A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

웜블러드 말에서 운동으로 인한 말초혈액의 백혈구 내 microRNA와 mRNA의 발현 영향에 대한 통합분석

Integrated analysis of microRNA and mRNA expression in peripheral blood leukocytes from warm-blood horses with exercise

2015년 8월

서울대학교 대학원

수의학과 임상수의학 (수의임상병리학)전공

김항아

웜블러드 말에서 운동으로 인한 말초혈액의 백혈구 내 microRNA와 mRNA의 발현 영향에 대한 통합분석

지도교수 김 용 백

이 논문을 수의학석사학위논문으로 제출함

2015 년 6 월

서울대학교 대학원

수의학과 임상수의학 (수의임상병리학)전공

김 항 아

김항아의 석사학위논문을 인준함

2015년 6월

위 원 장 (인)

부위원장 (인)

위 원 (인)

Abstract

Integrated analysis of microRNA and mRNA expression in peripheral blood leukocytes from warm-blood horses with exercise

Hang-Ah Kim

Major in Veterinary Clinical Pathology
Graduate School of Veterinary Medicine
Seoul National University

Since capacity of exercise and performance is the most valuable aspect of horses, they have been selected for strength, speed, and

endurance traits. Athletic phenotypes are markedly influenced by environment, management, and training. However, it has long been accepted that there are underlying genetic factors.

Exercise induces molecular homeostasis and adaptation but the mechanism is not fully understood. Because microRNA(miRNA)s particularly play important roles in post-transcriptional gene expression control, this study was to identify regulatory networks of miRNAs and mRNAs to adjust molecular changes induced by exercise. Total RNAs were isolated from leukocytes of four warm-blood horses before and after the exercise. Expression level of miRNA was investigated by New Generation Sequencing (NGS) and expression level of mRNA was measured using microarrays to identify the difference between pre-exercise and post-exercise. In NGS results, 4 known miRNAs and 2 novel miRNAs were found. Target prediction on mRNA of the miRNAs was processed and then the results were matched to mRNAs on microarray. Distinct networks for 5 genes associated with 3 miRNAs of the 6 miRNAs were identified. This study revealed regulatory networks of miRNAs and mRNAs and a subset of mRNAs and miRNAs in equine peripheral blood leukocytes affected by

exercise, suggesting background information for understanding molecular homeostasis and adaptation from exercise-associated physiology in horses.

Key words: Horse / microRNA / mRNA / Exercise / Blood leukocytes

Student number: 2013-23447

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ACTN3	Actin binding protein alpha actinin 3
AMH	Anti-müllerian hormone
AMP	Adenosine monophosphate
AMPD1	Adenosine monophosphate deaminase 1
AP	Antigen presentation
BMPs	Bone morphogenetic proteins
cAMP	Cyclic Adenosine monophosphate
cRNA	Complementary RNA
DGE	Differential Gene Expression
DRD	Dopamine receptors
EDTA	Ethylenediaminetetraacetic acid
FDR	False Discovery Ratio
GDFs	Growth and differentiation factors
GO	Gene Ontology
GYS1	Skeletal muscle glycogen synthase
HIF1A	Hypoxia inducible factor 1 α
IGF1R	Insulin-like Growth Factor 1
KEGG	Kyoto Encyclopedia of Genes and Genomes
MM	Mutant homozygotes

MN	Heterozygotes
mRNA	Messenger RNA
miRNA	MicroRNA
ncRNA	Non-coding RNA
NGS	Next Generation Sequencing
NN	Normal homozygotes
nt	Nucleotide
PBMC	Peripheral blood mononuclear cells
PCA	Principal Component Analysis
PCP	Planar cell polarity
rRNA	Ribosomal RNA
sRNA	Small RNA
scRNA	Small cytoplasmic RNA
snRNA	Small nuclear RNA
snoRNA	Small nucleolar RNA
TGFB	Transforming growth factor beta
tRNA	Transfer RNA
VEGF	Vascular endothelial growth factor
5 HT	5-hydroxytryptamine
5HTT	Serotonin transporter

Introduction

Since wild horses are grazers and fugitives from predators on fields, horses have been naturally selected for speed. Horses were domesticated for working, riding, recreation, sport, and racing (Levine 1999). The development of specific breeds has resulted in selection for athletic phenotypes. Thoroughbreds were selected to thrive in speed for racing and warm-bloods used for dressage and show jumping have been selected based on good performance (Emmeline W. *et al.* 2013).

Exercise and performance are important factors of a horse because the value of a horse is determined by its abilities. Superior racing and performance abilities can be developed by training, environmental factors, and management. However it has long been accepted that capabilities are influenced by underlying genetic factors. Genetics has been a focus in horse industries. Many studies tried to identify the genetic contribution or heritability of various equine performance traits (Gaffney *et al.* 1988). Molecular homeostasis and adaptation, induced by exercise, influence transcriptional and translational regulation of genes' encoding proteins (Russell 2010).Recently, messenger RNA (mRNA) and small RNAs (sRNA) including micro RNA

(miRNA) were focused. mRNAs contain genetic information from DNA to the ribosome for making the protein products during transcription and translation. miRNAs, approximately 22 nucleotides (nt) long, are short non-coding RNAs (ncRNAs), which play important roles in regulating target gene expression by mRNA degradation and translation inhibition (Flynt *et al.*2008).

Since genome research tools are developed, a high-capacity system like microarray was shown to identify the expression of many genes in parallel. Many performance studies for horses used microarray technology (Cappelli *et al.* 2007; McGivney *et al.* 2009). Next generation sequencing (NGS) has emerged as a major tool to scrutinize sRNAs including miRNAs. Due to its ability to generate millions of reads with determined lengths, NGS greatly improves the capacity to detect a large number of novel miRNAs on a genomic scale (Creighton *et al.* 2010; Fehniger *et al.* 2010).

There are studies about the effect of brief exercise on miRNA and/or mRNA expression in human neutrophils, serum, and peripheral blood mononuclear cells (PBMCs)(Radom-Aizik *et al.* 2010; Radom-Aizik *et al.* 2013; Tonevitsky *et al.* 2013). To date, no studies have examined the molecular adaptation of exercise on miRNA-mRNA network in

leukocytes of horses. Consequently, the purpose of this study was to investigate the genomic response of leukocytes obtained from healthy warm-blood horses performing exercise for 1 hour to identify regulatory networks between miRNAs and mRNAs (Figure 1).

Materials and Methods

Blood samples collection

Gelding warm-blood horses were selected (Table 1). The horses' results on physical exams and clinicopathologic tests are within normal limit. The horses were subjected to the trotting with alternative cantering for 60 min. The blood was sampled twice from the jugular vein to anti-coagulated ethylenediaminetetraacetic acid (EDTA) tubes immediately before and after exercise, and subsequently leukocytes from buffy coat were isolated by centrifugation at 3,000 rpm for 10 minutes at 4℃.

Total RNAs isolation

Total RNAs were isolated from leukocytes using a phenol chloroform method with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNAs were quantified by

absorbance at 260 nm and the integrity of the RNAs were checked by Bioanalyzer2100 (Agilent technologies, Santa Clara, USA). Total RNAs with high quality were subjected to NGS and microarray analysis performed at Theragen Bio Institute (Suwon-city, South Korea) (Kim *et al.* 2014).

DNA library of small RNA and NGS analysis

sRNAs of 18 ~ 30 nt isolated from leukocytes of three warm-blood horses were obtained by electrophoresis and ligated with 5' adapters and 3' adapters. Reverse transcription from sRNA into cDNA was carried out using a reverse transcription kit (Takara, Kyoto, Japan). After making a cDNA library of sRNAs, the 1 μg cDNA was loaded on Flowcell and then NGS was performed using by Illumina Hiseq 2000 machine (Illumina Inc, San Diego, USA) following the Illumina's protocols.

The sequence results were subject to a data cleaning process, which gets rid of the low quality reads, such as reads with 5' primer contaminants and poly A, reads without 3' primer and insert tag, and reads shorter than 18 nt. Sequencing quality control and distribution were carried out. sRNA sequences of all samples were compared to

each other to know common and specific reads. Standard bioinformatics analyses were performed to annotate the resultant clean tags into different categories. The reads map to equine genome by SOAP or bowtie to analyze the expression and distribution. The tags were matched to miRBase database using blast or bowtie to identify known miRNAs. Identified miRNAs were analyzed to get base bias on the first position and base bias on each position of all miRNAs respectively. sRNA mapping identified repeat. Repeat associated tags were screened and removed.

The sRNA reads were annotated using the Genbank database (http://www.ncbi.nlm.nih.gov/) and the Rfam RNA database (http://www.sanger.ac.uk/software/Rfam) with blast or bowtie to get rid of ribosomal RNA (rRNA), small cytoplasmic RNA (scRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), and transfer RNA (tRNA). The reads were performed to map to antisense exon, antisense intron, exon, and intron and the repeat of the reads were removed. sRNA annotation was carried out to investigate distribution of sRNA among different categories.

The sRNAs that can be annotated to exon, intron, antisense exon, antisense intron or intergenic region of equine genome and not map to

any other RNA category were subjected to novel miRNA.

The novel miRNAs' sequences were matched to those of the miRNAs in other species. The novel miRNAs were predicted by prediction software, Mireap (http://sourceforge.net/projects/mireap/).

Differential expression analysis, target prediction, and analysis of miRNAs

Expression levels of miRNAs in pre-exercise were compared to expression levels of miRNAs post-exercise by plotting Log2-ratio figure and Scatter plot. The procedures are as below:

Normalization formula: Normalized expression = Actual miRNA count/Total count of clean reads*1000000

Fold-change formula: Fold change = log 2 (Post-exercise/Pre-exercise)

Target prediction was performed for known miRNA and novel miRNA by computational tools like targetscan (provided by GeneSpring) (http://www.targetscan.org/) and miRanda (http://www.microrna.org/).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway with

results of target prediction of identified miRNAs were identified using DAVID Bioinformatics tools (http://david.abcc.ncifcrf.gov/) based on low functional annotation clustering, false discovery rate (FDR) < 5, and p-value < 0.05.

Microarray analysis and data analysis

Cyanine 3-labeled complementary RNA (cRNA) was generated by Agilent's Low RNA Input Linear Amplification kit with 500 ng total RNA. The quality and quantity of the labeled cRNA was also measured using Nanodrop spectrophotometer. The labeled cRNA was subjected to a microarray (Agilent technologies) using Agilent's Gene Expression Hybridization Kit for 17 h at 65 ℃. Hybridized microarray was washed with Agilent's Gene Expression Wash Buffer Kit.

The microarray chip was scanned using Agilent's DNA microarray scanner and the raw signal density was acquired from Agilent feature Extraction software. Threshold raw signal values were 1.0 and all raw signal values were normalized using percentile shift (75^{th}percentile). The results were analyzed by GeneSpring GX12 software (Agilent Technologies) for gene expression analysis. The differential gene expression (DGE) was classified by Benjamini-Hochberg's FDR

method. The results of the DGE based on the criteria of Log (fold change) ≥ 1 or ≤ -1 were differentially identified across the pre/post exercise leukocytes. The final list from DGE results further analyzed using the functional annotation tools provided by DAVID (http://david.abcc.ncifcrf.gov/summary.jsp) based on the *p*-value < 0.05.

mRNAs on microarray matching to mRNAs targeted from miRNAs on NGS

The results of genes on microarray were matched with mRNAs targeted from the miRNAs on NGS.

Results

Total RNAs, cDNA library of sRNA and NGS analysis

The results of RNAs were good and enough to perform the next procedure. Information for RNAs is shown at Table 2.

High-throughput short read sequencing of sRNAs from three warm-blood horses' leukocytes was performed. A number of reads, with counts ranging from 2511025 to 24070381, were obtained from the

cDNA libraries of sRNAs. Clean reads, approximately more than 85% high quality were subjected to further analyses using bioinformatics tools. The resultant sRNAs ranged from 18 to 30 nt in length, with the majority having a length of 21-23 nt. Comparative results of each sRNA sequence were 10-20% common reads which comprised more than 90% of all reads. The sRNAs against the equine genome database were identified. The unique sRNAs mapping to horse genome without repeats comprised from 7.36% to 12.25% and major sRNAs are scRNAs. Total sRNAs with repeats accounted for 54.84-57.13% of all reads and the greater part of the sRNAs is rRNA. Total sRNAs that were matched with Genebank and Rfam corresponding ncRNAs were sRNA except for rRNA, tRNA, snoRNA, and snRNA which comprised more than 99% of total sRNAs with repeats. miRNAs in the sRNAs were accounted for from 0.59 to 1.21% of all types and from 52.41 to 55.35% of the total reads. Identified known miRNAs in all samples are 170 miRNAs. After comparing to the first nucleotide bias of known miRNA in each sample, the first base pair was consistent. Predominant first base of 19 and 20 lengths in pre-exercise was different from those in post-exercise (Figure 2, A and C). The nucleotide at the 19th was predominantly A in pre-exercise but U in post-exercise. At 20th,

U was predominant in pre-exercise but C was predominant in post-exercise. Known miRNA alignment nucleotides at each position in all samples were consistent except for position 22 (Figure 2, B and D).

The sequences of unannotated sRNAs that could be mapped to the equine genome were subject to further analyses to identify novel miRNA candidates. Novel miRNAs found in samples are 99 miRNAs. First nucleotide bias of novel miRNAs in pre-exercise compared to that of novel miRNAs in post-exercise was evaluated (Figure 3, A and C). The base composition at each position of all mature miRNAs revealed clear differences (Figure 3, B and D).

Differential expression analysis

The expression profile of known miRNAs and novel miRNAs in horse leukocytes was analyzed. Subsets of exercise-specific miRNAs were identified: 4 known miRNAs and 2 novel miRNAs. Up-regulated miRNAs after exercise were eca-miR-423-5p. Down-regulated miRNAs after exercise were eca-miR-144, eca-miR33a, and eca-miR-545. Novel miR-14 was presented before exercise but not identified after exercise. Novel miR-95 was not detected pre-exercise but identified after exercise (Table 3).

Target prediction of miRNAs

In silico analysis such as targetscan and miRanda showed that the known 4 miRNAs and novel 2 miRNAs potentially targeted 1625 genes and 905 genes respectively; eca-miR-144 targeted 335genes, eca-miR-33a targeted 353 genes, eca-miR-423-5p targeted 367 genes, eca-miR-545 targeted 570 genes, novel-miR-14 targeted 115 genes, and novel-miR-95 targeted 790 genes.

Analysis of target prediction of miRNAs

KEGG pathway categorizes genes into functional groups. KEGG pathway analysis resulted in a total of 13 pathways from the genes targeted by known miRNAs and 16 pathways from the genes targeted by novel miRNAs. The significant pathways were identified based on *p*-value: Wnt signaling pathway, TGF-beta signaling pathway, and Adherens junction (Figures 4, 5, and 6).

Microarray data analysis

The gene expression data between pre-exercise and post-exercise in each horse was shown (Figure 7). The mean signal intensities for pre-exercise data were plotted against those of the post-exercise. A

mathematical procedure like Principal Component Analysis (PCA) identified two components such as pre-exercise part and post-exercise part in three horses (data not shown).

Exercise for 1 hour changed the expression levels of mRNAs in three horses based on Log (fold change) ≥ 1 or ≤ -1. Total 12,422 genes were differentially regulated between pre-exercise and post-exercise. A total of 9010 and 3412 genes were differentially up- and down-regulated in three horses, respectively. Exercise in three horses induced changes in gene expression. Among these changes, 87 up-regulated genes and 27 down-regulated genes were commonly identified (Figure 8, Table 4, and Table 5).

Gene functions were investigated using pathway provided by DAVID database. The increased genes after exercise are associated with two signaling pathways. The decreased genes are significantly involved in two signaling pathways and the down-regulated genes are significantly associated with antigen processing and presentation signaling pathway.

mRNAs on microarray matching to mRNAs targeted from miRNAs on NGS

mRNAs based on Log (fold change) ≥ 1 or ≤ -1 in three horses on microarray matched to mRNAs targeted from the 6 miRNAs on NGS. The results were shown at Figure 9.

Discussion

This report is the first study to analyze the relationships between mRNA and miRNA associated with exercise in warm-blood horses. mRNAs and miRNAs were analyzed separately and then the results were compared to each other.

In miRNAs analysis, substantial 170 known miRNAs and 99 novel miRNAs in circulating leukocytes were identified to be altered by exercise. Significantly, 4 known miRNAs and 2 novel miRNAs had common changes in expression between 3 horses following 1 hour of exercise, suggesting exercise specific miRNAs. The known miRNAs and novel miRNAs are eca-miR-144, eca-miR-33a, eca-miR-545, eca-miR-423-5p, novel-miR-14, and novel-miR-95.

miR-144 has been associated with tumors such as nasopharyngeal carcinoma(Zhang *et al.* 2013), bladder cancer(Guo *et al.* 2013), gastric cancer(Akiyoshi *et al.* 2012), and colorectal cancer(Kalimutho *et al.* 2011; Iwaya *et al.* 2012) and cholesterol metabolism(Ramirez*et al.*

2013). miR-33a involves in lipid metabolism and functions as a bone metastasis suppressor in lung cancer(Kuo *et al.* 2013). miR-545 suppresses cell growth in lung cancer(Du*et al.* 2014) and is a biomarker for Alzheimer's disease(Kumar *et al.* 2013). miR-423-5p is not a biomarker for heart failure and left ventricular remodeling after myocardial infarction(Kumarswamy*et al.* 2010; Bauters*et al.* 2013) but is related to heart disease and lupus nephritis(Te*et al.* 2010; Nabialek*et al.* 2013). miR-14 regulates cell death, fat metabolism, and insulin production and metabolism(Xu*et al.* 2003; Varghese*et al.* 2010)and modulate a positive auto-regulatory loop controlling steroid hormone signaling(Varghese *et al.* 2007). miR-95 promotes cell proliferation in colorectal carcinoma(Huang *et al.* 2011) and induces radio-resistance in cancers(Huang *et al.* 2013; Chen*et al.* 2014).

miRNAs related to exercise in humans did not match to the 6 miRNAs, thought to be exercise-specific in this study. However, some miRNAs matched exercise specific miRNAs in human of blood natural killer cells, mononuclear cells, and neutrophils(Radom-Aizik *et al.* 2010; Radom-Aizik *et al.* 2012; Radom-Aizik *et al.* 2013). The miRNAs were listed at Table 7.

miR-144 decreased in this study, was found increased in heart

muscles of rats after 1 hour of swimming exercise(Ma *et al.* 2013). Differences between the 2 studies, such as species, exercise type, and intensity of exercise may be the cause of the contrast in results. Also, miR-33a, which was down-regulated after the 1 hour of exercise in three warm-blood horses, increased after 30 minutes of trotting in thoroughbred horses. It might be resulted from different breed, type and duration of exercise(Gim *et al.* 2014).

The results of the target prediction for the identified miRNAs were analyzed by DAVID. In KEGG pathway, significant pathways such as wnt signaling pathway, TGF-beta signaling pathway, and adherens junction that were targeted from the miRNAs for the mRNAs were found.

Wnt signaling pathway is recognized as an important regulator of bone mass and bone cell function. β-catenin found in the study is associated with a protein kinase A pathway and known to increase its stability, promote nuclear translocation and LEF/TCF-mediated gene transcription and represent an exercise-induced signaling mechanism underlying skeletal muscle adaptation in rats(Aschenbach *et al.* 2006). Wnt signaling can serve as a negative regulator of mTOR via activation of the tumor suppressor in tuberous sclerosis complex(Inoki *et al.*

2006).

The transforming growth factor beta (TGFB) signaling pathway is involved in many cellular processes in both the adult organism and the developing embryo. One study found that exercise on miRNAs is associated with TGF-beta signaling(Radom-Aizik *et al.* 2012). Increased TGF-β_1 in circulation and tendon tissues after acute exercise regulates collagen type I synthesis in tendon-related connective tissues(Heinemeier *et al.* 2003). Increased expression of ventricular TGF-β_1 gene in physiological cardiac hypertrophy contributes to myocardial remodeling(Calderone*et al.* 2001). Regular exercise enhances regulatory T cell function by the increases of regulatory T-cell mediators TGF-β and IL-10 from mononuclear leucocytes(Yeh*et al.* 2006). TGF- β receptor signaling is important for producing systemic IgA, and is thought to be an important effector mechanism for Ag neutralization, prevention of microbial attachment to the epithelium, elimination of excessive Ag load, and the overall maintenance of mucosal homeostasis(Underdown저자 확인 *et al.* 1986; Borsutzky *et al.* 2004).

Adherens junction performs multiple functions including initiation and stabilization of cell-cell adhesion, regulation of the actin cytoskeleton,

intracellular signaling and transcriptional regulation(Hartsock *et al.* 2008). One study found that brief exercise on peripheral blood NK cell is associated with adherens junction(Radom-Aizik *et al.* 2013).

In mRNAs analysis, significant 87 up-regulated mRNAs and 27 down-regulated mRNAs, altered by exercise, were identified in circulating leukocytes. These genes can be exercises-specific genes in warm-blood horses. Significant KEGG pathway like antigen processing and presentation for down-regulated genes was found.

Antigen presentation induces a generation of specific T cell clones capable of recognizing and removing infectious microorganisms(Watts 1997). Some studies shown that exercise reduces the antigen-presenting capacity in mice's macrophages(Ceddia *et al.* 1999; Ceddia *et al.* 2000).

One study suggests 28 candidate performance genes in the equine muscle(Schroder*et al.* 2011). The significant genes that are focused in microarray analysis are not included in the 28 performance genes (Figure 6). The differences in samples may be the cause of differences in results. However, some of the 28 genes are identified in three warm-blood leukocytes. Upregulated genes such as ACTN3, IGF1R,

AMPD1, DRD1 and 3, and 5HTT were found. HIF1A, GYS1, and VEGFA were down-regulated.

ACTN gene was upregulated after exercise. The ACTN is present in type 2 (fast and glycolytic) muscle fibers for a pivotal role and muscle strength at high speed(Yang *et al.* 2003; Clarkson *et al.* 2005). IGF1 was increased. IGF1 induces nerve growth and differentiation, synthesis and release of neurotransmitters(Anlar*et al.* 1999). IGF plays a role in mediating exercise-induced neuronal and cognitive enhancement(Ding *et al.* 2006). AMP deaminase encoded by AMPD1 is activated in skeletal muscle after adenosine monophosphate (AMP) accumulation during exercise. Exercise performances were similar across the AMPD1 genotypes, whereas significant differences in several descriptors of energy metabolism were observed(Norman *et al.* 2001). HIF1A gene was decreased after exercise. HIF1A gene is up-regulated in states such as hypoxia or severe and repetitive oxygen stress. The gene is also involved in red cell production, angiogenesis, glucose metabolism(Maxwell *et al.* 1997; Mason *et al.* 2004; Walmsley *et al.* 2005). HIF1A gene in human leukocytes was reduced in training groups but exercise induced an up-regulation of HIF1 gene in human muscles(Mounier *et al.* 2006). Exercise showed a

decrease in GYS1 gene expression. Muscle glycogen synthase is an important enzyme for glycogen synthesis in skeletal muscles. GYS1 is a key candidate for diagnosing polysaccharide storage myopathy in horses(McCue *et al.* 2008). VEGFA gene is involved in endothelial cell proliferation and migration in peripheral circulation. VEGF gene expression of human skeletal muscle in hypoxic or normoxic training groups were up-regulated but the gene expressions of human leukocytes in the same conditions were down-regulated(Mounier*et al.* 2009). DRD1 and DRD3 genes were over-expressed. Dopamine receptors pass signal from one nerve cell to an adjacent cell. D1 is a D1-like family which increases cyclic adenosine monophosphate (cAMP), whereas D3 is a D2-like family which decreases cAMP. Dopaminergic transmission is associated with reward dependence and persistence of exercise(Kulikova*et al.* 2007). This study results from sampling the buffy coat, which contains leukocytes and platelets. Therefore an increase in 5 HTT gene expression may be caused by multiple biochemical and pharmacological traits of 5-hydroxytryptamine (5HT), which is similar to those of platelets(Da Prada*et al.* 1988). Serotonin transporter transports serotonin from synaptic cleft to the presynaptic neuron to remove and recycle.

Serotonin biosynthesis in the brain results from exercise to control emotion(Dye *et al.* 1992; Rethorst *et al.* 2010).

The mRNAs targeted from miRNAs using NGS were matched with mRNAs on microarray. Some regulated miRNA-mRNA networks were identified including mRNA known to be regulated by 2 miRNAs described. These have probability of being associated with the regulation of exercise-related physiological processes such as molecular adaptation and homeostasis.

Conclusion

Networks for5 mRNA genes involved in 3 miRNAs of the 6 miRNAs including mRNA known to be regulated by 2 miRNA were identified. This study revealed regulatory networks of miRNAs and mRNAs, and a subset of mRNAs and miRNAs in equine peripheral blood leukocytes affected by exercise, suggesting background information for understanding molecular homeostasis and adaptation from exercise-associated physiology in horses.

References

A. Calderone, R.J. Murphy, J. Lavoie, F. Colombo, L. Beliveau. 2001. TGF-beta(1) and prepro-ANP mRNAs are differentially regulated in exercise-induced cardiac hypertrophy. J Appl Physiol (1985) **91**(2): 771-776.

A.G. Tonevitsky, D.V. Maltseva, A. Abbasi, T.R. Samatov, D.A. Sakharov, M.U. Shkurnikov, A.E. Lebedev, V.V. Galatenko, A.I. Grigoriev, H. Northoff. 2013. Dynamically regulated miRNA-mRNA networks revealed by exercise. BMC Physiol **13**: 9.

B. Anlar, K.A. Sullivan, E.L. Feldman. 1999. Insulin-like growth factor-I and central nervous system development. Horm Metab Res **31**(2-3): 120-125.

B. Du, Z. Wang, X. Zhang, S. Feng, G. Wang, J. He, B. Zhang. 2014. MicroRNA-545 suppresses cell proliferation by targeting cyclin D1 and CDK4 in lung cancer cells. PLoS One **9**(2): e88022.

B. Norman, R.L. Sabina, E. Jansson. 2001. Regulation of skeletal muscle ATP catabolism by AMPD1 genotype during sprint exercise in asymptomatic subjects. J Appl Physiol (1985) 91(1): 258-264.

B.A. McGivney, S.S. Eivers, D.E. MacHugh, J.N. MacLeod, G.M. O'Gorman, S.D. Park, L.M. Katz, E.W. Hill. 2009. Transcriptional adaptations following exercise in thoroughbred horse skeletal muscle highlights molecular mechanisms that lead to muscle hypertrophy. BMC Genomics 10: 638.

C. Bauters, R. Kumarswamy, A. Holzmann, J. Bretthauer, S.D. Anker, F. Pinet, T. Thum. 2013. Circulating miR-133a and miR-423-5p fail as biomarkers for left ventricular remodeling after myocardial infarction. Int J Cardiol 168(3): 1837-1840.

C.D. Rethorst, D.M. Landers, C.T. Nagoshi, J.T. Ross. 2010. Efficacy of exercise in reducing depressive symptoms across 5-HTTLPR

genotypes. Med Sci Sports Exerc 42(11): 2141-2147.

C.J. Creighton, A.L. Benham, H. Zhu, M.F. Khan, J.G. Reid, A.K. Nagaraja, M.D. Fountain, O. Dziadek, D. Han, L. Ma, J. Kim, S.M. Hawkins, M.L. Anderson, M.M. Matzuk, P.H. Gunaratne. 2010. Discovery of novel microRNAs in female reproductive tract using next generation sequencing. PLoS One 5(3): e9637.

C.M. Ramirez, N. Rotllan, A.V. Vlassov, A. Davalos, M. Li, L. Goedeke, J.F. Aranda, D. Cirera-Salinas, E. Araldi, A. Salerno, A. Wanschel, J. Zavadil, A. Castrillo, J. Kim, Y. Suarez, C. Fernandez-Hernando. 2013. Control of cholesterol metabolism and plasma high-density lipoprotein levels by microRNA-144. Circ Res 112(12): 1592-1601.

Ceddia, M. A. and J. A. Woods. 1999. Exercise suppresses macrophage antigen presentation. J Appl Physiol (1985) 87(6): 2253-2258.

E. Nabialek, W. Wanha, D. Kula, T. Jadczyk, M. Krajewska, A. Kowalowka, S. Dworowy, E. Hrycek, W. Wludarczyk, Z. Parma, A. Michalewska-Wludarczyk, T. Pawlowski, B. Ochala, B. Jarzab, M. Tendera, W. Wojakowski. 2013. Circulating microRNAs (miR-423-5p, miR-208a and miR-1) in acute myocardial infarction and stable coronary heart disease. Minerva Cardioangiol 61(6): 627-637.

Emmeline W. Hill, Lisa M. Katz, and David E. MacHugh. 2013. Genomics of performance. Equine genomics 1: 265-283."

Flynt, A. S. and E. C. Lai. 2008. Biological principles of microRNA-mediated regulation: shared themes amid diversity. Nat Rev Genet 9(11): 831-842.

Gaffney, B. and E. P. Cunningham. 1988. Estimation of genetic trend in racing performance of thoroughbred horses. Nature 332(6166): 722-724.

Hartsock, A. and W. J. Nelson. 2008. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. Biochim Biophys Acta **1778**(3): 660-669.

J. Varghese, S.F. Lim, S.M. Cohen. 2010. Drosophila miR-14 regulates insulin production and metabolism through its target, sugarbabe. Genes Dev **24**(24): 2748-2753.

J.A. Gim, S. Ayarpadikannan, J. Eo, Y.J. Kwon, Y. Choi, H.K. Lee, K.D. Park, Y.M. Yang, B.W. Cho, H.S. Kim. 2014. Transcriptional expression changes of glucose metabolism genes after exercise in thoroughbred horses. Gene **547**(1): 152-158.

J.L. Te, I.M. Dozmorov, J.M. Guthridge, K.L. Nguyen, J.W. Cavett, J.A. Kelly, G.R. Bruner, J.B. Harley, J.O. Ojwang. 2010. Identification of unique microRNA signature associated with lupus nephritis. PLoS One **5**(5): e10344.

K. Cappelli, A. Verini-Supplizi, S. Capomaccio, M. Silvestrelli. 2007. Analysis of peripheral blood mononuclear cells gene expression in endurance horses by cDNA-AFLP technique. Res Vet Sci 82(3): 335-343.

K. Heinemeier, H. Langberg, J.L. Olesen, M. Kjaer. 2003. Role of TGF-beta1 in relation to exercise-induced type I collagen synthesis in human tendinous tissue. J Appl Physiol (1985) 95(6): 2390-2397.

K. Inoki, H. Ouyang, T. Zhu, C. Lindvall, Y. Wang, X. Zhang, Q. Yang, C. Bennett, Y. Harada, K. Stankunas, C.Y. Wang, X. He, O.A. MacDougald, M. You, B.O. Williams, K.L. Guan. 2006. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. Cell 126(5): 955-968.

L.Y. Zhang, V. Ho-Fun Lee, A.M. Wong, D.L. Kwong, Y.H. Zhu, S.S. Dong, K.L. Kong, J. Chen, S.W. Tsao, X.Y. Guan, L. Fu. 2013. MicroRNA-144 promotes cell proliferation, migration and invasion in

nasopharyngeal carcinoma through repression of PTEN. Carcinogenesis 34(2): 454-463.

Levine, M. A. 1999. Investigating the origins of horse domestication. Equine Vet J Suppl(28): 6-14.

M. Da Prada, A.M. Cesura, J.M. Launay, J.G. Richards. 1988. Platelets as a model for neurones? Experientia 44(2): 115-126.

M. Kalimutho, G. Del Vecchio Blanco, S. Di Cecilia, P. Sileri, M. Cretella, F. Pallone, G. Federici, S. Bernardini. 2011. Differential expression of miR-144* as a novel fecal-based diagnostic marker for colorectal cancer. J Gastroenterol 46(12): 1391-1402.

M.A. Ceddia, E.W. Voss, Jr., J.A. Woods. 2000. Intracellular mechanisms responsible for exercise-induced suppression of macrophage antigen presentation. J Appl Physiol (1985) 88(2): 804-

810.

M.A. Kulikova, N.V. Maliuchenko, M.A. Timofeeva, V.A. Shleptsova, A. Tschegol'kova Iu, A.M. Vediakov, A.G. Tonevitskii. 2007. Polymorphisms of the main genes of neurotransmitter systems: I. the dopaminergic system. Fiziol Cheloveka 33(6): 105-112.

M.C. Kim, S.W. Lee, D.Y. Ryu, F.J. Cui, J. Bhak, Y. Kim. 2014. Identification and characterization of microRNAs in normal equine tissues by Next Generation Sequencing. PLoS One 9(4): e93662.

M.E. McCue, S.J. Valberg, M. Lucio, J.R. Mickelson. 2008. Glycogen synthase 1 (GYS1) mutation in diverse breeds with polysaccharide storage myopathy. J Vet Intern Med 22(5): 1228-1233.

N. Yang, D.G. MacArthur, J.P. Gulbin, A.G. Hahn, A.H. Beggs, S. Easteal, K. North. 2003. ACTN3 genotype is associated with human elite

athletic performance. Am J Hum Genet 73(3): 627-631.

P. Kumar, Z. Dezso, C. MacKenzie, J. Oestreicher, S. Agoulnik, M. Byrne, F. Bernier, M. Yanagimachi, K. Aoshima, Y. Oda. 2013. Circulating miRNA biomarkers for Alzheimer's disease. PLoS One 8(7): e69807.

P. Xu, S.Y. Vernooy, M. Guo, B.A. Hay. 2003. The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. Curr Biol 13(9): 790-795.

P.H. Maxwell, G.U. Dachs, J.M. Gleadle, L.G. Nicholls, A.L. Harris, I.J. Stratford, O. Hankinson, C.W. Pugh, P.J. Ratcliffe. 1997. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci U S A 94(15): 8104-8109.

P.M. Clarkson, J.M. Devaney, H. Gordish-Dressman, P.D. Thompson, M.J. Hubal, M. Urso, T.B. Price, T.J. Angelopoulos, P.M. Gordon, N.M. Moyna, L.S. Pescatello, P.S. Visich, R.F. Zoeller, R.L. Seip, E.P. Hoffman. 2005. ACTN3 genotype is associated with increases in muscle strength in response to resistance training in women. J Appl Physiol (1985) 99(1): 154-163.

Q. Ding, S. Vaynman, M. Akhavan, Z. Ying, F. Gomez-Pinilla. 2006. Insulin-like growth factor I interfaces with brain-derived neurotrophic factor-mediated synaptic plasticity to modulate aspects of exercise-induced cognitive function. Neuroscience 140(3): 823-833.

R. Kumarswamy, S.D. Anker, T. Thum. 2010. MicroRNAs as circulating biomarkers for heart failure: questions about MiR-423-5p. Circ Res 106(9): e8; author reply e9.

R. Mounier, V. Pialoux, A. Cayre, L. Schmitt, J.P. Richalet, P. Robach, F. Lasne, B. Roels, G. Millet, J. Coudert, E. Clottes, N. Fellmann. 2006.

Leukocyte's Hif-1 expression and training-induced erythropoietic response in swimmers. Med Sci Sports Exerc 38(8): 1410-1417.

R. Mounier, V. Pialoux, B. Roels, C. Thomas, G. Millet, J. Mercier, J. Coudert, N. Fellmann, E. Clottes. 2009. "Effect of intermittent hypoxic training on HIF gene expression in human skeletal muscle and leukocytes." Eur J Appl Physiol 105(4): 515-524.

Russell, A. P. 2010. Molecular regulation of skeletal muscle mass. Clin Exp Pharmacol Physiol 37(3): 378-384.

S. Akiyoshi, T. Fukagawa, H. Ueo, M. Ishibashi, Y. Takahashi, M. Fabbri, M. Sasako, Y. Maehara, K. Mimori, M. Mori. 2012. Clinical significance of miR-144-ZFX axis in disseminated tumour cells in bone marrow in gastric cancer cases. Br J Cancer 107(8): 1345-1353.

S. Borsutzky, B.B. Cazac, J. Roes, C.A. Guzman. 2004. TGF-beta

receptor signaling is critical for mucosal IgA responses. J Immunol 173(5): 3305-3309.

S. Dey, R.H. Singh, P.K. Dey. 1992. Exercise training: significance of regional alterations in serotonin metabolism of rat brain in relation to antidepressant effect of exercise. Physiol Behav 52(6): 1095-1099.

S. Radom-Aizik, F. Zaldivar, F. Haddad, D.M. Cooper. 2013. Impact of brief exercise on peripheral blood NK cell gene and microRNA expression in young adults. J Appl Physiol (1985) 114(5): 628-636.

S. Radom-Aizik, F. Zaldivar, Jr., S. Oliver, P. Galassetti, D.M. Cooper. 2010. Evidence for microRNA involvement in exercise-associated neutrophil gene expression changes. J Appl Physiol (1985) 109(1): 252-261.

S. Radom-Aizik, F. Zaldivar, Jr., S.Y. Leu, G.R. Adams, S. Oliver, D.M.

Cooper. 2012. Effects of exercise on microRNA expression in young males peripheral blood mononuclear cells. Clin Transl Sci 5(1): 32-38.

S.D. Mason, R.A. Howlett, M.J. Kim, I.M. Olfert, M.C. Hogan, W. McNulty, R.P. Hickey, P.D. Wagner, C.R. Kahn, F.J. Giordano, R.S. Johnson. 2004. Loss of skeletal muscle HIF-1alpha results in altered exercise endurance. PLoS Biol 2(10): e288.

S.H. Yeh, H. Chuang, L.W. Lin, C.Y. Hsiao, H.L. Eng. 2006. Regular tai chi chuan exercise enhances functional mobility and CD4CD25 regulatory T cells. Br J Sports Med 40(3): 239-243.

S.R. Walmsley, C. Print, N. Farahi, C. Peyssonnaux, R.S. Johnson, T. Cramer, A. Sobolewski, A.M. Condliffe, A.S. Cowburn, N. Johnson, E.R. Chilvers. 2005. Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. J Exp Med 201(1): 105-115.

T. Iwaya, T. Yokobori, N. Nishida, R. Kogo, T. Sudo, F. Tanaka, K. Shibata, G. Sawada, Y. Takahashi, M. Ishibashi, G. Wakabayashi, M. Mori, K. Mimori. 2012. Downregulation of miR-144 is associated with colorectal cancer progression via activation of mTOR signaling pathway. Carcinogenesis 33(12): 2391-2397.

T.A. Fehniger, T. Wylie, E. Germino, J.W. Leong, V.J. Magrini, S. Koul, C.R. Keppel, S.E. Schneider, D.C. Koboldt, R.P. Sullivan, M.E. Heinz, S.D. Crosby, R. Nagarajan, G. Ramsingh, D.C. Link, T.J. Ley, E.R. Mardis. 2010. Next-generation sequencing identifies the natural killer cell microRNA transcriptome. Genome Res 20(11): 1590-1604.

Underdown, B. J. and J. M. Schiff. 1986. Immunoglobulin A: strategic defense initiative at the mucosal surface. Annu Rev Immunol 4: 389-417.

Varghese, J. and S. M. Cohen. 2007. microRNA miR-14 acts to modulate a positive autoregulatory loop controlling steroid hormone

signaling in Drosophila. Genes Dev 21(18): 2277-2282.

W. Schroder, A. Klostermann, O. Distl. 2011. Candidate genes for physical performance in the horse. Vet J 190(1): 39-48.

W.G. Aschenbach, R.C. Ho, K. Sakamoto, N. Fujii, Y. Li, Y.B. Kim, M.F. Hirshman, L.J. Goodyear. 2006. Regulation of disheveled and beta-catenin in rat skeletal muscle: an alternative exercise-induced GSK-3beta signaling pathway. Am J Physiol Endocrinol Metab 291(1): E152-158.

Watts, C. 1997. Capture and processing of exogenous antigens for presentation on MHC molecules. Annu Rev Immunol 15: 821-850.

X. Chen, S. Chen, W. Hang, H. Huang, H. Ma. 2014. MiR-95 induces proliferation and chemo- or radioresistance through directly targeting sorting nexin1 (SNX1) in non-small cell lung cancer. Biomed

Pharmacother 68(5): 589-595.

X. Huang, S. Taeb, S. Jahangiri, U. Emmenegger, E. Tran, J. Bruce, A. Mesci, E. Korpela, D. Vesprini, C.S. Wong, R.G. Bristow, F.F. Liu, S.K. Liu. 2013. miRNA-95 mediates radioresistance in tumors by targeting the sphingolipid phosphatase SGPP1. Cancer Res 73(23): 6972-6986.

Y. Guo, L. Ying, Y. Tian, P. Yang, Y. Zhu, Z. Wang, F. Qiu, J. Lin. 2013. miR-144 downregulation increases bladder cancer cell proliferation by targeting EZH2 and regulating Wnt signaling. Febs j 280(18): 4531-4538.

Z. Huang, S. Huang, Q. Wang, L. Liang, S. Ni, L. Wang, W. Sheng, X. He, X. Du. 2011. MicroRNA-95 promotes cell proliferation and targets sorting Nexin 1 in human colorectal carcinoma. Cancer Res 71(7): 2582-2589.

Z. Ma, J. Qi, S. Meng, B. Wen, J. Zhang. 2013. Swimming exercise training-induced left ventricular hypertrophy involves microRNAs and synergistic regulation of the PI3K/AKT/mTOR signaling pathway. Eur J Appl Physiol **113**(10): 2473-2486.

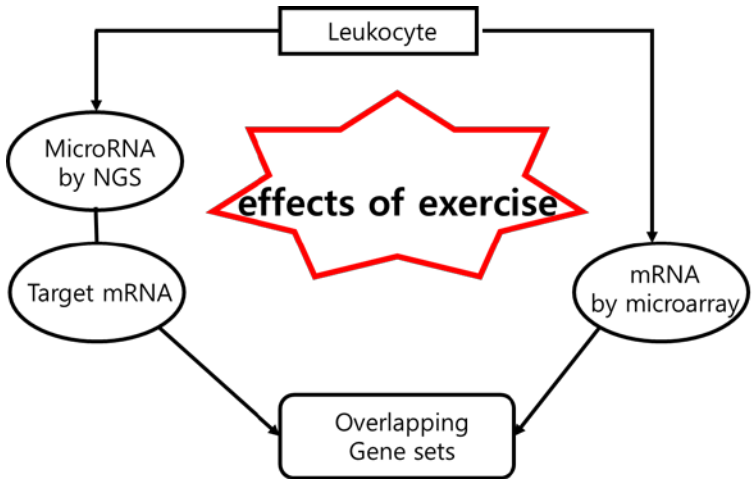


Figure 1. An analysis of the leukocyte miRNA and mRNAs whose expressions were changed by exercise.

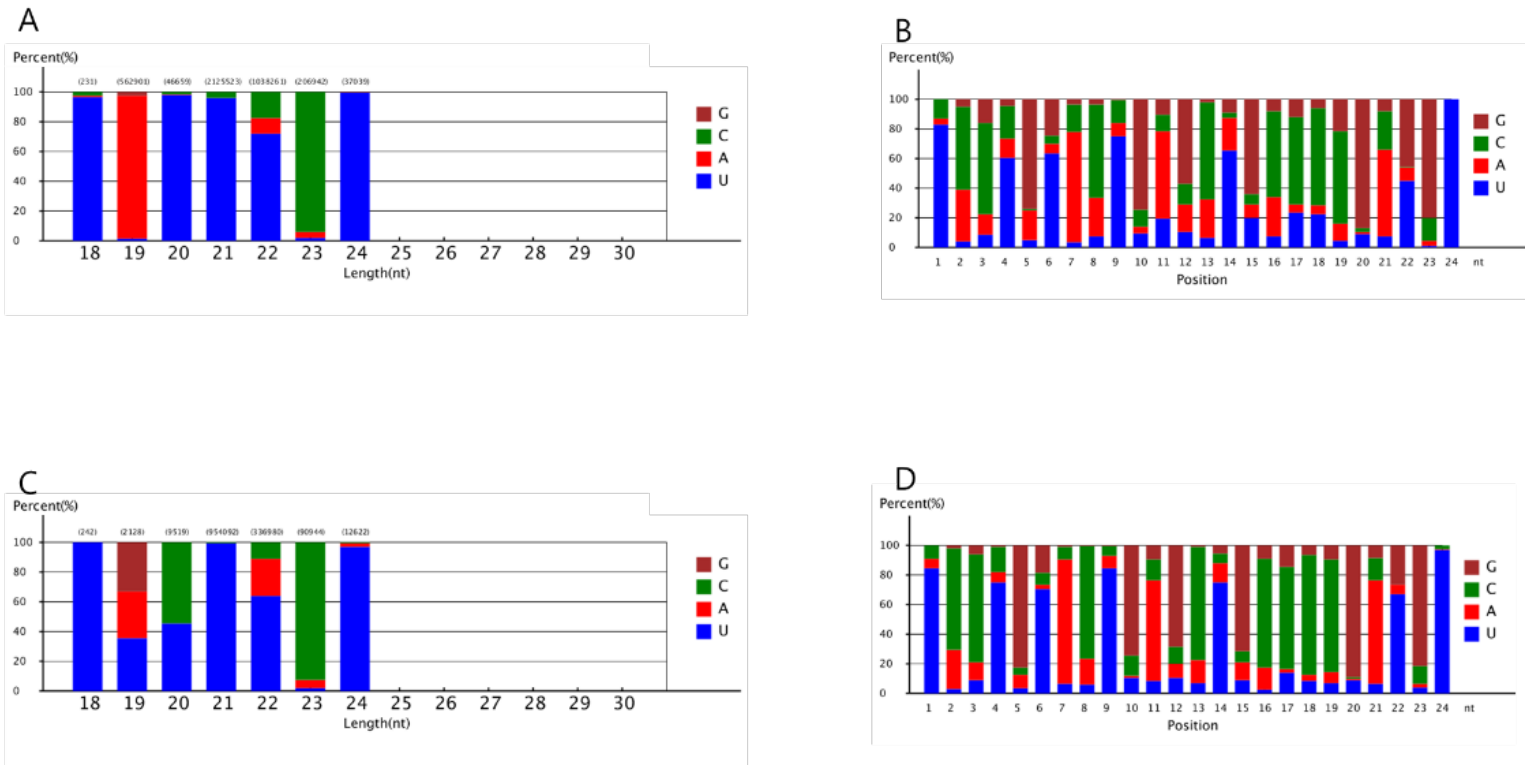


Figure 2. Known miRNA sequences analysis on NGS. First nucleotide bias in pre-exercise (A) and post-exercise (C). miRNA nucleotide bias at each position in pre-exercise (B) and post-exercise (D).

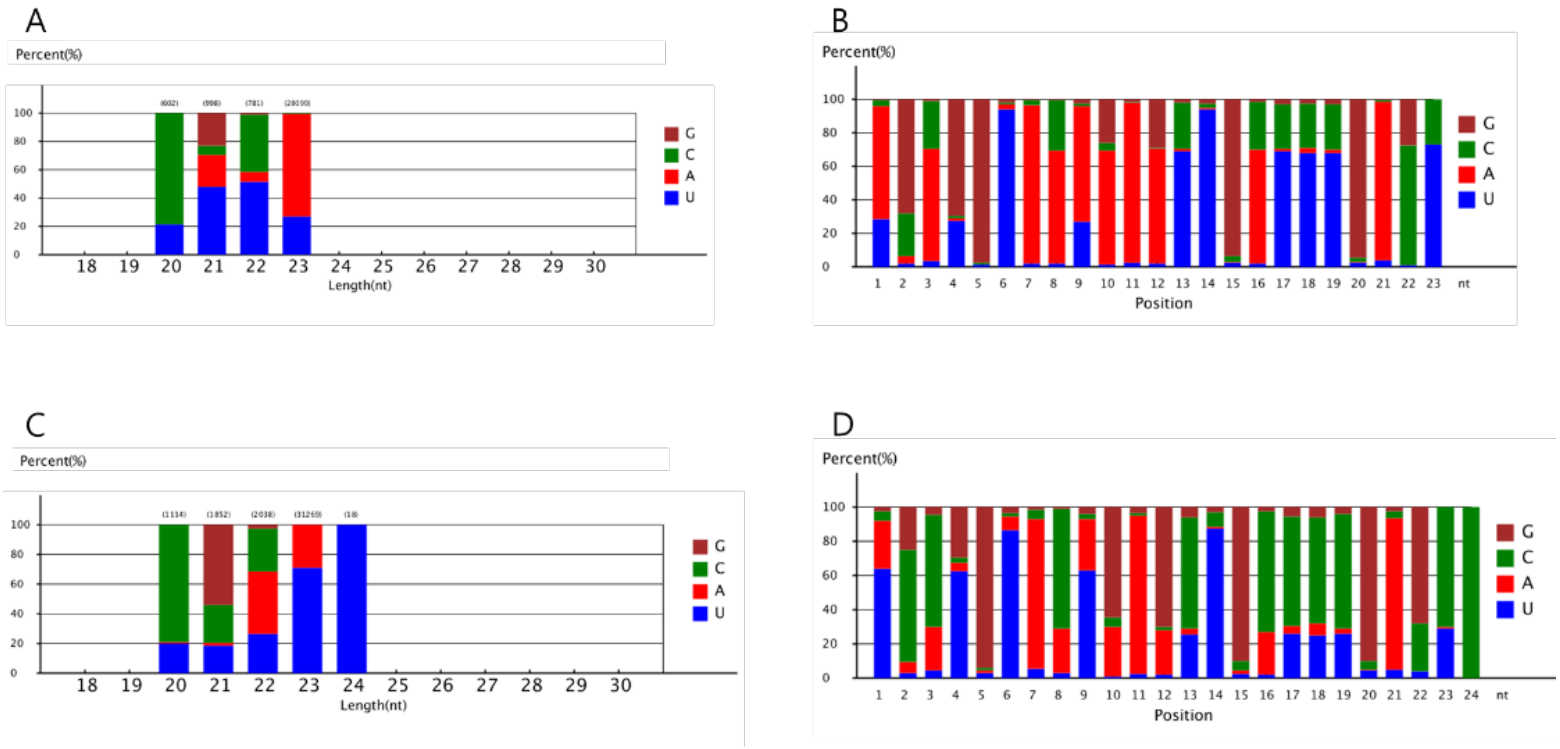


Figure 3. Novel miRNA sequences analysis on NGS. First nucleotide bias in pre-exercise (A) and post-exercise (C). miRNA nucleotide bias at each position in pre-exercise (B) and post-exercise (D).

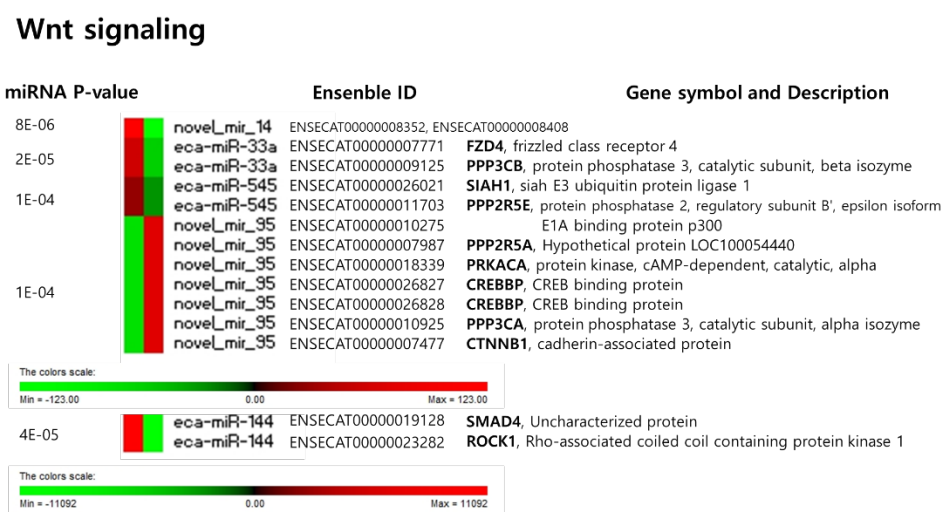


Figure 4. Wnt signaling pathway involved in significant miRNAs on NGS and target prediction on mRNA for the miRNA. Red color indicates over-expression of miRNA and green color represents down-expression of miRNA.

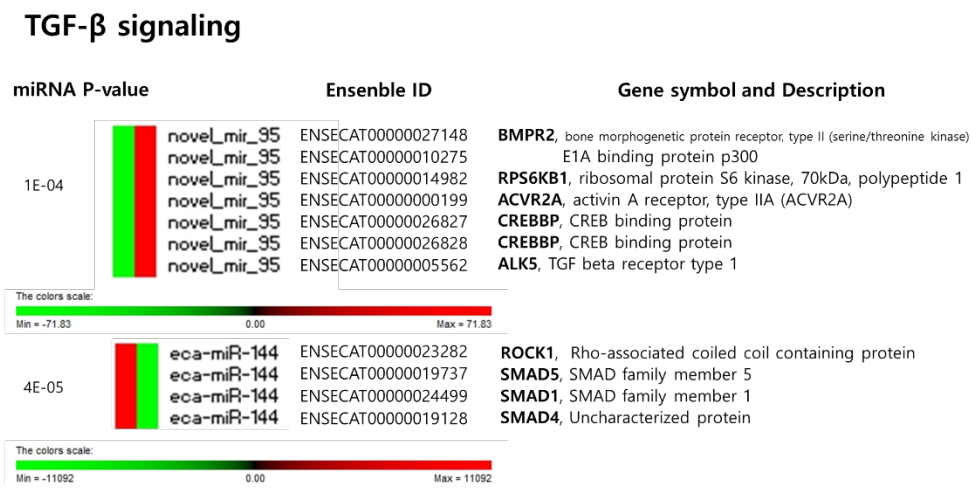


Figure 5.TGF-β signaling pathway involved in significant miRNAs on NGS and target prediction on mRNA for the miRNA. Red color indicates over-expression of miRNA and green color represents down-expression of miRNA.

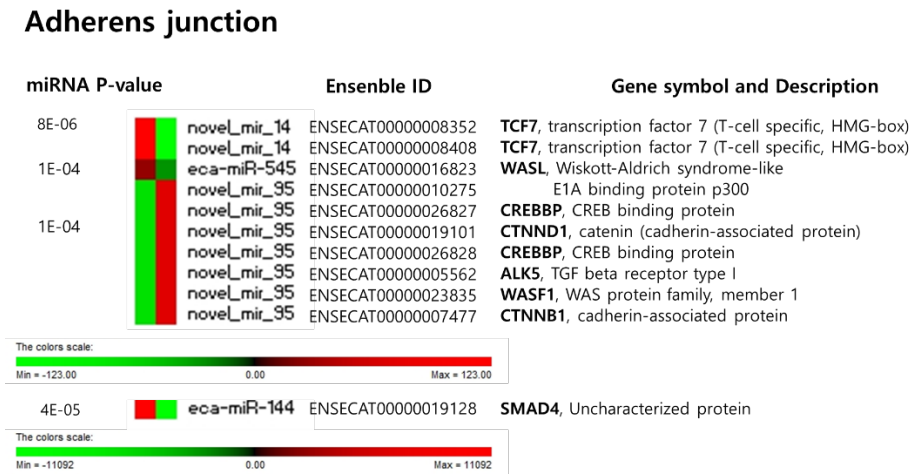


Figure 6. Adherens junction pathway involved in significant miRNAs on NGS and target prediction on mRNA for the miRNA. Red color indicates over-expression of miRNA and green color represents down-expression of miRNA.

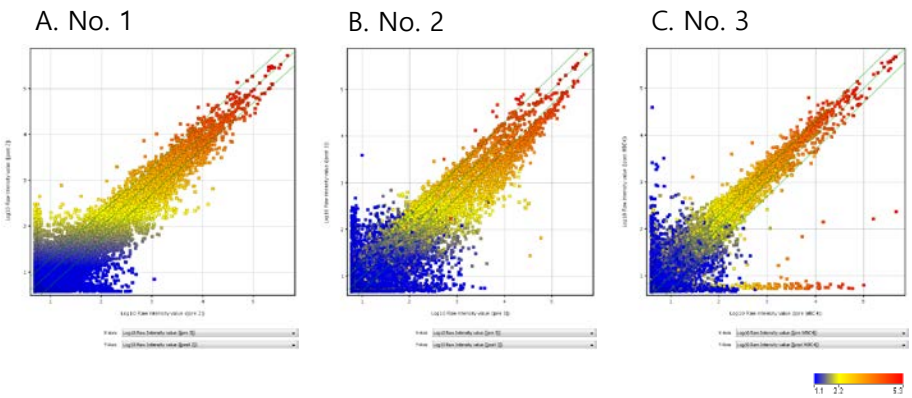
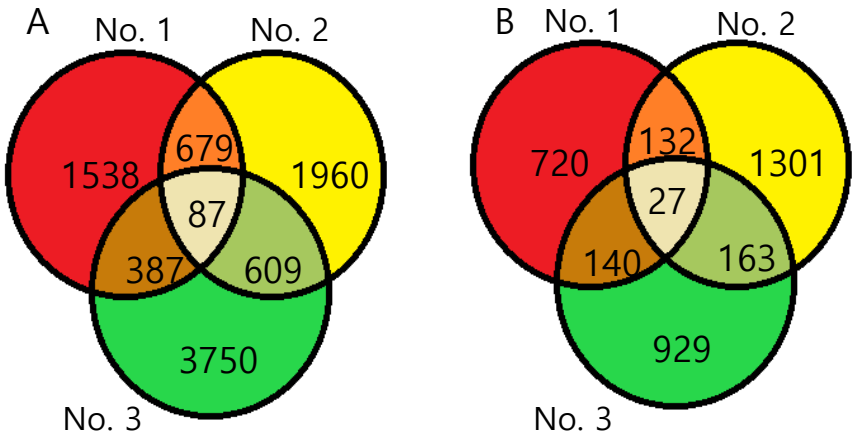


Figure 7. Scatter plots visualizing mean raw gene expression data on microarray. Comparison of the mean gene expression levels between pre-exercise and post-exercise of horse No. 1(A), horse No. 2 (B), and horse No. 3 (C).

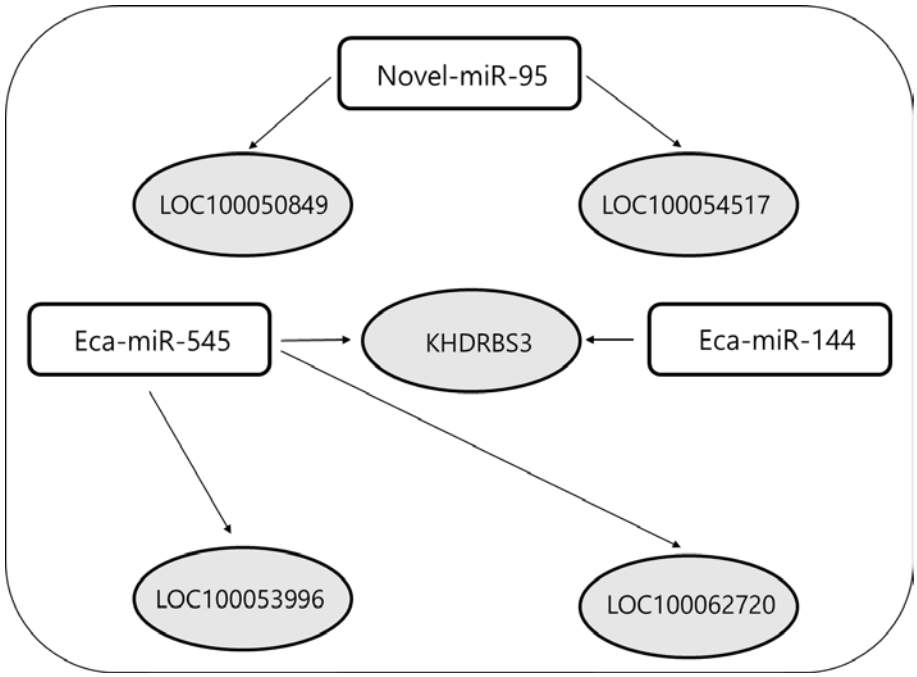


C

Number (before VS after exercise)	Up-regulated gene	Down-regulated gene
No. 1	2691	1019
No. 2	3335	1623
No. 3	4833	1259

Figure 8. Differentially expressed genes before and after exercise in horses on microarray. A. Significantly increased genes based on Log (Fold Change) > 1 or < −1. B. Significantly decreased genes based on Log (Fold Change) > 1 or < −1. Red, yellow, and green circles each indicate the number of genes differentially expressed in peripheral leukocyte of horse No. 2, horse No. 3, and horse No. 4, respectively. C. The number of the differential expression levels in 3 horses.

A



B

miRNA	UP/DOWN of miRNA	Gene symbol	UP/DOWN of mRNA
novel_mir_95	UP	LOC100050849	DOWN
		LOC100054517	DOWN
eca-miR-545	DOWN	LOC100053996	UP
		LOC100062720	UP
eca-miR-144	DOWN	KHDRBS3	UP

Figure 9. Relationship of miRNAs and mRNAs altered by exercise.

Figure (A) and table (B) illustrate the relationship between the differentially expressed mRNAs on microarray and mRNAs targeted from the differentially expressed miRNAs on NGS induced by exercise.

Table 1. Information of the horses used in this study

No.	Breed	Country	Age (years old)	Gender	NGS/Microarray	
1	Warm-blood	Germany	13	Gelding	NGS	-
2	Warm-blood	Germany	9	Gelding	NGS	Microarray
3	Warm-blood	Germany	14	Gelding	NGS	Microarray
4	Warm-blood	Germany	17	Gelding	-	Microarray

Table 2. Results of the RNAs from leukocytes before and after exercise

No.	Pre exercise			Post exercise		
	Con. (ng/μl)	Purity (260/280)	Total RNA amount (μg)	Con. (ng/μl)	Purity (260/280)	Total RNA amount (μg)
1	972.2	1.86	48.61	1053.4	1.83	52.67
2	283.4	1.65	5.668	310.1	1.65	6.202
3	895.8	1.82	26.874	1319.8	1.81	39.594
4	291.3	1.52	5.826	274.5	1.52	8.235

Table 3. Differential expression analysis of known miRNAs and novel miRNAs on NGS

miRNA	Pre exercise: reads mean	Post exercise: reads mean	log2Ratio (sample)	*p*-value	FDR
Known miRNAs					
eca-miR-144	23264.66	1081.66	-3.72	4E-05	3E-03
eca-miR-423-5p	841.66	13131	5.03	8E-08	1E-05
eca-miR-545	55.66	1.33	-4.51	1E-04	6E-03
eca-miR-33a	118	3	-4.39	2E-05	2E-03
Novel miRNAs					
novel_mir_14	246	0	-17.51	8E-06	1E-03
novel_mir_95	0	143.66	-16.60	1E-04	8E-03

Table 4. A list of up-regulated common genes after exercise in three horses on microarray

Gene Symbol	Gene name	Log (Fold Change)		
		No. 1	No. 2	No. 3
LOC100063970	endoplasmic reticulum resident protein 27-like	2.06	1.50	1.31
LOC100055780	olfactory receptor 5W2-like	1.85	2.47	1.27
LOC100053996	tetratricopeptide repeat protein 23-like	2.16	2.58	1.28
LOC100058490	transmembrane 4 L6 family member 18-like	2.92	1.95	1.31
LOC100073221	protein limb expression 1 homolog	2.63	3.22	1.32
LOC100067725	olfactory receptor 10W1-like	3.54	2.56	1.31
ADAMTS6	ADAM metallopeptidase with thrombospondin type 1 motif, 6	1.73	1.68	3.65
TF	transferrin	2.63	4.15	3.65
ADAMTS12	ADAM metallopeptidase with thrombospondin type 1 motif, 12	1.82	2.17	1.48
LOC100050742	transmembrane 4 L6 family member 1-like	2.49	3.95	1.23
LOC100053463	similar to proline arginine-rich end leucine-rich repeat protein	1.54	2.94	1.58
LOC100062850	similar to olfactory receptor MOR256-12	1.56	2.79	1.14
SLC26A3	solute carrier family 26, member 3	1.64	2.20	1.22
LOC100068137	similar to olfactory receptor Olr87	2.89	1.59	1.27
CDKL3	cyclin-dependent kinase-like 3	1.89	1.72	1.19
LOC100066473	similar to wax synthase	1.51	4.15	1.29
SLC8A1	solute carrier family 8 (sodium/calcium exchanger), member 1	3.21	2.31	1.30
LOC100065634	olfactory receptor 5M8-like	2.30	2.86	1.31
JAKMIP3	janus kinase and microtubule interacting protein 3	1.62	2.15	1.30
LOC100058155	similar to semaphorin III/collapsin-1	2.36	2.57	1.25
LOC100062480	similar to sprouty (Drosophila) homolog 3	1.77	1.92	2.03
GAB1	GRB2-associated binding protein 1	1.10	1.35	1.30
LOC100064336	similar to Multimerin 2	1.68	1.36	1.31
LOC100067873	olfactory receptor 51G1-like	1.17	1.18	2.22
LOC100061578	hypothetical protein LOC100061578	1.34	5.52	3.27

LOC100059542	similar to programmed death ligand 2	2.35	1.15	1.23
LOC100071790	similar to Fibroblast growth factor 16 (FGF-16)	1.22	2.21	1.56
HTR7	5-hydroxytryptamine (serotonin) receptor 7	1.44	1.66	3.29
LOC100070326	similar to paired box gene 2	1.05	3.75	1.80
LOC100057148	interferon-inducible protein AIM2-like	1.42	1.38	1.27
CTNNA2	catenin (cadherin-associated protein), alpha 2	1.93	1.26	1.30
GRIN1	glutamate receptor, ionotropic, N-methyl D-aspartate 1	1.01	2.59	4.48
LOC100053496	similar to Homeobox D9	1.29	1.55	1.30
FGFR2	fibroblast growth factor receptor	1.79	1.28	1.30
LOC100067877	olfactory receptor 9I1-like	2.91	1.01	1.30
LOC100051505	similar to Coiled-coil domain containing 65	1.24	2.17	1.31
LOC100051073	similar to pancreatic amylase B	1.05	1.02	1.14
MYO1H	myosin IH	1.32	4.78	2.37
LOC100062666	protein FAM13C-like	1.18	1.43	1.24
LOC100063215	keratin, type II cytoskeletal 2 epidermal-like	1.39	2.98	1.23
SLC36A3	solute carrier family 36 (proton/amino acid symporter), member 3	1.35	1.50	1.30
LOC100053699	hypothetical protein LOC100053699	1.14	3.88	1.24
LOC100063705	keratocan-like	1.40	1.33	2.17
LOC100069504	similar to transmembrane leptin receptor	4.51	1.27	1.24
LOC100062720	potassium voltage-gated channel subfamily E member 2-like	1.10	2.26	1.31
LOC100061071	similar to growth differentiation factor 3	1.59	1.25	1.18
DHX9	DEAH (Asp-Glu-Ala-His) box polypeptide 9	1.13	1.59	1.83
LOC100061513	Carboxypeptidase E-like	1.29	2.96	1.28
LOC100059030	similar to CTCL tumor antigen L14-2	1.25	1.16	1.15
EQUCABV1R935	similar to vomeronasal V1r-type receptor V1rk1	1.60	1.30	1.03
CENPE	centromere protein E, 312kDa	1.47	2.20	2.43
PEG10	paternally expressed 10	1.35	1.27	1.30
FBN2	fibrillin 2 (congenital contractural arachnodactyly)	1.01	2.76	1.02
IL4	interleukin 4	1.14	1.23	1.40
LOC100070477	PIH1 domain-containing protein 2-like	1.02	1.09	1.21

LOC100067174	similar to annexin XIIIb	2.79	1.24	1.18
LOC100056782	protein FAM124B-like	1.28	1.44	1.30
LOC100065162	similar to UDP-glucuronosyltransferase UGT1A6	1.46	1.34	1.31
SLFN11	schlafen family member 11	2.31	1.31	6.74
POU4F2	POU class 4 homeobox 2	2.86	1.99	1.74
LOC100629781	lysophosphatidic acid receptor 6-like	1.41	1.37	3.35
LOC100630059	interleukin-3-like	3.19	1.24	1.23
LOC100629214	uncharacterized LOC100629214	1.87	1.52	1.24
HAPLN2	hyaluronan and proteoglycan link protein 2	3.64	3.14	1.24
LOC100629377	uncharacterized LOC100629377	1.39	1.46	2.79
GPR126	G protein-coupled receptor 126	1.62	1.27	1.28
GALNTL2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	4.80	3.23	1.24
PLCE1	phospholipase C, epsilon 1	3.47	1.91	1.29
SATB2	SATB homeobox 2	2.40	1.69	1.30
LOC100629147	uncharacterized protein C2orf66-like	2.62	1.73	1.30
LOC100629443	uncharacterized LOC100629443	1.51	1.62	4.24
LOC100629614	uncharacterized LOC100629614	1.53	1.17	1.58
LOC100630062	keratinocyte differentiation-associated protein-like	1.32	2.44	1.31
CTSD	cathepsin D	1.28	1.04	1.29
LAMA4	laminin, alpha 4	1.73	1.79	3.21
DEFA3	defensin, alpha 3, neutrophil-specific	2.56	1.77	1.31
LOC100630442	uncharacterized LOC100630442	1.82	2.87	1.29
LOC100630298	uncharacterized LOC100630298	1.89	2.57	4.12
KHDRBS3	KH domain containing, RNA binding, signal transduction associated 3	1.50	1.30	3.28
CORIN	corin, serine peptidase	1.06	1.04	1.14
LOC100629950	beta-defensin 122-like	2.14	2.27	1.35
PRSS35	protease, serine, 35	2.02	2.23	2.61
AKAP9	A kinase (PRKA) anchor protein (yotiao) 9	1.34	1.70	1.31
ZNF518A	zinc finger protein 518A	1.22	1.08	1.14
LOC100629165	TOMM20-like protein 1-like	1.01	1.37	1.31
AGTR2	angiotensin II receptor, type 2	2.26	1.56	1.16
LOC100629125	uncharacterized LOC100629125	2.87	3.60	1.08

Table 5. A list of down-regulated common genes after exercise in three horses on microarray

Gene Symbol	Gene name	Log (Fold Change)		
		No. 1	No. 2	No. 3
LOC100051632	similar to c-Fos	-2.17	-2.96	-2.38
LOC100067425	hypothetical LOC100067425	-1.99	-1.99	-1.80
LOC100062643	tetratricopeptide repeat protein 39A-like	-2.56	-1.89	-1.73
LOC100050849	cyclic AMP-dependent transcription factor ATF-3-like	-1.18	-1.50	-1.1
LOC100054003	similar to chromosome transmission fidelity factor 8 protein	-1.28	-1.30	-2.71
LOC100071246	hepatitis A virus cellular receptor 2-like	-1.00	-1.57	-1.15
LOC100065961	tRNA pseudouridine synthase-like 1-like	-1.19	-1.11	-1.15
LOC100054517	transmembrane protein 163-like	-1.30	-1.43	-1.20
LOC100058421	similar to Tubulin beta-5 chain (Beta-tubulin class-V)	-1.20	-1.05	-1.19
LOC100063668	similar to epithelial membrane protein-1	-1.02	-1.57	-1.48
KIR3DL	killer cell immunoglobulin-like receptor with three domains and long cytoplasmic tail	-1.01	-1.32	-1.28
LOC100062983	hypothetical protein LOC100062983	-1.06	-2.03	-1.55
LOC100058582	similar to Lysozyme C, milk isozyme (1,4-beta-N-acetylmuramidase C)	-1.96	-1.04	-1.31
TRPM3	transient receptor potential cation channel, subfamily M, member 3	-4.51	-1.89	-1.18
LOC100147630	similar to DDB1- and CUL4-associated factor 15	-1.20	-2.56	-1.19
LOC100056508	protein Smaug homolog 1-like	-1.47	-3.73	-3.78
LOC100064195	hypothetical protein LOC100064195	-1.39	-1.77	-3.21
EEF1A2	eukaryotic translation elongation factor 1 alpha 2	-1.44	-1.39	-2.14
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	-1.08	-2.31	-1.11
PDE12	phosphodiesterase 12	-1.11	-1.03	-1.06
LOC100066774	similar to CD8 antigen	-1.09	-1.23	-1.40
LOC100066008	tubulin polymerization-promoting protein family member 3-like	-1.55	-1.01	-1.25
ZSCAN2	zinc finger and SCAN domain	-1.45	-1.81	-1.42

	containing 2			
ADAM12	ADAM metallopeptidase domain 12	-1.38	-2.50	-2.20
LMNA	lamin A/C	-1.64	-1.03	-1.27
SLC6A14	solute carrier family 6 (amino acid transporter), member 14	-1.86	-1.22	-1.16
PHLPP1	PH domain and leucine rich repeat protein phosphatase 1	-1.56	-1.40	-1.14

Table 6. Candidate genes for physical performance in the horse on microarray

Gene symbol	LogFC(pre2 vs post2)	LogFC(pre3 vs post3)	LogFC(pre4 vs post4)	Up/Down-regulation
Muscle strength and composition				
ACTN3	0.10735035	0.900885	1.336878	UP
IGF1R	0.16848326	0.060797	1.208437	UP
Muscle metabolism and exercise intolerance				
HIF1AN	-0.4743495	-1.20888	-0.528215	DOWN
GYS1	-0.17807436	-1.376169	-1.028878	DOWN
Haemodynamic and aerobic metabolism capacity				
VEGFA	-0.2651019	-1.041175	-0.052207	DOWN
VEGFA	-1.450016	-0.532534	-0.421213	DOWN
LOC100055952	0.66964245	0.548272	3.835264	UP
AMPD1	0.46920204	0.115521	1.221402	UP
AMPD1	0.3681717	0.046904	1.22941	UP
Tendon and ligament physiology				
DRD1	0.5742254	0.143991	1.244536	UP
DRD1	0.35477638	0.054341	1.317338	UP
DRD1	0.61072063	0.207029	1.267082	UP
DRD1	0.41914368	0.079347	1.247829	UP
DRD1	0.41110992	0.13381	1.303052	UP
DRD3	1.1515098	1.231298	0.012099	UP
5HTT	0.1302824	0.662432	1.27192	UP

Table 7. miRNA list for human exercise specific miRNA on NGS

miR_name	Pre 1	Pre 2	Pre 3	Post 1	Post 2	Post 3	*P*-value
Blood natural killer cells							
eca-miR-130a	210	62	219	61	43	114	0.1744
eca-miR-151-5p	5246	1489	3571	1094	765	2291	0.1574
eca-miR-199a-5p	4	2	2	1	0	0	0.0351
eca-miR-199a-3p	171	26	118	14	13	29	0.1131
eca-miR-221	2731	626	2043	485	551	766	0.1276
Blood mononuclear cells							
eca-miR-130a	210	62	219	61	43	114	0.1744
eca-miR-199b-3p	170	26	118	14	13	29	0.1125
eca-miR-23b	738	274	620	272	145	607	0.3593
eca-miR-221	2731	626	2043	485	551	766	0.1276
eca-miR-199a-5p	4	2	2	1	0	0	0.0351
eca-miR-151-5p	5246	1489	3571	1094	765	2291	0.1574
Neutrophils							
eca-miR-20b	21	4	21	1	1	2	0.0692
eca-miR-93	20116	7998	41161	9781	7706	32752	0.6405
eca-miR-130a	210	62	219	61	43	114	0.1744
eca-miR-151-5p	5246	1489	3571	1094	765	2291	0.1574
eca-miR-22	39610	14095	52463	14317	9806	45707	0.4900
eca-miR-363	644	141	386	84	133	187	0.1596

국 문 초 록

웜블러드 말에서 운동으로 인한 말초혈액의 백혈구 내 microRNA와 mRNA의 발현 영향에 대한 통합분석

김 항 아

(지도교수; 김 용 백)

서울대학교 대학원

수의과대학 수의임상병리학 전공

운동능력은 말에서 가장 중요한 요소로, 사람들은 운동에 필요한 힘,

스피드, 그리고 지구력이 좋은 말들을 키워왔다. 운동 능력은 환경, 관리, 그리고 훈련에 의해 영향을 받지만 근본적인 유전 요소가 중요하다는 것은 오랫동전부터 알려져 왔다. 운동 할 때 몸에서는 생체의 기능이 유지되기 위해 항상성과 적응과정이 일어나지만, 그 기전은 완전히 밝혀지지 않았다. 특히 miRNA는 전사 후의 유전자 발현 조절에 중요한 역할을 하, 운동으로 인해 말의 신체에서 일어나는 변화에 적응하기 위한 miRNA와 mRNA 사이의 조절 네트워크에 대해 조사하였다.

유전자 발현 분석을 위해 운동하기 전과 운동 후, 네 마리 웜블러드 말의 백혈구에서 RNA를 채취하여, 운동 전과 운동 후의 miRNA 발현 차이를 New Generation Sequencing (NGS)를 통해 확인했고, 운동으로 인한 mRNA의 발현 차이는 microarray를 통해 알아보았다. NGS 결과에서는 운동으로 인해 4개의 known miRNA와 2개의 novel miRNA에서 유의 있는 변화가 일어났다. 이 miRNA들을 이용해 어떤 mRNA와 관련이 있는지 targetscan과 miRanda 소프트웨어를 통해 알아 본 결과와 microarray를 이용한 mRNA결과가 매칭되었다. 그 결과, 6개의 miRNA 중 3개와 관련이 있는 5개의 mRNA가 확인되었다.

따라서 본 연구로 밝혀진 운동으로 변화된 말의 백혈구 내 miRNA로 인한 mRNA 조절 네트워크 기전은 운동으로 인한 생리학적 적응과정을 이해하는데 도움을 줄 것으로 판단된다.

주요어:말 / miRNA / mRNA / 운동 / 백혈구

학번:2013-23447