Transcriptome blood profile of the Yili horse before and after training

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Abstract

Proper training improves the horse's performance. In the present study, blood transcriptome of the Yili horse was analysed before and after training. Overall, six Yili horses were trained for 3 months; blood samples at rest were collected before and after the training period. Transcriptome libraries were created from the blood samples using high-throughput sequencing; Gene Ontology functional enrichment and Kyoto Encyclopaedia of Genes and Genomes pathway analyses were performed for differentially expressed genes to identify the pathways and candidate genes associated with training and locomotor performance. In total, transcriptome analysis screened 33 significantly differentially expressed genes; of these, 23 and 10 were up-regulated and down-regulated after training, respectively. These differentially expressed genes were significantly enriched in metabolic pathways associated with exercise physiology, including sphingolipid metabolism, tryptophan metabolism, and the cGMP-PKG signalling pathway. The results of this study provide a theoretical basis for exploring the molecular mechanisms underlying locomotor performance of the Yili horse as well as a novel insight into the kinematics of this breed.

Equine, daily training, differentially expressed genes

Conditioning training accelerates the horse's metabolism and improves coordination between respiration, blood circulation, thermoregulation, excretion, and other vital bodily processes (Castejon-Riber et al. 2017). In higher animals, the phenotypic and physiological changes following exercise training have been studied extensively, with adaptive responses to training favouring exercise-induced changes in the muscle load, energy requirements, and calcium flux. During exercise, oxygen demand in the body increases substantially. Therefore, the most important aspect of training is improving oxygen transport in the body by enhancing vascular endothelial function and increasing mitochondrial function (Jacobs et al. 2011).

Transcriptome sequencing, also known as RNA-seq, facilitates comprehensive analysis and quantification of all RNA types expressed in tissues and/or cells (Hrdlickova et al. 2017). Ropka-Molik et al. (2017) performed blood transcriptome analysis in 12 Arabian horses before and after training and found that interleukins (*IL-6ST*, *IL-6R*, and *IL-7R*) were expressed and integrin (*ITGA4*) was significantly up-regulated, thereby laying the foundation for further studies on genes related to athletic performance in Arabian horses. Farries et al. (2019) quantified gene expression in skeletal muscle of 111 Thoroughbred horses (47 stallions; 64 mares) by RNA-seq and identified genes (*SLC2A1*, *SELENBP1*) that may play a role in muscle and motor performance in Thoroughbreds. Ekici and Ozmen (2020) investigated microRNA expression in the saliva of Arabian horses before and after a race and suggested that the lipid metabolism of Arabian horses is influenced by ecamiR-33a and its target gene, thereby affecting the energy supply of the horses during racing.

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The Yili horse is a light riding and driving horse from the north-western region of China, also used for meat and milk production. Few studies have focused on the mechanism underlying the locomotion of the Yili horse (Zeng et al. 2019) and the effect of training on the blood transcriptome of this breed remains unclear. Therefore, in the present study, transcriptome sequencing technology and real-time fluorescence quantification were used to analyse the differentially expressed genes (DEG) in the blood transcriptome of Yili horses before and after training. This study will enrich our understanding of the molecular genetics and regulatory mechanisms of the Yili horse, thereby helping to ensure the welfare of the breed and providing a basis for early breeding and training programs.

Materials and Methods

Animals

A total of six 3-year-old Yili horses (geldings, not trained for speed racing) from Zhaosu Stud, Yili Kazakh Autonomous Prefecture, Xinjiang Uygur Autonomous Region, China, were selected for the study. All horses were bred at the same level and were of similar body size (body height: 145.85 ± 1.68 cm; body length: 141.22 ± 2.90 cm; chest circumference: 158.67 ± 1.63 cm; canno round: 18.75 ± 0.88 cm), in good health, and uniformly trained.

All procedures in this study were approved by the Animal Experiment Ethics Committee of the Xinjiang Agricultural University (protocol permit number: 2022058, 2 March 2022).

Table 1. Tuning training plan for special speed competition.

Time/d	Training content
1–7	Conditioning circle training for 30 min/day; complete saddle preparation training and mounting training for horses.
8–14	Conditioning circle training for 30 min/day; thereafter, both left and right ligament. Riding training for 1,200 m/day.
15–21	Tune-up lap training for 30 min/day and riding for 1,200 m/day in track trot ride training to acclimatize to the track.
22–28	Conditioning circle training for 30 min/day; riding training trot for 1,200 m/day. Organize a 1,600-m test race.
29–63	Conditioning circle training for 20 min/day; riding training (trotter + canter) for 1,200 m/day.
64	Training as usual; organize a 1,600-m test race.
65–92	Conditioning circle training for 20 min/day; riding training (trotter + canter) for 1,200 m/day.
93	Training as usual; organize a 1,600-m test race.

Study design

The experiment was conducted on a sandy track (width: 28 m; perimeter: 2,000 m). The conditioning training program used in the experiment was developed by Zhaosu Horse Farm (see Table 1 for the training plan). The horses were trained in the morning. At the beginning of the training, the horses were warmed up with a 20-min saddle preparation walk before training. In the middle and at the end of the training, the horses were warmed up with a 30-min saddle preparation walk. In the afternoon, the horses were allowed to move freely and were brushed.

Blood of the six horses was collected from their jugular vein at rest at A1 (the period before training) and at A2 (the period after three months' training). Specifically, 0.6 ml of blood was collected in a 5 ml blood sampling vessel without additives, and then blood was mixed in proportion with TRIzol (Thermo Fisher Scientific, Shanghai, China). This mixture was placed in a 5 ml frozen storage tube, which was marked and sealed with sealing film. Each sample was divided into 4 tubes, rapidly frozen in liquid nitrogen, and stored in liquid nitrogen at −196 °C until used for total RNA extraction.

A 1,600 m speed race was organized every month, and a stopwatch was used to record the performance of the horses in each race.

Total RNA extraction and library creation

Total RNA was extracted from whole blood using a TRIzol Reagent Extraction Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. RNA quality was assessed using 0.8% agarose gel; RNA purity following extraction (OD_{260}/OD_{280} ratio) was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). RNA concentration was accurately quantified and RNA quality was interpreted using an Agilent 4200 system (Agilent Technologies, Shanghai, China). An mRNA-seq library was constructed; the library was initially quantified using Qubit 3.0 software, following which the effective concentration of the library was accurately quantified using quantitative PCR (qPCR). After the quality control assessment of the library, PE150 double-end sequencing was performed using an Illumina NovaSeq 6000 platform (Illumina [China] Scientific Equipment, Shanghai, China).

RNA quality testing and data quality assessment

The blood of Yili horses for transcriptome analysis was collected separately before and after training; the preliminary quantification of RNA concentration using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) revealed that the OD260/OD280 ratio of the RNA samples was 1.7–2.0 and that the RIN values were all > 9. The RIN value, also known as RNA integrity number, is a digital parameter to represent RNA integrity. Therefore, the extracted samples exhibited high purity and good integrity of total RNA, thereby meeting library sequencing requirements.

RNA-seq data processing and analysis

Following high-throughput sequencing of the cDNA libraries using the Illumina NovaSeq 6000 platform (Illumina [China] Scientific Equipment), use Trimmatic software to filter the raw data output (N reads-containing filter, low-quality reads filter, and splice-containing reads filter). Valid data (clean reads) were obtained after the abovementioned filtering; subsequent analysis was performed based on the clean reads. The sequencing data of each sample were compared and annotated with the equine genome (EquCab3.0) published in the NCBI Genome database (https://www.ncbi.nlm.nih.gov/genome/?term=Equus+caballus) using the HISAT2 software.

Transcriptome sequencing library preparation

After the QC procedure, the RNA with poly-A in eukaryotic total RNA was enriched by TIANSeq mRNA Capture Kit (Tiangen, Beijing, China). Then, using the captured RNA as the starting sample, TIANSeq Fast RNA Library Kit (Illumina [China] Scientific Equipment) was used to construct the transcriptome sequencing libraries. Briefly, the transcriptome sequencing library was constructed through RNA randomly fragmentation, cDNA strand 1/strand 2 synthesis, end repair, A-tailing, ligation of sequencing adapters, size selection and library PCR enrichment.

Differential gene expression analysis was performed using edgeR on 6 samples each in A1 and A2 groups (12 samples in total). Further, *P* values were determined according to the model and corrected for multiple hypothesis testing to obtain q values. Differentially expressed genes were screened and selected using the q value combined with fold change, and the criteria for determining differential expression were as follows: $|\log_2(\text{Fold Change})| > 1$ and q value < 0.05. Subsequently, differentially expressed genes were analysed using the Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) (September 1st, 2021) databases to obtain information on annotation enrichment.

Statistics and data analysis

The obtained data were digitally processed, organized, and summarized using Excel. Independent sample *t*-tests were performed on the test match scores of different training stages using the SPSS 26.0 software; the results were expressed as mean ± standard error values. Multiple comparisons were performed using Duncan's test. *P* values of < 0.05 and < 0.01 were considered to indicate significance and high significance, respectively.

Real-time fluorescence qPCR

Seven differentially expressed genes were randomly selected from the transcriptome sequencing results for real-time quantitative reverse transcription PCR (qRT-PCR) analysis (ABI 7900HT Fluorescence Quantitative Analyser, Shanghai, China) with *GAPDH* being used as the internal reference gene. Gene primers were designed according to Primer 3.0 and synthesized by Xinjiang Compson Biotechnology Co., Ltd. (Xinjiang, China). Reverse transcription of RNA into cDNA was performed using a Reverse First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Specifically, 1 µl of upstream and downstream primers (10 µM/l) were added to 20 µl of the reaction system containing 5 µl of 2 × S6 Universal SYBR qPCR Mix (NovaBio, China, Shanghai) and 1 µl of cDNA. The reaction conditions were as follows: predenaturation at 95 °C for 30 s, followed by 40 cycles of amplification at 95 °C for 10 s and at 60 °C for 30 s. The $2^{-\Delta\Delta CT}$ method was used for relative quantitative analysis of the data; the results were used to evaluate the accuracy and repeatability of transcriptome sequencing (Table 2).

Table 2. Primers used in real-time fluorescence quantitative PCR.

Gene	Primer sequences	Product size (bp)	Annealing temperature (°C)
DDC	CCGAAGCCAGCACATCTCAT	84	60.32
	GACTGGGCTTTGTTGGAGGT		
PRDM16	CCCCCACATCCCAACAGAAA	208	60.07
	ACGGTGAAACTCAGTGACCG		
PTCD3	GGCCCCCAGTTGCAGATTT	165	60.33
	GCAGCTGTGGTATCCCTGTT		
CTSH	TCCGTGAGCCCCTTAGAGAA	98	59.97
	AACGTCTGTAGCCTGTGGTG		
IL1R2	GTGTCTATGGAGGATGCGGG	73	59.93
	TGATGTTGACTCGTGTGCCA		
CASR	TTCTCCAGGGAGGTGCCTTT	133	60.40
	TCTCATCGCTGTACTCCCCA		
VAT1L	AGTTGCCAACCTCCGAGAAG	170	59.93
	TGTCAAAGAGGTGGGTCACG		

Results

Before training, the horses' performance in the 1,600 m speed race was 135.09 ± 3.66 s; after training, the horses' performance in the 1,600 m speed race was 123.86 ± 2.74 s. The race time after the 1,600 m speed race training was significantly lower than that before training ($P < 0.05$).

Screening of differentially expressed genes

In total, the transcriptome analysis screened 33 differentially expressed genes, including 23 up-regulated and 10 down-regulated genes, by comparing the sequencing data of the A1 and A2 groups (Table 3; Plate V, Fig. 1).

The GO terms used for the functional annotation of differentially expressed genes in the pre- and post-training blood transcriptomes of the Yili horse were classified into biological process, cellular component, and molecular function, with adjusted P value of < 0.05 as the significance level. These genes were significantly enriched in the following terms: neuropeptide catabolic process, peptide metabolic process, positive regulation of ion transmembrane transporter activity, aromatic L-amino acid decarboxylase activity, L-dopa decarboxylase activity, MHC class I receptor activity (MHC class I: Major Histocompatibility Complex class I), positive regulation of ATPase activity (ATP - adenosinetriphosphate), regulation of metal ion transport, positive regulation of potassium ion import, and negative regulation of endothelial cell differentiation, among other terms (Plate V, Fig 2).

To further investigate the functions of the differentially expressed genes in the blood transcriptomes of Yili horses before and after conditioning training, KEGG pathway enrichment analysis was performed (Plate VI, Fig 3).

The differentially expressed genes were mainly associated with tryptophan metabolism, the sphingolipid metabolism signalling pathway, and the cGMP-PKG signalling pathway. These results indicated that the differentially expressed genes were linked to metabolic pathways associated primarily with glycolipid metabolic processes, cell cycle processes, and inflammatory regulatory pathways.

Table 3. Differentially expressed genes in the blood transcriptomes of Yili horses before and after training.

Gene	Log_2FoldChange	Q-value
ADGRE3	-1.450254537	0.0000579
LOC100050560	-1.358610164	0.000187369
DDC	2.157686484	0.000199486
IL5RA	-1.336923052	0.000453018
IL1R2	-1.738149085	0.001142175
CEBPE	-1.46492114	0.002158807
TP53INP2	-1.028204349	0.002335385
S1PR3	-1.56268941	0.01132425
C1QTNF12	-1.247792977	0.012816529
CASR	-2.684141394	0.012816529
LOC100071991	-1.610192025	0.015958815
KRT7	4.977584686	0.015958815
PRDM16	3.073335683	0.021080394
AATK	-1.006388922	0.022690762
FAM109A	-1.206418081	0.022690762
CUEDC1	-1.138115629	0.022690762
MMP1	-1.046170885	0.023052187
PTCD3	3.27444666	0.023052187
SMPD3	-1.341396599	0.023052187
VAT1L	-3.385280151	0.023068921
LOC111771832	5.14823036	0.032697286
IL18	-1.03156877	0.03463783
SERINC2	-1.02722752	0.037690997
LOC111772029	2.482562044	0.038899945
LOC111772887	2.898438318	0.039993271
LOC111767490	2.904452881	0.041100428
LOC100147370	1.125570834	0.041100428
ADGRG6	-1.028684119	0.041100428
KLHDC7B	-1.622546039	0.041100428
ATP1B2	-1.316441471	0.041100428
OLIG1	-1.834891022	0.043458332
TRPC6	-1.175692184	0.043458332
CTSH	3.881753309	0.046917725

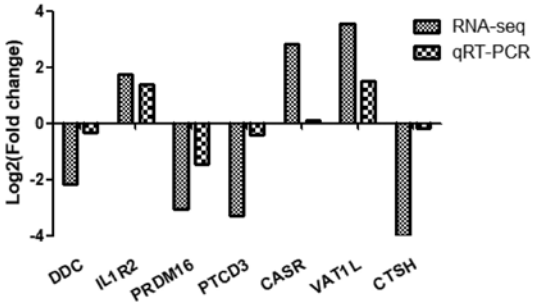

Fig. 4. Results of qRT-PCR analysis of selected significantly differentially expressed genes

The RNA samples used for qRT-PCR in this experiment were the same as those used for transcriptome sequencing. Seven known differentially expressed genes *DDC*, *PTCD3*, *IL1R2*, *CASR*, *VAT1L*, *CTSH*, and *PRDM16* were randomly selected from the transcriptome sequencing results and validated using qRT-PCR, with GAPDH being used as the internal reference gene. According to the validation results, the expression trends of these genes before and after training in Yili horses were consistent with the RNA-seq results (Fig. 4), indicating that the RNA-seq results were credible.

Discussion

During conditioning training, horses activate and inhibit several signalling pathways for adapting to exercise and restoring the dynamic homeostasis of the body. In the present study, transcriptome analysis screened 33 differentially expressed genes before and after conditioning training in Yili horses. GO annotation and KEGG enrichment analyses of these differentially expressed genes revealed that some were significantly enriched in sphingolipid metabolism, tryptophan metabolism, and the cGMP-PKG signalling pathway as well as other metabolic pathways related to exercise physiology.

To maintain homeostasis in an organism and as a manifestation of adaptation to exercise training, the expression levels of some genes associated with metabolism and cell cycle processes are altered. *TP53INP2* is associated with ubiquitin binding in the GO functional annotation; it is a dual regulator of transcription and autophagy, acts as a coactivator of the thyroid hormone receptor in the nucleus, and regulates the expression

of thyroid hormone-related genes. *TP53INP2* reportedly can interact with ubiquitin and ubiquitinated proteins through the ubiquitin interaction motif (UIM); however, in cells lacking the UIM, *TP53INP2* overexpression leads to ubiquitinated protein accumulation and apoptosis under stress conditions (Xu and Wan 2019). Further, Ivanova et al. (2019) found that *TP53INP2* inhibits cell proliferation, promotes apoptosis, and is involved in the cellular stress response. In the present study, *TP53INP2* was associated with the ubiquitin binding biological function and was upregulated after conditioning training in Yili horses, presumably in relation to cell proliferation.

Sphingosine-1-phosphate (S1P) is a biologically active sphingolipid mediator involved in numerous physiological processes (Intapad 2019), including those affecting vascular permeability and vascular tone (McGinley and Cohen 2021). S1P has five specific G protein-coupled receptors, i.e., *S1PR1–5*; of these, *S1PR1*, *S1PR2*, and *S1PR3* are expressed in endothelial cells and regulate the development and function of the vascular system (Cartier and Hla 2019). Exercise increases the number and function of endothelial progenitor cells in circulating peripheral blood (Xia et al. 2012). The *S1PR3* signalling pathway in endothelial cells contributes to vasodilation and helps mediate S1P-induced endothelial progenitor cell proliferation (Wang et al. 2018). Sammani et al. (2010) found that intratracheal administration of S1P at high doses in mice significantly disrupted the alveolar–capillary barrier; however, *S1PR3* silencing prevented barrier damage. Inhibiting *S1PR3* expression may be an effective method to prevent inflammation in other diseases associated with endothelial barrier disruption (Li et al. 2021). In the present study, *S1PR3* was associated with the GO-enriched biological function of the negative regulation of endothelial cell development and negative regulation of endothelial barrier establishment; *S1PR3* gene was upregulated after training in Yili horses, probably owing to cell cycle regulation and apoptosis induction, thereby increasing endothelial cell proliferation and vasodilation.

The genes associated with the GO-enriched function of histone methyltransferase activity in the present study included *PRDM16*. The PRDM family possesses a conserved PR structural domain at the N terminus. *PRDM16* is the 16th member of the PR structural domain; its N-terminal PR structural domain exerts histone methyltransferase activity and methylates histone H3K9, which plays a key role in the differentiation of brown fat from skeletal muscle methylation. Higher animals possess white adipose tissue which stores energy, and brown adipose tissue (BAT), which consumes energy through thermogenesis. The stimulation of BAT thermogenesis includes cold, sympathetic, and cytosolic factors (Valente et al. 2015). The white adipocyte browning-related transcription factor *PRDM16* acts as a downstream signal for sympathetic nerves and induces the expression of browning-related genes, such as *UCP1* (Ishibashi and Seale 2015). *PRDM16* positively regulates BAT differentiation and promotes BAT formation (Gu et al. 2019) as well as affects adipocyte and muscle cell formation and development (Cohen et al. 2014). Furthermore, a decrease in *PRDM16* gene expression increases MyoD and MyoG gene expression and MLC, MCK, and MyHC protein expression (Yang et al. 2010). Exercise training increases muscle mass and decreases BAT mass and activity in human athletes (Singhal et al. 2016), probably owing to the increase in muscle mass, which generates a greater thermogenic capacity. In the present study, the down-regulation of *PRDM16* in Yili horses after conditioning training may be attributed to the sudden increase in thermogenic capacity as a physiological mechanism to compensate for exercise; however, further investigation is required to explain this finding. Nevertheless, we hypothesize that *PRDM16* is involved in energy metabolism, lipid mobilization, and muscle cell formation and development that occurs as a physiological response to exercise.

In the present study, 23 significantly enriched KEGG pathways were associated with the differentially expressed genes, 10 of which were associated with DOPA decarboxylase

(DDC) and were involved in energy production and metabolic processes. The key to optimizing training efficiency is maintaining the dynamic balance of the glycolipid energy supply. DDC was first identified in the kidney tissues of higher animals, and its main function is promoting adrenaline synthesis, which is considered a widespread endogenous regulator of neurotransmission in tissues (Song et al. 2017). In the present study, the *DDC* gene was associated with aromatic-L-amino acid carboxylase activity and levodopa carboxylase activity. *DDC* catalyses the conversion of L-DOPA to dopamine (DA) and 5-hydroxytryptamine (5-HT). Reduced levels of DDC lead to lower levels of biogenic amines downstream of catecholamine (CA), including DA, norepinephrine, and adrenaline (Zhu et al. 2013). Therefore, the *DDC* gene plays a key role in the CA pathway and can regulate CA expression. Catecholamine causes physiological changes, such as changes in the heart rate, blood pressure, and blood glucose levels (Sellami et al. 2014). The content of 5-HT and the expression of receptor proteins in the central nervous system are affected by long-term training; therefore, they influence the ability of the central nervous system to regulate the organism's locomotion (Cordeiro et al. 2017). In the present study, the *DDC* gene was down-regulated in Yili horses after conditioning training, probably because the horses were in a state of motor fatigue during the late training period; however, the exact cause ought to be determined in further studies. The *DDC* gene is significantly enriched in various pathways such as tryptophan metabolism and phenylalanine metabolism; it plays an important regulatory role in endogenous central neurotransmitter production.

Exercise causes substantial physiological changes in the body, including increased blood flow and body temperature and oxidative stress along with the production of interleukins and their receptors as well as several other substances associated with inflammation. Inflammatory cytokines are typically considered as stress factors (Purvis et al. 2010). In the present study, differentially expressed genes related to inflammation-related KEGG pathways, such as receptor activity between cytokines and disease, were identified. Interleukin 1 (IL-1) is one of the most potent proinflammatory cytokines, IL-1 receptor type II (IL-1R2) is a negative regulator of IL-1 action (Zhang et al. 2012), and interleukin-18 (IL-18) is a proinflammatory factor produced by interferon-γ induction (Yamanishi et al. 2018). In the present study, IL-18 was related to the GO-enriched biological function of cytokine production. In previous studies on humans, prolonged heavy exercise was found to increase inflammation levels (Shi et al. 2014). In the present study, IL-18 and IL-1R2 were upregulated after training in Yili horses; this may be attributed to the unconventional stress stimulus caused by conditioning training and the induction of inflammation in the horses.

In conclusion, in the present study, the blood transcriptomes of Yili horses before and after conditioning training were comparatively analysed using transcriptome sequencing technology; 33 differentially expressed genes were screened. These genes were significantly enriched in GO terms such as ATPase activity, cell cycle process, and ion transmembrane transporter protein activity. Additionally, the genes were related to KEGG pathways such as amino acid biosynthesis and the cGMP-PKG signalling pathway. Most other important genes are related to the body's immunity, cell apoptosis, and so on. The genes *DDC*, *PRDM16*, *TP53INP2*, and *S1PR3* were significantly differentially expressed after training; we hypothesize that these genes are involved in regulating the physiological processes of conditioning training in Yili horses.

Conflict of Interest

The authors declare no conflict of interest.

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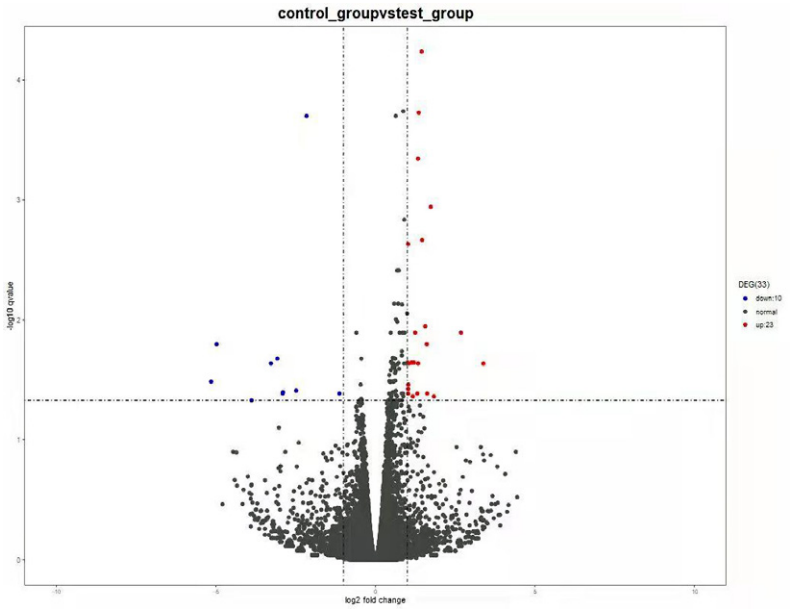

Fig.1. The horizontal axis represents the fold change of gene expression in different samples, whereas the vertical axis represents the significance level of the gene expression difference. Significantly differentially expressed genes are indicated by red dots (up-regulated) and blue dots (down-regulated), whereas non-significantly differentially expressed genes are indicated by grey dots.

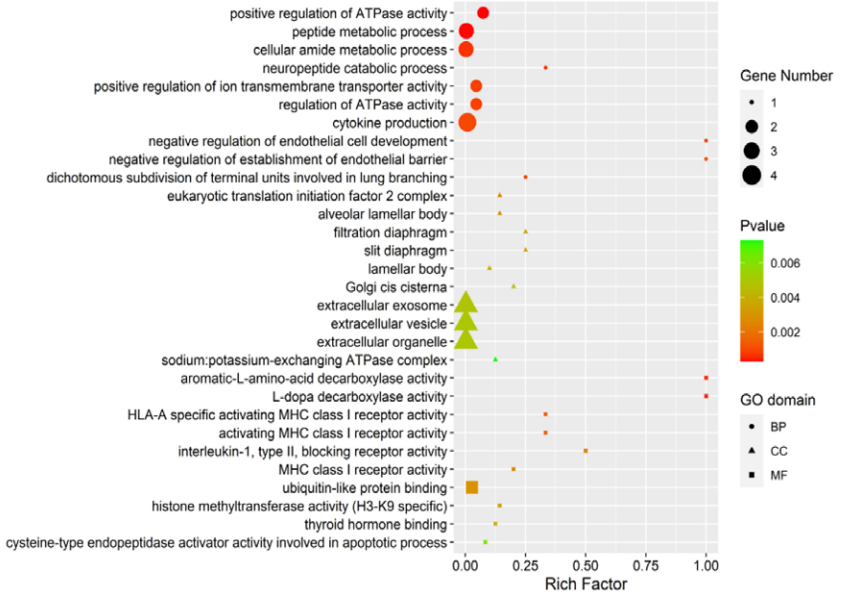

Fig. 2. Gene Ontology (GO) enrichment histogram of differentially expressed genes (top 30). The vertical axis represents the enriched GO terms, while the horizontal axis represents the Rich factor, indicating the proportion of enriched genes in the pathway to all genes in the pathway. Different colours are used to distinguish biological processes, cellular components, and molecular functions. The vertical axis represents the enriched GO terms, and the shape size represents the number of genes enriched by the GO terms.

Plate VI

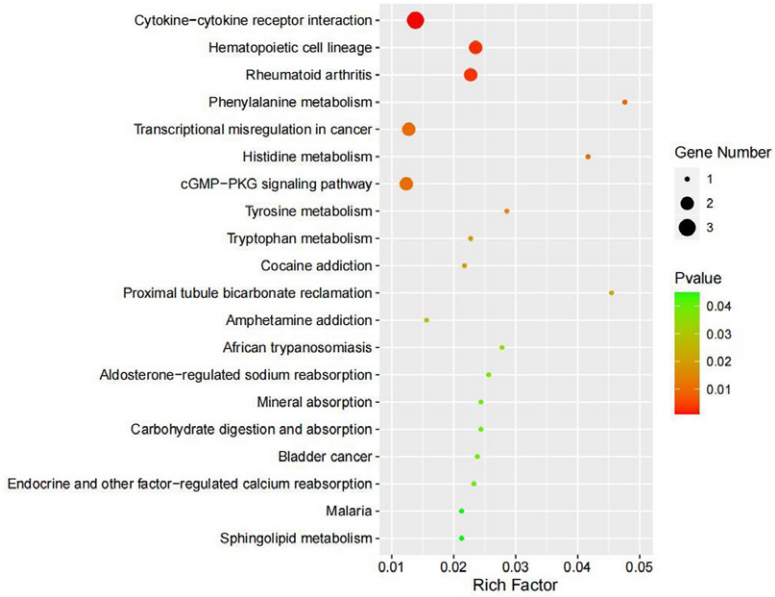

Fig. 3. Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment histogram of differentially expressed genes (top 20). The abscissa is the ratio of the number of differentially expressed genes annotated to the KEGG pathway to the total number of differentially expressed genes, whereas the ordinate is the KEGG pathway. The size of the point represents the number of genes annotated to the KEGG pathway, whereas the colour (from green to red) represents the significance of enrichment.