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ORIGINAL ARTICLE

Transcriptional Profile Alteration of Peripheral Blood in Chronic Hypoxia

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Objective Many physiological and pathological conditions, including cyanotic congenital heart diseases (CCHD), are accompanied by chronic hypoxia, which might interfere with the transcription process. However, the transcriptome profile in peripheral blood under hypoxia is still unidentified. The present work aimed to explore the transcriptional profile alteration of peripheral blood in chronic hypoxia.

Methods The present study used a chronic hypoxia rat model to simulate the hypoxic state of CCHD patients. Two groups of Sprague-Dawley rats ($n = 6$ per group) were either exposed to hypoxia (10% O_2) or normoxia (21% O_2) for 3 weeks. Body weight was measured weekly. Peripheral blood was collected and total RNA was extracted for RNA-Seq at the end of the hypoxia treatment. After quality assessment, the library was sequenced by the Illumina Hiseq platform. The differentially expressed genes were screened (false discovery rate < 0.05 and fold change > 2). The functional annotation analysis and cluster analysis of differentially expressed genes were performed based on the adjusted *P*-value (padj < 0.05).

Results Compared with the control group, the body weight of the rats in the hypoxia group was significantly

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lowered ($P<0.01$). RNA-Seq results showed that the transcriptome patterns of the two groups had significant differences. In total, 872 genes were identified as differentially expressed. Among all, 803 genes were down-regulated, while only 69 genes were up-regulated in the hypoxia group. The functional enrichment analysis of the 872 genes showed that multiple biological processes involved, such as porphyrin-containing compound metabolic process, hemoglobin complex and oxygen transporter activity.

Conclusions Our study demonstrated the transcriptional profile alteration in peripheral blood of chronic hypoxia rat model. This study provided basic data and directions to further understand the physiological and pathological changes in patients with CCHD.

OXYGEN is a basic factor involved in numerous developmental processes and tissue homeostasis, and it is essential for oxidative metabolism, energy supply, and cell survival.[1] Insufficient oxygen supply inevitably results in disturbance of energy metabolism, tissue function, or morphological structure.[2] When the oxygen demand and oxygen supply are unbalanced, the organism will suffer from hypoxia. Different from acute hypoxia, chronic hypoxia is a general stress that involved in many disease,[3, 4] such as cyanotic congenital heart diseases (CCHD),[5] myocardial infarction,[6] stroke,[7] cancer,[8] bronchopulmonary dysplasia[9] and pulmonary obstruction.[10] Instead of passing through the lungs for oxygenation, parts of the venous blood were mixed with the systemic arterial blood through abnormal shunts, resulting in long-term chronic hypoxia in children with CCHD.[11]

Gene transcription is a strictly controlled process. In line with the principle of economy and efficiency, only the genes needed by the tissues or cells could be expressed.[12] There is no doubt that chronic hypoxia affects transcriptional processes, and a lot of evidence support this concept.[13, 14] Herein, we introduced the transcriptome analysis to elucidate the pathological process under hypoxia.

We choose the peripheral blood as research material since blood flowed through the whole body and associated with all tissues. Genes expression pattern in peripheral blood is more than 80% similar to those expressed in other tissues or cells.[15] Additionally, blood samples are convenient to obtain from subjects and cause less damage to subjects. Currently, most studies of chronic hypoxia were carried on tumors[16] and high altitude populations.[17] The effect of chronic hypoxia alone on the whole blood transcriptome is undetermined.

Here, 2 weeks old rats were exposed to 10% O_2 for 3 weeks to simulate the chronic hypoxic state of CCHD patients.[18, 19] Given that hypoxia animal models have been widely demonstrated to share similarities in both physiological and myocardial metabolism with CCHD,[20] this study may help us to unveil the alteration of peripheral blood-derived gene expressions in rat models undergoing hypoxia exposure, whereby a list of homologous genes changing in expression likely vary analogously in CCHD.

MATERIALS AND METHODS

Study design and animal model

Two weeks old Sprague-Dawley rats were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and randomly divided into 2 groups (n=6 per group, female:male=3:3). They were either exposed to hypoxia (10% O_2, Biospherix, USA) or normoxia (21% O_2) for 3 weeks. The body weight was measured weekly. All treatments were conducted in accordance with humane animal care standards approved by the Institutional Animal Care Committee of Fuwai Hospital and Fuwai Hospital Ethics Committee (Beijing, China).

Blood sampling and RNA extraction

At the end of the experiment, whole blood samples were collected in tubes containing heparin (Vacuette, Austria). Total RNA was extracted from the blood samples with Trizol LS (Invitrogen, USA) following the manufacturer's instructions. In detail, 150 μl of whole blood was pipetted and diluted with RNase free water at a volume ratio of 1:1 to make a 300 μl sample. Then 900 μl of Trizol LS (Invitrogen, USA) was added into the mixed sample and homogenized. Two minutes later, 240 μl of chloroform was added and mixed by vortex. After 5 minutes' incubation at room temperature, the sample was centrifuged at 12 000×g for 15 minutes at 4°C. The upper transparent liquid was transferred to a new tube, followed by adding 600 μl

of isopropanol. After 10 minutes' incubation at room temperature, the sample was centrifuged at 12 000×*g* for 10 minutes at 4°C. The liquid was removed and the pellet was washed with 75% ethanol. At last, the RNA pellet was dissolved in 50 μl of RNase free water. The concentration of the RNA was determined by NanoDrop spectrophotometer (Thermo Fisher, USA). RNA quality was confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Samples with RNA integrity number (RIN) ⩾ 8.5 were used for further analysis.

RNA library preparation and deep sequencing

The NEBNext® Ultra™ RNA Library Prep Kit (New England BioLabs, USA) was used for sequencing library preparation. In brief, mRNA was purified by magnetic beads with polyT attachment. The mRNA was fragmented and used as the template for the first strand synthesis of cDNA. The RNA strand was degraded with RNaseH and then the second strand of cDNA was synthesized. The double-stranded cDNA was purified, and subjected to end repair and adaptor connection. The cDNA fragments of 200-300 bp in length were filtered with AMPure XP beads (Beckman Coulter, UK). After library preparation, the quantity was measured by Qubit 2.0 Fluorometer (Life Technologies, USA), and all samples were diluted to the same concentration. Library quality was assessed on the Agilent 2100 Bioanalyzer system (Agilent Technologies, USA). The library was sequenced on an Illumina Hiseq platform (Illumina, USA) by Novogene Bioinformatics Technology Co., Ltd., (Beijing, China) and 150 bp paired-end reads were generated.

Quality control and reads mapping

The raw sequencing data were filtered by removing the reads with adapters, reads containing N (N means that base information cannot be determined), and low quality reads. Through the above process, clean data was obtained. All the downstream analyses were based on clean data with high quality. The reference genome of rat (RGSC6.0/rn6) was downloaded from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov). All the clean data reads were aligned against the reference genome annotation file to get the BAM format file.

Quantification of gene expression level and differential expression analysis

FeatureCounts software (v1.5.0-p3, https://sourceforge.net/projects/subread/) was used to quantify the gene expression level. And the FPKM (fragments per kilobase of transcript per million mapped reads) of each gene was calculated based on the length of the gene and the reads count mapped to this gene. Differential expression analysis between the two groups was performed using the DESeq2 package (Version 1.16.1) in R software (https://www.r-project.org/). And false discovery rate (FDR) < 0.05 & fold change (FC) > 2 were considered significantly different.

Functional annotation and enrichment of differentially expressed genes

Gene Ontology (GO) analysis (molecular function, cellular component, and biological process) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis of differential expressed genes (DEGs) were performed by the clusterProfiler package in R software. And FDR<0.05 was considered to be significantly different.

Protein-Protein Interaction analysis of DEGs

Protein-protein interaction analysis of DEGs was based on the STRING database (https://string-db.org/), which is known for predicted protein-protein interactions. We chose two types of evidence (evidence comes from actual experiments or databases) as evidence sources for protein-protein interaction analysis. Confidence scores (scaled between 0 and 1) were provided to weigh the interaction. The confidence score was divided into four grades: highest confidence (>0.900), high confidence (0.700-0.900), medium confidence (0.400-0.700) and low confidence (<0.400).[21] The analysis result was visualized with the Cytoscape software (Version 3.3.0, https://cytoscape.org/). Meanwhile, the complex network was clustered by the MCODE (Molecular Complex Detection) plug-in to build the functional modules.[22]

Statistical analysis

Statistical analyses related to animal information were performed by SPSS Statistics 22.0 (IBM Corp. USA). The differences between groups were analyzed by Student's *t*-test. All data were presented as the mean ± standard deviation. $P < 0.05$ was considered to be significantly different.

Statistical analyses related to gene expression were performed in R software (R Version 3.5.2). Benjamini-Hochberg (BH) procedure was applied to con-

trol the false positive rate in multiple comparisons. FDR<0.05 was considered to be significantly different.

RESULTS

The characteristics of the studied rats

Rats were exposed to hypoxia ($n=6$) or normoxia ($n=6$) for 3 weeks. As shown in **Figure 1**, the growth rate of the hypoxia group rats was significantly lowered during the hypoxia exposure (3 weeks, $t=-9.271$, $P<0.001$; 4 weeks, $t=-9.132$, $P<0.001$; 5 weeks, $t=-6.225$, $P<0.001$). There was no difference between the two groups at the baseline ($t=-0.628$, $P=0.544$). These data indicated that the weight gain of the rat in the hypoxia group was limited by oxygen deficiency.

Quality of RNA-Seq analysis

The raw base data was more than 12 GB. And the Q20 (ratio of bases with a Phred quality value greater than 20 to the total base) was greater than 91%. The distribution of the raw reads in the two groups was exhibited in **Figure 2**. The clean reads in the two groups were approximate 97%, while low quality reads accounted for 1% only. More than 99% reads distributed in the exon region of the gene (**Table 1**). To sum up, the quality of the RNA-Seq data was reliable.

Differential gene expression analysis between two groups

To quantify the expression level of the gene, the gene reads counts were normalized and expressed in FPKM value. Principal Component Analysis (PCA) and hierarchical cluster analysis were performed based on the FPKM value. As shown in **Figure 3A**, the first two principal components of PCA were 63.5% and 8.9% respectively. Hierarchical cluster analysis (**Figure 3B**) showed that the samples in the same group were clustered together, and similar gene expression patterns were displayed in the same groups, while the expression patterns of the two groups were significantly different from each other.

Further analysis showed that a total of 872 genes displayed significantly different between the two groups (FDR<0.05 and FC>2). As shown in **Figure 3C**, the red dot represented down-regulated genes and the blue dot represented up-regulated genes in the

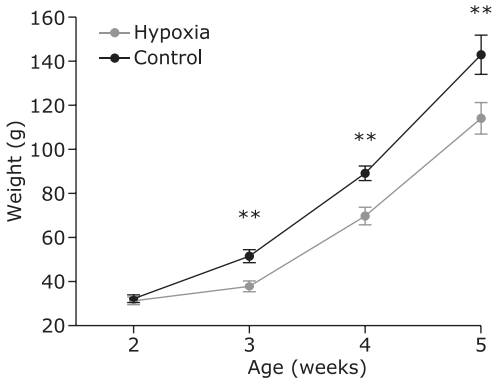

Figure 1. The dynamic change of body weight.
Data were shown as mean±SD, and statistical significance was analyzed by Student's *t*-test ($n=6$ per group, $^{**}P<0.01$).

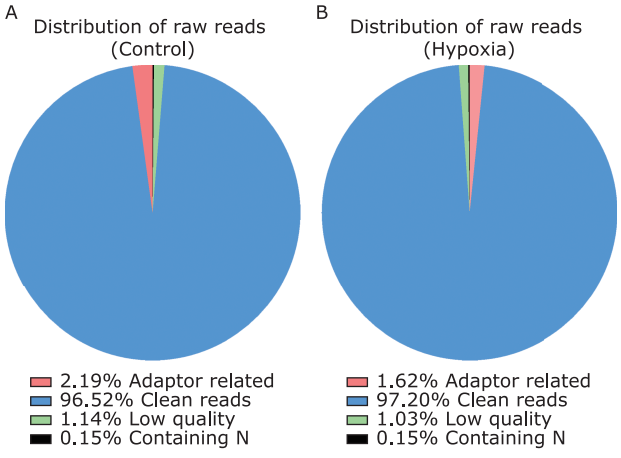

Figure 2. Distribution of raw reads in the control group (A) and the hypoxia group (B).

Table 1. Distribution of clean reads in genes (%)

Sample ID	Exon	Intron	Intergenic	Sample ID	Exon	Intron	Intergenic
C1	99.61	0.18	0.20	H1	99.26	0.40	0.34
C2	99.22	0.44	0.34	H2	99.26	0.38	0.36
C3	99.48	0.27	0.25	H3	99.26	0.40	0.34
C4	99.45	0.28	0.27	H4	99.34	0.33	0.32
C5	99.07	0.54	0.39	H5	99.06	0.53	0.41
C6	99.48	0.27	0.25	H6	99.03	0.51	0.46

C1-6 for control 1-6 and H1-6 for hypoxia 1-6.

hypoxia group. The number of down-regulated genes was 92.09% of the total DEGs (803/872), while the up-regulated genes only accounted for 7.91% (69/872; **Figure 3D**). In sum, chronic hypoxia inhibits gene transcription at the whole transcriptome level.

Functional enrichment analysis of DEGs

GO analysis showed that the DEGs were mainly enriched in oxygen transporter activity, hemoglobin complex and porphyrin-containing compound metabolic process, respectively (**Figure 4A**, marked in red box). These genes were also involved in iron ion binding, organelle disassembly, and antigen processing and presentation (**Figure 4A**, marked in black box). The KEGG pathway analysis demonstrated that these genes were involved in mitophagy, porphyrin and chlorophyll metabolism and ferroptosis (**Figure 4B**, marked in black box).

Gene interaction network and functional modules

Further analysis removed 11 new genes, 137 non-protein coding genes or pseudogenes, and 1 mitochondrial from 872 DEGs, 723 protein-coding genes remained for the protein-protein interaction network analysis. With the confidence score cutoff of 0.7, the final network contained 283 nodes (genes in the network) and 908 edges (interactions between genes) (**Figure 5A**). The top 30 high-degree (the number of adjacent links of nodes) nodes and their FC was shown in **Table 2.** The top six functional modules are demonstrated in **Figure 5B-G**. They might be related to protein degradation, cytoskeleton, transcriptional regulation, immunity, cell cycle and body weight, respectively.

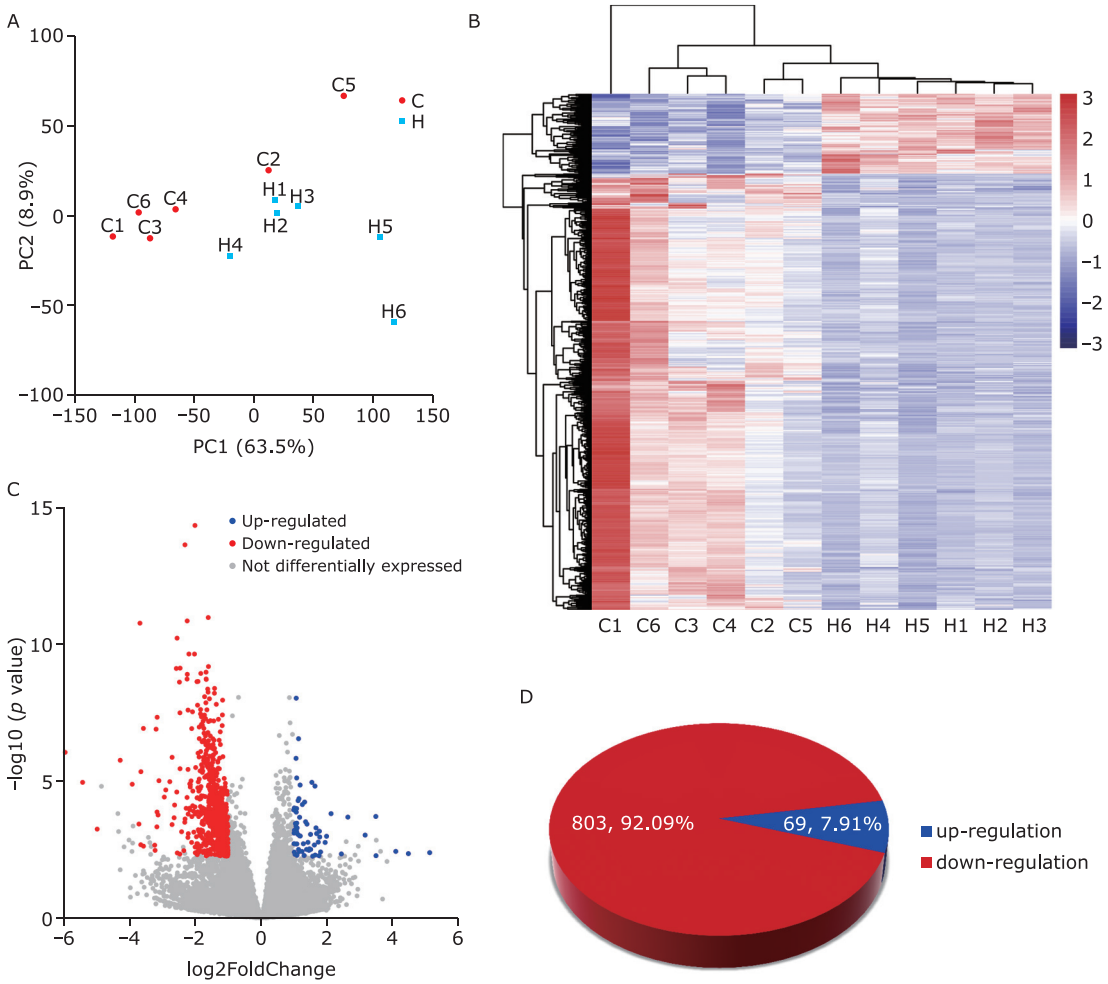

Figure 3. Differential analysis of gene expression.

A. Principal Component Analysis (PCA) of transcriptome data. B. Heatmap of the differential expressed genes (DEGs) for the two groups. C. Volcano plot of the DEGs for the two groups. Red dot represents down-regulated genes, blue dot represents up-regulated genes and gray dot represents not changed genes in the hypoxia group. D. The pie chart of DEGs.

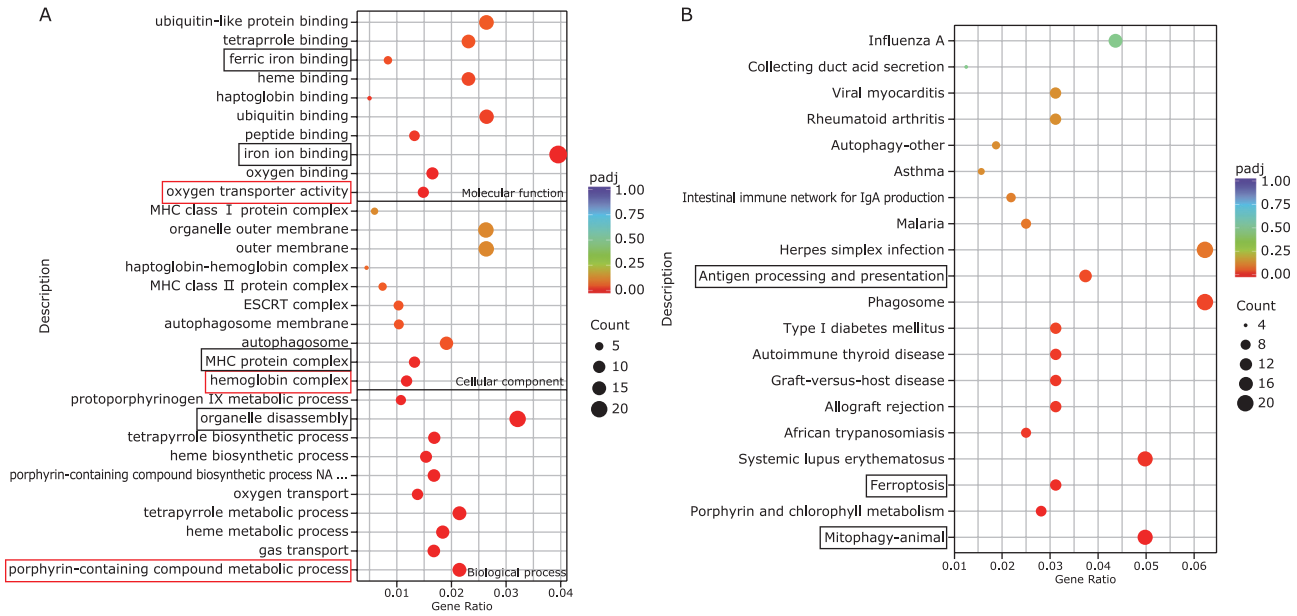

Figure 4. Functional enrichment of DEGs.

A. Gene Ontology (GO) terms (biological process, cellular component and molecular function) for DEGs. B. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for DEGs.

padj: adjusted *P*-value.

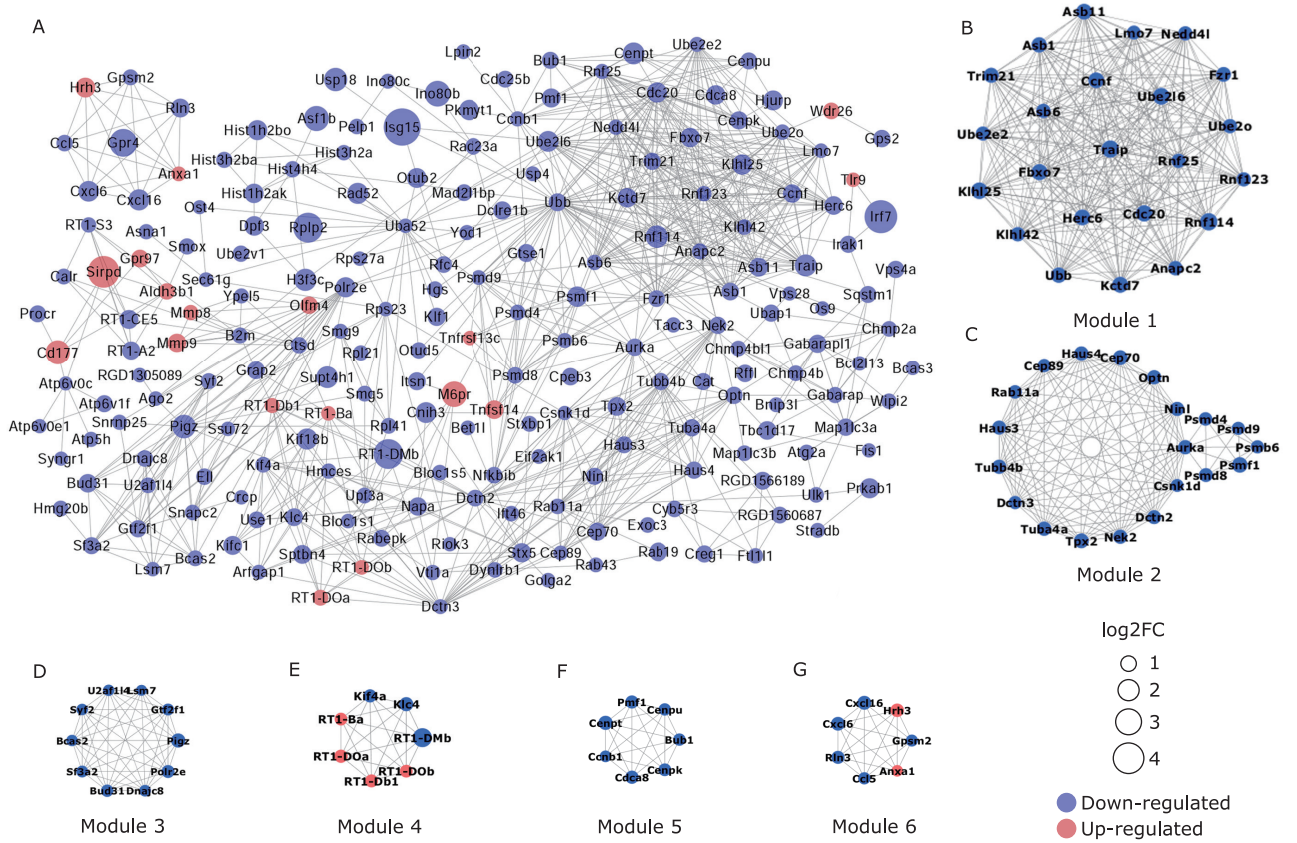

Figure 5. Gene protein-protein interaction network analysis.

A. The network of DEGs. B-G. The top 6 functional modules identified from the network. Red circles represent up-regulated genes. Blue circles represent down-regulated genes. The circle size reflects the fold change (FC) of genes.

Table 2. The top 30 high-degree nodes in the protein-protein interaction network

Genes	Degree	Log_2FC	Genes	Degree	Log_2FC
Ubb	56	1.54	Kctd7	22	1.74
Cdc20	37	1.84	Asb1	22	1.51
Anapc2	31	1.25	Trim21	22	1.44
Fzr1	30	1.36	Rnf25	22	1.15
Dctn2	26	1.37	Asb6	22	1.18
Dctn3	25	1.11	Ccnf	22	1.61
Ube2l6	25	1.94	Ube2e2	22	1.12
Aurka	23	1.48	Lmo7	22	1.07
Ube2o	23	1.20	Herc6	22	1.64
Nedd4l	23	1.17	Rnf123	22	1.17
Asb11	22	1.66	Traip	22	2.11
Fbxo7	22	1.75	Uba52	22	1.05
Rnf114	22	2.17	Pigz	20	2.35
Klhl25	22	1.88	Polr2e	19	1.85
Klhl42	22	1.21	Tubb4b	19	1.47

DISCUSSION

In the present study, we described the characteristics of the transcriptome profile in peripheral blood from chronic hypoxia juvenile rats. The rats' growth was limited by chronic hypoxia exposure, with the transcriptome pattern changed dramatically. GO, KEGG pathway and protein-protein interaction analysis revealed a series of related alterations to further understanding the effects of chronic hypoxia.

In this study, the growth of rats in the hypoxia group was significantly inhibited. The results are consistent with previous studies.[23] Under the condition of chronic hypoxia, the rats often have insufficient energy intake due to feeding difficulties.[24] The energy production switches from aerobic oxidation to glycolysis, which depletes less oxygen with inefficient energy production. Many studies have shown that patients with CCHD are accompanied by poor growth[25, 26] and neurodevelopmental disorders.[27] In addition to insufficient energy intake and low metabolic efficiency, the transportation of the patient's oxygen and nutrient is abnormal due to structural malformations.[28] These are considered to be the main causes of deficient growth and development in the condition of chronic hypoxia.

Compared to the normoxia group, the down-regulated genes in the hypoxia group accounted for more than 90% of all DEGs. This indicated that hypoxia reduced the activity of global transcription. Similar findings have been reported in previous studies, hypoxia significantly inhibited total RNA and mRNA synthesis in mouse hepatoma cells, which is independent of *Hif1*. The extent of inhibition was determined by both the duration and the severity of hypoxia.[29] Under hypoxia, ATP production is insufficient to support growth requirements, the body preferentially provides ATP to the housekeeping gene to maintain normal function instead of other luxury genes.[30] This helps the body survives under low ATP conditions and is an adaptive response of the body to hypoxia.

The functional enrichment analysis revealed that the down-regulated DEGs were associated with the hemoglobin biosynthesis, which is contradicting with other studies in which chronic hypoxia is often accompanied by higher production of hemoglobin. Modification of hemoglobin function usually plays a key role in hypoxia adaptation.[31] Under hypoxic conditions, arterial oxygen saturation was reduced. In order to provide sufficient oxygen to the body, the volume of blood cells would be increased with the enhanced affinity of hemoglobin to oxygen.[32, 33] In addition, the hemoglobin concentration increases due to the decreased plasma volume.[34] The transcription level of hemoglobin will decrease rapidly at the initial stage of hypoxia, then rise briefly, and continue to decline slowly.[35] In sum, the high level of hemoglobin in hypoxia patients is not due to increased transcription of hemoglobin related genes, but rather due to the decrease of the plasma volume.

Hemoglobin is an iron-bound protein. It is taken for granted that many DEGs may associate with iron homeostasis. GO and KEGG analysis revealed that iron homeostasis was disturbed after long-term hypoxia exposure. Studies have shown that maintenance of iron homeostasis depends on proper oxygen supply.[36] Under hypoxic conditions, the iron storage protein in the skeletal muscle was down-regulated, and the utilization of iron by other cells and tissues was increased to upregulate the synthesis of heme.[37] Besides, iron deficiency exacerbated the increase in pulmonary artery systolic pressure under hypoxia exposure, while iron supplement effectively reversed the increase of pulmonary artery systolic pressure.[38] In summary, hypoxia disturbed the iron homeostasis, and the disequilibration affected the response of organisms to hypoxia.

Another interesting finding from this study was that hypoxia affects many major histocompatibility complex (MHC) genes. The MHC of rats, also known as the RT1 complex, is primarily involved in the antigen processing and presentation and plays an important role in inflammatory responses and immune regulation.[39] In our study, many MHC class I family genes (mainly involved in endogenous antigen presentation) were downregulated, while lots of MHC class II family genes (mainly involved in exogenous antigen presentation) were upregulated, indicated that hypoxia has different impacts on the presentation of different types of antigens. In some tumor-related studies, it was found that hypoxia inhibits upregulation of IFN-γ-dependent MHC class I expression, and hyperoxia upregulated MHC class I at the transcriptional level. Downregulation of MHC class I affects anti-tumor immune responses and helps tumor cells to escape from the recognition of anti-tumor cells and cause immune escape. And radiotherapy can increase the anti-tumor immune response by alleviating hypoxia in the tumor.[40, 41] However, other tumor-related studies have shown that hypoxia enhances the antigen presentation of MHC class I.[42] The relationship between hypoxia and the MHC class II family is not identified. MHC class II expression was inhibited in most short-term hypoxia, such as in trauma or *in-vitro* studies.[43-46] The reasons for these contradictions perhaps due to differences in cell lines or the severity of hypoxia. Therefore, further exploration is needed to address this issue.

Hypoxia affects the function of oxygen-related organelles, such as mitochondria, endoplasmic reticulum and peroxisomes.[47] Our study also showed that many DEGs are related to organelle disassembly and enriched in the mitophagy pathway. Under chronic hypoxic conditions, mitochondrial assembly is disrupted.[48, 49] Mitophagy is an adaptive response of cells to hypoxia, which prevents cell death by degrading damaged mitochondria.[50] Previous studies have revealed that mitophagy is activated during hypoxia, and related gene is up-regulated. With the increase of hypoxia, mitophagy is inhibited.[51, 52] Our study revealed that the mitophagy-related genes, such as *Map1lc3a*, *Map1lc3b*, *Bnip3l* or *Pink1* were widely down-regulated after a long period of hypoxia, which coincides with previous studies.[51] It also shows that chronic hypoxia severely affects the normal function of organelles. For patients with chronic hypoxia, timely correction of hypoxia might be extremely important.

Gene network analysis showed that the functions of DEGs were mainly involved in protein degradation, cytoskeleton remodeling, and cell cycle regulation. Under anaerobic conditions, protein degradation was blocked due to insufficient energy supply. The accumulation of metabolites in glycolysis might further inhibit energy production and protein degradation.[53] As a result, the organism cannot rely on protein degradation to repair damage caused by hypoxia, resulting in long-term irreversible changes. Another classic response to hypoxia is cytoskeleton disruption and remodeling. Hypoxia affects the dynamic process of actin, which has been verified to occur in various cells.[54-56] Compared with cells cultured in normoxia, the cell morphology, adhesion, and movement of hypoxic cells have changed dramatically.[57-59] During prolonged hypoxia, the destruction of the microtubule gradually increased,[60] and the formation of the centrosome is blocked, leading to the dysfunction of cell mitotic activity.[61] In our study, microtubule-associated genes, such as *Tubb4b*, *Tuba4a*, *Dctn2*, *Dctn3* were inhibited, together with alteration of cell cycle-related genes, leading to cell cycle arrest. Inhibition of protein degradation, mitotic dysfunction, and cell cycle arrest may be another important reason for the slow growth of young rats in the hypoxic group.

Our study focused on the young chronic hypoxia model to simulate the hypoxic state of children with CCHD, while most previous research explored the process in adult animal models or cells.[40-46] Since the children are in the process of growth and development, sharing different physiological and pathological processes with the adults, the study is necessary and important.[62-64] In addition, our research uses whole blood as the research material which could reflect the state of the whole body and is convenient to obtain in the clinic for further study. Furthermore, in our study, subjects experienced long-term persistent hypoxia, the situation is more similar to that experienced by patients with CCHD.

The study still has limitations. In order to fully understand the impact of hypoxia on the transcription, larger sample size studies are necessary. In addition, the subject of this study is peripheral blood only. Although different tissues share more than 80% of the genes, the transcriptome profiles are tissue-specific, and whether peripheral blood can mirror systemic transcriptome patterns change remains to be further studied. The third limitation of our study is the lack of

further validation, and only bioinformatics analysis of the sequencing data was performed.

In conclusion, we demonstrated the transcriptional profile alteration in peripheral blood of rat chronic hypoxia model. Our research firstly revealed the effect of hypoxia on the whole blood transcriptome. Meanwhile, the study provided basic data and directions for the study of chronic hypoxia. In addition, the study helps clinical researchers to further understand the physiological and pathological changes in patients with CCHD.

Conflict of Interests Statement

The authors declare that they have no competing interests.

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