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Comparison of the proteomes in sera between healthy Thoroughbreds and Thoroughbreds with respiratory disease associated with transport using mass spectrometry-based proteomics

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In the past decade, mass spectrometry has become an important technology for protein identification. Recent developments in mass spectrometry allow a large number of identifications in samples; therefore, mass-spectrometry-based techniques have been applied to the discovery of biomarkers. Here, we conducted a proteomic study to compare the proteomes in sera between healthy Thoroughbreds and Thoroughbreds with respiratory disease associated with transport (RDT). We found that four proteins, apolipoprotein F, lipopolysaccharide binding protein, lysozyme and protein S100-A8, were upregulated, while keratin 1 was downregulated in the RDT group. It is assumed that inflammation and immune response are involved in the changes of these proteins. The findings suggested that these proteins are potentially useful for elucidating the mechanism of development of RDT.

Key words: *liquid chromatography-tandem mass spectrometry, proteome, respiratory disease associated with transport, Thoroughbred*

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Monitoring serum protein concentrations can provide useful information for making a diagnosis, and the changes are helpful in determining the conditions of horses. In the field of equine veterinary medicine, serum proteins such as C-reactive protein, fibrinogen, haptoglobin and serum amyloid A protein (SAA) are common indicators for inflammatory diseases [14, 17, 22, 26]. To measure these proteins, immunoassays such as ELISA are used because of their high sensitivity and specificity [4, 23, 27]. However, they are not suitable methods for simultaneous detection of multiple proteins because they are labor-intensive and not cost-effective.

Measurement of proteins using mass spectrometry has proven to be an indispensable tool in biological research. This technology provides comprehensive data which enable us to trace the various changes of proteins to be traced. With the recent dramatic improvement of its sensitivity, a single shot analysis generates more than a hundred protein identifications, and quantitative data for them are obtainable [11]. Taking this proteomic approach, many studies have been conducted to discover clinically relevant biomarkers [9]. In horses, a recent serum proteomic study reported that candidate diagnostic biomarkers have been discovered for equine glandular gastric disease [21]. Furthermore, proteomic analysis of equine chondrocytes provided useful information for determining the onset and progression of equine osteochondritis [3].

Respiratory disease associated with transport (RDT) is known as a malady typically found in horses after long-distance transport. It is caused by stress factors, including some irritants, such as particulate hay, dust, carbon and exhaust chemicals [15]. RDT can be a serious issue for the horseracing industry since it can affect training and racing schedules. Additionally, in some cases it can lead to fatal diseases, such as pneumonia or pleuritis [5].

The changes of serum proteins are thought to be impor-

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tant for a better understanding of the mechanism of development of RDT. Previous studies reported that haptoglobin and SAA were upregulated after long-distance transport [2, 13]. However, aside from these common inflammatory markers, proteomic data for serum proteins in RDT are not available. For the above reason, we performed a comprehensive proteomic analysis of the serum proteins in healthy Thoroughbreds and Thoroughbreds with RDT using a mass-spectrometry-based method and compared the levels of expression of serum proteins between the two groups.

Approval from the Animal Care and Use Committee of the Japan Racing Association (20180425; approved on 25 April 2018) and the consent of the owners were obtained for all experiments in this study. Blood samples were collected from 36 clinically healthy Thoroughbreds (16 males, 18 females and 2 geldings; aged from four to ten) and from 12 Thoroughbreds with RDT (6 males, 5 females and 1 gelding, aged from two to five). For Thoroughbreds with RDT, samples were collected from horses that presented fever with a recent history of long-distance transport and were diagnosed as having conditions in need of treatment by JRA veterinarians. The average duration of transport was approximately 18 hr, and all samples were collected within 24 hr after transport. The average duration of transportation was approximately 18 hr. For serum separation, a conventional blood collection tube was used. Centrifugation was carried out at 1,940 × *g* for 10 min. After centrifugation, the serum samples were stored at −80°C until proteomic analysis.

The proteins in serum samples were enzymatically digested with trypsin. To 5 μl of serum sample, 10 μl of 10 mM dithiothreitol and 5 μl of 5% (w/v) sodium deoxycholate were added and the mixture was incubated at 80°C for 10 min. The sample was reduced by the addition of 5 μl of 50 mM dithiothreitol and incubated at 60°C for 20 min. Then, the sample was alkylated by the addition of 5 μl of 100 mM iodoacetamide and incubated at room temperature for 30 min in the dark. Five microliters of 100 μg/m*l* trypsin (from porcine pancreas, mass spectrometry grade, Fujifilm Wako, Tokyo, Japan) in 100 mM ammonium bicarbonate was added, and incubation was carried out at 37°C overnight. Then, 5 μl of 10% (v/v) trifluoroacetic acid (TFA) was added to terminate the enzyme reaction. Five hundred microliters of ethyl acetate was added, and after shaking for 1 min and centrifugation at 14,000 × *g* at 4°C for 5 min, the organic phase was removed. The sample solution was dried under vacuum and reconstituted in 200 μl of 4% (v/v) acetonitrile in 0.1% (v/v) TFA, and the digested peptides were purified by SPE C-tip (Nikkyo Technos, Tokyo, Japan) according to the manufacturer's protocol. The obtained peptides were eluted from the SPE C-tip with 5 μl of 60% (v/v) acetonitrile in 0.1% (v/v) TFA and diluted with 35 μl of 4% (v/v) acetonitrile in 0.1% (v/v) TFA. Then, 5 μl of sample was injected into a liquid chromatograph coupled with a mass spectrometer (LC-MS/MS). Peptides were analyzed by an EASY-nLC 1200 system coupled with a Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.) with Acclaim PepMap 100 C18 columns (75 μm × 20 mm, 3 μm particle, 100 Å pore size, Thermo Fisher Scientific). An elution gradient over 120 min from 1.5% to 40% of acetonitrile was used on an Acclaim PepMap RSLC C18 analytical column (75 μm × 500 mm, 2 μm particle, 100 Å pore size, Thermo Fisher Scientific) at a flow rate of 300 n*l*/min. Data acquisition was executed with a data-dependent analysis with 27 V of collision energy and the scan range set between 400 and 2,000 *m/z*, followed by a data-dependent MS/MS scan with a mass range between 200 to 2,000 *m/z* of the 20 most intense ions.

The data files were processed by Proteome Discoverer version 2.2 (Thermo Fisher Scientific) using the UniProt database with *Equus caballus* taxonomy restriction (49800 computationally analyzed sequences in total, downloaded on 27 December 2019). Proteins were successfully identified based on the MS and MS/MS spectra with a false discovery rate (FDR) for peptides of 0.05. Quantification was performed for identified proteins using the sum of the precursor ion intensities. Based on the quantification results, a principal component analysis (PCA) was performed, and the Mann-Whitney *U*-test was conducted on the identified proteins in samples to compare the proteomes of healthy Thoroughbreds and Thoroughbreds with RDT.

Sera from two independent groups, the healthy group (n=36) and RDT group (n=12), were prepared and analyzed; 239 proteins were identified and quantified throughout our experiment in total. PCA revealed a clustering of RDT group members compared with the healthy group in Fig. 1. One Thoroughbred in the healthy group was considered a potential outlier on PC2 (4.5%); however, PC1 (16.7%) showed a clear separation between the healthy and RDT groups. Therefore, all samples were included in the following analysis. To discover the candidate proteins manually, a ± 2-fold change in relative protein expression was set as a cutoff. The Mann-Whitney *U*-test was applied to calculate the *P*-value for comparisons of each protein between the two groups. An adjusted $P<0.05$ was considered statistically significant following multiple testing correction. A summary is graphically depicted in the form of a volcano plot in Fig. 2. Out of more than two hundred identified proteins, comparisons between the RDT and healthy groups revealed four proteins, apolipoprotein F, lipopolysaccharide binding protein (LBP), lysozyme and protein S100, that were significantly upregulated and one protein, keratin 1, that was significantly downregulated in the RDT group. The fold-changes and *P*-values are listed in Table 1.

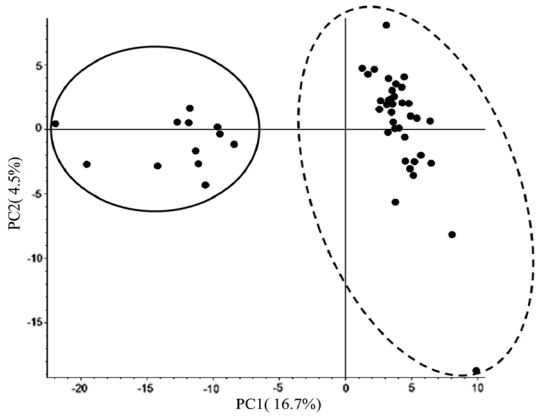

Fig. 1. The respiratory disease associated with transport (RDT) and healthy groups were distinctively separated on principal component analysis (PCA). Solid circles indicate the RDT group (n=12), and dotted circles indicate the healthy group (n=36).

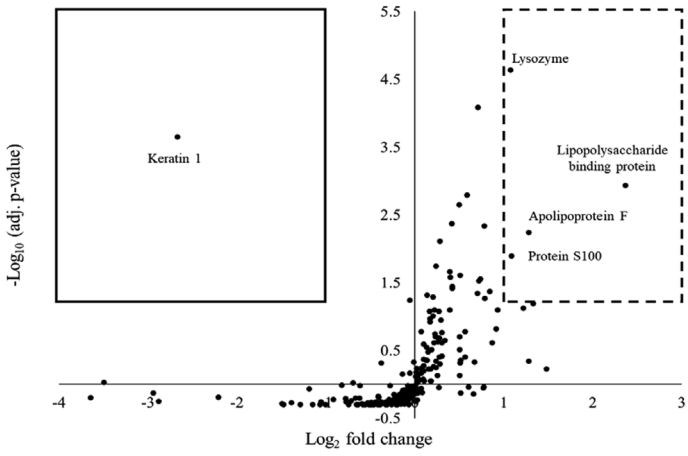

Fig. 2. Volcano plot showing differentially expressed proteins between the respiratory disease associated with transport (RDT) and healthy groups. Significantly upregulated proteins in the RDT group are surrounded by a dotted line, and the downregulated protein is surrounded by a solid line.

Table 1. Differentially expressed proteins (>2-fold change of intensity and *P*-value <0.05) for the healthy vs. respiratory disease associated with transport (RDT)

Accession number	Protein	Healthy vs. RDT	
		Log_2 fold change	*P*-value
F7B504	Apolipoprotein F	1.262	0.00580
F7C0D2	Lipopolysaccharide binding protein	3.293	0.00120
F7CU94	Lysozyme	1.898	0.00002
F6SN37	Protein S100-A8	1.225	0.01280
F7B504	Keratin 1	−2.669	0.00020

Information concerning the molecular functions of the detected proteins was obtained from human orthologues. Apolipoprotein F is classified as a component of plasma lipoprotein and known as a minor apolipoprotein which inhibits lipid transfer protein (LTIP) [24]. It has been reported that apolipoprotein F could be a potential biomarker for liver diseases, such as non-alcoholic fatty liver disease or liver fibrosis, in humans. This may suggest that the upregulation of apolipoprotein F indicates disorders in liver lipid metabolism caused by RDT [12].

LBP and lysozyme are already known as immune-responsive proteins. LBP binds with lipopolysaccharide, a glycolipid present on the outer membrane of Gram-negative bacteria, to elicit immune responses. A previous study reported that horses with colic showed great increases in serum LBP [18]. Lysozyme is an antimicrobial enzyme that catalyzes the hydrolysis of peptidoglycan, a component of the Gram-positive bacterial cell wall. It is normally secreted by macrophages and neutrophils in response to bacterial infection [1, 16]. It has been reported that the plasma level of lysozyme is associated with inflammatory conditions such as experimentally induced bacterial endometritis and uterine flushing [19]. Taken together, an increase in both proteins is seemingly induced by bacterial infections. These findings are consistent with the fact that the major causative factor of RDT is a respiratory tract infection with bacteria such as *Streptococcus zooepidemicus* [7].

Protein S100, a family of calcium-binding proteins, has a broad range of intracellular and extracellular functions, such as calcium balance, cell apoptosis, migration, proliferation, differentiation, energy metabolism and inflammation [6]. For an inflammatory response, protein S100 acts as a damage-associated molecular pattern (DAMP) released from damaged or stressed cells [8]. Therefore, the extracellular S100 protein level could be a possible marker for serious symptoms or activation of immune cells. It was previously reported that protein S100-A8 was upregulated after exercise in equine blood cells at the transcriptomic level [22]. Regarding the results from the previous report, the upregulated protein S100-A8 might have been caused by

leakage from peripheral blood cells as a result of hemolysis.

Keratin 1 is a member of the keratin family known as cytoskeletal proteins and may regulate the activity of protein kinases [26]. In a human study, keratin 1 reportedly participated in an inflammatory network, and the absence of keratin 1 caused the upregulation of protein S100-A8 [20]. This finding is consistent with our results; however, contamination during sample preparation should be considered as a possibility, since keratin is a well-known contaminant originating from skin, hair and dust in the laboratory [10].

Previous studies reported that common inflammatory markers, such as haptoglobin and SAA, were upregulated after transport [2, 13]. In this study, haptoglobin did not show a significant change between the healthy and RDT groups. The influences of sampling time, duration and distance of transport and physical condition before transport could be possible reasons for this.

The fold-changes of the five proteins that showed significance in this study ranged from approximately two-fold to eight-fold. On the other hand, SAA showed the highest elevation (an approximately 250-fold increase) among all detected proteins in the RDT group compared with the healthy group, even though SAA did not meet the criteria for protein identification due to the lack of unique peptides. To ensure the presence of SAA, immunoassays such as ELISA are necessary for the identification of SAA; however, the comprehensive proteomic results indicated that SAA might be the most responsive biomarker to RDT.

In this study, applying the mass spectrometry-based proteomic method to Thoroughbred serum enabled us to measure more than 200 proteins simultaneously, and the result showed that five candidate biomarkers (apolipoprotein F, LBP, lysozyme, protein S100-A8 and keratin 1) were significantly upregulated in the RDT group. Considering the molecular functions of these five proteins, inflammation and immune response are likely to be involved in RDT. This assumption corresponds with the pathology of RDT, such as respiratory tract infection with bacteria [15]. Additionally, to the best of our knowledge, the changes in these five proteins have not been reported in RDT, whereas many candidate biomarkers for RDT, including blood biochemistry parameters such as packed cell volume and white blood cells, have been investigated [13, 25]. For the above reasons, mass spectrometry-based proteomic analysis would be a powerful tool for new biomarker discovery, and the five proteins found in this study can be new diagnostic biomarkers for RDT. Furthermore, monitoring these proteins might be helpful in elucidating the mechanism of development of RDT. However, it should be noted that it is still unclear whether the five proteins are RDT-specific markers, so comparative proteomic studies between RDT and other inflammatory diseases must be conducted. Furthermore, in order to prove the availability of the five proteins as diagnostic markers, a larger number of samples is needed for further validation.

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