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ORIGINAL RESEARCH ARTICLE

Effects of DNA methylation and gene expression on rats with protein malnutrition in early life

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Abstract

Although the mechanisms underlying how malnutrition in early life affects the susceptibility to diseases later in life remain unclear, there is considerable interest in the potential role of DNA methylation in the intrauterine programming of diseases. In this study, digital gene expression profiles were used to analyze changes in gene expression of protein-restricted rats early in life, while intergenerational rat models were used to explore differences in whole blood genomic DNA methylation in an environment stimulated by maternal protein intervention. Nine rats were randomly divided into early-life low-protein group (LPE), fetal low-protein group (LPF), and normal control group (CON). The LPE group was fed a low-protein diet on the 1st day of pregnancy until the end of lactation. The LPF group was given low-protein diet during pregnancy. The CON group was given a 20% protein diet from the 1st day of pregnancy. Total mRNA was extracted from the sacrificed rats at the 48th week. The number of differentially expressed genes for LPE versus CON, LPF versus CON, and LPF versus LPE was 178, 223, and 302, respectively. Comparing LPE versus CON and LPF versus CON, the upregulated genes common to the two groups were *Gimap-9*, *Serinc-4*, *Dnah-2*, *Sf3b-5*, and *Sat-2*, and the downregulated genes were *Ppp1r-3*. Comparing LPF versus CON and LPF versus LPE, the upregulated genes were *Mgat2* and *Cars*, and the downregulated genes were *Ddx28* and *Slc12a9*. The differentially expressed genes were mainly related to cell metabolism, immune response, signaling pathway, endocrine metabolism, stress response, ATP binding, and other functions. Early-life protein malnutrition affects gene expression of rat offspring and involves multiple aspects of growth and development, with different stages of early-life malnutrition leading to altered DNA methylation expression of corresponding genes, mainly in mitochondrial genes.

Keywords: Rats; Early life; Protein; Malnutrition; Gene expression; Genome-wide DNA methylation

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1. Introduction

The prevalence of chronic non-communicable diseases[1], such as obesity, diabetes, hypertension, gout, and cardiovascular diseases, has been increasing, which not only causes great suffering and a heavy financial burden on patients but also seriously affects

the quality of health. Numerous studies have shown that the occurrence of chronic diseases is related to a variety of factors, such as genetic[2], environmental, and behavioral factors. In recent years, epigenetic inheritance has been postulated as a bridge between gene expression and the environment[3] and is an important mechanism for the development of complex diseases[4,5]. Barker *et al.* found the association between fetal growth restriction[6,7] and adult disease through large-scale epidemiological investigations[8,9] and proposed the "fetal origin hypothesis" in 1993. The "fetal origin hypothesis" holds that the fetus is highly sensitive to nutrient supply[10]. Adaptive changes[11], including reduced blood flow to visceral tissues such as kidneys to ensure sufficient blood supply to the heart and brain and reduced hormone secretion to reduce the body's sensitivity to hormones[12], occur to the fetus if the pregnant mother is unable to absorb adequate nutrients or the uterus is underdeveloped. These adaptive changes affect the development and metabolic types of peripheral tissues (liver, fat, skeletal muscle, etc.), resulting in "programmed" changes, which can affect the differentiation, proliferation, and/or function of fetal cells, thereby resulting in diseases later in life[13].

Epigenetics regulates gene promoter regions and maintains their action throughout the life of the organism, ensuring their transcriptional expression and termination. In recent years, a large number of studies have confirmed that epigenetic inheritance is assumed to be a bridge between gene expression and the environment, and an important mechanism underlying the occurrence of complex diseases[14]. As a heritable epigenetic mark, DNA methylation is particularly sensitive to environmental factors, and is the most important form of epigenetic modification studied thus far.

Boubred *et al.* constructed a rat model of intrauterine nutrition restriction using a 9% low-protein diet[15], and compared with the normal group, they found that damaged kidney, reduced glomerular filtration rate, and increased glomerular sclerosis in intrauterine undernourished offspring may lead to renal disease in adulthood. Pond *et al.* found that the body weight of rats born with energy restriction and free feeding during the first two trimesters of pregnancy were 10 – 20% higher than that of the control group[16]. However, studies by Tomi *et al.* showed that the catch-up growth of intrauterine growth restriction (IUGR) infants after birth may destroy the blood–brain barrier, resulting in metabolic dysfunction, reduced glucose content in cerebrospinal fluid and reduced secretion of brain-derived neurotrophic factor, and may lead to an increased risk of Alzheimer's disease in adulthood[17,18]. Many studies have shown that early fetal malnutrition acquired from the mother is the earliest environmental factor, leading to the subsequent chronic diseases, and the permanent change of the offspring phenotype suggests that intrauterine growth retardation[19] may be related to some stable gene expression changes.

In this study, we used the digital gene expression profile to investigate the gene expression of adult offspring of malnourished rats at different times of life[20] and to explore the differences in DNA methylation in early life[21]. Therefore, it is of great significance to identify the functional genes of malnutrition in early life to explore the pathogenesis of related diseases. Studying the DNA methylation in the early-life malnutrition environment will provide a reference for exploring the mechanism of early-life malnutrition environment and adult diseases as well as the genetic factors of disease development[22].

2. Materials and methods

2.1. Experimental animals

In this study, 12 SD rats were purchased from the Medical Experimental Center of Zhengzhou University, including nine female rats and three male rats. Each rat weighed 200–250 g. Adaptive feeding was performed before mating. The female and male mice were put in the same cage for mating at around 5 pm every day. Vaginal swab smear was taken the next morning. The day when sperm was found by microscopic examination was determined as the 1st day of pregnancy. Female rats were kept in a cage (three pregnant rats per cage) from 1 to 15 days of gestation, and then were kept in a protective cage after 15 days of gestation until they gave birth. Until the young rats grew to 21 days old, they were separated from the cages after weaning. During the whole experiment, all rats were fed in the laboratory animal feeding room of Medical College of Henan University in a quiet environment, with the temperature ranging from 21°C to 22°C and the relative humidity ranging from 50% to 70%. The environment was well-ventilated and clean, and the animals were given fixed portions of food and free drinking water during the experiment.

2.2. Animal grouping

The newborn mice were classified as IUGR newborn mice if their average weight were less than 2 times the standard deviation, and if there were more than 10 pups per litter. Some pups were randomly excluded. The specific grouping of the pregnant rats is as follows.

Using the random number table method, nine pregnant rats were randomly and evenly allocated to three groups, with three rats in each group:

i. Normal control (CON) group. The mother rats were given normal protein diet (20% protein) during the course of pregnancy, lactation, and after cage separation.

ii. Early-life low-protein (LPE) group. From the 1st day after conception, the mother rats were given a low-protein diet containing 6% protein to establish the intrauterine malnutrition LPE rat model. The mother rats were given a 20% protein diet until after weaning.
iii. Fetal low-protein (LPF) group. From the 1st day after conception, the mother rats were given a low-protein diet containing 6% protein to establish the intrauterine malnutrition LPF rat model until the birth of offspring. After that, the mother and young mice were given a 20% protein content[23].

In the process of animal experiments, the welfare and ethical guidelines for experimental animals issued by the Ethics Committee of Animal Experiments of Henan University were strictly implemented, and the pain of experimental animals was minimized throughout the experiment.

2.3. Animal observation and collection of specimens

During the experiment, litter size, IUGR occurrence, and perinatal mortality were recorded in each litter, and the litter birth status of pregnant rats was observed at 8 am, 12 pm, 4 pm, and 8 pm every day when the rats were close to childbirth. Newborn rats born during the daytime were weighed within 4 h. For nocturnal births, the newborn rats were weighed no later than 8 a.m. the next day. At the experimental time point, the weight of the young mice was weighed at a fixed time point (8–9 am).

The rats in the three groups were anesthetized by intraperitoneal injection of 0.3 mL/100 g 10% chloral hydrate at the 48th week, and then, 8 mL blood was collected from the abdominal aorta using a disposable negative pressure vacuums tube (K2 EDTA anticoagulant tube). The animals were then sacrificed. RNA was extracted by TRIzol method and stored at −80°C for future use. After all the animals were sampled, the samples were stored on dry ice and transported to Hangzhou Lianchuan Biological Company for sample analysis.

2.4. Sample acquisition and preservation

Rats in the three groups were reared until the 48th week, and 8–10 mL blood samples were collected through abdominal aorta puncture before the animals were sacrificed. DNA was extracted by TRIzol method. Blood specimens from four male and female samples were randomly selected from each group, with 16 samples in total. The blood specimens were stored at −80°C.

2.5. mRNA library construction and sequencing

Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA) following the manufacturer's procedure. The total RNA quantity and purity were analyzed using Agilent 2100 Bioanalyzer Instrument and RNA 1000 Nano LabChip Kit (Agilent, CA, USA), and the RIN number >7.0. Poly(A) RNA was purified from total RNA (5 μg) using poly-T oligo-attached magnetic beads using two rounds of purification. Following purification, the mRNA was fragmented into small pieces using divalent cations under elevated temperature. Then, the cleaved RNA fragments were reverse transcribed to create the final cDNA library in accordance with the protocol for the mRNASeq sample preparation kit (Illumina, San Diego, USA). The average insert size for the paired-end libraries was 300 bp (±50 bp). Then, we performed the paired-end sequencing on an IlluminaHiseq4000 at the LC Sciences, USA, following the vendor's recommended protocol.

2.6. Sequence and primary analysis

Illumina Hiseq4000 was used for sequencing, and the read length of sequencing was double-ended 2 × 150 bp. This yielded gigabases (Gb) of sequence. Before assembly, the low-quality reads (1, reads containing sequencing adaptors; 2, reads containing sequencing primer; nucleotide with q quality score lower than 20) were removed.

2.7. Study on differential expression of genes

The study of gene-level expression differences was carried out based on the data analysis procedure described above. Ic-bio used Hisat software to compare the sequencing data to the reference genome and used the alignments to assemble the transcripts. StringTie, developed at Johns Hopkins University in association with the University of Texas Southwestern Medical Center, assembles transcripts and predicts expression levels. It applies network flow algorithms and the optional *de novo* assembly to assemble complex datasets into transcripts. Compared to programs such as Cufflinks, StringTie achieves more complete and accurate gene reconstruction and better prediction of expression levels when analyzing simulated and real datasets. Then, edgeR was used for differential expression analysis, and R language was used for graphical display of differential expression results, including differential expression gene heat map, scatter plot, volcano map, and principal component analysis map.

2.8. Clustering analysis of differential gene expression levels

We used log10(FPKM+1) for gene expression demonstration. At the same time, the gene expression of differentially expressed FPKM (fragments per kilobase of exon per million mapped fragments) can be displayed by Z-value method[24], where the abscissa is the sample, the

ordinate is the gene, and different colors indicate different gene expression levels.

2.9. RNA-seq reads mapping

We aligned the reads of sample A and sample B to the UCSC (http://genome.ucsc.edu/) *Homo sapiens* reference genome using HISAT package, which initially removed a portion of the reads based on quality information accompanying each read and then mapped the reads to the reference genome. HISAT allows multiple alignments PE read (up to 20 by default) and a maximum of two mismatches when mapping the reads to the reference. HISAT builds a database of potential splice junctions and confirms these by comparing the previously unmapped reads against the database of putative junctions.

2.10. Transcript abundance estimation and differentially expressed testing

The mapped read of each sample was assembled using StringTie. Then, all transcriptomes from samples were merged to reconstruct a comprehensive transcriptome using perl scripts. After the final transcriptome was generated, StringTie and edgeR were used to estimate the expression levels of all transcripts. StringTie was used to perform expression level for mRNAs by calculating FPKM. The differentially expressed mRNAs and genes were selected with log2 (fold change) >1 or log2 (fold change) <−1 and with statistical significance ($P < 0.01$) by R package.

2.11. Genome-wide DNA methylation assay

Total DNA was extracted using QIAamp Fast DNA Tissue Kit (Qiagen, Dusseldorf, Germany). The bisulfate sequence libraries were constructed using the Acegen Bisulfite-Seq Library Prep Kit (AceGen, Cat. No. AG0311), according to the manufacturer's protocol. Briefly, the genomic DNA spiked with methylated Lambda DNA was fragmented by sonication (for whole-genome bisulfite sequencing) or using MspI (NEB, USA, for reduced representation bisulfite sequencing) to a mean size of approximately 200–500 bp, then end-repaired, 5'-phosphorylated, 3'-dA-tailed, and ligated to 5-methylcytosine-modified adapters. After bisulfate treatment, the DNA was amplified with 10 cycles of polymerase chain reaction (PCR). The constructed libraries were then analyzed by Agilent 2100 Bioanalyzer and finally sequenced by Illumina platforms using a 2×150 bp paired-end sequence protocol.

2.12. Single-nucleotide polymorphism and indel analysis

We analyzed single-nucleotide polymorphism (SNP) sites in coding region at transcriptomic level. Samtools software was used for MPileUP processing according to the Hisat comparison results of each sample and the reference genome, and the possible SNP and indel information of each sample were then annotated with Annovar.

2.13. Statistical analysis

The methylKit software was used to analyze the differentially methylated regions (DMRs) between groups. A 1000 bp Windows and 500 bp overlap were selected by default. P <0.01 was the difference screening threshold. GoMiner database was used to analyze the enrichment of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) for the DMR-related genes obtained from the difference comparison of each group. The number of DMR-related genes included in each GO (or KEGG entry) was counted, and the P-value of enrichment significance of DMR-related genes in each GO (or KEGG pathway entry) was calculated by hypergeometric distribution test. t-test was used to screen the different methylation sites between groups after data processing.

3. Results

3.1. The quality of raw sequencing data and differentially expressed analysis

All the raw sequence data were eligible for further analysis, and the results of quality control are shown in Table 1. The results of mapping to genome through Hisat2 had a higher concordant rate (Table 2). Regional distribution of reference genome alignment is shown in Figure 1. Valid data that can be compared to the reference genome can be subjected to the comparisons of exon, intron, and intergeneric regions based on the region information of the reference genome. Under normal circumstances, the percentage content of sequence localization in exon region should be the highest, while reads in intron and intergeneric region are compared, which may be caused by the shearing event of pre-mRNA, incomplete genome annotation, DNA pollution and background noise, etc.

3.2. Analysis of total gene expression level

The distribution statistics of expression values in the Table 3 can be further expressed by the sample FPKM box diagram (Figure 2), so as to understand the gene expression level from the overall level. For samples of biological duplication, the reproducibility of design samples can also be preliminarily judged by the box diagram. The x-coordinate is the sample name, the y-coordinate is log10 (FPKM), and the box chart for each region corresponds to five statistics (maximum, upper quartile, median, lower quartile, and minimum from top to bottom).

Table 1. Sequence statistics and quality control.

Sample	Raw data		Valid data		Valid ratio (reads)	Q20%	Q30%	GC content%
	Read	Base	Read	Base				
LPE_1	50,110,990	7.52G	49,585,840	7.44G	98.95	99.78	98.20	55
LPE_2	48,159,708	7.22G	47,586,754	7.14G	98.81	99.74	98.02	54.50
LPE_3	52,007,858	7.80G	51,336,482	7.70G	98.71	99.69	97.96	55.50
LPE_4	40,309,918	6.05G	39,767,412	5.97G	98.65	99.72	98.08	55
LPF_1	49,703,862	7.46G	49,125,664	7.37G	98.84	99.67	97.94	55.50
LPF_2	43,420,462	6.51G	42,969,142	6.45G	98.96	99.72	97.97	55
LPF_3	49,434,484	7.42G	48,855,946	7.33G	98.83	99.76	98.13	55
LPF_4	40,791,036	6.12G	39,541,740	5.93G	96.94	99.89	98.15	54
CON_1	49,544,160	7.43G	47,851,296	7.18G	96.58	99.19	97.24	55
CON_2	45,720,378	6.86G	45,212,860	6.78G	98.89	99.76	98.09	55.50
CON_3	40,445,068	6.07G	39,991,978	6.00G	98.88	99.77	98.06	55
CON_4	46,744,408	7.01G	45,959,300	6.89G	98.32	99.78	98.18	52

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

Table 2. The sequencing raw and trimmed data mapping to the reference genome for each group.

Sample	Valid reads	Mapped reads	Unique mapped reads	Multi mapped reads	PE mapped reads	Reads map to sense strand	Reads map to antisense strand	Non-splice reads	Splice reads
LPE_1	49,585,840	47,535,165 (95.86%)	25,374,438 (51.17%)	22,160,727 (44.69%)	44,425,314 (89.59%)	16,893,012 (34.07%)	17,926,921 (36.15%)	9,816,663 (19.80%)	25,003,270 (50.42%)
LPE_2	47,586,754	45,554,047 (95.73%)	25,821,477 (54.26%)	19,732,570 (41.47%)	42,853,620 (90.05%)	17,176,552 (36.10%)	17,944,361 (37.71%)	11,322,327 (23.79%)	23,798,586 (50.01%)
LPE_3	51,336,482	49,194,876 (95.83%)	24,722,802 (48.16%)	24,472,074 (47.67%)	45,955,892 (89.52%)	16,861,421 (32.84%)	17,863,308 (34.80%)	9,183,794 (17.89%)	25,540,935 (49.75%)
LPE_4	39,767,412	38,063,647 (95.72%)	20,594,994 (51.79%)	17,468,653 (43.93%)	35,499,230 (89.27%)	13,812,681 (34.73%)	14,547,644 (36.58%)	8,703,040 (21.88%)	19,657,285 (49.43%)
LPF_1	49,125,664	47,138,541 (95.96%)	25,090,583 (51.07%)	22,047,958 (44.88%)	44,623,268 (90.83%)	16,832,181 (34.26%)	17,251,928 (35.12%)	9,062,872 (18.45%)	25,021,237 (50.93%)
LPF_2	42,969,142	41,103,823 (95.66%)	21,990,657 (51.18%)	19,113,166 (44.48%)	38,391,118 (89.35%)	14,773,806 (34.38%)	15,553,045 (36.20%)	8,792,928 (20.46%)	21,533,923 (50.11%)
LPF_3	48,855,946	46,727,599 (95.64%)	25,360,279 (51.91%)	21,367,320 (43.74%)	43,579,804 (89.20%)	16,726,508 (34.24%)	17,700,034 (36.23%)	10,203,384 (20.88%)	24,223,158 (49.58%)
LPF_4	39,541,740	37,913,410 (95.88%)	20,566,967 (52.01%)	17,346,443 (43.87%)	35,434,888 (89.61%)	14,135,611 (35.75%)	14,275,674 (36.10%)	9,322,817 (23.58%)	19,088,468 (48.27%)
CON_1	47,851,296	44,924,433 (93.88%)	24,842,903 (51.92%)	20,081,530 (41.97%)	41,396,402 (86.51%)	16,611,513 (34.71%)	17,424,875 (36.41%)	10,739,480 (22.44%)	23,296,908 (48.69%)
CON_2	45,212,860	43,239,128 (95.63%)	22,380,112 (49.50%)	20,859,016 (46.14%)	40,178,906 (88.87%)	15,072,207 (33.34%)	15,858,565 (35.08%)	7,861,707 (17.39%)	23,069,065 (51.02%)
CON_3	39,991,978	38,283,877 (95.73%)	20,607,038 (51.53%)	17,676,839 (44.20%)	35,748,418 (89.39%)	13,794,624 (34.49%)	14,535,738 (36.35%)	8,268,306 (20.67%)	20,062,056 (50.17%)
CON_4	45,959,300	43,757,403 (95.21%)	28,236,788 (61.44%)	15,520,615 (33.77%)	41,800,784 (90.95%)	18,267,361 (39.75%)	18,722,174 (40.74%)	16,610,634 (36.14%)	20,378,901 (44.34%)

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

Due to the differences in gene expression number and distribution of gene expression value in the samples, the sample expression value (FPKM) can be divided into different intervals and the number of genes expressed in different expression intervals can be calculated. The percentage in Table 4 indicates the proportion of the number of genes expressed in the expression interval between all the genes expressed in the sample. Coverage

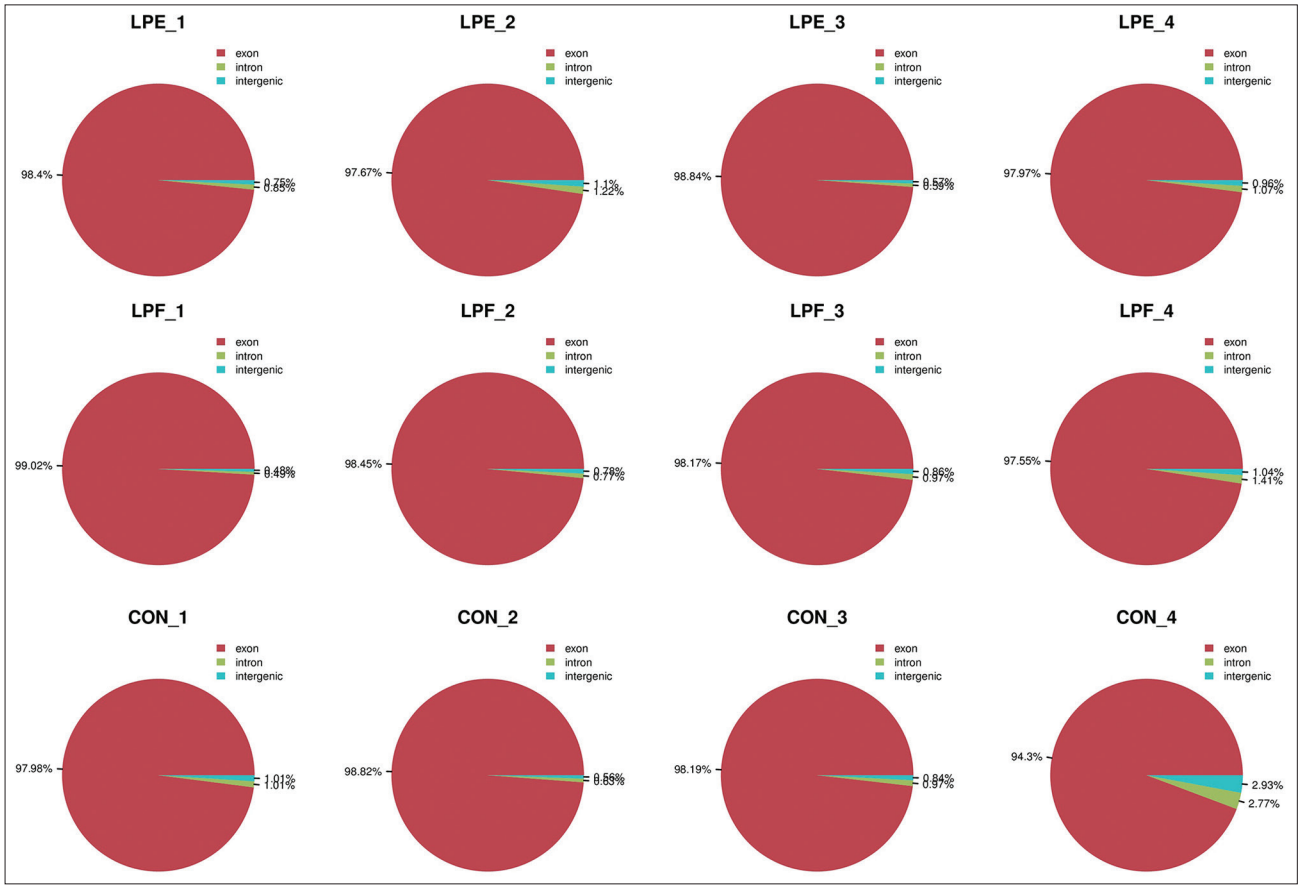

Figure 1. Regional distribution of reference genome alignment.

Table 3. Statistical table of gene expression value distribution in each sample.

Sample	Exp gene	Min.	1st Qu.	Median	Mean	3rd Qu.	Max.	Sd.	Sum.
LPE_1	13,897	0.00	0.17	0.68	20.92	2.12	100,118.16	944.65	290,677.27
LPE_2	14,566	0.00	0.25	1.20	20.94	3.83	79,119.20	782.82	304,980.25
LPE_3	12,791	0.00	0.09	0.28	22.73	0.93	103,934.75	1047.15	290,738.28
LPE_4	13,908	0.00	0.22	0.88	21.48	2.70	95,642.32	912.56	298,781.54
LPF_1	12,113	0.00	0.07	0.21	24.51	0.73	124,902.09	1245.18	296,859.96
LPF_2	13,894	0.00	0.19	0.75	21.24	2.25	90,630.45	899.69	295,102.61
LPF_3	13,937	0.00	0.17	0.71	22.50	2.17	114,011.71	1079.30	313,581.54
LPF_4	14,273	0.00	0.23	0.96	22.13	2.89	107,773.62	977.68	315,828.50
CON_1	14,332	0.00	0.22	1.03	21.81	3.28	110,772.82	998.48	312,629.31
CON_2	13,366	0.00	0.14	0.50	20.85	1.49	98,315.52	965.55	278,649.78
CON_3	13,951	0.00	0.20	0.85	20.87	2.54	96,050.62	922.73	291,113.76
CON_4	15,626	0.00	0.38	2.78	21.20	9.32	49,694.29	473.42	331,285.81

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

of gene transcripts of each sample was measured by the concept of coverage (Table 5).

The expression density map of log10 (FPKM) after the treatment of different samples can be used to compare the change of expression trend among different samples (Figure 3). Ideally, the expression density map of each sample should conform to the normal distribution, and the expression trend of biological repeat samples should

Table 4. The interval distribution statistics of different gene expression values in samples.

Sample	0-0.1 FI	0.1-0.3 FI	0.3-3.57 FI	3.57-15 FI	15-60 FI	>60 FI
LPE_1	2574 (18.52%)	2096 (15.08%)	6919 (49.79%)	1635 (11.77%)	447 (3.22%)	226 (1.63%)
LPE_2	2315 (15.89%)	1664 (11.42%)	6742 (46.29%)	2817 (19.34%)	733 (5.03%)	295 (2.03%)
LPE_3	3476 (27.18%)	3101 (24.24%)	4895 (38.27%)	805 (6.29%)	309 (2.42%)	205 (1.60%)
LPE_4	2244 (16.13%)	1870 (13.45%)	6985 (50.22%)	2047 (14.72%)	513 (3.69%)	249 (1.79%)
LPF_1	3964 (32.73%)	3071 (25.35%)	3895 (32.16%)	709 (5.85%)	283 (2.34%)	191 (1.58%)
LPF_2	2363 (17.01%)	1995 (14.36%)	7132 (51.33%)	1711 (12.31%)	457 (3.29%)	236 (1.70%)
LPF_3	2532 (18.17%)	2021 (14.50%)	7052 (50.60%)	1657 (11.89%)	441 (3.16%)	234 (1.68%)
LPF_4	2273 (15.93%)	1824 (12.78%)	7162 (50.18%)	2198 (15.40%)	530 (3.71%)	286 (2.00%)
CON_1	2339 (16.32%)	1800 (12.56%)	6835 (47.69%)	2468 (17.22%)	613 (4.28%)	277 (1.93%)
CON_2	2642 (19.77%)	2487 (18.61%)	6523 (48.80%)	1171 (8.76%)	367 (2.75%)	176 (1.32%)
CON_3	2298 (16.47%)	1962 (14.06%)	7003 (50.20%)	1940 (13.91%)	504 (3.61%)	244 (1.75%)
CON_4	2188 (14.00%)	1395 (8.93%)	4960 (31.74%)	4576 (29.28%)	1975 (12.64%)	532 (3.40%)

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

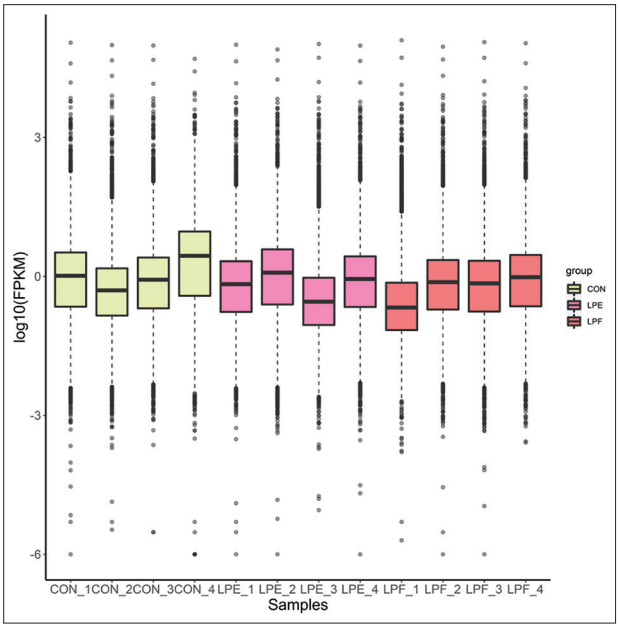

Figure 2. The distribution of gene expression values in each sample.

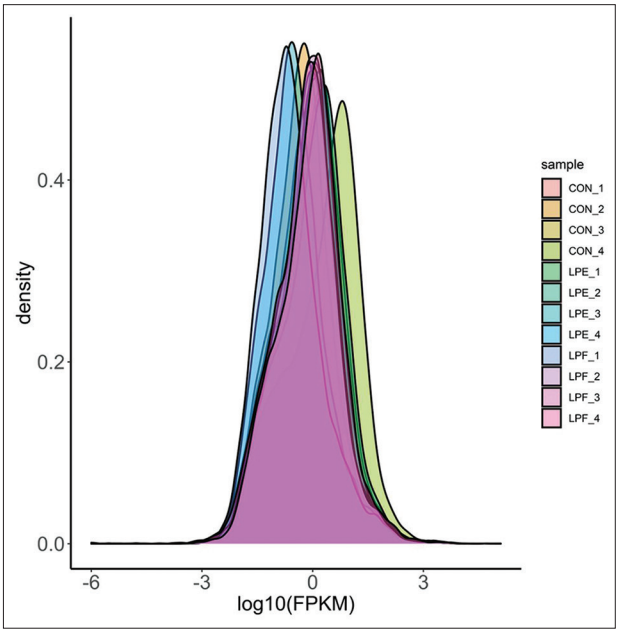

Figure 3. Density of gene expression value.

be consistent. The x-coordinate is log10 (FPKM) and the y-coordinate is the density of genes.

3.3. Volcano map analysis of the levels of differentially expressed genes

The overall distribution of differentially expressed genes can be understood by mapping volcanoes (Figure 4). Taking log2 (fold change) as the x-coordinate and -log10 (*P*-value) as the y-coordinate, volcano maps were drawn for all genes in differential expression analysis. The abscissa represents the multiple change of differential expression of genes in different samples. The ordinate represents the statistical significance of the variation on gene expression. Red represents significantly upregulated differentially expressed genes, blue represents significantly downregulated differentially expressed genes, and gray dots represent non-significant differentially expressed genes.

Among the number of significantly differentially expressed genes in LPE versus CON, LPF versus CON, and LPF versus LPE groups, there were 302 significantly differentially expressed genes in the LPF versus LPE group, of which 195 were upregulated and 107 were downregulated. Details of the other two groups are shown in Figure 5.

Table 5. Sample gene transcript coverage depth statistics.

Coverage	LPE_1	LPE_2	LPE_3	LPE_4	LPF_1	LPF_2	LPF_3	LPF_4	CON_1	CON_2	CON_3	CON_4
0–1	26.57%	23.46%	36.30%	27.12%	42.70%	26.98%	27.31%	26.59%	24.52%	30.72%	27.31%	20.55%
2–5	28.79%	22.83%	34.57%	27.81%	31.92%	29.48%	28.08%	27.15%	24.29%	33.42%	28.21%	17.64%
6–10	14.65%	13.23%	10.26%	14.86%	7.92%	15.03%	14.57%	15.32%	13.61%	13.32%	14.87%	9.39%
11–15	7.55%	8.32%	3.98%	7.97%	3.57%	7.24%	7.59%	7.80%	8.30%	5.69%	7.79%	6.09%
16–20	4.62%	5.65%	2.52%	4.51%	1.89%	4.27%	4.43%	4.76%	5.60%	3.18%	4.46%	4.91%
21–25	2.89%	4.31%	1.72%	3.20%	1.46%	2.89%	3.05%	3.19%	3.62%	2.27%	2.95%	4.17%
26–30	2.17%	3.16%	1.26%	2.05%	1.05%	2.06%	2.17%	2.17%	2.87%	1.60%	2.01%	3.25%
>30	12.76%	19.03%	9.39%	12.48%	9.49%	12.07%	12.80%	13.01%	17.19%	9.80%	12.41%	34.00%

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

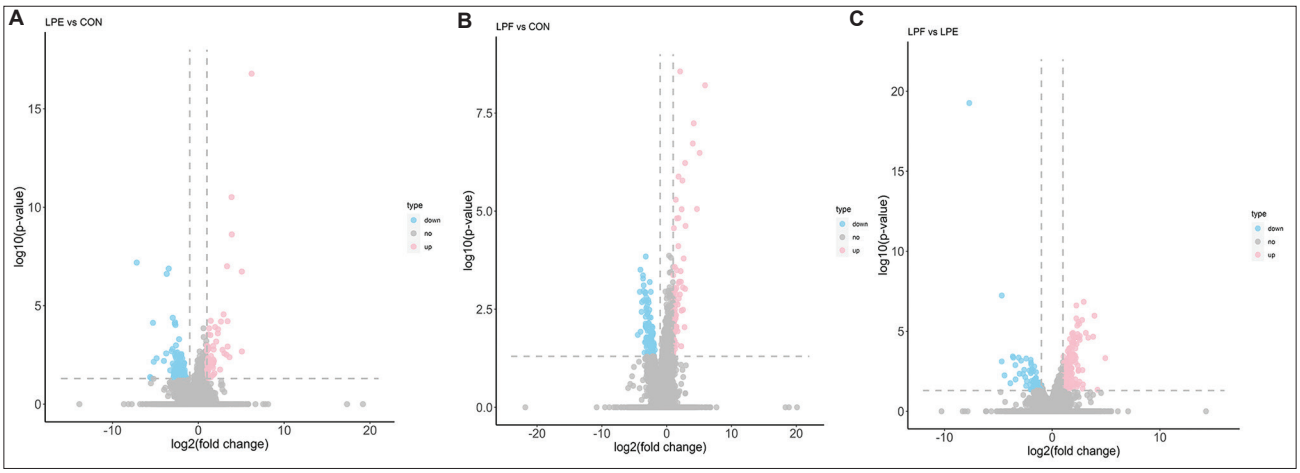

Figure 4. Volcano map analysis of the expression level of differentially expressed genes. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; and (C) is for LPF versus LPE.

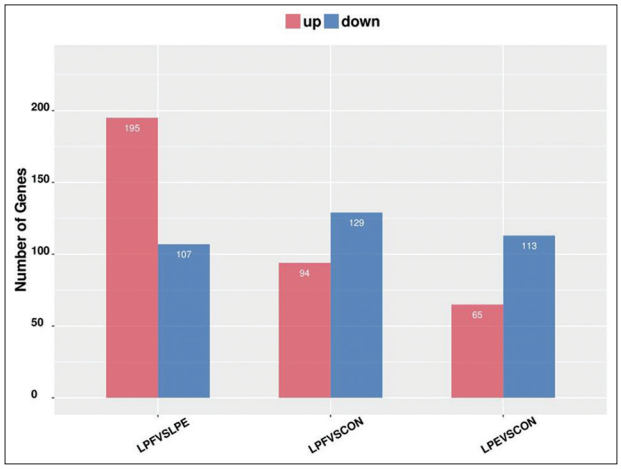

Figure 5. Up- and down-regulation of significantly differentially expressed genes in fetal low-protein group (LPF) versus early-life low-protein group (LPE), LPF versus control group (CON), and LPE versus CON groups.

According to the similarity degree of gene expression profile, the gene cluster analysis was carried out (Figure 6). The comparison between LPE versus CON and LPF versus CON revealed that the two groups share a selection of overlapping upregulated genes, such as *Gimap-9*, AABR07010705.1, AABR07031521.1, AABR07032888.1, *Serinc-4*, *Dnah-2*, *Sf3b-5*, and *Sat-2*. Comparing LPF versus CON and LPF versus LPE, *Mgat2* and *Cars* were common genes in the two groups. Comparing LPE versus CON with LPF versus CON, the overlapping downregulated gene of the two groups was *Ppp1r3d*. Comparing LPF versus CON with LPF versus LPE, the two groups shared *Ddx28* and *Slc12a9* genes.

3.4. GO enrichment analysis of differentially expressed genes

GO enrichment analysis was performed on the differentially expressed genes in the GO enrichment analysis database (http://geneontology.org/)[25], and significant differences were defined with $P < 0.01$ and the number of enriched genes ≥5. The GO items enriched by differentially expressed genes and the number of enriched genes in each GO item is shown in Tables S1-S3 of the Raw Data file. Bar chart and scatter plot of GO enrichment of differentially expressed genes are shown in Figure 7.

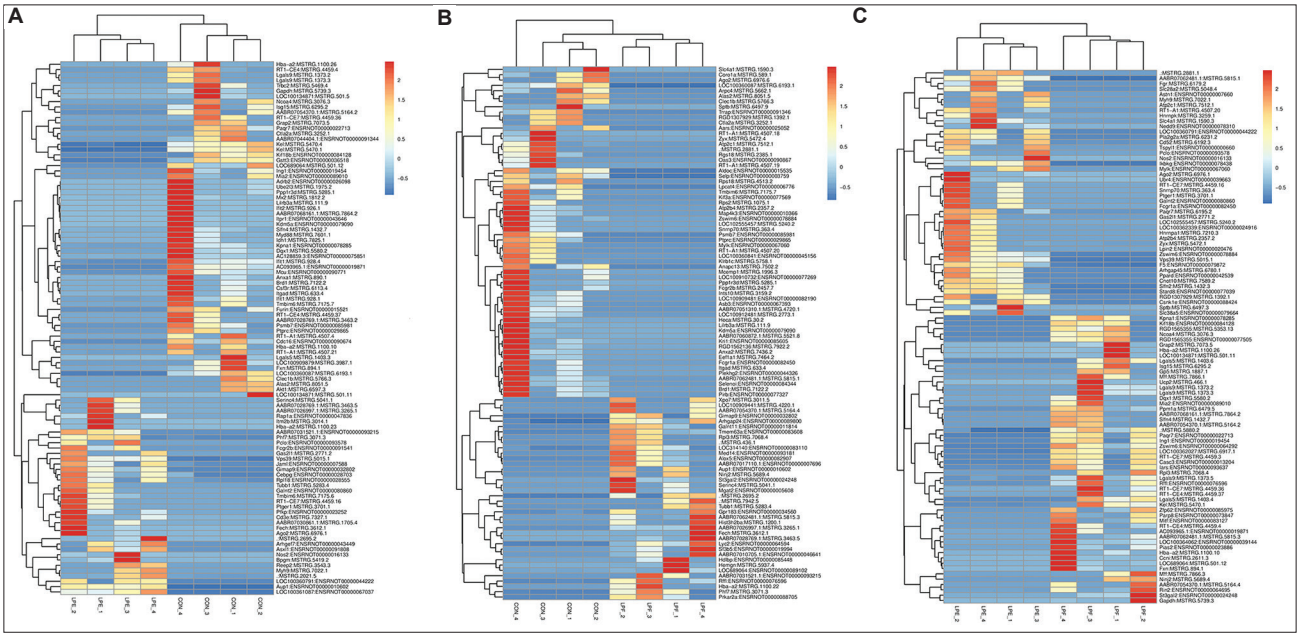

Figure 6. Cluster analysis of the expression level of differentially expressed genes. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; and (C) is for LPF versus LPE.

3.5. KEGG enrichment analysis of differential gene

In KEGG database (https://www.kegg.jp/kegg/), the difference in genes involved in the metabolic pathways of further analysis, according to the $P < 0.01$, the number of genes involved in three or more screenings, and the metabolic pathways mainly regulated by early-life malnutrition in the later growth and development of rats are shown in Tables S4-S6 of the Raw Data file. Scatter plots of KEGG enrichment of differentially expressed genes are shown in Figures 8 and 9.

3.6. SNP and indel analysis

We analyzed SNP sites in the coding region at the transcriptomic level. According to the Hisat results of each sample and the reference genome, the Samtools software was used for MPileup processing, and the possible SNP and indel information about each sample were then annotated with Annovar (Tables 6-9).

3.7. Variable shear analysis

We used ASprofile software to perform qualitative analysis of variable shear events for each sample using the gene model predicted by Stringtie (Figure 11 and Table 10).

3.8. Pre-processing of sequencing data

In general, data required include volume of sequencing data (e.g., the volume of sequencing data of sample T1 was 61,004,256 reads), average sequencing depth (=6.10 g/species genome size), and sequencing quality (98.8% of Q20), as shown in Table 9.

WALT, which is a fast and accurate read mapping for bisulfite sequencing available under the GPL v3 license and downloadable from https://github.com/smithlabcode/walt, was used to perform reference genome sequence alignment on the pre-processed valid data, and the results of genome alignment as well as the depth of sequencing were statistically analyzed (Table 11).

3.9. Sample correlation analysis

Principal component analysis (PCA) analysis was used to investigate the distribution of samples to evaluate the uniformity of biological replicates (Figure 12).

3.10. Statistical distribution of methylation on chromosomes

The number of occurrences of methylated CpG, CHG, and CHH on each chromosome was counted according to the results of genome analysis (Figure 13).

3.11. Distribution of DMRs in different segments of the genome

Compared with normal group, there were 48,149 DMRs in the LPF group, among which 29,033 (60.3%) were hypermethylated DMRs. Compared with the normal group, there were 52,185 DMRs in the LPE group, among which 32,947 (62.3%) were hypermethylated DMRs. There were

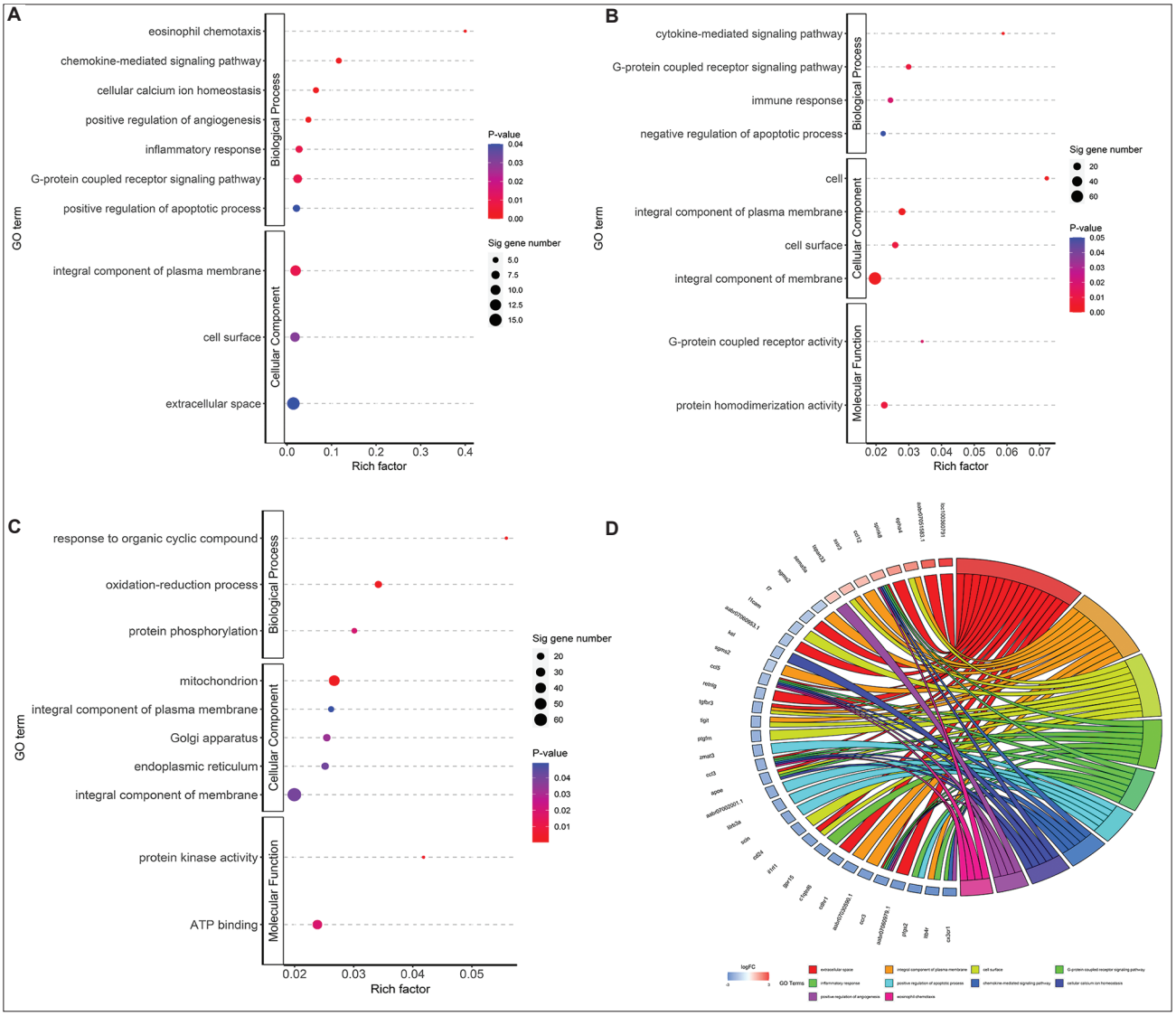

Figure 7. Scatter plot of GO enrichment of differentially expressed genes. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; (C) is for LPF versus LPE. (D) The correlation between LPE and CON in immune checkpoint.

53,410 DMRs in the LPF group compared with the LPE group, among which 25,365 were hypermethylated DMRs, accounting for 47.5%, as shown in Table 12. According to the distribution of DMRs in the genome promoter, exon, intron, and intergenic regions, the comparison of DMRs in the intergenic region among the three groups was much higher than that of the other three regions. The number of hypermethylated DMRs in the LPF group was higher than that in the normal group than that in the LPF group, especially in the intergenic region. Compared with the LPE group, the number of hypermethylated DMRs in the exonic and intergenic regions was higher than that in the demethylated DMRs, while the number of hypermethylated and demethylated DMRs in the promoter and intronic regions was similar.

3.12. GO enrichment analysis of DMR-related genes

GO enrichment analysis was carried out according to the differential DMRs screened by intergroup comparison, including molecular function (MF), cellular component (CC), and biological process (BP). According to the number of genes enriched in GO Term, the first 25, 15, and 10 items of GO function enrichment of differentially expressed genes that were methylated were statistically plotted. The results showed that the differentially expressed genes were mainly involved in the biological processes of DNA-

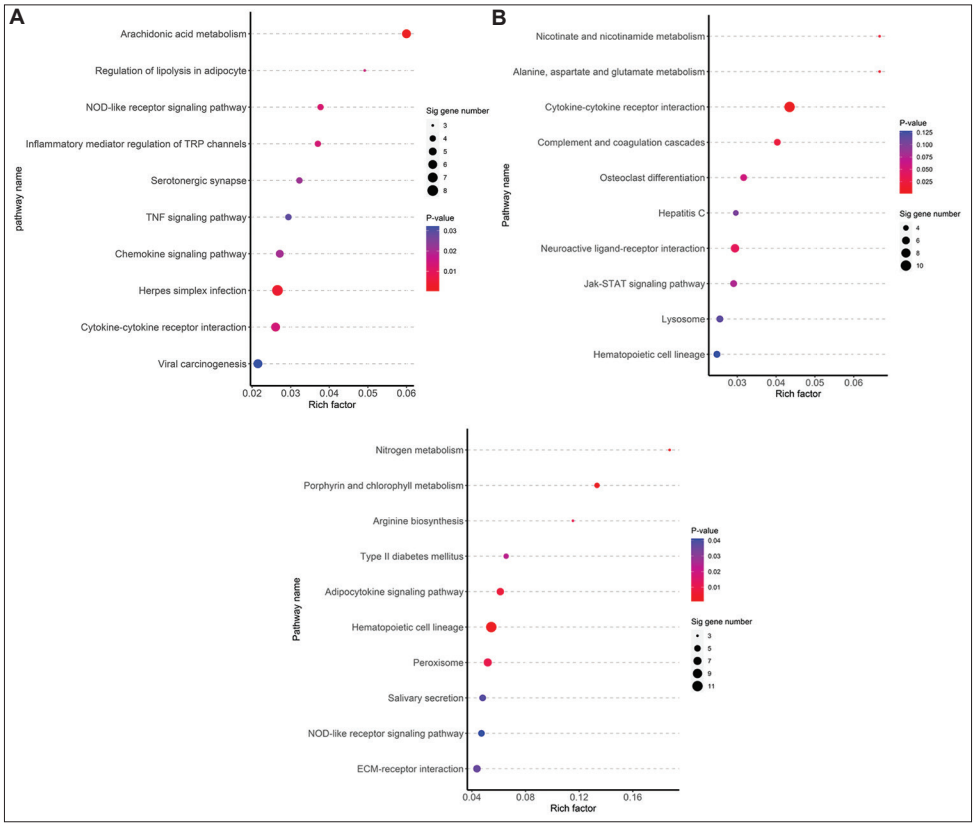

Figure 8. Scatter plot of KEGG enrichment of differentially expressed gene. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; and (C) is for LPF versus LPE.

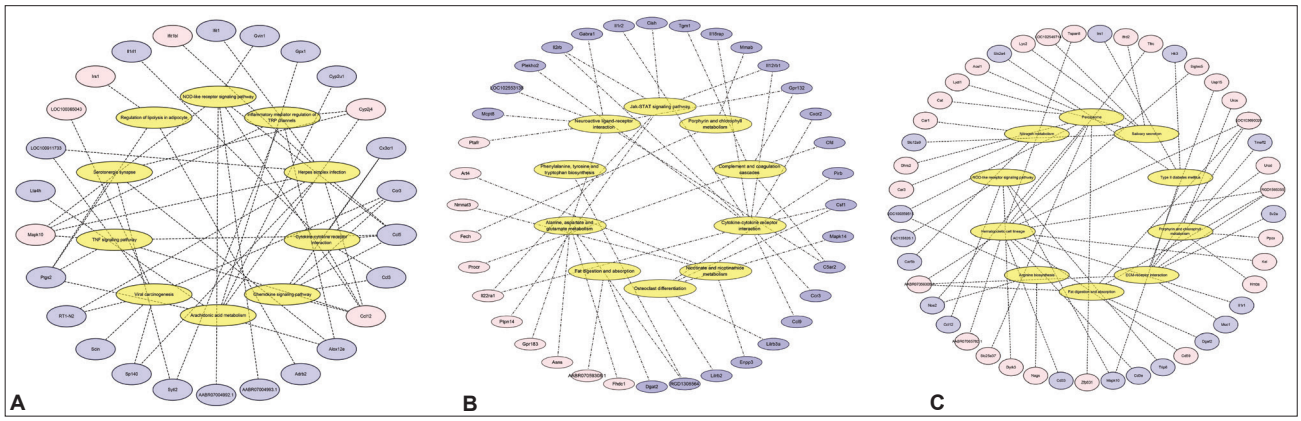

Figure 9. Molecular regulatory network of differentially expressed genes of KEGG. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; and (C) is for LPF versus LPE.

templated transcription. They are located in extracellular exosome, integral components of membrane, extracellular space, and cellular components of plasma membrane and molecular function, etc. (Figure 14).

According to the *P*-value of pair comparison among the three groups, the most significantly enriched GO terms ($P < 0.01$), 20 in total, were selected as the significant enrichment. Compared with the normal group, the LPF group contained 3 MF, 11 BP, and 6 CC, while the LPE group contained 3 MF, 7 BP, and 10 CC. Compared with the LPE group, the LPF group contained 4 MF, 6 BP, and 10 CC. The differentially expressed genes were mainly in the enrichment of transcription, poly(A) RNA binding, and transcriptional regulatory function of RNA polymerase II promoter (Tables S7-S9).

3.13. KEGG pathway enrichment analysis of differentially methylated genes

According to the KEGG annotation, the KEGG pathway contains the following intermolecular interactions and reaction networks: Metabolism, genetic information processing, environmental information processing, cellular processes, and biological systems. Overall evaluation and pairwise comparison of the three groups showed that differential genes were mainly enriched in the pathways of metabolism and organismal systems (Figure 15).

With a significant enrichment at $P < 0.01$, the differentially expressed genes were mainly enriched in the following pathways. The enrichment of differentially expressed genes in 57 pathways in the LPF group was different from that in the CON group. The differentially methylated genes were mainly involved in endocytosis under the cell process category. Differentially methylated genes are mainly involved in endocytosis under the cell process category, and in protein processing, spliceosome, and ubiquitin-mediated proteolytic pathways in the endoplasmic reticulum under the genetic information processing category. The enrichment of differentially expressed genes in 50 pathways in the LPE group was significantly different from that in the CON group, and more genes were significantly enriched in endocytosis, protein processing in endoplasmic reticulum, spliceosome, and cell adhesion molecules (CAMs), which belong to the environmental information processing class. The enrichment of differentially expressed genes in 53 pathways in LPF group was different from that in the LPE group, and the major significantly enriched pathways were similar to those in the LPE group than in the CON group, as shown in Table S10 and S11.

Table 6. Statistical table of SNP and INDEL of each sample.

Sample	SNV	SNV (gene region)	Indel	Indel (gene region)
LPE_1	152,729	40,923	7323	599
LPE_2	192,390	41,932	10,052	745
LPE_3	88,619	31,351	3745	403
LPE_4	157,613	41,109	7577	630
LPF_1	62,352	24,571	2381	262
LPF_2	128,068	40,725	5884	597
LPF_3	156,719	38,749	7638	570
LPF_4	189,352	57,741	8654	910
CON_1	160,696	41,970	8118	709
CON_2	112,760	37,478	5082	535
CON_3	151,412	41,289	7239	622
CON_4	292,764	38,766	16,292	791

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

3.14. Genetic profiles of significant differences in DMRs

The R package methylKit was used to analyze DMR. A 1000 bp windows, 500 bp overlap, and $P < 0.01$ were selected as the different screening threshold for DMRs analysis. In the three pairwise comparisons, methylation was mainly differentially expressed in *MT-Cyb*, *Vom2r75*, *Htr5a*, *Mt-Nd1*, *Mt-Co1*, *Mt-Co2,* and *Mt-Co3* genes, which were mainly mitochondrial genes. *Ces2a*, AABR07065970.1, and AABR07042565.1 were the differentially expressed methylated genes unique to the LPF group relative to the CON group, and AC239701.1 was the differentially expressed methylated gene unique to the LPE

Table 7. SNP location classification table.

Sample (SNV)	Intergenic	Intronic	Exonic	UTR3	UTR5	Upstream	Downstream	Splicing
LPE_1	26,662	51,622	40,904	18,791	2577	2384	7098	271
LPE_2	35,828	74,638	41,908	21,125	3009	3187	9302	344
LPE_3	13,845	20,630	31,332	13,455	1784	1419	4485	152
LPE_4	27,041	54,739	41,092	19,320	2619	2470	7656	235
LPF_1	9100	11,250	24,558	10,362	1498	1101	3231	142
LPF_2	21,044	34,378	40,707	18,252	2569	2060	6881	221
LPF_3	27,432	57,086	38,731	18,469	2458	2437	7433	257
LPF_4	30,812	56,480	57,724	25,377	3461	3096	9273	283
CON_1	28,978	51,940	41,953	20,566	2842	2885	8644	315
CON_2	17,609	29,859	37,458	16,130	2238	1910	5688	204
CON_3	24,740	51,849	41,271	18,648	2671	2426	7220	257
CON_4	65,176	139,697	38,742	22,063	3539	5422	12,797	536

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

Table 8. Indel location classification table.

Sample (indel)	Intergenic	Intronic	Exonic	UTR3	UTR5	Upstream	Downstream	Splicing
LPE_1	1509	2774	591	1480	122	152	566	38
LPE_2	2125	4111	738	1775	147	203	779	42
LPE_3	734	1068	399	968	65	89	341	22
LPE_4	1577	2897	624	1483	117	147	573	41
LPF_1	467	549	260	704	52	58	228	16
LPF_2	1188	1810	592	1362	115	140	538	33
LPF_3	1560	3148	566	1388	115	140	568	35
LPF_4	1734	3078	902	1740	146	201	691	38
CON_1	1751	2741	700	1752	156	178	685	40
CON_2	999	1631	529	1155	104	105	450	27
CON_3	1456	2770	613	1396	126	144	604	39
CON_4	4132	7109	781	2229	217	362	1159	54

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

Table 9. Quality pre-processing of sequencing data.

Sample	Raw data	Valid data	Q20%	Q30%	GC%
	Base	Base			
CON-1	7.75G	6.19G	98.59	96.6	22.72
CON-2	6.14G	4.95G	98.63	96.69	22.45
CON-3	8.14G	5.60G	98.45	96.19	22.7
CON-4	9.56G	5.68G	98.24	95.69	22.6
LPE-1	9.19G	6.20G	98.69	96.81	22.47
LPE-2	8.36G	5.80G	98.7	96.84	22.39
LPE-3	11.57G	6.40G	98.5	96.39	23.54
LPE-4	6.84G	5.35G	98.6	96.62	23.51
LPF-1	13.07G	5.60G	98.51	96.38	23.42
LPF-2	8.74G	6.04G	98.62	96.66	23.08
LPF-3	10.28G	5.82G	98.59	96.59	23.24
LPF-4	10.31G	5.56G	98.6	96.64	22.13

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

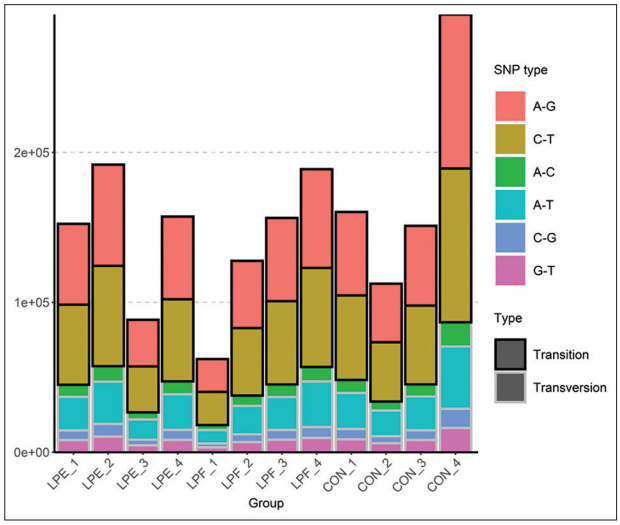

Figure 10. SNP type statistics.

group compared with the CON group. AABR07046628.1 was a unique gene that was differentially expressed in DNA methylation in the LPF group compared with the LPE group. *Mt-nd2*, *Mt-nd3*, *Mt-nd4*, *Mt-nd5*, *Mt-nd6*, *Mt-nd4l*, *Mt-atp6*, *Mt-atp8*, AABR07034833.1, and AY172581.24 were specific in the LPE group (Table 13).

4. Discussion

Malnutrition in early life refers to insufficient nutrient intake leading to nutrient deficiency in the fetus or developmental retardation of the mother's uterus, which affects the nutrition intake of the fetus[26]. Early life (generally refers to the fetal period, infant period) is the key period of growth and development, as this stage corresponds to the mature period of the formation and development of organs and tissues as well as cell differentiation. The plasticity of this stage is strong, and the development of most organs and systems is sensitive and variable to the environment during this "time window." Adverse stress can lead to increased susceptibility to the occurrence and development of diseases. A large number of epidemiological investigations, animal experiments, and clinical trials have shown that malnutrition in early life is an important environmental factor for various chronic non-communicable diseases in adulthood, but most of the relevant studies are still limited to the study of morphology[27]. With the development of genomics technology, it is possible to explore the occurrence and development of intrauterine nutritional

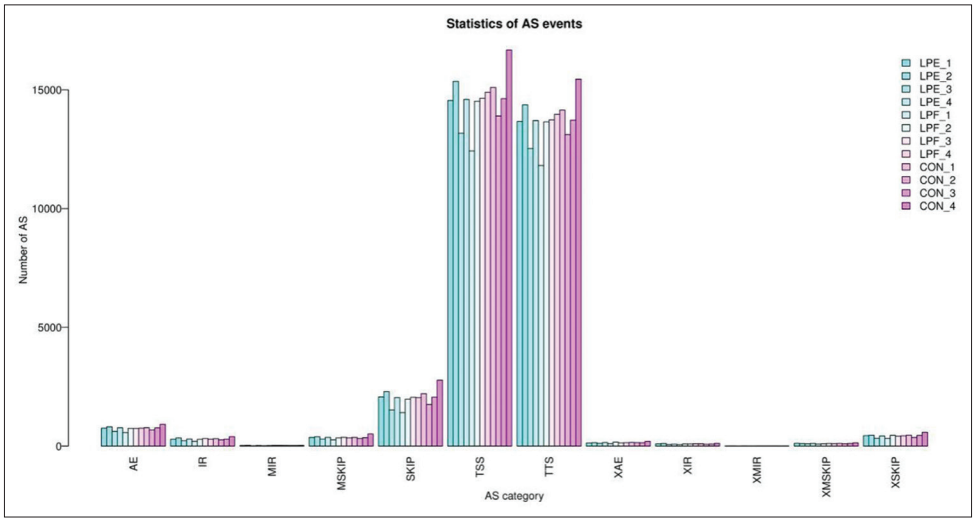

Figure 11. The species and its sequenced samples were classified and counted for variable shear events.

Table 10. Variable shear type statistics.

AS_ category	LPE_1	LPE_2	LPE_3	LPE_4	LPF_1	LPF_2	LPF_3	LPF_4	CON_1	CON_2	CON_3	CON_4
AE	756 (2.33)	815 (2.37)	620 (2.14)	770 (2.36)	571 (2.09)	744 (2.30)	743 (2.28)	756 (2.28)	775 (2.30)	677 (2.20)	768 (2.35)	916 (2.42)
IR	290 (0.89)	344 (1.00)	222 (0.77)	298 (0.91)	202 (0.74)	286 (0.88)	318 (0.97)	294 (0.89)	312 (0.92)	264 (0.86)	292 (0.89)	398 (1.05)
MIR	24 (0.07)	30 (0.09)	12 (0.04)	24 (0.07)	14 (0.05)	20 (0.06)	28 (0.09)	28 (0.08)	26 (0.08)	24 (0.08)	24 (0.07)	28 (0.07)
MSKIP	367 (1.13)	397 (1.15)	297 (1.02)	370 (1.14)	264 (0.97)	345 (1.07)	371 (1.14)	345 (1.04)	369 (1.09)	319 (1.04)	355 (1.09)	511 (1.35)
SKIP	2072 (6.37)	2292 (6.66)	1522 (5.25)	2044 (6.27)	1410 (5.17)	1978 (6.11)	2054 (6.29)	2042 (6.17)	2208 (6.54)	1754 (5.71)	2058 (6.31)	2776 (7.35)
TSS	14,550 (44.76)	15,358 (44.61)	13,170 (45.45)	14,593 (44.80)	12,426 (45.55)	14,519 (44.88)	14,645 (44.85)	14,894 (44.97)	15,098 (44.72)	13,898 (45.21)	14,625 (44.82)	16,676 (44.12)
TTS	13,664 (42.04)	14,368 (41.74)	12,526 (43.23)	13,703 (42.06)	11,814 (43.31)	13,651 (42.19)	13,736 (42.07)	13,967 (42.17)	14,146 (41.90)	13,113 (42.65)	13,719 (42.04)	15,448 (40.87)
XAE	131 (0.40)	143 (0.42)	116 (0.40)	148 (0.45)	106 (0.39)	169 (0.52)	137 (0.42)	144 (0.43)	152 (0.45)	146 (0.47)	141 (0.43)	199 (0.53)
XIR	92 (0.28)	109 (0.32)	67 (0.23)	81 (0.25)	62 (0.23)	89 (0.28)	87 (0.27)	101 (0.30)	98 (0.29)	78 (0.25)	85 (0.26)	113 (0.30)
XMIR	4 (0.01)	6 (0.02)	2 (0.01)	6 (0.02)	4 (0.01)	4 (0.01)	6 (0.02)	6 (0.02)	6 (0.02)	8 (0.03)	4 (0.01)	6 (0.02)
XMSKIP	117 (0.36)	107 (0.31)	94 (0.32)	111 (0.34)	84 (0.31)	94 (0.29)	110 (0.34)	106 (0.32)	112 (0.33)	97 (0.32)	111 (0.34)	139 (0.37)
XSKIP	438 (1.35)	457 (1.33)	330 (1.14)	428 (1.31)	321 (1.18)	455 (1.41)	415 (1.27)	434 (1.31)	459 (1.36)	366 (1.19)	449 (1.38)	584 (1.55)

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

restriction and subsequently chronic diseases at genetic level. The changes in gene expression profile detected by high-throughput sequencing technology are based on the changes in mRNA transcription level, which can screen for the upregulated or downregulated differentially expressed genes from tens of thousands of genes and then search for the candidate gene groups from the key differentially expressed genes[28]. This study compared the whole gene

Table 11. Refer to the results of genome alignment.

Sample	Total read pairs	Unique mapped reads	Unique reads mapping rate (%)	Duplication reads	Duplication rate (%)	Mean of C coverage (%)	≥ 2 × C coverage (%)	≥ 5 × C coverage (%)	≥ 10 × C coverage (%)	≥ 15 × C coverage (%)
CON-1	46,427,704	31,127,342	67.04	8,045,485	17.33	8.85	2.71	0.15	0.03	0.01
CON-2	37,189,180	24,980,322	67.17	6,539,296	17.58	7.38	2	0.09	0.02	0.01
CON-3	42,626,476	28,805,019	67.58	5,712,456	13.4	9.62	2.85	0.12	0.02	0.01
CON-4	43,577,094	29,600,141	67.93	3,928,370	9.01	11.33	3.55	0.12	0.01	0
LPF-1	41,677,970	27,091,665	65	7,416,428	17.79	7.58	2.15	0.16	0.03	0.01
LPF-2	45,268,204	30,224,062	66.77	8,009,661	17.69	9.4	2.52	0.08	0.01	0
LPF-3	43,834,898	29,552,657	67.42	7,616,973	17.38	8.14	2.44	0.2	0.05	0.02
LPF-4	41,643,906	27,741,918	66.62	7,459,192	17.91	7.92	2.29	0.1	0.02	0.01
LPE-1	46,665,754	32,188,404	68.98	8,342,186	17.88	9.82	2.75	0.07	0.01	0
LPE-2	43,751,478	30,338,359	69.34	7,986,753	18.25	9.5	2.46	0.04	0	0
LPE-3	47,565,118	29,962,681	62.99	9,229,541	19.4	7.65	2.18	0.11	0.01	0.01
LPE-4	40,483,496	26,193,485	64.7	7,182,350	17.74	7.27	2	0.12	0.03	0.01

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

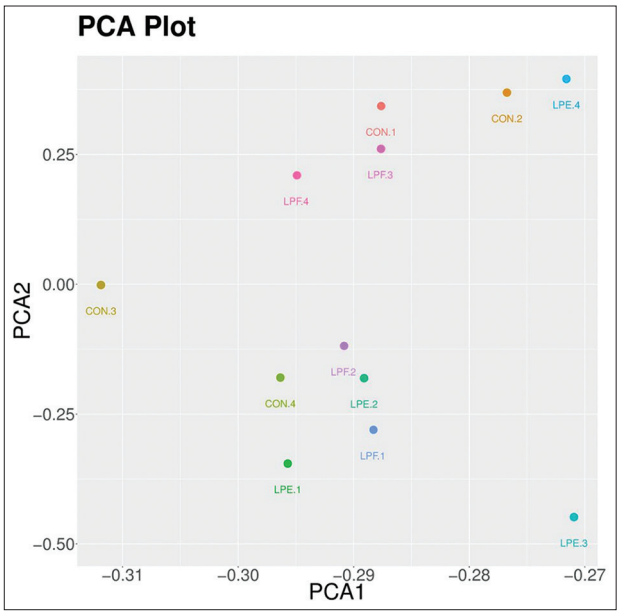

Figure 12. Analysis of PCA_2d.

expression profiles of early malnourished rats with those of normal rats, while observing the effects on the whole genome of DNA methylation in newborns[29,30]. The results showed altered gene expression of many cytokines, cellular components, and transcriptional regulatory molecules, which are involved in a variety of biological processes, such as metabolism, sensation, response, and regulation[31].

Through the intervention of protein intake in the early diet for the rats[32], the whole course low-protein group in the early life, the low-protein group in the fetal period and the normal control group were established. The results of gene detection in this study showed that the number of significantly altered gene expression in the LPE versus CON group, the LPF versus CON group, and the LPF versus LPE group was 178, 223, and 302, respectively. Compared with the control group, the number of differentially expressed genes in the offspring of the rats fed with low-protein diet during pregnancy was higher than that of the mice fed with low-protein diet during both the pregnancy and lactation periods, suggesting that nutrition during pregnancy plays a more important role in gene expression. The "thrifty gene hypothesis"[33] postulated that in the offspring rats affected by prenatal malnutrition during pregnancy, a "thrifty gene" is gradually formed and the protein content was immediately increased to 20% after birth to preserve the development of important organs such as kidney[34]; however, the beneficial effects of thrifty genes would become a burden, thereby inducing the occurrence of diseases and more changes in gene expression[35]. However, for the offspring of rats fed with 6% protein diet during pregnancy and lactation[36], the expression of organs, tissues, and genes in the body may be relatively stable due to the long period of malnutrition, and the gene does not change significantly after the replacement with normal diet. However, the number of differentially expressed genes compared between the LPE and LPF groups was 302, further indicating that the different feeding time of the low-protein diet had inconsistent effects on the genes of the offspring[37].

GO enrichment analysis showed that the differential genes in the LPE versus CON group and the LPF versus CON group were mainly related to signaling

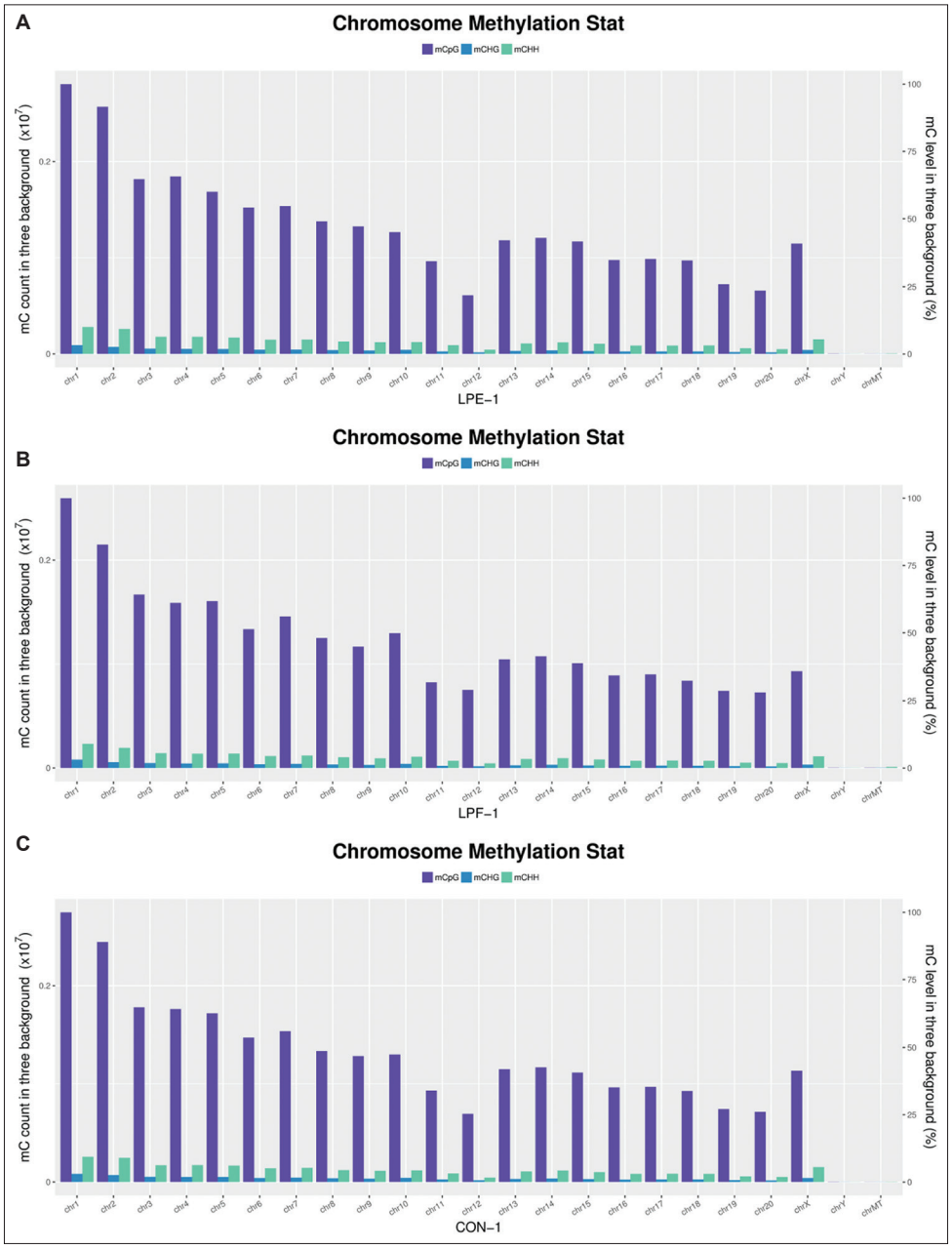

Figure 13. (A-C) Analysis of chromosome methylation levels.

pathway, cell metabolism, immune response, and other processes. The differentially expressed genes in the LPF versus LPE group were mainly related to endocrine metabolism, cell division and stress response, and their molecular functions were mainly ATP binding, protein kinase activity, and heme binding. According to KEGG enrichment results, the differentially expressed genes in the LPE versus CON group were mainly involved in viral infection, lipid metabolism regulation, signaling pathway regulation of various factors, and nervous system regulation. Differentially expressed genes in the LPE versus CON group mainly involved signaling molecule interaction, immune system, amino acid metabolism, and cofactor metabolism pathways[38]. The differentially expressed genes in the LPF versus LPE group were mainly involved in the regulation of immune system, cytokine signal regulation, tissue system, endocrine and metabolic diseases, lipid metabolism, energy metabolism, and cofactor metabolism. Therefore, malnutrition in early life can affect a number of biological functions

Table 12. Differential DMR screening.

Content	LPF versus CON		LPE versus CON		LPF versus LPE	
	Hyper-methylated	Hypo-methylated	Hyper-methylated	Hypo-methylated	Hyper-methylated	Hypo-methylated
Whole genome	29,033	119,116	32,947	19,238	25,365	28,045
Total promoters	3158	2096	3283	2042	2785	2920
Proximal	1568	1028	1682	985	1340	1491
Intermediate	998	661	1033	638	880	919
Distant	592	407	568	419	565	510
Total exons	5170	2879	5958	2869	4111	4728
First exon	1423	882	1542	846	1179	1338
Internal exon	2276	1358	2699	1347	1854	2191
Last exon	1471	639	1717	676	1078	1199
Total intron	3098	1853	3649	1793	2540	2903
First intron	643	443	748	434	557	619
Internal intron	1757	1065	2093	1040	1492	1685
Last intron	698	345	808	319	491	599
Total intergenic	17,607	12,288	20,057	12,534	15,929	17,494

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

and physiological activities through the change of gene expression[39].

The impact of early-life malnutrition on growth and development later in life has attracted a great deal of attention, and research has been devoted to investigating the genetic factors that contribute to the development of chronic diseases[40]. In this study, we found that differential expression of genome-wide DNA methylation was mainly concentrated on mitochondrial genes[41], including *Mt-cyb*, *Mt-co1*, *Mt-co3*, *Mt-co2,* and *Mt-nd1*[42], in response to environmental stimuli at different stages of early-life malnutrition. Mitochondrial DNA has an extra-nuclear genetic function and its methylation, although limited in proportion, plays a crucial role in the development of disease[43]. Tang reported that the expression of *Notch1* gene in pulmonary vascular endothelial cells of 3 and 9 weeks IUGR mice was significantly decreased compared with the control group, and the expression of the downstream gene *Hes-1* was also significantly decreased[44]. Kuang pointed out that a low-protein diet during pregnancy in rats may lead to IUGR in offspring and significant renal impairment in adulthood. The results showed that the abnormal expression of *Wt1* and *Igf2* may be involved in the reduction of glomerulus in IUGR rats and the occurrence of adult proteinuria, and normal protein feeding in adulthood cannot correct the abnormal methylation state of *Wt1* gene nor prevent kidney damage[45]. Chen *et al.* reported that early-life malnutrition affects the cognitive function of young mice in the senile stage, and the mechanism may be related to the abnormal expression of *PI3Kp110α* in hippocampus[46]. Da Costa *et al.* gave one group of pups a 0% protein diet and the other a standard diet during lactation. The expression of *CYP* gene was significantly upregulated in both 60- and 90-day-old rats[47]. Nutritional status may affect the metabolism of drugs and other substances through the regulation of the expression of CYP enzyme, leading to disrupted hormone homeostasis and various chronic diseases. These studies shed light on a number of ways that malnutrition caused by protein restriction early in life can lead to genetic changes in fetuses that have important effects on later growth and development. However, these gene changes were not detected in this study, which may be related to protein content[48], feeding time, rat species, and test samples. Some genes exist only in certain tissue structures but not in blood.

By comparing and analyzing the significantly differentially expressed genes between LPE versus CON and LPF versus CON, this study found that the two groups shared a number of upregulated genes[49], such as *Gimap-9*, AABR07010705.1, AABR07031521.1, AABR07032888.1, *Serinc-4*, *Dnah-2*, *Sf3b-5,* and *Sat-2*. Comparing LPF versus CON with LPF versus LPE, *Mgat2* and *Car3* were common genes in the two groups. Comparing LPE versus CON with LPF versus CON, the common downregulated gene of the two groups was *Ppp1r3d*. Comparing LPF versus CON with LPF versus LPE, we found that the two groups shared *Ddx28* and *Slc12a9* genes. These gene expression changes may play a significant role in the growth and development of early malnourished rats[50]. For example, *Mgat2* is mainly

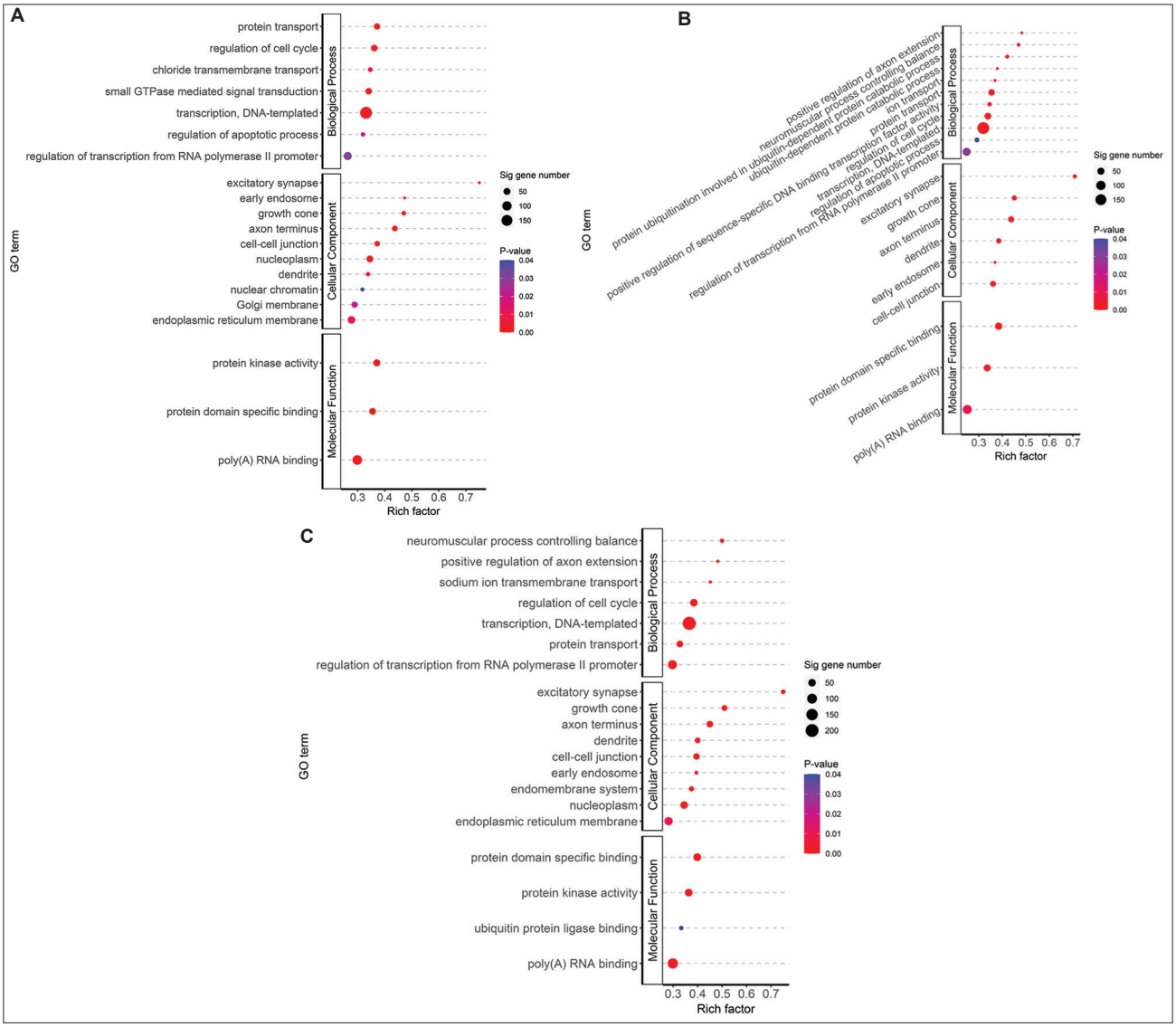

Figure 14. Scatter plot of GO enrichment of methylation levels. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; and (C) is for LPF versus LPE.

expressed in small intestines, and its role is to catalyze the synthesis of diacylglycerol (DAG) from free fatty acids (FFA) and Sn-monoacylglycerol (MG) from dietary fats. Mgat2-deficient mice may delay FFA and Sn-MG absorption from the proximal to distal parts of the small intestine, increase energy consumption and regulate diet-induced thermal energy production, and show resistance to obesity, glucose tolerance, hypercholesterolemia, and hepatic steatosis. However, in this study, the expression of *Mgat2* was upregulated in both the LPF versus CON and LPF versus LPE groups, which means that the offspring of early protein-malnourished rats may develop chronic diseases, such as obesity and hepatic steatosis, due to the increased expression of *Mgat2*. Another example is PPPLR3D, a glycogen-targeting subunit of protein phosphohydrolase 1 family, which is mainly distributed in liver, skeletal muscle, pancreas, and brain. Recent studies have shown that the glycogen activity of PPPLR3D is regulated by ubiquitination, and PPPLR3D is associated with a specific phosphate-epileptic protein involved in Lafora disease[51], a type of progressive myoclonic epilepsy. In this study, the expression of *Ppplr3d* was downregulated in both LPE versus CON and LPF versus CON groups, so it was speculated that this gene variation might also lead to neuro-related diseases in early malnourished adult rats.

This study found that *Mt-nd* (2, 3, 4L, 4, 5, 6, ATP-6, and ATP-8) and DNA methylation were differentially

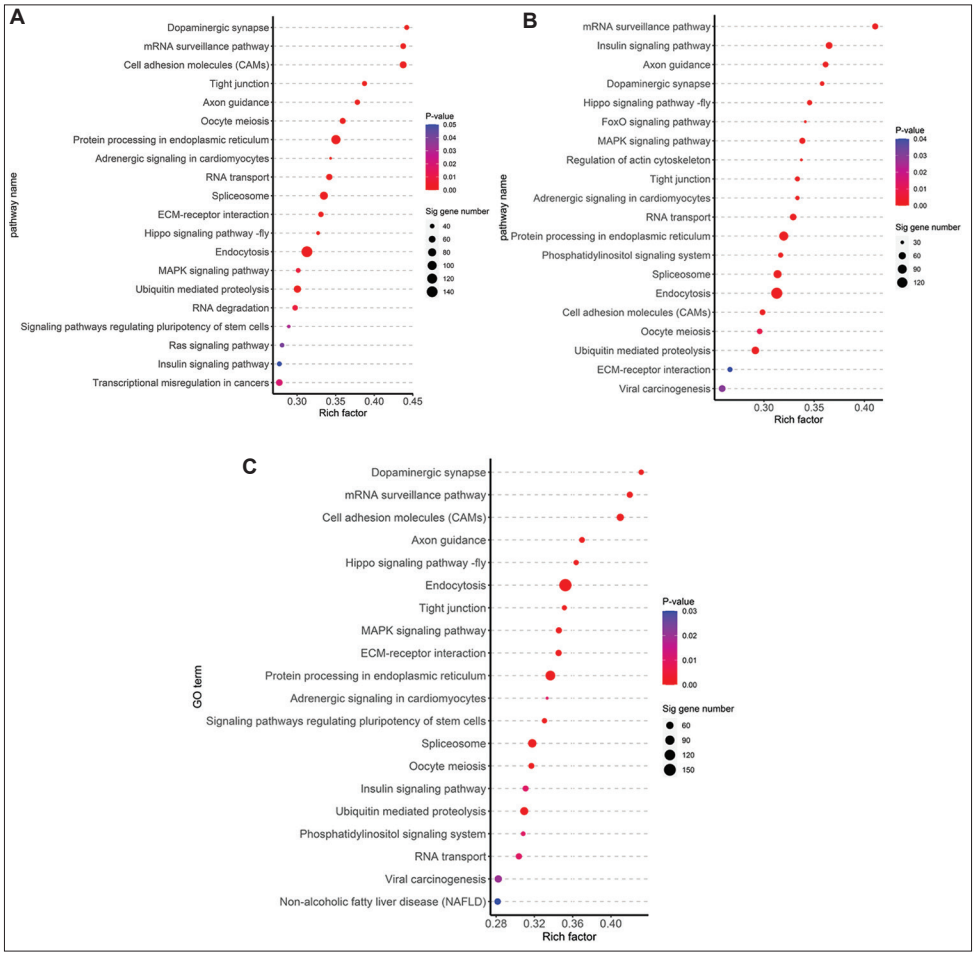

Figure 15. Scatter plot of KEGG enrichment of methylation levels. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; and (C) is for LPF versus LPE.

expressed in rats at the early stage of life compared with malnourished rats during pregnancy. Therefore, whether the methylation of *MT-ND* (1, 2, 3, 4L, 4, 5, and 6) gene is associated with frontotemporal lobe degeneration diseases deserves further exploration.

5. Conclusion

Malnutrition in early life can regulate the expression of genes related to endocrine metabolism, inflammatory factors, immune function, viral response, and signal transduction, thus regulating the body's metabolism, cell proliferation, division, apoptosis, and inflammatory response. The previous studies have proven that genetic mutations caused by malnutrition in early life are closely related to the occurrence of chronic diseases, such as cardiovascular disease, kidney disease, and neurological disease in adulthood, which indicate that exploring genetic changes provide a new direction for the diagnosis and treatment of a variety of diseases. However, the genes with significant differences excavated in this study are rarely reported. According to the KEGG pathway and GO enrichment analysis, they must be related to the occurrence and development of later diseases, which also opens up new prospects for future experimental research.

Whole-genome DNA methylation sequencing was performed on the whole blood of the offspring rats of the LPE and LPF groups, and it was found that there were significant differences in the methylation sites and levels of the genes, especially the mitochondrial genes.

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Table 13. Genetic profiles of significant differences in DMRs.

chr	Gene symbol	Methylation level		Fold-change	Methylation type	p	q
		LPE	CON				
chrMT	*Mt-cyb*	0.52	0.1	5.28	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd5*	0.52	0.1	5.28	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd3*	0.52	0.1	5.28	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd4l*	0.52	0.1	5.28	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd4*	0.52	0.1	5.28	Hyper-methylated	< 0.001	< 0.001
chr4	*Htr5a*	12.71	4.63	2.74	Hyper-methylated	0.01	0.37
chr12	AABR07034833.1	6.67	3.89	1.72	Hyper-methylated	0.01	0.17
chr18	*Vom2r75*	5.88	3.9	1.51	Hyper-methylated	0.02	0.42
chrMT	*Mt-co2*	0.36	0.31	1.17	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-co3*	0.36	0.31	1.17	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-co1*	0.36	0.31	1.17	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd2*	0.36	0.31	1.17	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-atp8*	0.36	0.31	1.17	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-atp6*	0.36	0.31	1.17	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd6*	0.36	0.34	1.06	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd1*	1.48	1.71	0.87	Hypo-methylated	< 0.001	< 0.001
chrMT	AY172581.24	1.48	1.71	0.87	Hypo-methylated	< 0.001	< 0.001
chr3	AABR07051177.1	2.36	3.51	0.67	Hypo-methylated	0.03	0.48
chr15	*Dlg5*	0.69	1.04	0.67	Hypo-methylated	0.04	0.55
chrY	AC239701.1	31.56	48.5	0.65	Hypo-methylated	< 0.001	0.04
chr9	*Efhb*	3.23	6.43	0.5	Hypo-methylated	0.03	0.48
chr20	*RT1-CE10*	0.95	2.77	0.34	Hypo-methylated	< 0.001	0.02
chrMT	*Mt-nd1*	3.69	1.82	2.03	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd2*	3.69	1.82	2.03	Hyper-methylated	< 0.001	< 0.001
chrMT	AY172581.24	3.69	1.82	2.03	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-co1*	2.84	1.48	1.92	Hyper-methylated	< 0.001	< 0.001
chr20	*Rt1-ce10*	1.75	1	1.76	Hyper-methylated	0.01	0.21
chr5	*Tipinl1*	12.83	8.55	1.5	Hyper-methylated	< 0.001	0.11
chr15	*Dlg5*	1.03	0.69	1.5	Hyper-methylated	0.04	0.53
chr12	AABR07034833.1	14.75	11.11	1.33	Hyper-methylated	0.02	0.33
chrMT	*Mt-co3*	0.41	0.31	1.32	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd4l*	0.41	0.31	1.32	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd4*	0.41	0.31	1.32	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd5*	0.41	0.31	1.32	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd3*	0.41	0.31	1.32	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-atp6*	0.41	0.31	1.32	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-co2*	0.37	0.31	1.22	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-atp8*	0.52	0.45	1.16	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-cyb*	0.36	0.32	1.13	Hyper-methylated	< 0.001	< 0.001
chrMT	*Mt-nd6*	0.36	0.32	1.13	Hyper-methylated	< 0.001	< 0.001
chr4	*Htr5a*	4.43	5.77	0.77	Hypo-methylated	< 0.001	0.23
chr18	*Vom2r75*	4.01	5.88	0.68	Hypo-methylated	0.05	0.55
chr5	AABR07046628.1	1.09	2.23	0.49	Hypo-methylated	0.02	0.44
chr19	LOC679149	1.64	3.64	0.45	Hypo-methylated	0.02	0.41

LPE: Early-life low-protein group, LPF: Fetal low-protein group, CON: Control group

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Conflict of interest

The authors declare no competing interests.

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Investigation: Zhi Qu, Liying Fu, Chenchen Wang, and Suting Liu
Supervision: Zhi Qu and Bo Li
Writing – original draft: Zhi Qu, Liying Fu, Chenchen Wang, Suting Liu, and Bo Li
Writing – review & editing: Zhi Qu and Bo Li

Ethics approval and consent to participate

The study was reviewed and approved by the Ethics Committee of Medicine and Scientific Research of Henan University (HUSOM-2019-112).

Consent for publication

Not applicable.

Availability of data

Source data are provided with this paper.

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