—Original Article—

Profiling of exercise-induced transcripts in the peripheral blood cells of Thoroughbred horses

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Transcriptome analyses based on DNA microarray technology have been used to investigate gene expression profiles in horses. In this study, we aimed to identify exercise-induced changes in the expression profiles of genes in the peripheral blood of Thoroughbred horses using DNA microarray technology (15,429 genes on 43,603 probes). Blood samples from the jugular vein were collected from six horses before and 1 min, 4 hr, and 24 hr after all-out running on a treadmill. After the normalization of microarray data, a total of 26,830 probes were clustered into four groups and 11 subgroups showing similar expression changes based on k-mean clustering. The expression level of inflammation-related genes, including interleukin-1 receptor type II (IL-1R2), matrix metallopeptidase 8 (MMP8), protein S100-A8 (S100-A8), and serum amyloid A (SAA), increased at 4 hr after exercise, whereas that of c-Fos (FOS) increased at 1 min after exercise. These results indicated that the inflammatory response increased in the peripheral blood cells after exercise. Our study also revealed the presence of genes that may not be affected by all-out exercise. In conclusion, transcriptome analysis of peripheral blood cells could be used to monitor physiological changes induced by various external stress factors, including exercise, in Thoroughbred racehorses.

Key words: *exercise, horses, Thoroughbred, transcriptome*

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The Thoroughbred horse is one of the most popular breeds for horseracing, which is a sport that is popular worldwide. The racing performance of Thoroughbred horses, such as speed and stamina, has been improved by over 300 years of selective breeding based on racing records [3, 6, 9, 36].

Transcriptome analyses based on DNA microarrays and RNA sequencing provide a snapshot of actively expressed genes and transcripts under various conditions [4, 19, 29] and can characterize all transcriptional phenomena such as several biological processes in cells. DNA microarray technology allows the investigation of gene expression profiles [28] and has enabled the parallel detection of thousands of genes in a single analysis. Gene expression microarray systems are commercially available for several species, including horses (https://earray.chem.agilent.com/earray/).

A high-quality draft sequence of the horse genome is available through the Broad Institute (http://www.broad.mit.edu/mammals/horse/) as a result of the international efforts on the Horse Genome Project. The Horse Genome Project mapped 2.7 billion base pairs and over 1.1 million single nucleotide polymorphisms (SNPs), and 20,322 protein-coding genes were annotated by the Ensembl pipeline (Ensembl build 52.2b) [34]. These high-quality draft sequences have enabled transcriptome analyses in horses [7, 35].

Skeletal muscle mass is higher in Thoroughbred horses [18, 25] than in other horse breeds at over 55% of total body mass [11], suggesting that this is the major locomotor organ in racehorses. Therefore, skeletal muscle samples collected by muscle biopsy have been used in several transcriptome analyses to identify genes expressed during exercise.

Although transcriptome analyses are useful for under-

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standing the biological processes in skeletal muscle cells that are activated by exercise in humans and horses [18, 25, 33], muscle biopsy for sample collection includes some risk and may cause tissue damage. Liquid biopsy involving the collection of peripheral blood samples has attracted a lot of attention as an alternative to muscle biopsy and has been applied for the diagnosis of cancer in humans [16, 38]. Liquid biopsy lowers the risk of sample collection and thus might be a suitable technique for transcriptome analyses in Thoroughbred racehorses.

In this study, we investigated the influence of exercise on gene expression in peripheral blood cells of Thoroughbred horses by employing DNA microarrays that cover a total of 15,429 genes.

Materials and Methods

Ethics

All the experimental procedures were approved by the Animal Care Committee of the Equine Research Institute of the Japan Racing Association.

Animals

Six Thoroughbred horses (three geldings and three females; age, 5 years; weight, 475–524 kg) were used. The horses ran to exhaustion, following a standardized incremental-step exercise regime (walk, 4 m sec^{-1}; trot, 6 m sec^{-1}; canter, 8 m sec^{-1}, 10 m sec^{-1}, 12 m sec^{-1}, and 13 m sec^{-1}), on a treadmill with an incline of 6%. Venous blood samples were collected from the jugular vein before exercise (T0) and 1 min (T1), 4 hr (T4), and 24 hr (T24) after exercise. All the horses used in this study were healthy and well trained for treadmill exercise.

White blood cell counts

Venous blood (3.0 m*l*) was collected into a heparinized tube. Within 2 hr after collection, white blood cell (WBC) counts were measured using a K-4500 system (Sysmex, Kobe, Japan). WBC counts at T1, T4, and T24 were compared with those at T0 using a paired *t*-test with Bonferroni correction (corrected *P*-value <0.0166).

Microarray analyses

Venous blood samples (2.5 m*l*) were collected in PAXgene Blood RNA Tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.), kept at 18–25°C for 3 hr, and stored at −80°C. Total RNA was extracted using a PAXgene Blood RNA Kit (Qiagen, Valencia, CA, U.S.A.), according to the manufacturer's protocol.

Sample preparation and hybridization were performed using the One-color Microarray-based Gene Expression Analysis protocol (Agilent Technologies, Santa Clara, CA, U.S.A.). Briefly, one-color spike mix was added to 100 ng extracted RNA and labeled with Cy3-CTP using an Agilent Quick Amp Labeling Kit (Agilent Technologies). The labeled cRNA was then fragmented and hybridized on a 44K microarray platform (15,429 genes on 43,603 probes) at 65°C for 17 hr using an Agilent Gene Expression Hybridization Kit (Agilent Technologies). After washing, microarray intensity values were measured by SureScan Microarray Scanner (Agilent Technologies). No protocols for the elimination of hemoglobin mRNA were adopted.

Data analysis

The microarray intensity values were processed by the quantile method using GeneSpring GX12 (Agilent Technologies) in order to normalize the deviation of expression intensities for the inter-microarray error. Then, probe level data were normalized against the expression intensity at T0. Only probes with expression data available in all the 24 microarrays (four time points × six horses) were used for further analysis.

Data obtained at T0, T1, T4, and T24 from six horses were assembled into a matrix. The maximum cluster separation was performed using the K-means method with the Davies-Bouldin Validity Index and repeated for further clustering the subgroups within each group. The analysis was conducted using the cclust package in R v. 3.1.2 (http://cran.at.r-project.org). Next, only the probes that clustered into a common subgroup at each time point in all horses were only selected for further analysis.

Gene annotation

The Basic Local Alignment Search Tool (BLAST) v.2.2.22 of the National Center for Biotechnology Information (NCBI) was used to annotate horse genes based on reciprocal sequence comparisons. All sequence comparisons were performed by testing human genes (27,876 genes) and horse genes (35,847 sequences) as queries and subjects or as subjects and queries, respectively. An e-value threshold of 1.0E-4 was used for the reciprocal sequence comparisons performed with BLAST. A gene identified by the bidirectional best hit was defined as being orthologous between humans and horses.

Gene ontology (GO) analyses

The structure of GO terms (biological processes, molecular functions, and cellular components) was determined using Ensembl 56 (Description and Annotation v. 5). The GO option in GeneSpring GX12 was used to identify the most significant biological process, molecular function, and cellular component (corrected *P*-value <0.1).

Table 1. White blood cell (WBC) counts at T0, T1, T4, and T24

Parameter	T0	T1	T4	T24
WBC ($\times 10^2/\mu l$)	68.3 ± 12.1	88.0 ± 14.8	82.3 ± 11.3	60.3 ± 11.1

T0, before exercise; T1, 1 min after exercise; T4, 4 hr after exercise; and T24, 24 hr after exercise. Values are expressed as means ± standard deviation (n=6).

Results

WBC counts

WBC counts were higher at T1 and T4 than at T0; however, the differences were not significant. Changes in WBC counts were not uniform among horses; WBC counts reached a peak at T1 in four horses and at T4 in two horses (Table 1).

Analysis of expression profiles

After the normalization of microarray data, a total of 160,980 probes were available for further analysis. The probes were clustered using the Davies-Bouldin Validity Index into four groups based on gene expression patterns (CL1, downregulation at T4; CL2, upregulation at T4; CL3, upregulation at T1; and CL4, no change at any time point) and then into 11 subgroups by further clustering within each group. The number of probes and genes clustered into subgroups were as follows: two subgroups for CL1 (CL1A, one probe, one gene; and CL1B, 11 probes, six genes); two subgroups for CL2 (CL2A, 48 probes, 21 genes; and CL2B, 565 probes, 205 genes); two subgroups for CL3 (CL3A, one probe, one gene; and CL3B, 20 probes, nine genes); and five subgroups for CL4 (CL4A, two probes, two genes; CL4B, 30 probes, 21 genes; CL4C, 13 probes, five genes; CL4D, two probes, one gene; and CL4E, one probe, one gene) (Fig. 1). The other probes and genes were not clustered into a common group based on the used criteria.

The expression of genes in CL2A was higher by 4.5- to 24.1-fold at T4 than at T0 (Table 2). Specifically, the expression of the inflammation-related genes, interleukin-1 receptor type II (*IL-1R2*), matrix metallopeptidase 8 (*MMP8*), protein S100-A8 (*S100-A8*), and serum amyloid A (*SAA*) was higher by 14.2-fold, 12.6-fold, 15.8-fold, and 5.7-fold at T4 than at T0, respectively. In CL3A, the expression of c-Fos (*FOS*) was higher by 3.7-fold at T1 than at T0. The expression of genes in the other subgroups remained almost stable, indicating that they were not affected by exercise.

Gene ontology

GO term enrichment analysis was performed to explore the association of the identified subgroups with specific mechanisms. CL2A showed a significant correlation with biological processes and molecular functions (Table 3), and CL4B showed a significant correlation with cellular components (data not shown). In particular, CL2A was correlated with GO:0030574, GO:0032963, GO:0044243, GO:0044259, and GO:0044236 for biological processes, as well as with GO:0004222, GO:0008237, and GO:0005053 for molecular functions.

Discussion

In the present study, the transcriptome analysis of peripheral blood cells in Thoroughbred horses using DNA microarrays allowed us to identify genes whose expression levels were altered after all-out exercise. These genes were clustered into four groups (CL1, CL2, CL3, and CL4) and 11 subgroups (CL1A, B; CL2A, B; CL3A, B; and CL4A–E). CL2A included *IL-1R2*, *MMP8*, *S100-A8*, and *SAA*, which had significantly higher expression levels at T4 than at T0. IL-1R2 suppresses the inflammatory cytokine IL-1 [8, 23]; MMP8 acts as a collagenase in neutrophils [15, 26]; and protein S100-A8 is known as a neutrophil membrane surface protein involved in the acute inflammatory response [27].

Previous studies have demonstrated that *IL-1R2*, *MMP8*, *S100-A8*, and *SAA* are expressed in neutrophils at the early stage of the inflammatory response. Therefore, it was assumed that WBCs, when stimulated by intense exercise, could activate the expression of these genes. It is well known that muscle damage induced by exercise is closely related to inflammatory responses in humans [22, 31]; thus, the increased expression levels of *IL-1R2*, *MMP8*, *S100-A8*, and *SAA* observed in the present study might be related to muscle damage induced by all-out exercise. The increased expression level of *SAA* after exercise might reflect an acute inflammatory response, since muscle injuries can cause a moderate increase in blood SAA concentration in horses [32].

The secretion of catecholamine and cortisol depends on exercise intensity and time [12, 17, 39] and leads to an increase in the number of neutrophils. Thus, the upward trends of WBC counts after exercise might be related to the secretion of catecholamine and cortisol. However, the increase in WBC counts post exercise was not significant (Table 1), although several genes in CL2A showed dynamic changes in expression level (Table 2). These findings

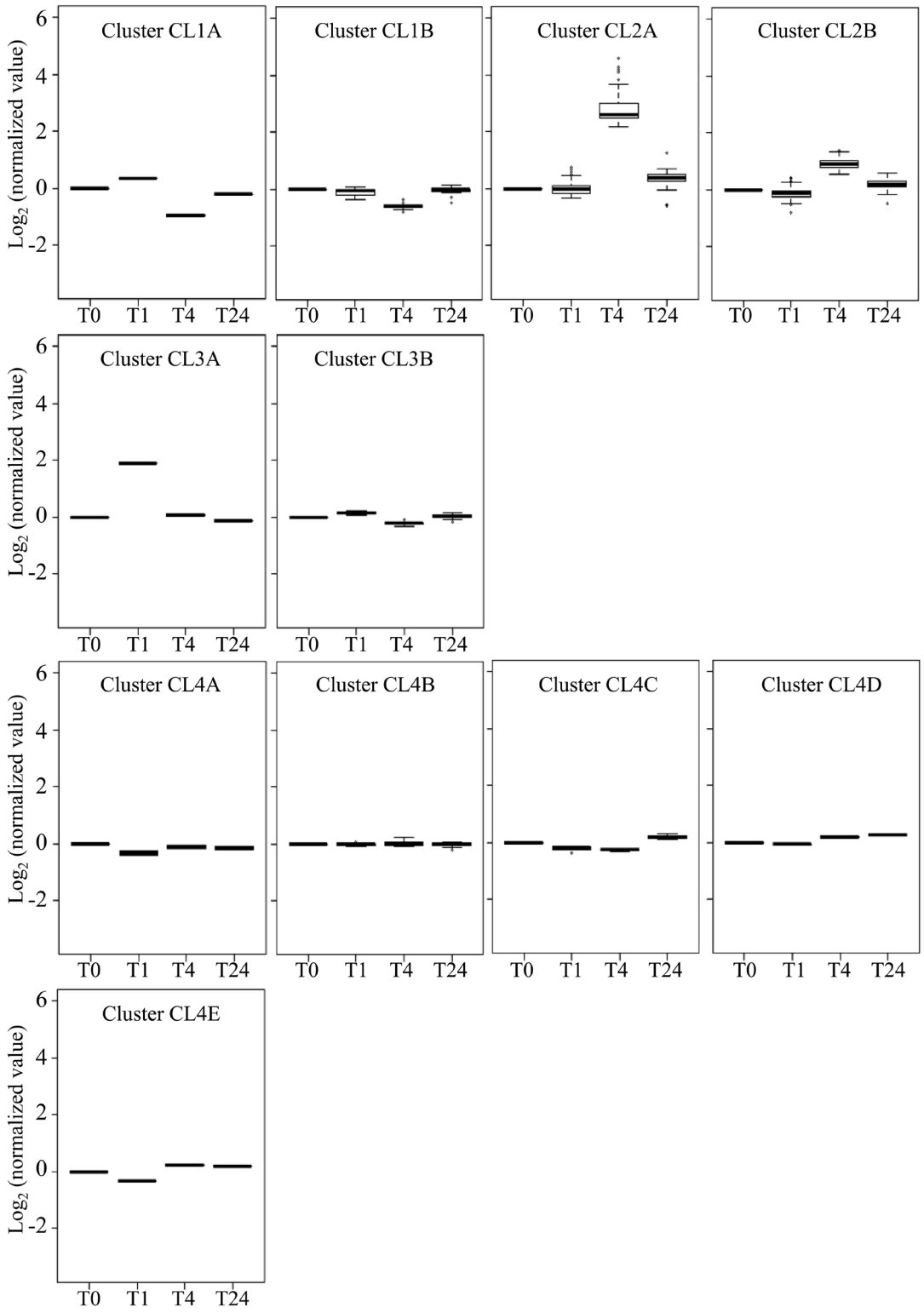

Fig. 1. Normalized gene expression in each gene cluster at T0, T1, T4, and T24. A total of 160,980 probes were clustered into four groups based on gene expression pattern (CL1, downregulation at T4; CL2, upregulation at T4; CL3, upregulation at T1; and CL4, no change at any time point) and then into 11 subgroups for maximum cluster separation (CL1A, one probe; CL1B, 11 probes; CL2A, 48 probes; CL2B, 565 probes; CL3A, one probe; CL3B, 20 probes; CL4A, two probes; CL4B, 30 probes; CL4C, 13 probes; CL4D, two probes; and CL4E, one probe). T0, before exercise; T1, 1 min after exercise; T4, 4 hr after exercise; and T24, 24 hr after exercise. The scale of the vertical axes is the binary logarithm of the normalized microarray intensity value.

Table 2. Genes in peripheral blood cells highly responsive to exercise stimulation in Thoroughbred racehorses

Clusters	Genes	Fold change
CL2A	resistin-like	24.1
	protein S100-A8-like	15.8
	interleukin-1 receptor type II	14.2
	haptoglobin-like	12.8
	matrix metallopeptidase 8 (neutrophil collagenase)	12.6
	TBC1 domain family member 2A-like	8.2
	scrapie-responsive protein 1-like	8.2
	Thy-1 cell surface antigen	8.0
	myc target 1	7.2
	peptidoglycan recognition protein 1	6.8
	2-acylglycerol O-acyltransferase 1-like	6.7
	uridine phosphorylase 1-like	6.2
	protein S100-A9-like	5.9
	serum amyloid A1	5.7
	uncharacterized LOC100066570	5.7
	matrix metallopeptidase 1 (interstitial collagenase)	5.7
	matrix metallopeptidase 27	5.3
	maltase-glucoamylase (alpha-glucosidase)	5.2
	interferon-induced transmembrane protein 1-like	5.2
	inositol monophosphatase 2-like	4.8
	n-formyl peptide receptor 2-like	4.5
CL3A	proto-oncogene c-Fos-like	3.7

Fold changes were calculated as T3/T0 for CL2A and T2/T0 for CL3A using normalized expression data (see Fig. 1). T0, before exercise; T1, 1 min after exercise; T4, 4 hr after exercise; and T24, 24 hr after exercise. The fold change of each gene was calculated as the average of the fold change in probes that were annotated to the same gene.

Table 3. Functional gene groups identified in the equine blood transcriptome

AmiGO accession	Term	Corrected *P*-value
CL2A		
BP GO:0030574	collagen catabolic process	6.66E-04
BP GO:0032963	collagen metabolic process	6.66E-04
BP GO:0044243	multicellular organismal catabolic process	6.66E-04
BP GO:0044259	multicellular organismal macromolecule metabolic process	6.73E-04
BP GO:0044236	multicellular organismal metabolic process	1.02E-03
MF GO:0004222	metalloendopeptidase activity	2.06E-02
MF GO:0008237	metallopeptidase activity	8.94E-02
MF GO:0005053	peroxisome matrix targeting signal-2 binding	1.84E-02

BP, biological process; MF, molecular function.

suggested that changes in the intensity of probes were probably caused by gene expression rather than the migration of neutrophils.

The expression of *MMP-1* and *IL-8* has been reported to increase in endurance competitions [5], in which horses run long distances of 50 to 200 km in a day. Although all-out running and running during endurance competition differ in intensity, both types of exercise induce the upregulation of similar inflammation-related genes in peripheral blood cells, suggesting that these genes might be involved in exercise-induced stress responses in horses.

Our findings indicate that transcriptome analysis using peripheral blood samples collected via liquid biopsy is a useful analytical approach to understand changes in body condition induced by various external stressors, including exercise. Although many laboratory tests have been developed for evaluating body condition in racehorses [10, 13], the genes identified in the present study might be good biomarkers for assessing body condition during exercise.

Evaluation of body condition in racehorses is important

for ensuring their health and welfare, but doping control is also a critical issue for ensuring fair play in horseracing [37]. Gene doping, defined as the "abuse or misuse of gene therapy", has recently raised concerns, since many medical technologies have been developed for cellular and gene therapy. Although the detection of low-molecular-weight substances used in conventional doping is possible by chemical analyses, such as mass spectrometry [14], gene doping that includes the introduction of genes using virus vectors is more difficult to identify.

Proteome analysis, which measures the expression of proteins in different tissues, is a similar approach to transcriptome analysis [24, 30]. Proteome analysis has been performed using liquid chromatography-tandem mass spectrometry for monitoring gene and protein doping in horses [1]; however, when conducting proteome analysis using peripheral blood, it is necessary to remove the excess amounts of albumin, which may otherwise mask proteins expressed at lower levels [20]. Transcriptome analysis using peripheral blood could also be used for the detection of gene doping by identifying changes in gene expression due to alien genes introduced into WBCs or viral vectors that are likely to induce immune responses [2, 21]. Of these two methods, the latter has a potential advantage, since it does not require the removal of albumin.

In the present study, we identified several genes with significantly increased expression levels after high-intensity exercise. We, therefore, suggested that the transcriptome analysis using peripheral blood might be used for monitoring physiological changes induced by various external stress factors, including exercise, in Thoroughbred racehorses.

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