of the *B3GNT5* gene. In addition, the genotype frequencies of non-synonymous SNPs (nsSNPs) were analyzed in *B3GNT5* to confirm the significance between the predicted and observed genotypes. We identified the characteristics of *B3GNT5* gene in horses through this study. Further analysis is needed to investigate the impact of the *B3GNT5* SNP on racehorses.

Keywords: Horse, *B3GNT5*, Single-nucleotide Polymorphism, Genotype, Racing

Introduction

The diversity of modern horses is rooted in the process of domestication that began 5,000 to 6,000 years ago in the Eurasian steppe. Horses are economically important and popular animal in many industries include agriculture, transportation and recreation (Lippold et al. 2011, Ludwig et al. 2009) Horses are practical animals that breed for endurance, strength, speed and metabolic efficiency (Olsen 2006) and the horses are exceptional model for study of musculoskeletal, cardiovascular and respiratory systems (McCue et al. 2012). The Thoroughbreds is one of famous breeds to use in the horse racing industry.

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Exercise capacity in Thoroughbred horses have resulted to the emergence of horse industry with worth many billions of dollars (Gordon 2001). Thoroughbred have been selected by breeders to produce a racehorses with the outstanding exercise performance phenotype. Although, exercise and nutrition management have a significant impact on the development of the elite Thoroughbred racehorse, a significant portion of variation in athletic abilities are inherited (Gaffney and Cunningham 1988). Various gene expression occurs in horse skeletal muscle before and after exercise (Eivers et al. 2010). During the development of Thoroughbred, it was confirmed that a genomic region has been selected containing over-representation genes involved in insulin signaling, fatty acid metabolism and muscle strengthening (Gu et al. 2009).

Genome sequencing projects completed in many species have able to conduct genomic selection studies in several species including horses (Venter et al. 2001, Wade et al. 2009). Single nucleotide polymorphisms (SNPs) have been identified in the completed genome-sequenced species. Mostly, SNPs were identified by sequence changes between two alleles (Eck et al. 2009). SNPs are important variations induced phenotypes, traits, diseases (Shastry 2009). Also, SNPs located in the promoter of the economic traits have been related in a variety of morphological traits in horses (Dall'Olio et al. 2012, Dall'Olio et al. 2014).

From previous study, we conducted RNA-sequencing in skeletal muscle of six Thoroughbred horses before and after exercise. As a result, we obtained mount of differentially expressed genes (DEGs) by exercise. Among them, we found the expression of UDP-GlcNAc:BetaGal Beta-1,3-N-Acetylglucosaminyltransferase 5 (*B3GNT5*) was induced by the exercise. *B3GNT5* expressed in almost the whole tissues, and *B3GNT5* expression immunocytes shows high expression level (Kuan et al. 2010, Togayachi 2015).

In current study, we focused on the characterization of the *B3GNT5* expression and genetic variation in Thoroughbred. The expression of *B3GNT5* was confirmed in the muscles and leukocytes before and after exercise. Subsequently, the transcription factor (TF) binding sites within the regulatory region of *B3GNT5* were predicted. In addition, we analyzed genotype frequency to non-synonymous SNP (nsSNP) in horse *B3GNT5*.

Materials and Method

Experimental animals and sample

Blood samples were collected from 98 domestic thoroughbred racehorses that had run a race at the Seoul Lets Run Park. To extract genomic DNA, 900 μL red blood cell (RBC) lysis solution (Solgent, Daejeon Korea) was added to 300 μL of blood, processed for 3 minutes, and centrifuged at 15,000 rpm for 30 seconds. The supernatant was removed, and 300 μL of cell lysis solution (Solgent, Daejeon Korea) and 100 μL of protein precipitation solution (Solgent, Daejeon, Korea) were added, and the solution was processed and mixed thoroughly. The DNA solution layer was collected by centrifuging the solution at 15,000 rpm for 5 minutes, and the supernatant was added to 300 μL isopropanol (Duksan, Seoul, Korea) and shaken slowly. The resulting solution was centrifuged at 15,000 rpm for 10 minutes, and the supernatant was

removed. 500 μL of ethanol was added to the supernatant, and the solution was shaken until it became clear and then centrifuged at 15,000 rpm for 3 minutes. DNA was extracted by volatilizing and removing ethanol. (protocol number : PNU-2017-1553)

Polymerase chain reaction analysis

NCBI (http://www.ncbi.nlm.nih.gov) and Ensembl Genome Browser (www.ensembl.org) were utilized to retrieve gene sequence information. The primers used to detect SNPs were synthesized using PRIMER3 software (http://bioinfo.ut.ee/primer3-0.4.0/), and the synthesized primers included *B3GNT5* Primer F (5`- CGTGGAGAAGTGTCAAGCAC -3`) and R (5` - TGCAGTTCTCTGTCCTGTGG -3`). To determine the genotype of *B3GNT5* SNPs, PCR was conducted on the genomic DNA of racehorses using the following conditions: initial denaturation at 94 °C for 10 minutes; 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 30 seconds; and final extension at 72 °C for 10 minutes. PCR products were separated in a 1.5% SeaKem® LE agarose gel (Lonza, Rockland, ME, USA), detected under UV light, and subjected to Sanger sequencing for confirmation after cloning. Cloning of PCR products was carried out using a pGEM®-T Easy Cloning Vector System (Promega, Madison, WI, USA), and each gene sequence was confirmed through Sanger sequencing. SNPs were checked by comparing the gene sequence obtained from sequencing with those obtained from a BLAST search (National Center for Biotechnology Information, Bethesda, MD, USA).

Transcription factor binding site prediction

Transcription factor binding sites were predicted using the ALGGEN PROMO software program v8.3 (http://alggen.lsi.upc.es).

Results and Discussion

Expression of *B3GNT5* and *B3GNT5*-associated genes in horse

The equine *B3GNT5* gene sequence was obtained from whole genome sequencing and RNA-sequencing (Kim et al. 2013, Park et al. 2012). The gene consists of one exon with a total length of 1,128 bp. We found that *B3GNT5* expression increased 3.37-fold after exercise compared to before exercise that response to Thoroughbred muscle movement. (Figure 1). To account for this expression pattern, the transcription factor (TF) binding site was predicted up to 600 bp upstream using PROMO in the region defined by *B3GNT5* (data not shown). We identified five overlapping genes by comparing between predicted TF and differentially expressed genes. *ATF3, IRF1, JUNB* and XBP1 were upregulated and *DBP* was downregulated. (Figure 2 and Table 2).

X-box binding protein 1 (*XBP1*) is regulates the gene expression of the immune system and important to the plasma cell differentiation (Reimold et al. 2001). B cells that differentiate into plasma cells that secrete immunoglobulin are regulated by the transcription factor XBP1 (Shaffer et al. 2004). B cells affect a variety of immune functions such as the production of immunoglobulins and cytokines (Harris et al. 2000). The cytokines of inflammatory and anti-inflammatory B cells are associated with autoimmunity

regulation (Mizoguchi et al. 2002). The expression of JunB proto-oncogene (*JUNB*) show that increase after acute exercise in humans (Trenerry et al. 2011). JunB protein regulates the expression of cytokines

Table 1. List of predicted binding sequence locations and fold change values

Transcription factor	Binding sequence	Start position	End position	Average log2 ratio
ATF3	GCTCCGTCA	165	173	5.62
DBP	CTCTGCT	127	133	-1.71
	GTTTGCT	206	212	
	AGCAAGC	540	546	
	GGAATTA	193	199	
	TAATAGG	267	273	
	GGCATTA	425	431	
	TAATCAC	474	480	
	TGTATTA	486	492	
IRF-1	TTTCCCAAG	16	24	2.47
	AAGAGGAAA	31	39	
	TTTCCCTGT	88	96	
JUNB	CCGTCA	168	173	5.11
	TTGTCA	592	597	
XBP1	ATGCCT	123	128	2.1
	TTGCAT	274	279	
	CTGCAT	312	317	
	ATGAGG	316	321	
	ATGAAG	338	343	
	ATGCCG	345	350	
	CCTCAT	379	384	
	ATGGCA	423	428	
	TGGCAT	424	429	
	GGACAT	497	502	
	ATGATG	585	590	

DEGs were selected from RNA-sequencing of Thoroughbred horses before and after exercise.

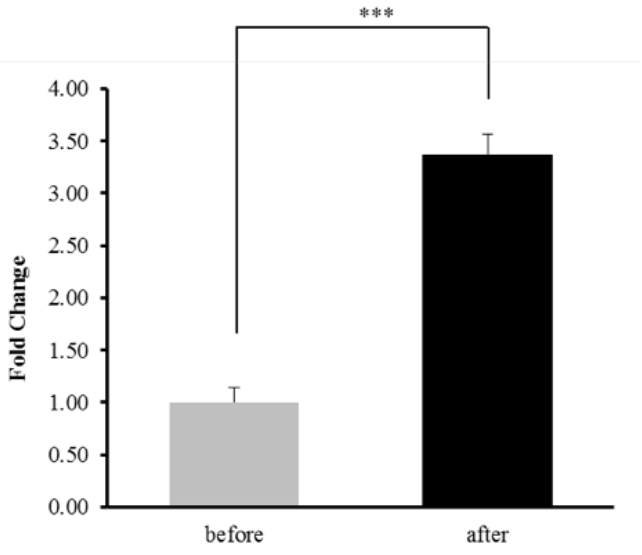

Figure 1. Gene expression of B3GNT5 before and exercise as decided via whole transcriptome analysis.
*** p<0.001

in Th2 cells (Gao et al. 2004). Th2 cells are related to humoral immunity and antibody production (Lakier Smith 2003). Activating transcription factor 3 (*ATF3*) is induced by a variety of signals, including those initiated by physiological stress and cytokines(Hai 2006). *ATF3* acts in a negative feedback system that is induced by many stimuli and limits excessive production of pro-inflammatory cytokines (Hai et al. 2010, Whitmore et al. 2007). Increased levels of Inflammatory and anti-inflammatory cytokines induced by Hard exercise (Pedersen 2000). Cytokines are nonspecific regulated proteins in inflammatory reactions, cell growth and differentiation (Oppenheim 2001). *B3GNT5* (beta-1,3-N-acetylglucosaminyltransferase family) is involved in lacto- or neolacto synthesis and carbohydrate structure biosynthesis of HNK-1 and Lewis X especially (Henion et al. 2001, Togayachi et al. 2001). Removal of *B3GNT5* in the lacto / neolacto series, which is responsible for Lc3 synthesis expression leads to multiple postnatal defects, including premature death, growth retardation, reproductive and B cell function defects (Biellmann et al. 2008, Kuan, Chang, Mansson, Li, Pegram, Fredman, McLendon and Bigner 2010). Thus, we supposed that *B3GNT5* could contribute as an important regulator of inflammatory responses which were caused by exercise.

Association analysis between candidate gene polymorphisms and racing traits of Thoroughbred horses

B3GNT5 nsSNP were retrieved from the Ensemble database. The nsSNP (rs69214296) exist the coding regions of *B3GNT5* in horse genome and locate in 1st exon (21,689,523th sequence of chromosome 19), where the base change from T to C cause the amino acid change from Tryptophan to Arginine on the 115th amino acid sequence (Figure 3A and B). We identified the frequency of each allele within the racehorse group of known nsSNPs using a blood sample from a race horses. When the genotyping for DNA samples of 98 Thoroughbreds was individually performed. As a result, we found TT, TC, and CC genotype were 4, 33, and 61 respectively and each allele frequency showed 20.92 % and 79.08 % for T

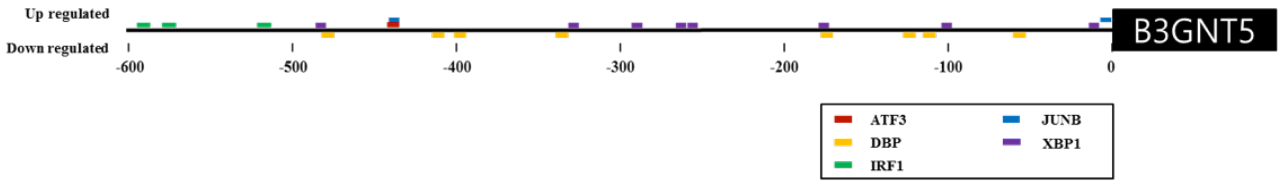

Figure 2. Transcription factor (TF) - binding site prediction in the region from B3GNT5 gene up to 600 bp upstream. The upper element represents the binding site of the upregulated transcription factor and the lower element represents the binding site of the regulatory element in Thoroughbred horses after exercise.

Table 2. Chi-squared and Hardy-Weinberg equilibrium test of SNP type distribution for B3GNT5 gene SNP (LOC21689523 T to C)

% (No.)	Overall	Genotype			Allele frequency		χ^2
		TT	TC	CC	T	C	
Observed	100 (98)	4.08 (4)	33.67 (33)	62.24 (61)	20.92	79.08	0.030985
Expected		4.29	32.42	61.29			

allele and C allele. The Hardy-Weinberg equilibrium analysis was performed to compare the genotyped allelic frequencies with the expected allelic frequencies. An assumption of this analysis method is that the alleles within each subject are statistically independent, at least when no association exists. This is equivalent, assuming that the frequencies of the genotypes in the general population comply with Hardy-Weinberg Equilibrium proportions. The result of χ^2 test indicates the significance (< 0.05) between expected values and observed values for the genotypes. As a result, the frequencies of all genotypes were in Hardy-Weinberg equilibrium (Table 2).

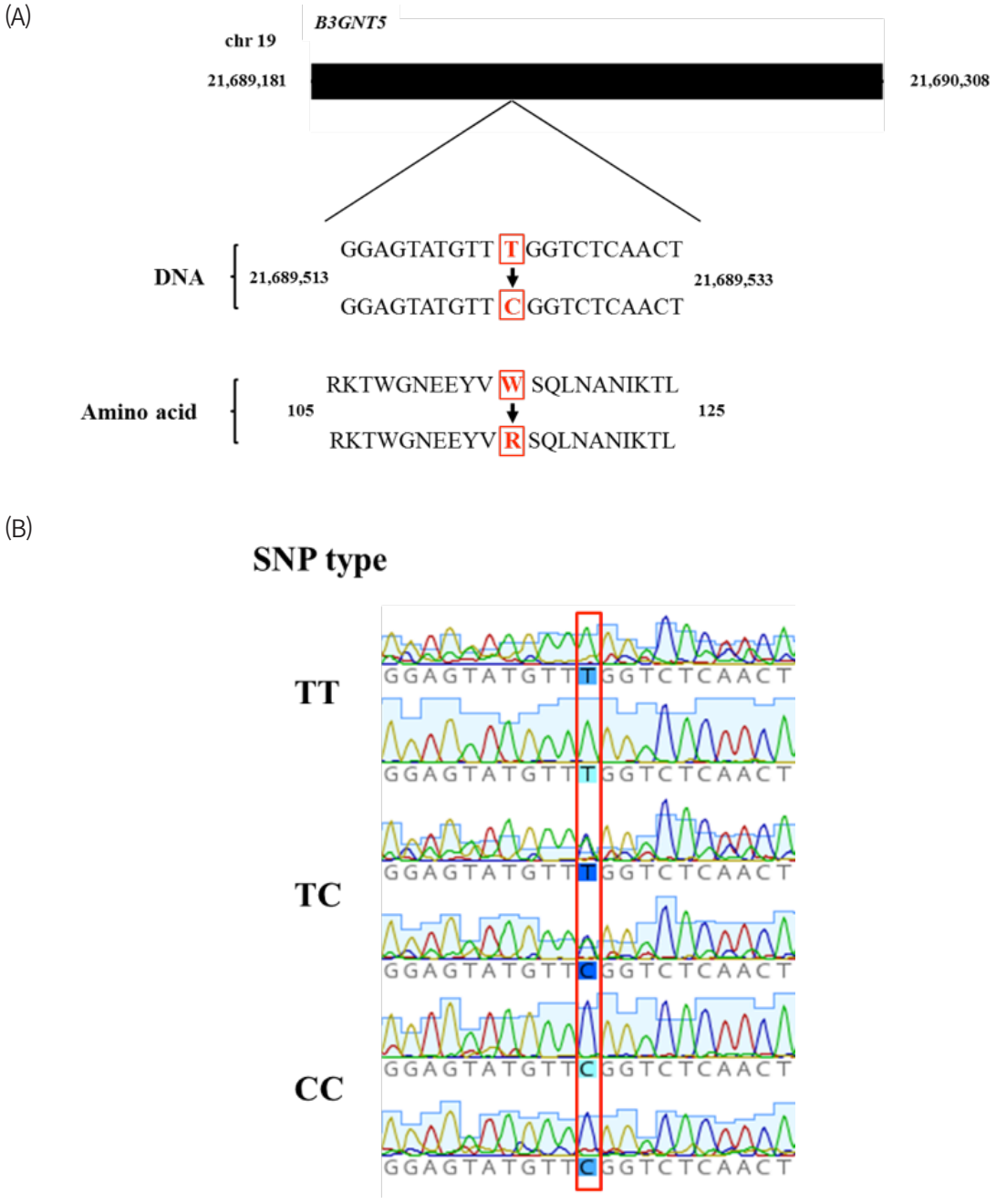

Figure 3. (A) Structure and location of the nsSNP of the B3GNT5 gene (LOC21689523 T to C). The filled box describes the B3GNT5 coding area. (B) Analysis of nsSNP in B3GNT5 gene.

Conclusively, we proposed the possibility to correlate between the induction of *B3GNT5* and the regulation of inflammatory responses by muscle damages in horse and subsequently nsSNP of horse *B3GNT5* and calculated the genotypes in Thoroughbred horse. This study could contribute to future research of the exercise ability of horse.

Conflict of Interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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