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A graphic illustration of double-stranded DNA

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Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

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

Zhi Qu[1], Liying Fu[1,2], Chenchen Wang[1], Suting Liu[1], and Bo Li[1]*

[1]Institute of Chronic Disease Risks Assessment, School of Nursing and Health, Henan University, Kaifeng, 475004, P. R. China

[2]Henan Provincial People's Hospital, Henan Provincial Eye Hospital, People's Hospital of Zhengzhou University, People's Hospital of Henan University, Zhengzhou, 450003, China

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

***Corresponding author:**
Bo Li
(10210022@vip.henu.edu.cn)

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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

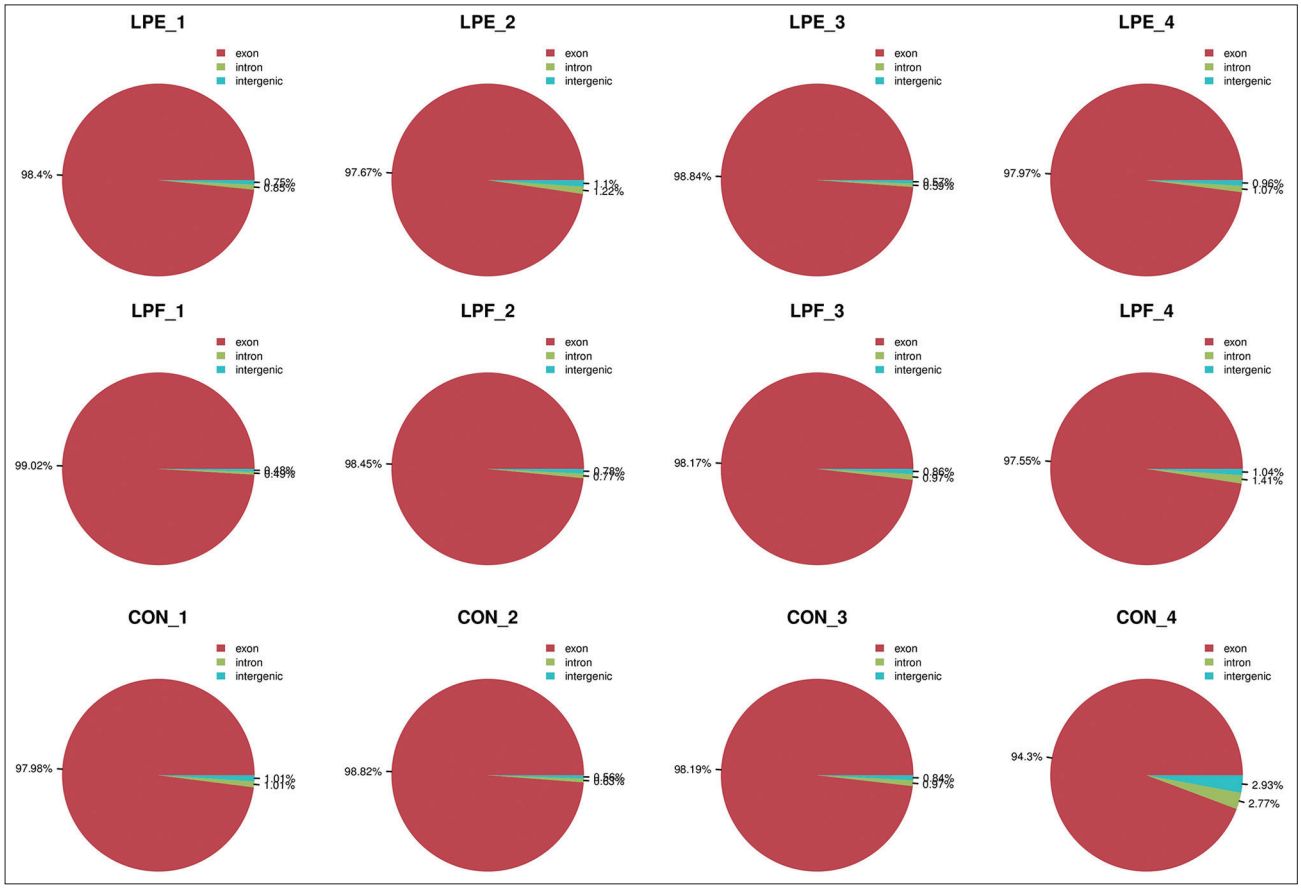

Figure 1. Regional distribution of reference genome alignment.

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

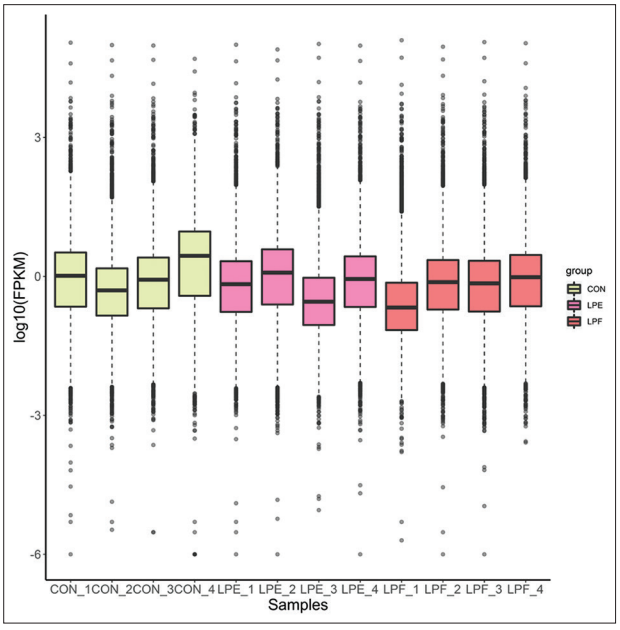

Figure 2. The distribution of gene expression values in each sample.

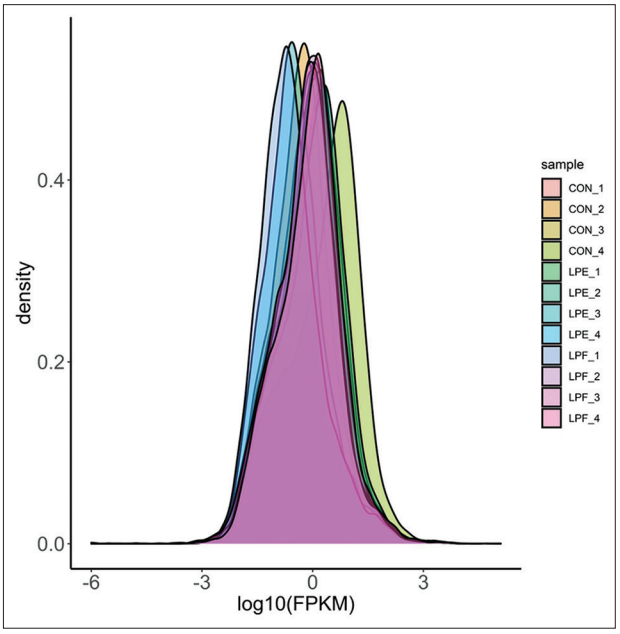

Figure 3. Density of gene expression value.

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

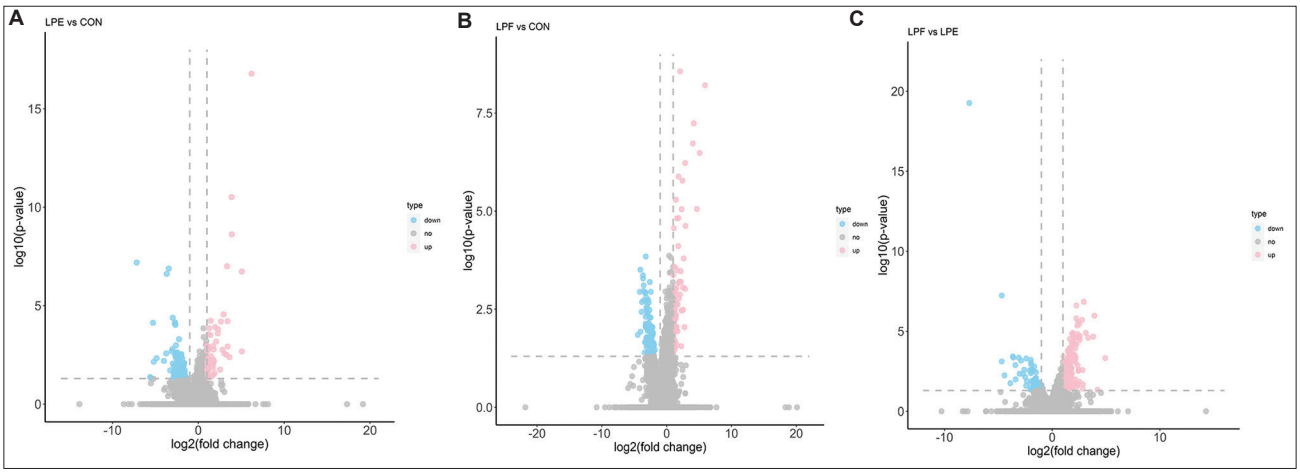

Figure 4. Volcano map analysis of the expression level of differentially expressed genes. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; and (C) is for LPF versus LPE.

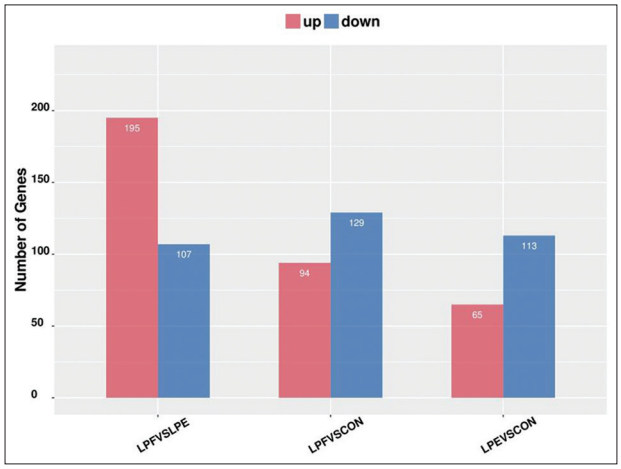

Figure 5. Up- and down-regulation of significantly differentially expressed genes in fetal low-protein group (LPF) versus early-life low-protein group (LPE), LPF versus control group (CON), and LPE versus CON groups.

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.

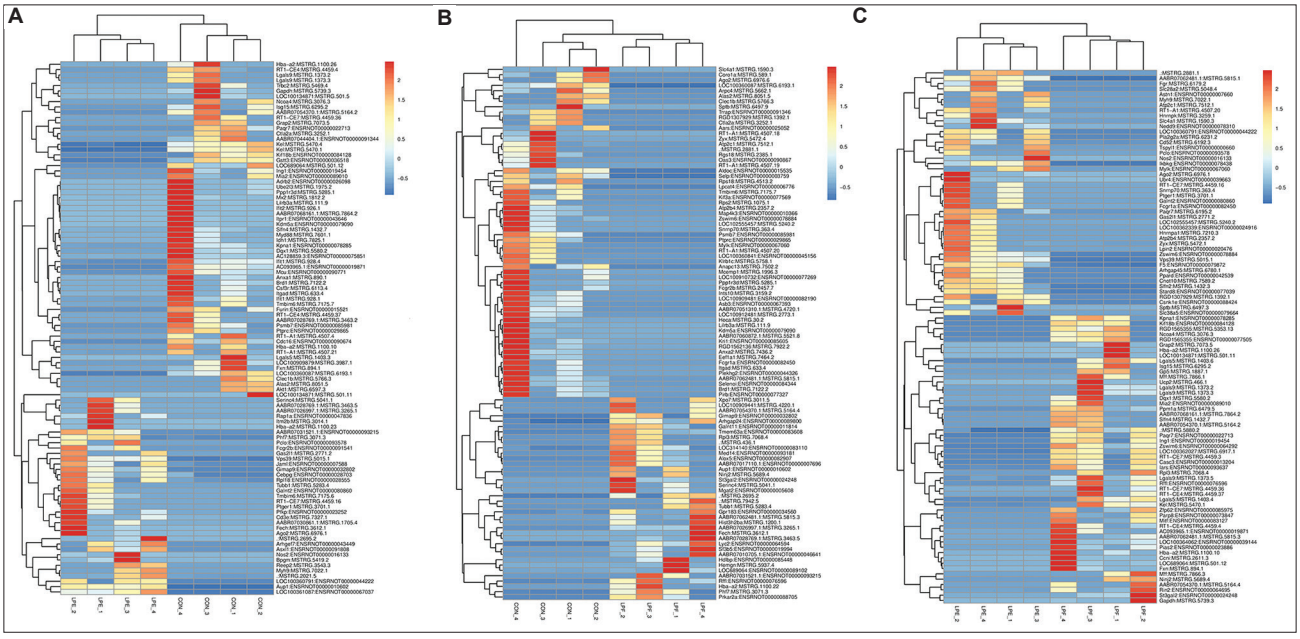

Figure 6. Cluster analysis of the expression level of differentially expressed genes. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; and (C) is for LPF versus LPE.

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

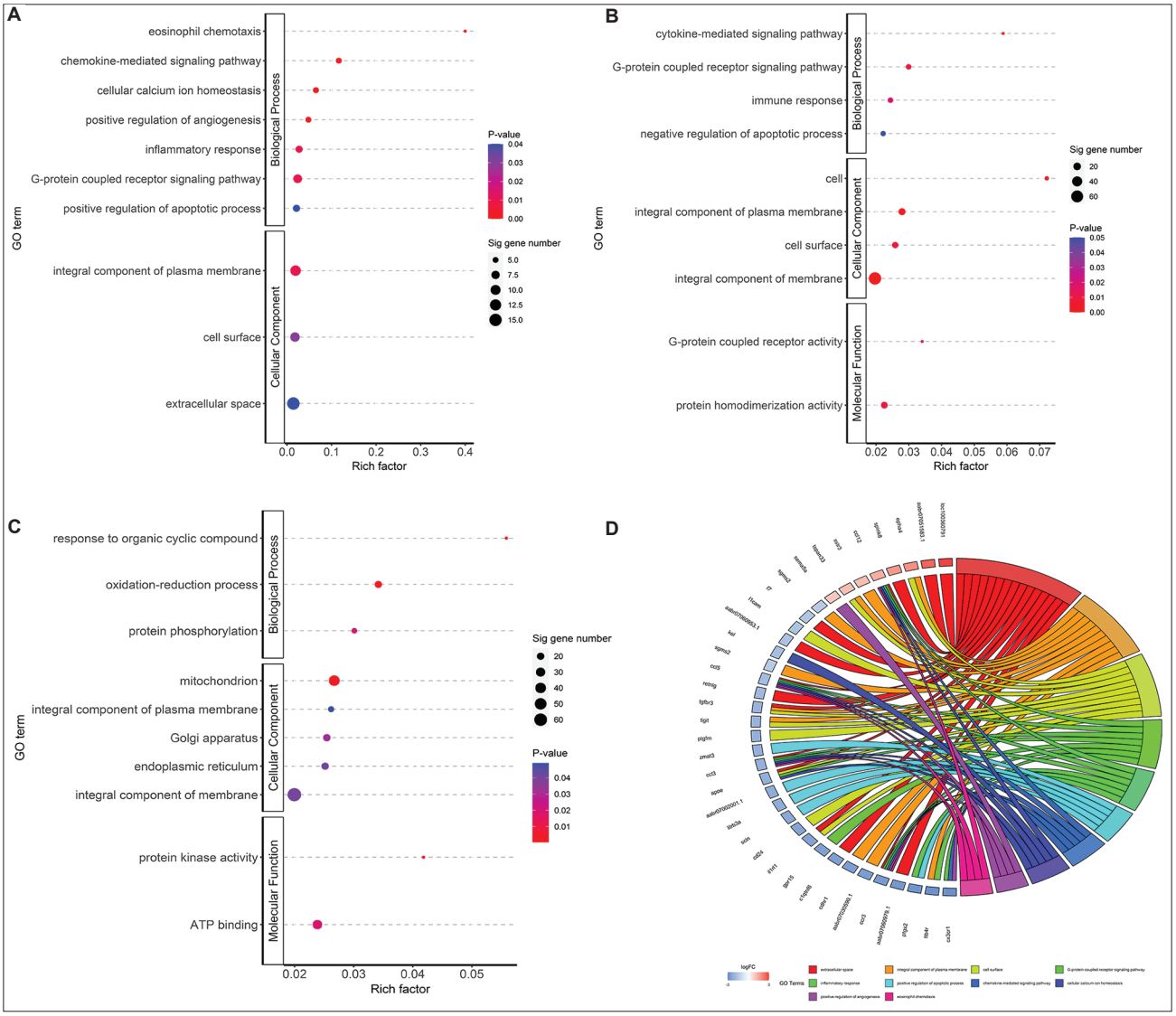

Figure 7. Scatter plot of GO enrichment of differentially expressed genes. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; (C) is for LPF versus LPE. (D) The correlation between LPE and CON in immune checkpoint.

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-

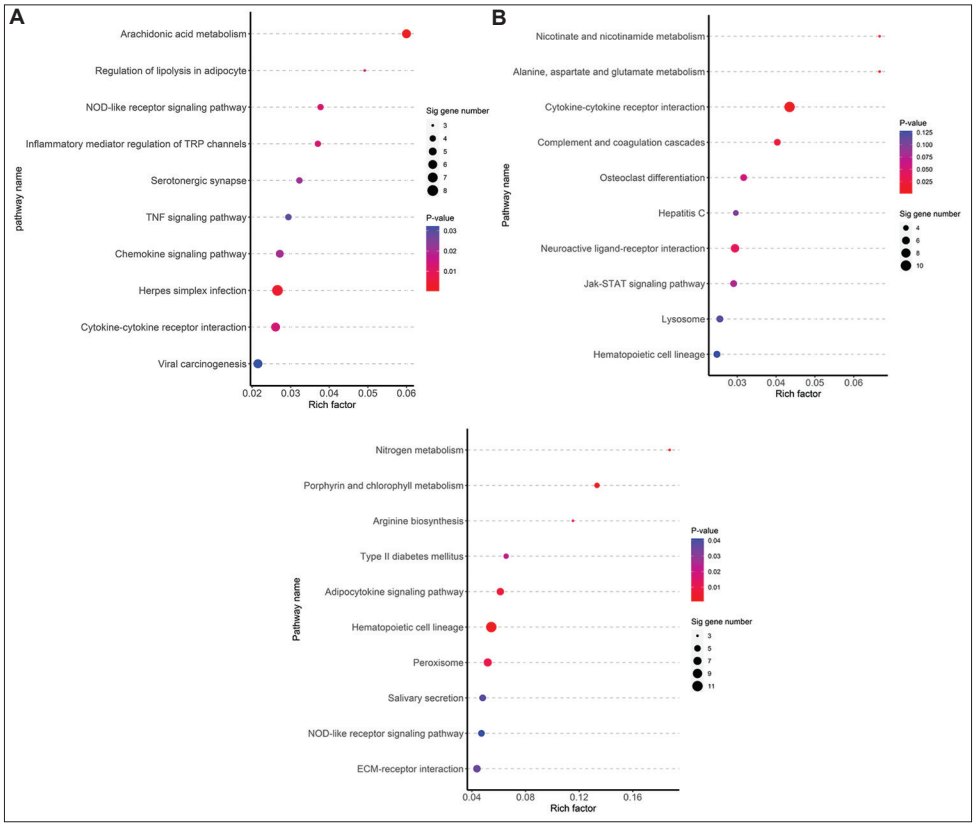

Figure 8. Scatter plot of KEGG enrichment of differentially expressed gene. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; and (C) is for LPF versus LPE.

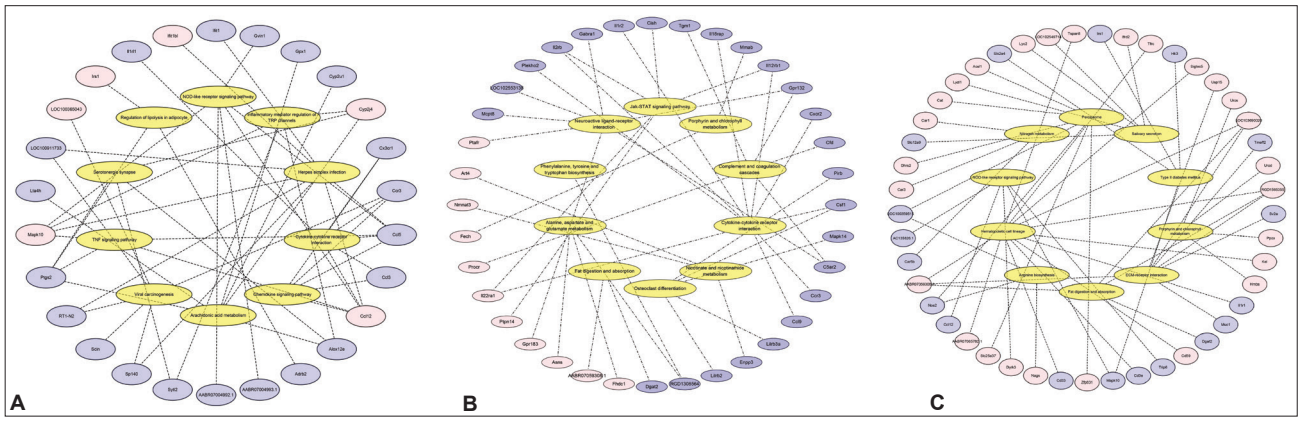

Figure 9. Molecular regulatory network of differentially expressed genes of KEGG. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; and (C) is for LPF versus LPE.

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

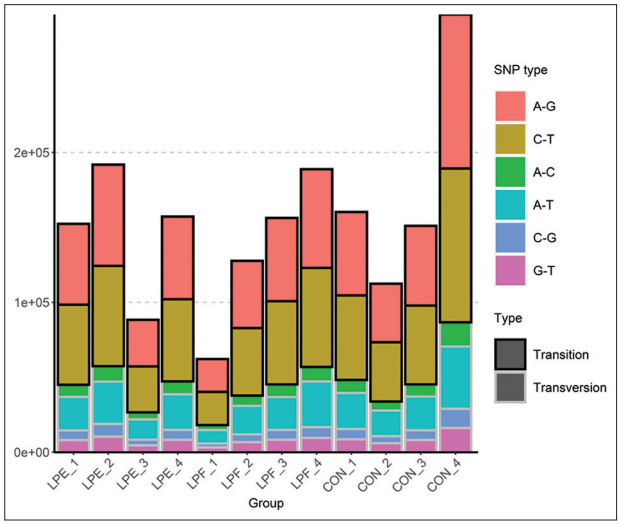

Figure 10. SNP type statistics.

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

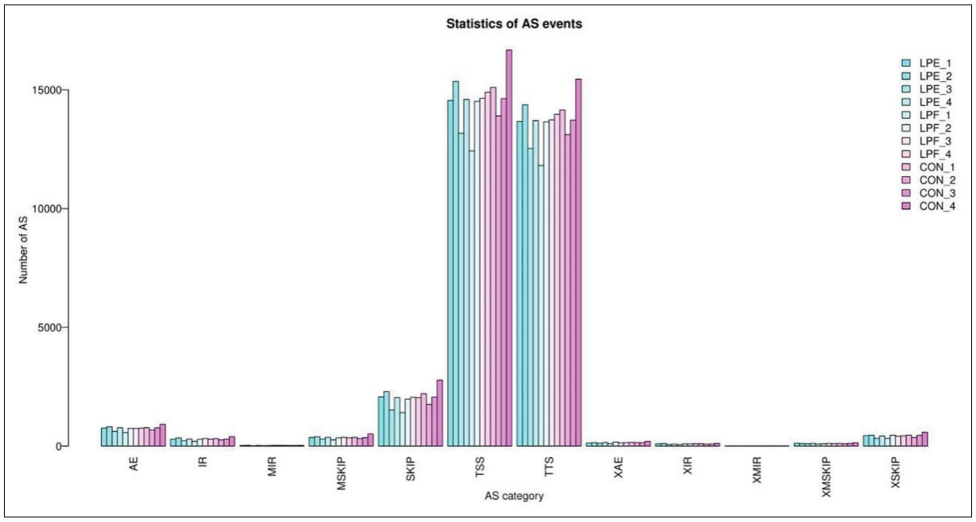

Figure 11. The species and its sequenced samples were classified and counted for variable shear events.

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

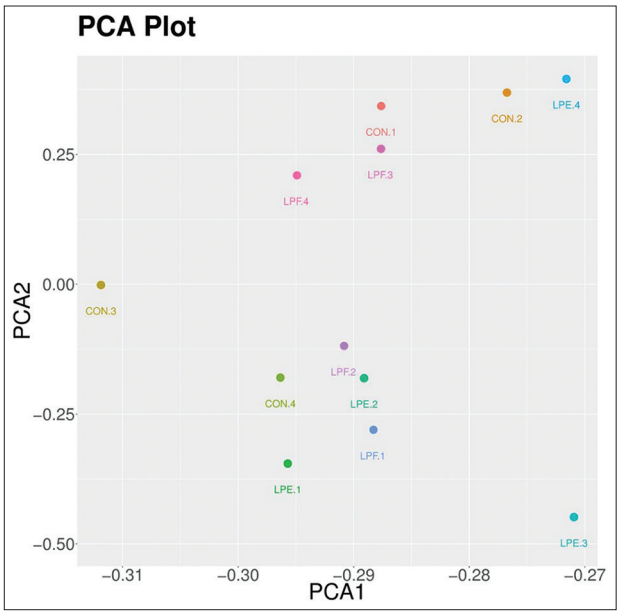

Figure 12. Analysis of PCA_2d.

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

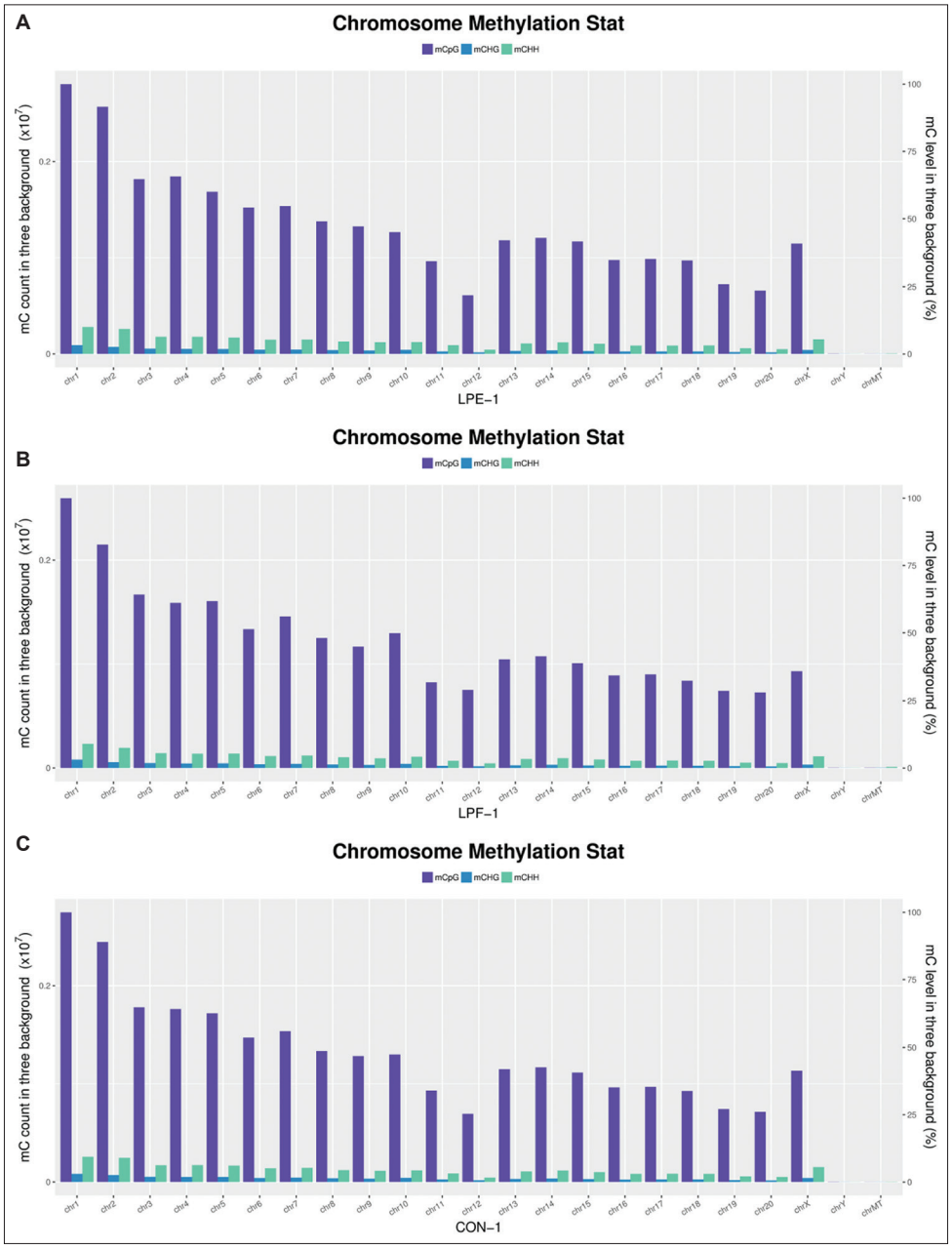

Figure 13. (A-C) Analysis of chromosome methylation levels.

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

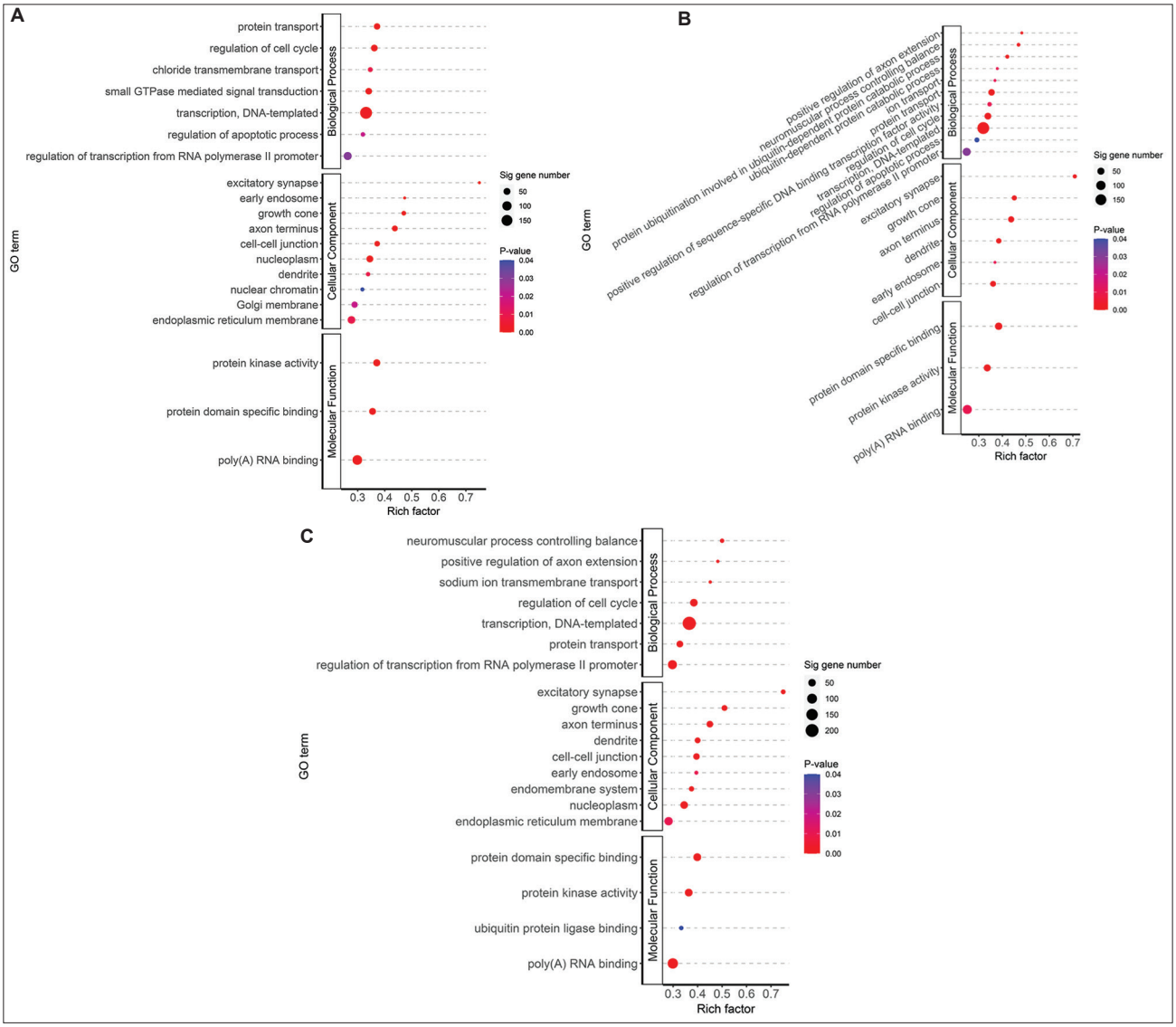

Figure 14. Scatter plot of GO enrichment of methylation levels. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; and (C) is for LPF versus LPE.

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

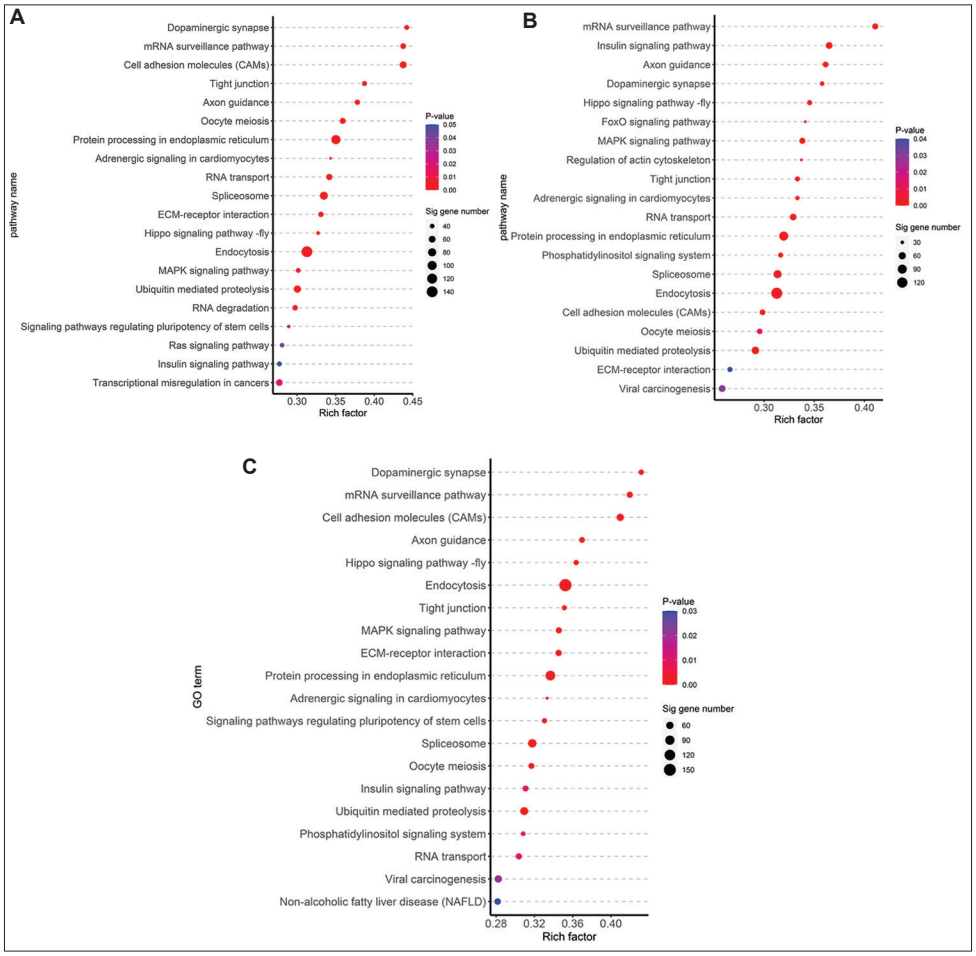

Figure 15. Scatter plot of KEGG enrichment of methylation levels. (A) is for early-life low-protein group (LPE) versus control group (CON); (B) is for fetal low-protein group (LPF) versus CON; and (C) is for LPF versus LPE.

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.

Acknowledgments

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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.

Author contributions

Conceptualization: Zhi Qu and Bo Li
Formal analysis: Zhi Qu, Liying Fu, Chenchen Wang, and Suting Liu
Funding acquisition: Zhi Qu and Bo Li
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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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

REVIEW ARTICLE

Insights into the role of *RUNX1* gene in female-related cancers

Sagarika Shahriar[1†], Zarin Tasnim Rafa[1†], Maiesha Samiha Mahmood[1†], Deera Mahasin[1†], Yusha Araf[2†], Md. Asad Ullah[3], MD. Hasanur Rahman[4], Md. Akkas Ali[5], Fatama Tous Zohora[6], Chunfu Zheng[7,8*], and Mohammad Jakir Hosen[5*]

[1]Biotechnology Program, Department of Mathematics and Natural Sciences, School of Data and Sciences, Brac University, Dhaka, Bangladesh

[2]Department of Biotechnology, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh

[3]Department of Biotechnology and Genetic Engineering, Faculty of Biological Sciences, Jahangirnagar University, Savar, Dhaka, Bangladesh

[4]Department of Biotechnology and Genetic Engineering, Faculty of Life Sciences, Bangabandhu Sheikh Mujibur Rahman Science and Technology University, Gopalganj, Bangladesh

[5]Department of Genetic Engineering and Biotechnology, School of Life Sciences, Shahjalal University of Science and Technology, Sylhet, Bangladesh

[6]Department of Biotechnology and Genetic Engineering, Faculty of Life Science, Mawlana Bhashani Science and Technology University, Tangail 1902, Bangladesh

[7]Department of Immunology, School of Basic Medical Sciences, Fujian Medical University, Fuzhou, China

[8]Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada

[†]These authors contributed equally to this work.

***Corresponding authors:**
Chunfu Zheng
(zheng.alan@gmail.com)
Mohammad Jakir Hosen
(jakir-gen@sust.edu)

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Abstract

The transcription factors of runt-related transcription factor (RUNX) are important regulators of various developmental pathways, with roles in proliferation, differentiation, apoptosis, and cell lineage. One of the core subclasses of the RUNX family that codes for a number of transcription-binding proteins is the *RUNX1* gene, which is located on chromosome 21q22.12. There has been extensive research on *RUNX1* mutations in hematological cancers, where the most conspicuous position of several chromosomal translocations has drawn interest as a tumor suppressor. In this paper, the malignancies triggered by *RUNX1* mutations, which are strongly associated with cancers of the female reproductive system, along with their diagnoses and potential treatments, are reviewed. It has been found that *RUNX1* mutation plays a pervasive function in female health, including sex determination, follicular development, steroidogenesis, and the interaction of the estrogen system. In contrast, chromosomal translocations in the gene linked to *RUNX1* mutation may lead to severe malignancies in females. Breast, ovarian, uterine, and cervical cancers have shown the highest frequency of genetic abnormalities in the *RUNX1* gene. The second most common cause of cancer-related mortality in women is breast cancer, which is also the most common cancer. There is an opposing relationship between uterine cancer and low-grade tumors that often remain confined to the uterus. Due to the regular occurrence of promoter hypermethylation and hypomethylation changes, ovarian cancer has become the most fatal of all gynecological tumors. Finally, despite being the cancer least likely to result from *RUNX1* mutation, cervical

cancer can directly impair natural killer cell activity. Both hematopoietic and non-hematopoietic cancer cells can form and become tumors when the *RUNX1* gene is mutated, with female malignancies being the primary target. Therefore, more research on *RUNX1* gene's pattern of expression, both *in vitro* and *in silico*, is needed to lower the incidence of female-related cancers.

Keywords: RUNX1; Chromosomal translocation; Breast cancer; Uterine cancer; Ovarian cancer; Cervical cancer

1. Introduction

Cancer is a disease where cells grow uncontrollably and spread throughout the body, affecting the body's immune system and eventually all the organs[1]. Gynecologic cancer is any cancer that begins in a woman's reproductive organs. Cervical, ovarian, uterine, vaginal, and vulvar cancers are the five most common kinds of gynecologic cancers[2]. According to the data obtained from Cancer Research UK in 2016, breast cancer is the most common female-related cancer in women and has the second highest mortality rate; uterine cancer is the fourth most common cancer in females and has the ninth highest mortality rate in women; ovarian cancer is the fifth most common cancer in women and the sixth most common cause of death in women[3]. According to the National Cancer Institute, the estimated number of new breast cancer cases and uterine cancer cases in 2022 is 287,850 and 65,950, respectively, while the estimated deaths of these cases in 2022 are 43,250 and 12,550, respectively[4]. The top four cancer-related causes of death in females are expected to see a shift over the next two decades to lung (34,000 deaths), breast (30,000 deaths), pancreatic (22,000 deaths), and uterine (18,000 deaths) by 2040[5]. Cancer diagnostics and treatments remain a challenge, because cancer consists of a group of several diseases, and many diverse genetic abnormalities underpin more than 200 different mutations[6]. Female-related cancers are caused by several factors, which can be genetic, epigenetic, viral, and environmental[7]. Among the susceptible genes, *RUNX1* is crucial as it has been found to be associated with most female-related cancers[8]. The RUNX family of transcription factors is evolutionarily conserved proteins that play an essential role in fundamental biological processes, including growth and development[9]. There are three members (RUNX1, RUNX2, and RUNX3) of the RUNX transcription family in humans, each of which is uniquely expressed in different tissues[10]. The *RUNX1* gene codes for a human protein, a transcription factor called runt-related transcription factor 1, is also known as acute myeloid leukemia (AML) 1 or core-binding factor subunit alpha-2, which regulates the differentiation of hematopoietic stem cells (HSCs) into mature blood cells[11]. The RUNX1 protein binds to specific DNA regions and helps control the function of specific genes. This protein interacts with the core-binding factor subunit beta or CBFβ (produced from the *CBF-β* gene), which, in turn, helps connect it to DNA and inhibits DNA from being degraded (Figure 1). These proteins, collectively, form one version of a CBF[11-13]. The RUNX1 protein turns on genes that help control blood cell growth (hematopoiesis)[14]. Most research related to females (including breast, ovarian, uterine, and cervical malignancies) indicates a prevalence of *RUNX1* gene changes in hormone-associated cancers[15]. Mutation in the *RUNX1* gene is often found in various hematological malignancies. With the advent of technological revolution, *RUNX1* mutation has been proven to play a more pervasive role in cancer than previously believed. Dysregulation of the *RUNX1* gene has been reported to be associated with gastric and hepatocellular carcinoma, while single nucleotide polymorphisms (SNPs) in *RUNX1* have been linked with human colorectal and prostate cancer[3,15-21]. In almost 15% of esophageal cancers, *RUNX1* mutation tended to be an essential factor for the etiology and development of squamous cell carcinoma in the skin and oral cavity; additionally, strongly focal *RUNX1* deletions have been reported[22].

RUNX1 gene was first discovered in acute myeloid leukemia gene 1 (*AML1*) in 1991 as it was linked to the translocation of AML[23]. Later in 1993, a murine version of *RUNX1* was discovered, leading to the development of *RUNX1* knockout mouse models[24]. In a screen that was performed to identify mutations affecting segment number and polarity in *Drosophila*, Nusslein-Volhard and Wieschaus identified the transcription factor RUNX[25]. The mutation that led to defects in pre-segmentation

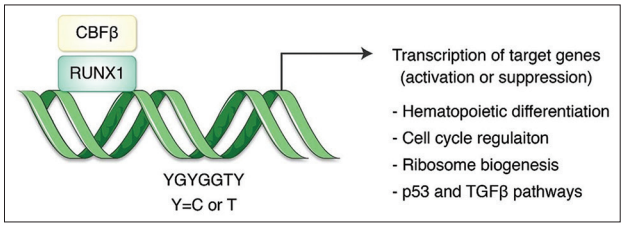

Figure 1. RUNX1 heterodimerization with its binding partner, CBF, and interaction with DNA at promoters of target genes that have the particular binding site YGYGGTY, where Y is C or T.

patterning and runted embryos was called runt. Later, Gergen *et al.* cloned the *Drosophila* segmentation gene runt[26]. Although the protein encoded by runt was demonstrated to exhibit nuclear translocation, its role as a transcription factor was yet to be established. Eventually, in 1991, the human *RUNX1* gene was cloned and observed to be rearranged from t(8;21)(q22;q22) AML patients in leukemic cell DNAs[27]. However, the role of the human *RUNX1* gene had not been identified. The function of the *RUNX1* gene was discovered shortly after the discovery of *Drosophila* runt protein and human RUNX1 protein. As a sequence-specific DNA-binding protein that monitored the disease specificity of the Moloney murine leukemia virus, RUNX1 was purified. Besides, Ito *et al.* purified RUNX2, the RUNX1 homolog. Two subunits, a DNA-binding CBF alpha chain (RUNX1 or RUNX2) and a non-DNA-binding subunit termed core binding factor β (CBFβ), were purified transcription factors; the binding capacity of RUNX1 and RUNX2 was significantly enhanced by the interaction with CBFβ[28].

2. *RUNX1* gene

2.1. Background of *RUNX1* gene

The human *RUNX1* gene is 260 kilobases (kb) long and is found on chromosome 21 (21q22.12)[29]. The gene can be transcribed from either promoter 1 (distal) or promoter 2 (proximal). As a result of alternative splicing, multiple *RUNX1* isoforms can be generated. The exons encode the full-length RUNX1 protein[30]. There are two distinct domains among the exons: the runt homology domain (RHD) or runt domain (exons 2, 3, and 4) and the transactivation domain (TAD) (exon 6). RUNX1 requires these domains to facilitate DNA binding and protein-protein interactions, respectively. RUNX1 protein has 453 amino acids. The runt domain (residues 50 – 177), which is homologous to the p53 family, encodes its DNA-binding capabilities as a transcription factor (TF)[13].

RUNX1 is mutated in 4.26% of breast cancer patients, with 1.55% of all breast cancer patients having *RUNX1* mutation[31]. *RUNX1* is mutated in 0.82% of ovarian cancer patients, with *RUNX1* mutation present in 0.56% of all ovarian cancer patients[32]. The disruption of gene regulation, which results in the loss or gain of genetic function, is known to play a significant role in carcinogenesis. The addition of a methyl group to cytosine-5 position within the context of a CpG dinucleotide, mediated by DNA methyltransferases, is the most studied epigenetic modification. The natural control of DNA methylation is disrupted in cancer, resulting in dramatic alterations in the distribution pattern of 5-methylcytosine. Heavy methylation in most chromatin is restricted, but unmethylated CpG islands in gene promoters and first exons often become hypermethylated. Aberrant DNA methylation is also implicated in the development of chemotherapeutic resistance. It occurs in ovarian cancer and contributes to carcinogenesis and chemoresistance pathways. In a study, *RUNX1* gene was significantly hypomethylated and overexpressed in post-chemotherapy (CT) primary cultures of ovarian cancer patients[33]. Using a similar approach (methylated DNA immunoprecipitation coupled to CpG island tiling arrays), the study demonstrated that DNA hypermethylation occurs in less invasive/early stages of ovarian tumorigenesis.

In contrast, advanced disease has been found to be associated with DNA hypomethylation of several oncogenes involved in cancer progression, invasion/metastasis, and likely chemoresistance[34]. Moreover, according to a study, mutations were observed in *RUNX1* and *CBFB* genes, in which about 8 *RUNX1* mutations were seen within the *RUNX1* coding region in breast cancer cases[35]. The runt domain, which is crucial for DNA binding and heterodimerization, has all six missense mutations, with four mutations occurring at two mutation hotspots (amino-acid positions 174 and 139/141/142). Notably, all *RUNX1* mutations associated with breast cancer appear to result in loss-of-function mutants[36].

2.2. Functions of *RUNX1* gene

Almost all adult mammalian, blood cells develop from HSCs in the bone marrow. HSCs are cells that may engraft adult transplant patients and develop from immature HSC precursors, which are known as pre-HSC. RUNX1 is required for the development of all embryonic blood cell lineages[27]. The trigeminal and dorsal root ganglia include nociceptors, specialized primary sensory neurons with high stimulus thresholds, and cell bodies. They express various ion channels that convert mechanical, thermal, or chemical inputs into electrical activity[37]. Several transcription factors regulate the growth of nociceptive sensory neurons. These runt proteins regulate developmental events by interacting with a common cofactor called CBF[38]. The trigeminal and dorsal root ganglia both express RUNX1 and RUNX3. The *RUNX1* gene is responsible for coordinating the phenotypic of a large number of nociceptors[39].

In adult skin, RUNX1 regulates the activation and proliferation of hair follicle stem cells (HFSCs). RUNX1 is found in three types of embryonic skin precursors: short-term hair follicle (HF) progenitors, adult HFSCs, and mesenchymal progenitors[40]. *RUNX1* gene is required for the development of adult HFSCs and short-term progenitors in the embryonic epithelium, but it is not required either. The *RUNX1* gene is rigidly sought in the embryonic mesenchyme for proper adult HFSC differentiation and long-term skin functioning[41] (Table 1).

Table 1. List of some cancer types where *RUNX1* gene functions as an oncogene or tumor suppressor gene.

Function of *RUNX1* gene	Cancer type	References
Oncogenic	Triple-negative breast cancer	[44,48,49]
	Ovarian cancer	[34,48,55]
	Uterine cancer	[47]
	Cervical cancer	[47,55]
	Prostate cancer	[47,48]
	Colorectal cancer	[47,48]
	Skin cancer	[48,55]
	Head and neck cancer	[48,56]
	Acute myeloid leukemia	[44,57]
Tumor suppressor	Breast cancer (excluding triple negative)	[48,58]
	Gastrointestinal cancer	[48,59]
	Lung adenocarcinomas	[48,60]
	Non-small cell lung cancer	[48,49]
	Glioblastoma multiforme	[48,61]
	Hepatocellular carcinoma	[54,62,63]
	Acute lymphoblastic leukemia	[64,65]

Although the genes associated with cancer development are generally either tumor suppressor genes or oncogenes, the *RUNX1* gene can function as both, depending on the context[42-44]. *RUNX* genes play a significant role as tumor suppressor genes by inactivating gene mutations, hypermethylation, and deletions in some cancers[42,45]. While in many other cases, these genes are transcriptionally activated by retroviral insertion, indicating dominant oncogenic potential[42,46]. The role of *RUNX1* gene in tumorigenesis differs with the tumor tissue, type, and stage of tumor development[44,47]. For instance, the *RUNX1* gene plays the role of a tumor promoter in ovarian and skin cancers[48], but it has been identified as a tumor suppressor in breast (excluding triple negative)[49], lung[48], and prostate cancers[48,50]. *RUNX1* mutations, which are mostly loss-of-function mutations occurring due to non-sense, frame shift, or missense mutations within the runt DNA-binding domain[8], take place almost exclusively in the ER-positive, luminal subtype of breast cancer, and pointing to a tumor suppressor role[35].

On the contrary, enhanced levels of *RUNX1* gene expression have been found to be associated with poor outcomes in triple-negative breast cancers (TNBCs), suggesting its role as an oncogenic gene in this breast cancer subtype[51]. Whether the *RUNX1* gene acts as an activator or repressor of target gene expression, it depends on the massive number of interacting coactivators, transcription factors, and corepressors[52]. The downregulation of the *RUNX1* gene may lead to constitutive gene abnormalities and, thus, result in tumorigenesis [53], but *RUNX1* gene amplification induces gene overexpression or upregulation, which has an immense potential to contribute to the transformation of hematopoietic cells into tumors[54].

2.2.1. Hematopoiesis

Hematopoiesis occurs during embryonic development and maturity to create and replenish the blood system. Through hematopoiesis research, the mechanisms that lead to blood diseases and malignancies can be better understood by scientists and doctors. HSCs can also be utilized as a model system to study tissue stem cells and their involvement in aging and cancer[66]. HSCs form in the human embryo at 1 month of gestation[67]. However, before HSCs appear, numerous other blood cells develop, some of which are required for embryonic survival and contribute to tissue macrophages in adults. HSCs and hematopoietic progenitors (cells that can develop into various blood cells but do not have long-term multiline age reconstitution capability) arise in three waves, as detailed below. *RUNX1* gene is required to differentiate all embryonic blood cell lineages, but it plays a particularly important role in differentiating blood cells from hemogenic endothelium in the second and third waves[68].

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been used to study human hematopoiesis, including the involvement of *RUNX1* gene, over the past decade. In a study, human CD34+ CD45+ hematopoietic stem and progenitor cells (HSPCs) were produced from human ESCs and iPSCs about 11–14 days following embryoid body (EB) formation using a feeder-free culture method. Multiple kinds of hematopoietic cells, including myeloid, erythroid, and polyploid megakaryocytic (MK) cells, were formed by CD34+ CD45+ HSPCs. The human iPSCs generated from familial platelet disorder (FPD) patients with heterozygous *RUNX1* mutation were similarly found to be deficient in MK production, with the targeted repair of the mutant *RUNX1* allele by genome editing restoring MK potential[69].

Since then, scientists have expanded their research using precise genomic targeting in human wildtype iPSCs to remove excess exon 5 of the *RUNX1* gene, shared by all three isoforms, or exon 1B, which is unique to the *RUNX1c* isoform. On days 11–14 following EB development, the bi-allelic knockout (KO) of *RUNX1* at exon 5 prevented the production of hematopoietic cells. In the presence of *RUNX1a* and *RUNX1b* isoforms, produced from the downstream P2 promoter, the complete deletion of exon 1B had no effect, thus revealing that the *RUNX1c* isoform is not required for definitive hematopoiesis. According to a detailed examination of EBs on days 6–8, the bi-allelic

KO of *RUNX1* at exon 5 (ablating all three isoforms) did not affect the development of CD34+/CD31+/CD144+ endothelial-like cells. However, no CD45+ hematopoietic cells developed from the endothelial-to-hematopoietic transition (EHT) culture that was treated with hematopoietic cytokines, indicating that the EHT was fully inhibited[27].

2.2.2. Nociceptive sensory neurons regulation

The transduction of noxious stimuli triggers the interpretation of pain in mammals through specialized ion channels and receptors expressed by nociceptive sensory neurons[70]. However, the molecular mechanisms responsible for specifying different sensory modalities remain poorly understood. RUNX1, a runt domain transcription factor, is expressed in most nociceptors during embryogenesis. RUNX1 controls the expression of several ion channels and receptors in these neurons, namely, thermal receptors of the transient receptor potential (TRP) class, Na^+-gated, ATP-gated, and H^+-gated channels, μ-opioid receptor (MOR), and protein-coupled receptors of Mas-related G-protein-coupled receptor (Mrgpr) class G[71]. RUNX1 also regulates the lamina-specific innervation pattern of the spinal cord's nociceptive afferents. In addition, mice that lack RUNX1 show certain defects in thermal and neuropathic pain[72], thus suggesting that the phenotype is coordinated by RUNX1. This finding has implications for pain treatment of a broad cohort of nociceptors. The mammalian genome encodes three runt domain transcription variables: RUNX1, RUNX2, and RUNX3. These runt proteins interact with CBFβ cofactor to regulate several developmental processes [73]. The trigeminal and dorsal root ganglia both express RUNX1 and RUNX3. It appears that RUNX1 expression is limited to nociceptors; the persistent *RUNX1* gene expression labels nociceptors undergoing the developmental transition from TrkA to Ret. The transition from TrkA to Ret is disrupted in mice that selectively lack *RUNX1* gene function in the peripheral nervous system[74]. Besides, we find that activating or suppressing the expression of a significant number of nociceptive ion channels and sensory receptors requires RUNX1 molecules.

Moreover, the *RUNX1* gene is necessary for the dorsal spinal cord to target afferent projections to the specific lamina[75]. Ultimately, behavioral research has demonstrated certain deficits in thermal and neuropathic pain in RUNX1-deficient mice. These results indicate that the phenotype of a broad collection of nociceptors is coordinated by RUNX1 molecules[40].

2.2.3. Hair follicle development

RUNX1 genes also regulate HFSC activation and proliferation in adult skin[76]. *RUNX1* gene expression is most common in the mesenchymal and epithelial compartments in hair follicles, where the three RUNX proteins are expressed[77]. The epithelial expression involves hair keratin, which forms layers of the hair shaft and the bulge. The *RUNX1* gene is notably coexpressed with keratin 15, which is a marker of hair follicle stem cells. *RUNX1* gene is expressed in a distinct dermal sub-epithelial layer in the hair mesenchyme during the initial stages of hair morphogenesis. At the same time, it is located in the dermal papilla at later stages in a hair cycle-dependent pattern. In experiments on knockout mice, the *RUNX1* gene was found to be expressed in several hair follicle chambers, along with the bulge and germ, but not in other skin epithelial structures, such as the sebaceous gland and the epidermis. The structure of the hair shaft, the activation of HFSC, and the onset of anagen are all affected by constitutive epithelial deletion of *RUNX1* gene through development. Besides, the role of *RUNX1* gene in skin cancers is yet to be explored[78]. It is necessary to clarify this stance because skin cancer is the most common human malignancy and HFSC is a well-recognized source of skin appendage tumors, such as basal cell carcinoma (Table 2).

3. *RUNX1* gene and female health

3.1. Sex determination

Sex determination is the process through which sexually reproducing organisms differentiate as male or female[79]. Although the process varies extensively between different organisms, numerous species have shown a physiological link between the *RUNX1* gene and the development of female reproductive tissues. Conceivably, this link was studied for the first time in *Drosophila melanogaster*. It can be seen that the *runt* gene is required to express *Sxl* or *Sex-lethal* gene in the blastoderm embryo, which is responsible for maintaining the on/female mode or off/male mode[80,81]. In mammals, the *RUNX1* gene was found to be strongly expressed in the ovarian stroma, suggesting that it is indeed related to female sex development[82]. RUNX1 protein was also detected in oocytes and granulosa cells of preantral follicles and theca cells of antral follicles. This was further bolstered by the fact that *RUNX1* is a Wnt-4 signaling target gene[83]. The absence of Wnt-4 results in partial reverts of a female embryo into a male embryo. In addition, Wnt-4 also suppresses testosterone-related genes, promotes the maturation of the Mullerian duct, and triggers the production of female germ cells. The knockout of Wnt-4 causes a reduction in *RUNX1* gene expression, suggesting that the *RUNX1* gene might play a crucial role in ovary organogenesis[84]. A significant expression of the *RUNX1* gene in the ovaries of various species (human, goat, mice, and trout) during early gonad differentiation indicates an evolutionarily conserved role of the gene in ovary differentiation.

Table 2. Summary of the functions of *RUNX1* gene and the phenotypes through which the functions are expressed.

Functions	Phenotypes	References
Hematopoiesis	Transcriptional transfer is essential for the trans-differentiation of endothelial cells into functional HSPCs. Pre-HSCs are differentiated into HSCs. Primitive macrophages are absent, the number of diploid megakaryocytes decreases, and primitive erythropoiesis is unusual in the absence of RUNX1.	[27,66-69]
Nociceptive sensory neurons regulation	Regulates the phenotype of several nociceptors, such as the expression of thermal receptors of the TRP class.	[40,67-73]
Hair follicle development	Controls the activation of HFSCs, and increases the number of adult skin cells.	[73-78]

HFSCs: Hair follicle stem cells; HSCs: Hematopoietic stem cells; HSPCs: Hematopoietic stem and progenitor cells; TRP: Transient receptor potential.

3.2. Follicular development and steroidogenesis

RUNX1 also plays pivotal roles in follicular development and steroidogenesis. According to research, it plays a role in the production and survival of periovulatory follicles in rat ovaries[85,86]. Studies have shown that RUNX1 protein is expressed particularly in granulosa cells of preovulatory follicles in rat ovaries following human chorionic gonadotropin (hCG) injection, which stimulates ovulation through luteinizing hormone (LH) surge (Figure 2)[87]. A similar result was observed in bovine follicles, where *RUNX1* gene was significantly upregulated by an LH surge in theca[88]. This aggregated evidence suggests that *RUNX1* gene might be hormonally regulated. It has also been observed that the reduction of *RUNX1* mRNA expression leads to a decrease in progesterone production. Moreover, the knockdown of *RUNX1* gene significantly decreases estradiol levels and several other steroidogenic enzymes in granulosa cells, such as cytochrome P450 family 11 subfamily A member 1 (CYP11A1), which is responsible for progesterone synthesis (Figure 2). However, the exact stage of follicular development in which hCG injection causes an increase in *RUNX1* expression has yet to be elucidated. Several studies have shown that it solely involves the LH-activated adenylate cyclase-mediated signaling pathway, while others have suggested that it involves protein kinase C (PKC) and P13K pathways[89]. Therefore, further studies are required to achieve definitive results.

3.3. Estrogen pathway interplay

Naturally, RUNX1 and its relation to the estrogen-estrogen receptor (ER) pathway significantly contribute to female sex development, given that estrogen is the primary female sex hormone. It is essential for the function and development of female reproductive tissues, mammary cell division, *etc*. Most estrogen activity is mediated by ER, which can be further classified into ER alpha (ERα) and ER beta (ERβ); ERα and ERβ are isoforms of each other, formed from separate genes[90]. Initially, estrogen response was thought to be the classical estrogen pathway, which

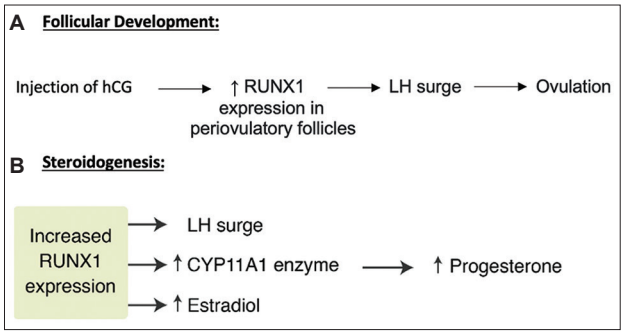

Figure 2. *RUNX1* gene and its effect on follicular development and steroidogenesis. (A) shows how the injection of hCG subsequently leads to increased *RUNX1* gene expression, leading to an LH surge, and ultimately causing ovulation. (B) shows how the increased *RUNX1* expression affects LH, progesterone, and estradiol levels.

solely depends on ligand activation, but, further, research has led to more pathway mechanisms, including ligand-independent ER activation, non-genomic activation, and ER element (ERE)-independent activation[91]. The mechanism of the putative role of *RUNX1* gene in the ER pathway has not been elucidated; however, some links have been deduced between the two (Figure 3).

3.4. Uterine development

In mice, the *RUNX1* gene is significantly expressed in the uterus's luminal and glandular epithelia and immune cells. Interestingly, studies in different mouse strains showed significant upregulation of *RUNX1* gene when exposed to estradiol. Based on a study that demonstrated estradiol-induced cellular responses in mouse models with greater *RUNX1* gene expression, it has been suggested that RUNX1 enhances estradiol, and thus uterine development[92]. Estradiol is the primary estrogen steroid female sex hormone that regulates the estrous and female menstrual cycles. This suggests that ERα might regulate gene expression by binding to RUNX1 (where RUNX1 acts as a tethering factor)[93]. RUNX1 mediates ERα localization in the chromatin and has been identified as a mediator of ERE-independent estrogen signaling[94]. The restriction of

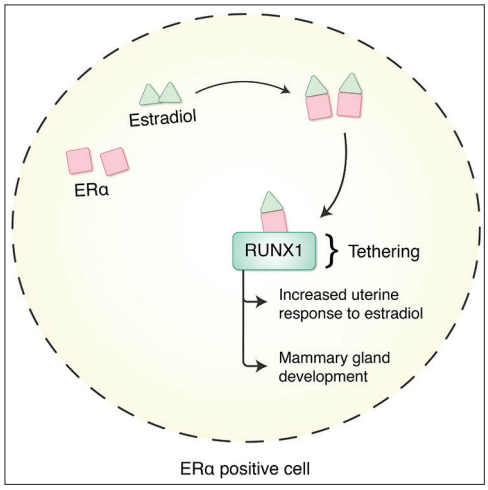

Figure 3. RUNX1 in the estrogen-ER pathway. Estradiol, bound to ERα, and tethers to RUNX1. This leads to a higher receptivity of the uterus to estradiol and develops the mammary glands.

estradiol to the tethered pathway often prevents increased uterine function[95].

In contrast, the tethered/non-ERE pathway is a typical uterine transcriptional response to estradiol[95]. It can be deduced from this that *RUNX1* gene plays a role in uterine development, even when a tethered/non-ERE pathway is used. Collectively, this concludes that RUNX1 has a putative role in epistatic interactions that lead to genetic variations in the responsiveness of the uterus to estradiol. However, further research is needed to fill the knowledge gaps of how this has a role in inheritance.

3.5. Mammary gland functioning

Mammary cells arise from the ectodermal bud and undergo postnatal ductal development to form alveolar structures up to lactogenesis after pregnancy[96]. Estradiol is a crucial regulator of this development. It acts on ERα found in the stroma and epithelium of mammary cells. ERα has been reported to play a role in the differentiation and proliferation of mammary cells[97]. Hence, ERα is essential for the development of the adult mammary gland. Studies have shown that ERα knockout leads to underdeveloped mammary glands in mice[98]. Genome-wide maps specific to ER-binding sites have shown that ERα tethering requires the RUNX1 transcription factor.

Moreover, *RUNX1* genes are usually present in the mammary gland. Their expression levels vary during different stages of pregnancy, lactation, and the female reproductive cycle. This reflects their specific roles in mediating mammary gland function. The RUNX1 protein is mainly found in the basal and luminal cell layers of epithelial cells[99]. On the contrary, there is lower expression of normal *RUNX1* gene in breast cancer cells, indicating that RUNX1 is essential for healthy cell proliferation, and sustaining the pivotal role of RUNX1 in the mammary gland.

4. Impact of *RUNX1* mutation

Chromosomal translocations are a specific type of mutation, in which abnormal exchanges occur between homologous chromosomes. According to numerous studies related to different forms of cancers, the *RUNX1* locus is a common site for multiple chromosomal translocations. Monoallelic point mutations such as the one that results in the loss of RUNX1-MTG16 contribute to breast cancer.

RUNX1-MTG16 protein is the result of a fusion between the RUNX1 at its N terminus (truncated at the runt domain) to the C terminus of MTG16 protein that plays a part in tumor suppression due to t(16;21)(q24;q22) translocation. This results in the loss of MTG16 tumor suppressor function through heterozygosity in breast neoplasms at 16q24- resulting in breast cancer[100]. Moreover, the *RUNX1* gene is downregulated in cancer cells at the metastasis stage and thought to be a tumor suppressor[101].

Other aberrations in this gene lead to different cancers with varying percentages of alteration frequencies (genes altered per case), some of which include acute myeloid leukemia (13.5%), esophageal cancer (8.24%), breast cancer (6%), and colorectal cancer (3.03%)[102]. Absolute counts of these mutations showed that hormone-related female cancers are prevalent with relatively high aberrations compared to male-related cancers (Table 3).

5. Role of *RUNX1* gene mutation(s) in female-related cancers

5.1. Breast cancer: Overview and development

Whole-genome/exome sequence studies have reported that point and deletion mutations in *RUNX1* gene could result in ER-positive, luminal, and basal subtype of breast cancer[104]. Besides, it has been found that *RUNX1* knockdown may cause hyperproliferation and abnormal morphogenesis of the human mammary epithelial cell line (MCF10A)[112]. Moreover, a Moroccan study found that *RUNX1* SNPs were firmly correlated with breast cancer risk[113].

On the other hand, several experiments have indicated that the *RUNX1* gene plays a pro-oncogenic role in breast cancer, which is interestingly related to the ER-negative and triple-negative (TN) subtypes. Different transcriptome studies have reported that *RUNX1* mRNA is significantly upregulated in the TN subtype group[114,115]. At the same time, the *RUNX1* gene has been found to be correlated with

Table 3. Summary of *RUNX1* gene mutations that lead to the development of different female-related cancers.

Cancer type	*RUNX1* mutation	Tumor stage/type	References
Breast cancer	Silent	Luminal B (ER-positive)	[35,103]
	Missense	Luminal B	[49]
	Homozygous deletion	Luminal A and B	[104]
	Monoallelic point	Luminal A and B	[8]
	Frameshift	Luminal A and B	[44]
	Non-sense	Luminal A and B	[49]
	Deletion	Luminal and basal	[44]
	Chromosomal translocation	-	[99]
	Mutation (driver)	Luminal and basal	[44]
Uterine cancer	Substitution	Endometrioid carcinoma	[105]
	mRNA upregulation	Endometrioid carcinoma	[106]
	mRNA upregulation	Myometric	[106]
	Chromosomal translocation	Endometrioid carcinoma	[106]
	Heterozygous point	Endometrioid carcinoma	[107]
Ovarian cancer	mRNA upregulation	Epithelial ovarian cancer	[8]
	Point	Epithelial ovarian cancer	[108]
	mRNA downregulation	Epithelial ovarian cancer	[109]
	Deletion	Epithelial ovarian cancer	[110]
	mRNA upregulation	Epithelial ovarian cancer	[110]
Cervical cancer	NK cell cytotoxicity	Squamous cell carcinoma, adenocarcinoma	[111]

super-enhancer elements that are connected to oncogenes and genes associated with cancer pathogenesis, precisely in an ER-negative breast cancer cell line[116]. In another study, *RUNX1* gene was found to be highly expressed with disease progression in patient samples and a mouse model of breast cancer[35].

RUNX1 has recently been recognized as a novel mutated gene in human luminal breast cancer. It is expressed in all murine mammary epithelial cells (MECs) subpopulations, except for secretory alveolar luminal cells. Moreover, a decrease in luminal cells is observed due to the conditional knockdown of *RUNX1* in MECs by MMTV-Cre. The reason behind this is the significant reduction of the ER-positive, mature luminal subpopulation. A master regulatory transcription factor for alveolar cells, Elf5, is repressed by RUNX1. The *RUNX1* gene also regulates mature luminal TF/co-factor genes (*e.g.*, *FOXA1* and *CITED1*) that are involved in the ER program (Figure 4). Besides, it is possible that the *RUNX1* gene also contributes to the loss of the cell-of-origin of luminal breast cancer, as its disruption reduces the ER-positive luminal MECs from where cancer originates[117]. Since a decrease in *RUNX1* expression leads to an increase in breast cancer aggression, higher levels of *RUNX1* expression are associated with good prognosis[103]. However, an excessive expression of *RUNX1* might be oncogenic as well. It has been found that in luminal breast cancer, four missense mutations take place in the *RUNX1* gene, of which three are located in the runt domain, gathered within the putative ATP-binding site, which contains eight amino acid residues[44]. Various studies have revealed RUNX1 suppression of breast cancer epithelial-to-mesenchymal transition and RUNX1 repression of cancer stem cells and tumor formation[8]. Apart from that, in many other studies, RUNX1 seemed to show monoallelic point mutations in different luminal and basal levels of human breast cancer[118].

5.2. Uterine cancer: Overview and development

According to the well-accepted dualistic model of endometrial tumorigenesis, uterine or endometrial cancer is generally classified into two major types based on histological and clinical characteristics, that is, endometrioid endometrial carcinoma (EEC) and non-endometrioid endometrial carcinoma[106]. Intriguingly, *RUNX1* appeared as one of the most highly upregulated genes from a list of 53 differentially expressed cDNA targets, while comparing gene expression profiles of normal versus EEC tissues[119]. Impressively, *RUNX1* gene expression is strongly associated with the Ets variant gene 5 (ETV5), also known as Ets-related molecule (ERM) transcription

factor, whose expression also seemed to be upregulated during the primary steps of EEC progression. Moreover, high levels of matrix metalloproteinase (MMP)-2 and -9 expression have also been found to colocalize with ERM/ETV5 and RUNX1 at the invasive front of endometrial cells, encouraging an interplay between these proteins during myometrial infiltration[120,121]. Besides, the *RUNX1* gene is also strongly associated with the expression of the double-strand-break repair protein rad21 homolog (RAD21), a crucial component of the cohesin complex that is involved in chromosome segregation and often dysregulated in solid tumors of the breast and ovary[122,123]. Surprisingly, experiments in zebrafish have revealed that RAD21 is a regulator of *RUNX1* gene (Figure 5).

In a study, RUNX1 levels were significantly increased in circulating tumor cells (CTCs) that were isolated from high-risk EEC patients presenting with more than 50% of myometrial infiltration. Besides, ectopic *RUNX1* gene expression increased the rate of metastasis in an orthotropic endometrial mouse model, implicating the gene as a putative inducer of metastasis[105]. Therefore, RUNX1 is a pro-oncogenic player in uterine cancer. The mutations observed in *RUNX1* gene are mostly due to myometrial infiltration, substitution, mRNA upregulation, or in very few cases, heterozygous point mutations[107]. EEC, or type I endometrioid endometrial carcinoma, is one of the two types of uterine cancer, in which the gene expression profile of RUNX1 has the highest value.

5.3. Ovarian cancer: Overview and development

Of all the female gynecological malignancies, ovarian cancer is the deadliest one. Its treatment is also complicated. According to the World Health Organization (WHO), each year, an estimated total of 140,200 patients are diagnosed with ovarian cancer, representing the 7th most common form of cancer and the 8th leading cause of cancer-

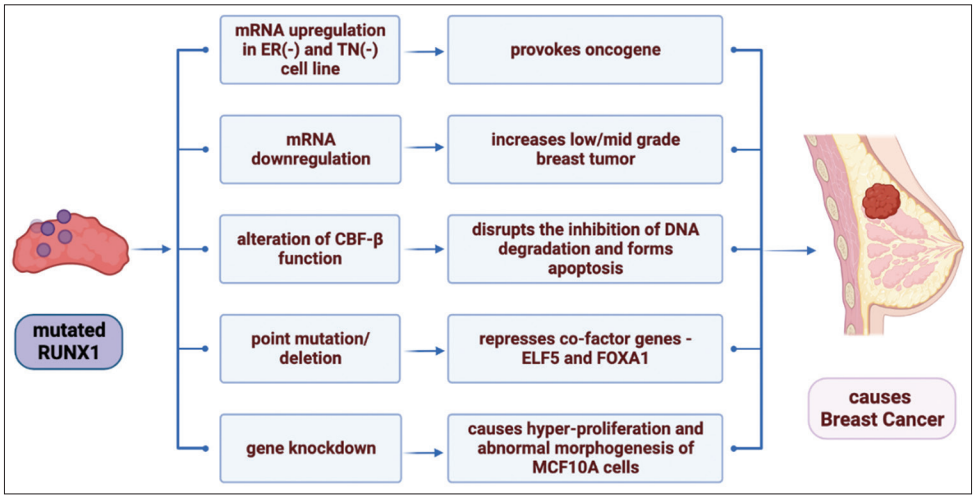

Figure 4. Formation of breast cancer by different mutated pathways of RUNX1.

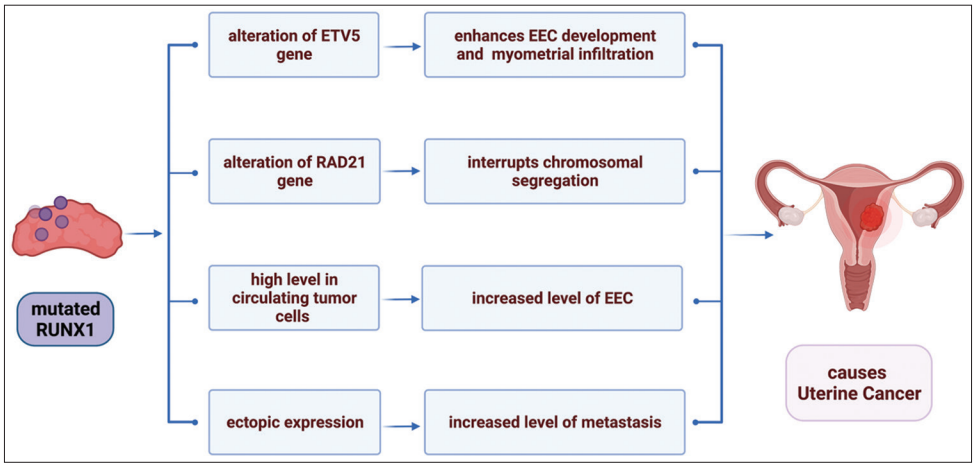

Figure 5. Formation of uterine cancer by different mutated pathways of RUNX1.

related deaths in women worldwide[124,125]. Moreover, around 300,000 new cases of ovarian cancer occur every year according to the World Cancer Research Fund[126]. Most tumors in this particular malignancy are usually diagnosed at a very late stage when patients present with upper abdominal and distant metastases. In such cases, life-saving surgery cannot be performed[127]. The disease may be caused by various genes being overexpressed, under-expressed, or even subjected to epigenetic changes besides somatic mutations[128]. In a study, hypomethylation was observed in 46 putative oncogenes in the primary cell culture, where a high occurrence of *RUNX1* mutation was detected in ovarian cancer progression[3]. Immunohistochemical analysis showed that *RUNX1* gene expression was significantly higher in high malignant potential and low malignant potential tumors, as well as in omental metastasis, compared to normal ovary[34]. Another report demonstrated that short hairpin (sh)RNA-mediated *RUNX1* knockdown significantly impacted the proliferative and migratory ability of ovarian cancer-causing SKOV3 cells (Figure 6). Similarly, *RUNX1* mRNA upregulation and RUNX1 protein overexpression are known to decrease miR-302b and increase the risk of ovarian cancer[110]. A reduced miR-302b can regulate RUNX1 pro-oncogenic activity, leading to signal transducer and activator of transcription 3 (STAT3) signaling pathway[129]. Likewise, in the case of ovarian cancer, it can have the same effect by activating invasive phenotypes by MMP-2 and MMP-9 signaling pathways[130]. Therefore, all the dysfunctional evidence hints at the correlation between the oncogenic functionality of *RUNX1* gene and a potential prognostic biomarker for developing ovarian cancer in the female population. A carcinoma cell biopsy followed by immunohistochemical analysis revealed significant upregulation of the *RUNX1* gene compared to the control, suggesting the use of *RUNX1* gene as a biomarker for ovarian cancer diagnosis[3].

5.4. Cervical cancer: Overview and development

Cervical cancer rates vary across the world, with Eastern Africa having the highest rate and Western Asia having the lowest. According to the WHO, it is the fourth most common type of cancer in women and a major cause of cancer-related deaths among low- and middle-income countries. In 2020, an estimated 604,237 women were diagnosed with cervical cancer globally, representing 6.5% of all female cancers[131]; new cases of cervical cancer have been estimated to be around 14,100 in 2022, with 4280 deaths[132]. One of the significant kinds of adenocarcinoma, developed from the influence of RUNX1, is the cervical that occurs in the ectocervix. In the lower Mullerian duct (MD), epithelial cells are obligated to become stratified squamous epithelium of the ectocervix and vagina, as the expression of ΔNp63 transcription factor is induced by vaginal mesenchyme[111]. In the MD epithelium (MDE), *SMAD4* gene is essential for the activation of ΔNp63. This transcription factor binds on the 5′ sequence adjacent to the transcription start site (TSS) of ΔNp63 in the future vaginal epithelium (VgE)[133]. This SMAD-dependent activation of the ΔNp63 locus requires the expression of RUNX, which activin A (ActA) activates through a SMAD-independent mechanism[134]. The ActA-RUNX1 pathway is independently required for the vaginal cell fate commitment of MDE. Inactivation of it in MDE results in uterine epithelial differentiation of MDE within the vagina, a congenital epithelial lesion called vaginal adenosis[133]. Vaginal adenocarcinomas (VACs) or cervical cancers are believed to arise from vaginal adenosis due to adenosis lesions at the primary site of VACs[135]. According to cBioPortal for Cancer Genomics, the *RUNX1* gene shows 2.3% genetic perturbations in cervical cancers. In their report, the genetic alterations of *RUNX1*, including amplification of 1%, deep deletion of 2%, and missense

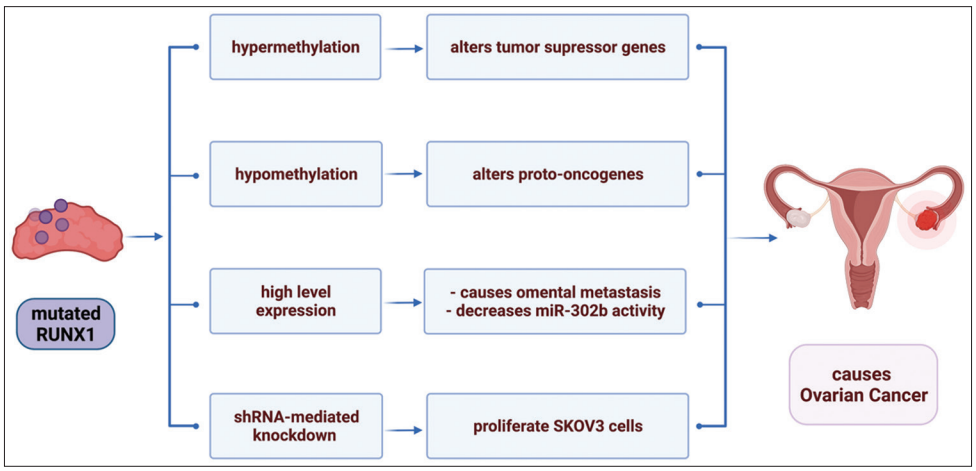

Figure 6. Formation of ovarian cancer by different mutated pathways of RUNX1.

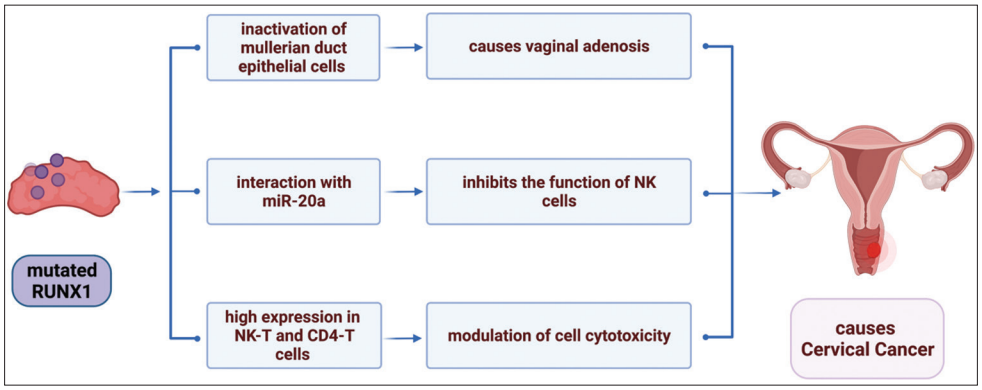

Figure 7. Formation of cervical cancer by different mutated pathways of RUNX1.

mutation (putative passenger) of 2.3%, have been documented in cervical cancer[136,137]. Several studies have concluded that micro (mi)RNAs could function as a major oncogene or tumor suppressor of various cancers by reprogramming NK cell-mediated cytotoxicity to tumor cells by different mechanisms[138,139]. miR-20a is a widely known oncomiR that is associated with the development of cervical cancer[140]. In a study, the *RUNX1* gene was identified as a direct target of miR-20a, inhibiting the killing effect of NK cells to cervical cancer cells by negatively regulating *RUNX1* gene expression (Figure 7)[141]. There is emerging evidence showing that *RUNX1* gene is highly expressed in NK T-cells and CD4 T-cells and is associated with NK differentiation. Cells participate in the progression of cervical cancer through the modulation of NK[142]. The treatment for cervical cancer, which includes surgery, radiation, and chemotherapy, depends on the stage of cancer, patients' health issues, and their personal preferences. Surgeries might include only removing the carcinoma if the cancer is at the first stage. Immunotherapy and drug-based treatments are also potential therapies, where cell-mediated drugs and various inhibitors against *RUNX1* gene are used to treat cervical cancer[143].

6. Conclusion

Both hematopoietic and non-hematopoietic cancer cells develop and undergo tumorigenesis as a result of *RUNX1* mutation or overexpression. However, the role of *RUNX1* gene in the activation or inhibition of different tumor cell growth is still unknown, which leaves room for additional research. Chromosomal abnormalities result in gene mutations, which have a statistically significant association with non-hematopoietic cancer in women in particular. In addition, the interaction of *RUNX1* gene with the estrogen-ER pathway shows that it has a tight connection with female reproductive development. Female health and reproduction are particularly vulnerable to disorders brought on by the *RUNX1* gene. Since the dawn of humanity, technology has been constantly improving and changing, and medical discoveries have not lagged behind. Even now, the number of diagnostic facilities, equipped with cutting-edge technology that speeds up the process and produces precise findings, is growing. Therefore, identifying mutations in malignancies, particularly those linked to RUNX1 disorders, have become much more effective. Every day, there are improvements in therapies, and genomic sequences are employed as guidelines for drug development. As a result, *RUNX1* mutations are now easy to identify and treat.

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Conflict of interest

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Author contributions

Conceptualization: Yusha Araf
Supervision: Yusha Araf, Mohammad Jakir Hosen, Chunfu Zheng
Visualization: MD. Hasanur Rahman, Sagarika Shahriar
Writing – original draft: Sagarika Shahriar, Zarin Tasnim Rafa, Maiesha Samiha Mahmood, Deera Mahasin
Writing - review & editing: Sagarika Shahriar, Zarin Tasnim Rafa, Maiesha Samiha Mahmood, Yusha Araf, Fatama Tous Zohora, Md. Asad Ullah, Md. Akkas Ali, Mohammad Jakir Hosen

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Ethics approval and consent to participate

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Gene & Protein in Disease

REVIEW ARTICLE

Differential expression profiles of circRNAs in cancers: Future clinical and diagnostic perspectives

Faiz Ali Khan[1,2,3], Bernard Nsengimana[1], Nazeer Hussain Khan[2,4], Jingjing Huang[1], Haojie Guo[1], Usman Ayub Awan[5], Weijuan Zhang[1]*, Wenqiang Wei[1]*, and Shaoping Ji[1]*

[1]Laboratory of Cell Signal Transduction, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Henan University, Kaifeng, China

[2]School of Life Sciences, Henan University, Kaifeng, China

[3]Department of Basic Sciences Research, Shaukat Khanum Memorial Cancer Hospital and Research Centre (SKMCH&RC), Lahore, Pakistan

[4]Henan International Joint Laboratory for Nuclear Protein Regulation, School of Basic Medical Sciences, Henan University, Kaifeng, Henan, China

[5]Department of Medical Laboratory Technology, The University of Haripur, Haripur, Khyber Pakhtunkhwa, Pakistan

Abstract

Since decades ago, circular RNAs (circRNAs) have been known for their critical role in RNA maturation, RNA transportation, epigenetic regulation, gene transcription, peptide/protein translation, and interaction with proteins to modulate their activities. At present, circRNAs are being extensively studied as oncotargets in the onset and development of several malignancies and treatment resistance by modulating various signaling pathways and cellular processes such as ubiquitination, degradation, invasion, proliferation, c-Myc oncoprotein stabilization, epithelial-mesenchymal transition, autophagy, and apoptosis. In addition, circRNAs are known to be highly conserved, have a longer life span than other RNAs, and their differential expressions are implicated in solid tumors and hematological malignancies. However, their potential diagnostic and therapeutic targets are not fully elucidated. Therefore, we sought to underline the opportunities and constraints associated with using these oncotargets in cancer therapy by outlining the functional mechanisms of circRNAs and their ectopic expression in distinct malignancies. The clinical applications of circRNAs in developing progressive markers like liquid biopsy biomarkers and for treating cancers are also prospected in this paper.

Keywords: CircRNAs; Upregulation; Down-regulation; Anticancer drugs resistance; Liquid biopsy biomarkers

***Corresponding authors:**
Weijuan Zhang
(zwjuan1965@henu.edu.cn)
Wenqiang Wei
(weiwq@henu.edu.cn)
Shaoping Ji
(shaopingji@henu.edu.cn)

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1. Introduction

Circular RNAs (circRNAs) are among the most thoroughly investigated ncRNAs in recent years, accounting for most of the human non-coding transcriptome[1,2]. Subsequent research has recognized the importance of circRNAs in regulating many cellular developmental and biological events, which involve critical molecular processes

such as chromatin modifications, DNA methylation, gene transcription, and messenger RNA (mRNA) destiny regulators splicing, translation, or decay[3-8].

Although circRNA biogenesis has been previously reviewed[9], the specific mechanisms of circRNAs formation are still poorly understood in terms of being explored and validated regularly. Most of the discovered exonic circRNAs (>90%) originated from protein-coding genes with one or several exons that have substantially longer intracellular half-lives, which implicates that circRNAs are resistant to exonuclease digestion[10-12]. Certain features of circRNAs include: (1) circRNAs are abundantly available in human fluids and tissues, and due to their stable covalently closed structure, certain circRNAs gather at a significant level compared to their canonical linear mRNAs[13]; (2) numerous circRNAs in eukaryotes are conserved evolutionarily[14]; and (3) many circRNAs are explicitly expressed in tissues or cells[14,15].

In addition, by competing for the particular miRNA, RNA species such as lncRNAs, pseudogene transcripts, mRNAs, and circRNAs that serve as competing endogenous RNAs (ceRNAs) affect genomic expression post-transcriptionally and impact the half-life or translation of target RNAs[16,17]. CircRNAs have been revealed to act like ceRNAs, regulating important biochemical events in cancer such as cell division, angiogenesis, and apoptosis. For example, it has been demonstrated that circRNAs can act as ceRNAs by controlling the expression of the GDNF family receptor alpha-1 (GFRA1) to change the expression of *miR-34a*, which subsequently inhibits the apoptosis of triple-negative breast cancer (TNBC)[17,18]. CircRNAs can also be utilized as RNA-binding protein (RBP) decoys to mediate its host gene expression or to subtly influence the activities of RBPs and associated proteins[14,19]. Furthermore, certain circRNAs containing IRES-like components and start codon (AUG) sites enable them to translate into particular small proteins/peptides[20].

Some circRNAs are obviously linked to cancer patients' clinical outcomes due to their crucial roles in biological pathways[21]. However, circRNAs can also play opposing roles to their linear counterparts. For example, the linear RNA of the human and mouse *ZBTB7A* genes works as a tumor suppressor, but the circRNA generated by *ZBTB7A* genes has a carcinogenic role in connective tissue malignancies[22]. On the other hand, the formation of *FOXO3*-encoded proteins, such as those caused by the mouse or human *FOXO3* gene, induces apoptosis and suppress tumor formation[23]. Moreover, circRNAs in exosomes and body fluids can serve as potential disease biomarkers[21,24,25].

Given the worth and interest of creating circRNAs-based knowledge, this paper aims to provide a detailed discussion on the functional mechanisms of circRNAs and their dysregulation in several cancers. In addition, this paper highlights the gaps, opportunities, and challenges in using circRNA-based approaches for cancer treatment. Moreover, the clinical applications of circRNAs in developing liquid biopsy biomarkers and identifying promising biomarkers for cancer diagnosis, prognosis, and therapy are also well discussed. Considering the need for literature and novelty aspects in this domain, we anticipate that the current review will be a resourceful addition of literature on circRNAs research and their future perspective regarding diagnosis and anticancer therapies.

2. Regulatory role of circRNAs in cancer

CircRNA altered expression is connected with diabetes, atherosclerosis, cardiovascular disease, and neurological illnesses based on its developing involvement in gene regulation[26]. However, recent research has demonstrated the aberrant production of circRNAs in several cell types of cancer, including gastric cancer (GC)[27], colorectal cancer (CRC)[28], hepatocellular carcinoma (HCC)[29], breast cancer (BC)[30], glioblastoma[31], and ovarian cancer (OC)[32]. Along the same lines, circRNAs participate in the modulation of many cellular signaling pathways, which can modulate tumorigenesis[33]. Most circRNAs have potential binding sites and act as sponges for the different miRNAs to regulate miRNA mediating the downstream activation of the target genes implicated in cancers. CircRNAs feature a covalently closed ring structure that is difficult to be degraded by cellular exonuclease degradation mechanism which typically readily recognizes the terminals of linear RNAs[12]. Exosomes are also enriched with stable circRNAs[25]. Considering the wide availability, cell-and-tissue-specific expression, and better stability, determining their function in human illness, particularly cancer, has been the main focus of many researchers.

2.1. BC

BC is a diverse illness and the world's most significant cause of mortality[34]. Evidence shows that oncogenic or tumor suppressor properties are influenced by circRNAs in BC[35,36]. Overexpression of circRNAs was found in estrogen-positive (ER+) adjacent normal tissues than in ER-negative tumor samples in the Cancer Genome Atlas (TCGA) database, implying that the frequency of circRNAs could serve as a possible cell proliferation indicator in BC[37]. Another highly expressed circRNA in BC, *circ-DNMT1* (hsa_circRNA_102439), was reported to increase cell proliferation and autophagy and inhibit senescence by binding directly to p53 and AUF1, resulting in the

promotion of nuclear translocation of these proteins[38]. Similarly, *FECR1*, a novel circular RNA generated from the FLI1 gene, regulates DNA methylation/demethylation of target genes to mediate BC cell metastasis[7,39]. In addition, the increase expression of circAGFG1 in TNBC increases cell proliferation, migration, invasion, tumor development, and metastasis by serving as ceRNA for *miR-195-5p* to diminish its target genes' inhibitory function[40,41].

On the contrary, *circFOXO3* is significantly lowered in BC tissues compared to the adjacent normal ones[36]. Abnormal *circ-FOXO3* triggers programmed death and prevents tumor cell development. On the other hand, overexpression of *circFOXO3* leads to the increase of its parental gene expression and upregulates the genes responsible for cell deaths. For example, *PUMA* inhibits p53 expression and promotes *MDM2*-induced p53 degradation. In addition, *circ-CCNB1* is significantly suppressed in BC. It has been demonstrated that *circ-CCNB1* in p53 mutant cells forms a compound with *H2AX* and *BCLAF1* mediating apoptosis[39]. In TNBC, the downregulation of *circTADA2A-E6* inhibits proliferation, motility, and infiltration. *circTADA2A-E6* serves as a *miR-203a-3p* sponge and restores the production of miRNA targeting the *SOCS3* gene, leading to the less invasive neoplastic phenotype. It concludes that circRNAs can mediate BC by acting as oncogenes or suppressors.

2.2. OC

OC is a highly aggressive tumor among gynecological malignancies and is primarily diagnosed in advance[42]. Multiple cancer-related signaling pathways, usually activated by linear mRNA, can be downregulated by circRNAs, indicating a potential role of circRNAs in tumor activation or inhibition[43]. CircRNAs exhibit differential expression among OC patients and healthy individuals, showing their potential roles as both the therapeutic and diagnostic biomarkers for this disease[44]. Ning *et al.* investigated circRNAs expression in EOC tissue samples and identified that 2556 circRNAs are upregulated, and 1832 circRNAs are downregulated compared with normal ovarian tissue samples[45]. Moreover, among the dysregulated circRNAs, *circEXOC6B* and *circN4BP2L2* were suggested to possess a potential prognostic disease-specific biomarker. Similarly, hsa_circ_0061140 has also been elevated in OC cells, which directly controls FOXM1 expression by binding to miR-370, thus promoting cell proliferation, migration, and epithelial-mesenchymal transition (EMT)[46]. However, *CircHIPK3* (hsa_circ_0000284) is substantially related to multiplication, motility, invasive, and apoptosis inhibition through binding to *miR-10-5p*[47].

2.3. GC

GC is a major source of mortality and a significant financial burden on health-care systems worldwide[48]. CircRNAs are reported to be stable in plasma and gastric juice, making them an ideal diagnostic marker for GC. Three circRNAs (has_circ_002059, hsa_circ_0000190, and circ-0001649) are variably expressed in the plasma of post-operative GC patients compared to pre-operative GC individuals. They are considerably downregulated in GC tissues compared to nearby tissues with average physiological properties[49]. The reduced expression of the above three circRNAs correlates significantly with gender, age, distal metastasis, and TNM stage[50-52]. However, circ*HIPK3*, but not *HIPK3* mRNA, is upregulated in GC and significantly affects cell proliferation. *CircHIPK3* expression is substantially higher than *HIPK3* mRNA, thus it demonstrates its crucial role as a product of pre-*HIPK3* mRNA[19]. *CircPVT1*, overexpressed in GC, has been identified to promote cell proliferation. Mortality risk and time without developing an illness positively correlate with its interaction with *miR-125* in GC patients[27]. *CircPVT1* mediates its function by upregulating c-Myc oncoprotein levels in GC cells and acts as a sponge for let-7b. CircRNAs are expressed differently, they interact with miRNAs through their binding sites to control the transcription of their downstream targets and may serve as a novel biomarker for GC[53].

2.4. CRC

A study showed a negative correlation between the total circRNAs abundance and cell proliferation in CRC, where its high expression suppressed cell proliferation[32]. The circ-ITCH was significantly lowered in CRC tissues. Its deficiency upregulated ITCH expression, which blocked the Wnt/beta-catenin pathway[54]. However, circ_0060745 was upregulated in CRC and linked with cellular propagation and metastasis through the sequestering of *miR-4736* and elevation of *CSE1L* levels[55]. Similarly, an analysis of 50 CRC tissue samples revealed that hsa_circ_0007142 is upregulated and linked with poor differentiation, and metastasis to the lymph nodes is mediated by its binding to miR-103a-2-5p[56]. Furthermore, circ-BANP is overexpressed in CRC tissues, and its silencing suppresses cellular multiplication[57]. Other circRNAs reported to be upregulated in CRC include *ciRS-7*[58], *circCCDC66*[28], and has_circ_0000069[59]. They are also associated with clinicopathological variables such as age and TNM stage. According to Zhang *et al.*'s research of the human circRNAs array in CRC tissues, hsa circRNAs 103809 and 104700 were dramatically downregulated and associated with lymph nodes, tumors, and distant metastasis[60]. CircRNAs are expressed differently to mediate cancer development.

2.5. HCC

Increasing evidence in scientific literatures implicate circRNAs in the development and progression of HCC, leading to a poor prognosis for HCC patients. For example, in comparison with neighboring normal liver tissue samples, hsa_circ_0001649 was significantly suppressed in 89 HCC tissue samples. In HCC, its expression was associated with both the size of the tumor and the tumor embolus, and knocking down the circRNA increased the mRNA level of matrix metallopeptidases (MMPs), promoting the spread of HCC[61]. Similar *circZKSCAN1* and its linear analog were suppressed in HCC tissues. Increased tumor size, cirrhosis, metastasis, and poor prognosis were linked to reduced expression of *circZKSCAN1*. Through several signaling pathways, the upregulation of *circZKSCAN1* and its linear mRNA can significantly prevent the growth and metastasis of HCC[62]. However, high expression of *circRHOT1* (hsa_circRNA_102034) in HCC tissue samples is associated with poor prognosis[63]. hsa_circ_100084 was more highly expressed in HCC clinical samples than in samples of healthy liver tissue. Sponging *miR-23a-5p* functions as a ceRNA to increase *IGF2* expression, enhancing HCC cell proliferation, invasion, and relocation[64]. Recently, Sun *et al.* observed three circRNAs (hsa_circ_0004001, hsa_circ_0004123, and hsa_circ_0075792) upregulated and positively correlated with tumor size and TNM stage in the blood of HCC patients. The underlying mechanism was the regulation of 35 distinct miRNAs by the Akt/mTOR, VEG, and Wnt signaling pathways[65].

2.6. Gliomas

Gliomas are a vast category of nervous system tumors and one of the most prevalent malignancies with a poor prognosis globally. Recent studies have revealed the transcription of circRNAs in mammalian brains and their role in the growth and spread of gliomas[66]. A total of 476 circRNAs with differential expression have been found in 46 GBM and normal brain tissue samples, among which 468 circRNAs were more upregulated in normal brain tissue than GBM tissues. Furthermore, eight circRNAs, including *circCOL1A2*, *circPTN*, *circVCAN*, *circSMO*, *circPLOD2*, *circGLIS3*, *circEPHB4*, and *circCLIP2*, had significantly higher expression in glioblastoma multiforme (GBM) samples compared to normal human tissues, raising the possibility that they could be used as biomarkers for the disease[67]. The oncogenic circRNA, *cZNF292*, influences the development of tube formation. By controlling the Wnt/beta-catenin biochemical pathway, the suppression of *cZNF292* prevents glioma cell proliferation, progression, and tube formation[68]. Another study determined differentially expressed (206 upregulated and 1205 downregulated) circRNAs in GBM patients and found a significant association between circRNAs with low expression and the development of GBM through ErbB and neurotrophin signaling pathways[69]. Besides, common expression of *circBRAF* has been identified in glioma patients with high-grade tumors[68]. However, *circTTBK2* has a higher expression in glioma tissues. Its effect is induced by impairing miR-217 and elevating HNF1β expression, which subsequently binds to Derlin-1 oncogene, thereby increasing its promoter activity and upregulating the cell proliferation, migration, and apoptotic repression by targeting PI3K/Akt and ERK signaling pathways[70]. Similarly, hsa_circ_0000177 also causes an increased expression of glioma tissues, which is linked to the poor prognosis of patients. The silenced hsa_circ_0000177 acts as a *miR-638* sponge to drastically reduce cell growth and metastasis. This causes the frizzled class receptor 7 (FZD7) to be upregulated, further regulating Wnt signaling and enhancing glioma cell development[71]. *CircMMP9* also has high expression in glioma cancer. Through *miR-124* sponging, increased expression of *circMMP9* regulates CDK4 and Aurora kinase A (AURKA), and glioblastoma multiforme cells are driven to proliferate, migrate, and invade[72]. Moreover, *circPRKCI* is overexpressed in glioma tissues and significantly stimulates *miR-545*, inhibiting cancer growth, survival, multiplication, and relocation[31]. Similarly, circ_001350 is highly expressed in tissue samples and cells from glioma patients, and by interacting with *miR-1236*, it reduces cell growth and metastasis while promoting apoptosis[73]. *Circ-MAPK4* (hsa_circ_0047688) upregulated and associated with a pathogenic form of gliomas, has been shown to impede the apoptosis of glioma cells by acting as a sponge of miR-125a-3p and regulating p38/MAPK signaling pathway[74].

2.7. Pancreatic ductal adenocarcinoma (PDAC)

More than 85% of cases of pancreatic cancer are caused by PDAC, which is also one of the main causes of death and poor prognosis globally[75]. Numerous circRNAs that are overexpressed in PDAC have been identified. It is crucial to find these circRNAs that express differently to better comprehend PDAC. In PDAC cancer patient samples, Li *et al.* discovered seven circRNAs using circRNA microarray analysis, of which two had upregulated expression levels and the remaining five had downregulated expression levels[76]. A total of 278 circRNAs were differentially expressed in PDAC tissues according to RNA sequencing and circRNA expression analysis. Among these, hsa_circ_0007334 was strongly upregulated and acts as a ceRNA by binding to *miR-144-3p* and *miR-577*, which boosted the expression and functionality of *MMP7* and collagen type I alpha 1 chain (*COL1A1*) in PDAC[77]. Likewise, pancreatic cancer (PC) tissues had higher levels of *circLDLRAD3*, which

was associated with a poor prognosis for PC patients. *CircLDLRAD3* directly binds to *miR-137-3p* as a sponge and promotes cellular multiplication, relocation, and infiltration of PC cells through targeting pleiotrophin (PTN)[78]. Chen *et al.* discovered 12,572 circRNAs in the exosomes of irradiated human PC cells using RNA-sequencing analysis. There were 196 circRNAs that showed varied expression and were connected to methylation. In addition, the upregulated gene hsa_circ_0002130 interacted with *miR-4482-3p* to enhance NBN expression, lowering the survival rate in PC patients[79]. Binding to *miR-34b-5p*, upregulating mesenchymal-epithelial transition factor, and phosphorylating Akt, a new circRNA (*circBFAR*, hsa_circ_0009065) derived from exon 2 of the *BFAR* gene, upregulated in 208 PDAC patients, was demonstrated to accelerate PDAC cells progression (Ser 473)[80]. *CircFOXK2* was upregulated in PDAC tissue samples according to another study. It stimulates Ankyrin 1 (*ANK1*), glial cell-derived neurotrophic factor (GDNF), paired box 6 (PAX6), NUF2 component of NDC80 kinetochore complex (NUF2), pyridoxal kinase (PDXK), Y-box-binding protein 1 (YBX1), and heterogeneous nuclear ribonucleoprotein K (hnRNPK) expression, as well as cell expansion, migratory property, and colony formation[81].

2.8. Lung cancer (LC)

Most deaths resulting from cancer are attributable to LC worldwide. The development of LC can be controlled by inappropriate circRNAs expression. In non-small-cell LC (NSCLC) tissue samples, *ciRS-7* was upregulated and was associated with a higher TNM stage, lymph node metastases, and a shorter overall survival time. Overexpressed *ciRS-7* inhibits *miR-7*, which causes the *EGFR*, *CCNE1*, and *PIK3CD* to be upregulated to promote cellular proliferation and prevent LC apoptosis[82]. Hsa_circ_0012673 upregulates lung adenocarcinoma (LAC) tissues compared to non-tumor tissues. The hsa_circ_0012673 promotes LAC cell propagation by inhibiting the expression of *miR-22* and targeting the erb-b2 receptor tyrosine kinase 3 (*ErbB3*)[83]. Similarly, increased expression of hsa_circ_0020123 in NSCLC tissues is connected to poor differentiation, lymph node metastases, and advanced TNM stage, all associated with malignancy. The inhibition of *miR-144* and the increase in the expression of *ZEB1* and *EZH2* encourages NSCLC cell proliferation, migration, and invasion[84]. *CircAGFG1*, another circRNA upregulated in NSCLC tissues, promotes cell proliferation and metastasis by suppressing *miR-203* and activating *ZNF281*[85]. The most current microarray research shows that the highly expressed new circRNA, *circSLC25A16* (hsa_circ_0018534), stimulates lactate dehydrogenase A and *sponges miR-488-3p/HIF-1* to activate glycolysis and promote cell growth in NSCLC cells (LDHA)[86]. In addition, circRNAs such as *circRNA 102231*, *circRNA 100146*, *circFARSA*, and *circPVT1* are elevated in NSCLC and enhance the advancement of NSCLC[87-91]. Therefore, these circRNAs may be employed as promising diagnostic and prognostic indicators to identify LC.

2.9. Prostate cancer (PCa)

PCa is among the main risk factors in males due to cancer-related causes[92]. A study conducted by Xia *et al.* found 1021 circRNAs with differential expression (117 upregulated and 904 downregulated) in PCa tissues sample and cell lines. Some circRNAs were overexpressed in PCa tissues and connected to PCa pathogenesis, specifically hsa_circ_0057558 and hsa_circ_0062019. While hsa_circ_0062019 operates as a sponge for *miR-5008-5p* to control the folate level and the development of PCa, hsa_circ_0057558 functions as a sponge for miR-6884-*3p*, thereby preventing lipid metabolism by supporting its downstream target genes[93]. Moreover, downregulation of hsa_circ_0004870 and its host gene, *RBM39*, in enzalutamide-resistant cells suggests that they play a critical role in the progression of castration-resistant PCa that is resistant to enzalutamide[94]. In addition, PCa tissues and serum samples had higher levels of *circFOXO3*, and its silencing prevented mitosis and promoted programmed cell death by acting as a reservoir for *miR-29-3p* and upregulated the expression of *SLC25A15*[95]. Through bioinformatics research, Wu *et al.* screened 60 circRNAs that were differentially expressed in PCa and healthy samples: One circRNA, hsa_cir_0024353, was downregulated; and two circRNAs, hsa_circ_0031408 and hsa_circ_0085494, were found to be upregulated among the differentially expressed circRNAs. These three circRNAs affect a number of metabolic processes, including phosphoinositide 3-kinase-Akt, P53, and hypoxia-inducible factor-1[96]. Another study discovered 749 circRNAs differentially expressed in PCa tumor and paracancerous tissues. Among them, 261 were upregulated in PCa tissues, whereas 487 were downregulated. According to analyses by KEGG pathways and gene ontologies, numerous circRNAs are connected to cancer development. The most significant alterations among the differentially expressed circRNAs were in hsa_circ_0033074 and hsa_circ_0016064[97]. Moreover, PCa cells proliferate and invade when *circHIPK3* is upregulated and acts as a miR-339-3p sponge, while circRNA *ITCH* works as a *miR-17-5p* sponge and slows PCa growth by increasing *HOXB13* expression[98,99].

2.10. Hematological malignancies

Hematological malignancies are life-threatening cancers that originate from genetic and epigenetic abnormalities[100].

Acute myeloid leukemia, chronic myeloid neoplasms, chronic lymphocytic leukemia (CML), B- and T-cell lymphoma, and multiple myeloma (MM) are hematological malignancies that exhibit circRNA expression. Hsa_circ_0004277 was downregulated in AML samples as opposed to controls and post-treatment subjects. By acting as a sponge for *miR-138-5p* and *miR-30c-1-3p*, hsa_circ_0004277 controls the expression of downstream genes implicated in cancer development[101]. *CircPVT1* was increased in acute myeloid leukemia compared to normal bone marrow cells, and it was discovered that it acts as a sponge for the miRNA *let-7*, showing that it might be a viable treatment focus for the disease[102]. Several circRNAs with abnormal expression in CML have been identified using high-throughput sequencing technology. Among these circRNAs, hsa_circ_0080145 was significantly overexpressed and can effectively bind to *miR-29b* to regulate cell proliferation in CML[103]. Besides, *circ-CBFB* was overexpressed in CLL patients' samples compared to normal controls. It promotes cell cycle progression and reduction by inhibiting *miR-607*, facilitating *FZD3* expression, and activating the Wnt/b-catenin pathway in CLL[104]. By performing RNA sequencing profiling, Dahl *et al.* recently identified a novel overexpressed circRNA from the *IKZF3* gene with oncogenesis functions[105,106].

Several other differentially expressed circRNAs have also been studied, and their upregulation is associated with the progression of different cancers. For instance, *circTCF25* in bladder cancer[107], hsa_circ_100855 in laryngeal cancer[108], *circUBAP2* in osteosarcoma[109], *circ-ZNF609* in renal cell carcinoma[110], *circSLC30A7* in oral squamous cell carcinomas[111], and all of which are upregulated and involved in the progression of cancers. In contrast, *Cir-ITCH* is less expressed in esophageal squamous cell carcinoma than in the adjacent normal tissues[50]. Therefore, based on the widespread presence of circRNAs in various parts of the human body, it is logical to propose that circRNAs can be used in cancer diagnosis.

3. CircRNAs as diagnostic biomarkers for cancer

Different sampling strategies can be used to diagnose the etiologic modulator of cancer. In a liquid biopsy, body fluid was used as a sample for diagnosis or the development of human diseases instead of tissue biopsy, with the advantages of being less intrusive, accurate, easy assessments, time serving, and with a lower morbidity rate[112]. Several liquid biopsy biomarkers have been proved helpful in the diagnosis of various malignancies, including cell-free DNA[113], circulating tumor DNA[112], and cell-free RNA[113-115]. NcRNAs, particularly circRNAs, have demonstrated significant potential as circulating diagnostic biomarkers for liquid biopsy using cell-free RNA-based liquid biopsy. CircRNAs have several essential features including stability, specificity, conservation, and abundance in bodily fluid, making them valuable biomarkers for human diseases, including cancer. CircRNAs can be found in various human body fluids, including saliva, brain/spinal fluid, blood, urine, free-floating cells (such as circulating blood cells/tumor cells), and exosomes[112,114,116]. Circulating cell-free RNAs released by various tissues and cells can be recognized in serum, plasma, and blood. In addition, the stability of circRNA is an important factor, leading to potential target for many diseases[117]. As a result, cell-free circRNAs have tissue-specific features, emphasizing their clinical significance for the respective tissues[118]. Microarray, NanoString technology, digital droplet PCR (ddPCR), or next-generation sequencing (NGS) technologies can be used to quantify circRNAs derived from different body fluids, circulating blood cells, or exosomes for early diagnosis, disease development and monitoring, or precise therapy selection for different types of cancers (Figure 1)[119-121]. In liver cancer cells and cell-derived exosomes, a 2-fold enrichment of circRNAs was recently discovered in exosomes compared to parental cells, and the expression can be persistent in serum after incubation at room temperature for up to 24 h[25]. *circFMN2* and *circIFT80* have recently been shown to be highly overexpressed in serum exosomes and have been linked to cell proliferation, invasion, and inhibition of apoptosis through the *circFMN2/miR-1182/hTERT* axis and the *circIFT80/miR-1236-3p/HOXB7* axis, respectively[122,123]. Cell-free circRNAs' roles in human bodily fluids and their implications in numerous diseases might be used as liquid biopsy biomarkers to identify tumors at an early stage. Table 1 enumerates the circRNAs identified to be dysregulated in different human body fluids.

4. CircRNAs involved in drug resistance

Many studies have linked ncRNAs (lncRNAs and miRNAs) to chemoresistance[150,151], paucity of data is available on the regulatory mechanisms of circRNAs in developing drug resistance in malignancies. Some studies have recently looked at the function of circRNAs in resistance to various anticancer medicines (chemotherapeutic drugs, targeted therapies, and immunotherapeutic drugs) in terms of concentration of drug, downstream signaling pathway regulation, and DNA repair ability[152,153]. Drug resistance phenotypes can have two mechanisms: intrinsic or acquired, depending on whether they were innately resistant to treatment or tolerated after drug exposure. Due to their high differentiation rates, cancer stem cells can also drive acquired resistance. As a result, tumor heterogeneity,

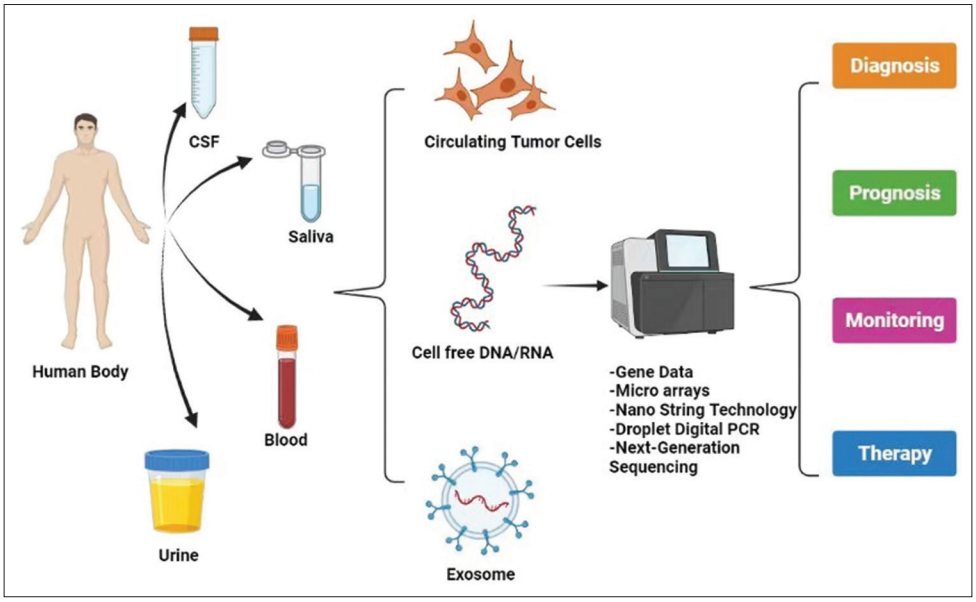

Figure 1. Orientation of circRNAs as a diagnostic biomarker for cancers through liquid biopsy. Using body fluids and other molecular biomarkers, effective management strategies for cancer diagnostic screening. The body fluids of cancer patients contain disease-specific circulating free DNA/RNA, circRNAs, and circulating tumor cells. Various molecular biology techniques, such as digital droplet PCR, NanoString technology, microarrays, and next-generation sequencing, can be used to analyze the nucleic acid extracted from a sample of body fluids.

EMT, drug transport and metabolism, autophagy, and apoptosis inhibition enhance drug resistance[154]. CircRNAs interact with these molecules and modify them to promote drug resistance[155].

Various drug response pathways, such as PI3K/AKT, MAPK, VEGFR, MEK/ERK, and the ATP-binding cassette (ABC) transport system, are regulated by circRNAs as well[156]. According to a recent study, *circNFIX* was overexpressed in temozolomide-resistant glioma individuals and conferred temozolomide resistance to recipient glioma cells through exosomes through inhibiting *miR-132*[151]. *CircPVT1* promotes treatment resistance to doxorubicin and cisplatin in osteosarcoma tissues, blood, and chemoresistant cell lines by increasing the expression of the *ABCB1* gene[157]. Furthermore, through the *miR-182-5p/FOXO3a* axis, hsa_circ_0025202 overexpression is frequently downregulated in BC tissues and tamoxifen-resistant cell lines, reduces cell division and movement, boosts cell apoptosis, and improves tamoxifen sensitivity[158]. The above findings suggest that circRNAs perform a vital part in chemoresistance. Figure 2 depicts certain anticancer drug-resistant circRNAs in various malignancies.

5. Implementation of new models in circRNA cancer research

The significance of circRNAs in the pathophysiology of specific cells is being studied using two-dimensional and three-dimensional cellular systems as research platforms. Due to their ability to faithfully mimic the evolution of cancer, inducible systems that overexpress genes are valuable models for observing circRNA expression and changes during tumor progression in two-dimensional biological systems[159]. In addition, to gain crucial knowledge about the pathways and processes impacted by circRNA entities, it is possible to experimentally assess the impact of circRNAs' potential qualitative/quantitative variations on other properties, such as RBPs and miRNAs. Similarly, three-dimensional cellular systems, including brain organoids made from iPSCs[159], have already been used in circRNA research. About 56% of the detected circRNAs in those organoid cultures overlapped circRNAs from the postmortem brain[160]. The highest in terms of ability and similarity to the original tissue are patient-derived organoids, indicating their broad applications in various malignancies, including GC[161-166]. Other approaches for studying circRNAs include animal models with manipulable loci encoding these molecules. Sensorimotor gating was disturbed in mice with a deletion in the *Cdr1* as circRNA gene, which substantially binds *miR-7* and *miR-671*, leading in neuropsychiatric problems due to the animals' inability to filter out redundant data[165]. In addition, a study utilized shRNAs to selectively downregulate five highly expressed circRNAs in *Drosophila* by targeting particular circRNA-back-splice junctions[166]. The downregulation of *circ–Ctrip* resulted in developmental lethality. These examples highlight

Table 1. circRNAs dysregulated in several cancers and also found in body fluids.

Cancer type	circRNA	Expression in disease	Body fluid	Function	Pathway/ miRNA sponge	Pathological status	Cases/ controls	References
BC	hsa_ circ_0001785	Up	Plasma	The expression level decreases significantly in post-operative patients than pre-operative patients. A potential biomarker for early detection of BC	-	Associated with histological grade, TNM stage, and DS	57/17	[124]
TC	hsa_ circ_007293, hsa_ circ_031752, and hsa_ circ_020135	Up	Serum exosomes	Potential diagnostic, prognostic, or therapeutic biomarkers	PI3K-Akt and AMPK signaling pathway	-	3/3	[125]
OC	*circMAN1A2*	Up	Serum	Could be a good diagnostic biomarker for malignant tumor	*miR-135a-3p*	-	36/121	[126]
	hsa_ circ_0001068	Up	Serum exosomes	Non-invasive biomarker for the diagnosis and treatment of OC	*miR-28-5p*	-	95/53	[127]
EC	hsa_ circ_0109046 & hsa_ circ_0002577	Up	Serum	EVs circRNAs served as biomarkers for EC diagnosis	Involved in multiple pathways	-	10/10	[128]
CRC	*circIFT80*	Up	Serum exosomes	Promotes CRC cell growth, proliferation, migration, and invasion	*circIFT80/miR-1236-3p/HOXB7*	Associated with advanced tumor stage and DS	58/58	[123]
	hsa_ circ_0001649	Down	Serum	A potential biomarker for CRC patients	-	Associated with pathological differentiation	64/64	[129]
	circVAPA	Up	Plasma	Promotes cell proliferation, migration, invasion, and inhibit apoptosis	*miR-101*/KEGG pathway	Associated with LNM, DS, and TNM stage	60/60	[130]
	circFMN2	Up	Serum exosomes	Promotes cell proliferation and migration	*circFMN2/miR-1182/hTERT*	Associated with tumor size, advanced tumor stage, DS, and TNM stage	88/88	[122]
	circ-CCDC66, circ-ABCC1 and *circ-STIL*	Down	Plasma	Promotes tumor growth and progression	-	No association observed	45/61	[131]
	hsa_ circ_0004771	Up	Serum	Associated with clinicopathological factors of GC patients	-	Associated with DS and TNM stage	110/70	[132]
GC	hsa_ circ_0000190	Down	Plasma	Associated with clinicopathological factors of GC patients	-	Associated with tumor diameter, LNM, DS, and TNM stage	104/014	[51]
	hsa_ circ_0000745	Down	Plasma	Might be a novel potential biomarker for diagnosing of GC	Bind to various miRNAs	Associated with TNM stage	60/60	[133]
	hsa_ circ_0001017, hsa_ circ_0061276	Down	Plasma	Associated with pathological factors and predictor for early diagnosis, prognosis of GC	-	Associated with higher mortality and TNM stage	121/121	[134]
	hsa_ circ_0000467	Up	Plasma	Promotes proliferation, migration, and invasion	Bind to various miRNAs	Associated with TNM stage	51/51	[136]
LC	*circFARSA*	Up	Plasma	Promotes cell migration and invasion	*miR-330-5p* and *miR-326*	No significant differences observed	10/10	[91]

(Cont'd...)

Table 1. ***(Continued).***

Cancer type	circRNA	Expression in disease	Body fluid	Function	Pathway/ miRNA sponge	Pathological status	Cases/ controls	References
UCB	*circPRMT5*	Up	Serum and urine exosomes	Promotes metastasis and aggressiveness of USB cells	Promotes EMT via circPRMT5/ *miR-30c*/SNAIL1/ E-cadherin	Associated with advanced tumor stage and worse patient survival	119/119	[137]
NPC	*circMAN1A2*	Up	Serum	Could be good diagnostic biomarker for malignant tumor	*miR-135a-3p*	-	100/121	[126]
	hsa_ circ_0000285	Up	Serum	Possibly regulates cell proliferation, metastasis, and radio sensitivity	*miR124*	Associated with tumor size, differentiation, LNM, DS, and TNM stage	150/100	[138]
ALL	*circPVT1*	Up	BM	Promotes proliferation and apoptosis	Through c-Myc and Bcl-2 expression in ALL	No significant differences observed	48/40	[139]
CLL	*circ-COX2*	Up	Plasma exosomes	Promotes CLL progression and prognosis	-	-	54/40	[140]
MM	*circMYC*	Up	Exosomes	Expression level circMYC associated with deletion 17p	-	Associated with higher relapse and mortality rates	122/54	[141]
	hsa_ circ_0007841	Up	BM plasma	Promote cell proliferation, cell cycle, metastasis, and inhibits apoptosis	Through the activation of PI3K/Akt signaling through *miR-338-3p*/ *BRD4* axis	-	41/41	[142]
HCC	*circSMARCA5*	Down	Plasma	Promotes proliferation, invasion, metastasis, and inhibits apoptosis	-	Associated with tumor differentiation and TNM stage	133/33	[143]
	circ-DB	Up	Plasma	Promotes rapid tumor progression by targeting deubiquitination-related USP7	*miR-34a*/ USP7/ Cyclin A2 signaling pathway	-	-	[144]
	circUHRF1	Up	Plasma	Decreases NK cell proportion and NK cell tumor infiltration and associated with poor prognosis	Through degradation of *miR-449c-5p*/ TIM-3	Associated with tumor size	240/240	[145]
PC	*circ-LDLRAD3*	Up	Plasma	Associated with venous, lymphatic invasion, and metastasis	-	No association found with pathological factors	31/31	[146]
OSCC	hsa_ circ_0001874, hsa_ circ_0001971,	Up	Salivary	The expression level decreases in the post-operative samples than pre-operative samples	*miR-107* and *miR-103a-3p*	Associated with TNM stage and tumor grade	163/85	[147]
OS	hsa_ circ_0000885	Up	Serum	High expression level associated with lower rates of disease-free survival and overall survival	-	Associated with Enneking stage IIB or III osteosarcoma and lung metastasis	50/50	[148]
LAC	hsa_ circ_0013958	Up	Plasma	Promotes cell proliferation and invasion and inhibited cell apoptosis	*miR-134*	Associated with TNM stage and LNM	49/49	[149]

BC: Breast cancer; TNM: Tumor node metastasis; DS: Distant metastasis; LNM: Lymph node metastasis; TC: Thyroid cancer; OC: Ovarian cancer; EC: Endometrial cancer; EVs: Extracellular vesicles; CRC: Colorectal cancer; GC: Gastric cancer; LC: Lung cancer; UCB: Urothelial carcinoma of the bladder; NPC: Nasopharyngeal carcinoma; ALL: Acute lymphoblastic leukemia; BM: Bone marrow; CLL: Chronic lymphocytic leukemia; MM: Multiple myeloma; HCC: Hepatocellular carcinoma; PC: Pancreatic cancer; OSCC: Oral squamous cell carcinoma; OS: Osteosarcoma; LAC: Lung adenocarcinoma

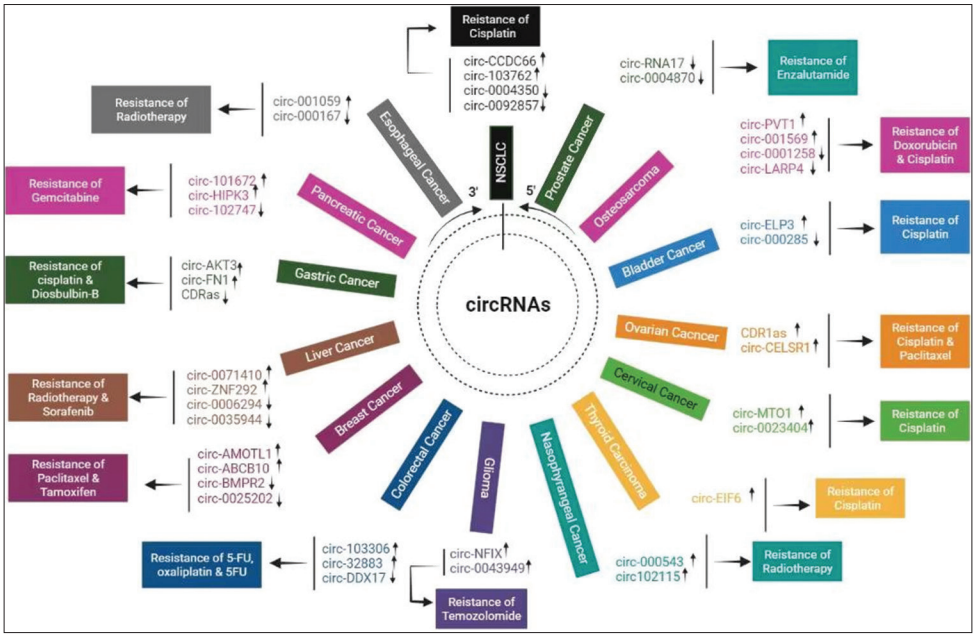

Figure 2. Anti-cancers drug-resistant circRNAs in various malignancies. Differential expression of each circRNA promotes the resistance to one or multiple drugs results in activation of the respective pathway and affects anticancer treatment efficacy, by altering cancer cell properties and/or drug distribution. The illustrated circRNAs act mainly as miRNA sponges, leading to up-/down-regulation of the downstream targets of these miRNAs. Other modes of action of circRNAs affecting the expression of key proteins implicated in therapy resistance.

the importance of such procedures and provide crucial information on how circRNAs function and are active.

6. CircRNAs: Toward a unified nomenclature

CircRNAs are becoming essential indicators of disease, particularly in cancer, necessitating a unified naming scheme. In addition, developing new biochemical methods and bioinformatic methodologies add to the evidence for distinguishing and classifying circRNAs. The current terminology is based on circBase, which includes a numeric code and species information. In contrast, circBank and circles build a circRNA annotation based on UCSC genomic coordinates (https://genome.ucsc.edu/) using the gene symbol of the transcript. CIRCpedia uses a distinctive name scheme that incorporates both the species and a circBase internal identification number. In addition, circRNAs have been classified using a unique vocabulary. The species is indicated by the first few letters of the gene name, the exons involved in the circularization, and the gene name: For example, exons of the hsa-circ gene (exons7-8)[167].

7. Conclusion and perspectives

circRNAs have a wide range of functional possibilities due to their active roles as miRNA sponges, RBP sponges, and transcriptional regulators in the modulation of protein-coding gene expression. The fact that circRNAs are abundantly available in saliva, exosomes, and blood samples makes them promising biomarkers for disease diagnosis, particularly for the onset, progression, and prognosis of cancer. Identification of circRNA biomarkers in blood and saliva and in other clinical samples such as urine and cerebrospinal fluid would be feasible in further research. More crucially, research on circRNAs opens up a new path for treating diseases, with the circularization of RNA being the eventual goal for treating illness. For instance, in the future, the overexpression of artificial circRNA can function as a "super-sponge" or can be used to silent the circRNAs in cells to remodel and alter the expression profile of miRNAs and other RNAs, or RBP levels can increase the activities of a suppressor gene in the context of cancer therapy. Although circRNAs have been the subject of several research studies, little is known about the biological and molecular processes by which circRNAs contribute to cancer development. Therefore, finding numerous additional circRNAs implicated in diseases and investigating their functional motifs and target locations will be essential.

The practical use of circRNAs in clinics still has a long way to go as the field of circRNA research is still in its infancy. CircRNAs alone will not be sufficient to serve as precise biomarkers for any particular malignancy. Despite some reports that certain circRNAs are specific for individual cancers, the level of these circRNAs in the other cancer types has not been determined by any studies, and this underlies the current uncertainty and specificity of

these circRNAs for a particular cancer type. Theoretically, although circRNA acts as a miRNA sponge, the miRNA may target several genes, indicating that a circRNA may modulate the expression of hundreds of genes. As a result, it is doubtful that a circRNA would be entirely specific for a particular cancer type. The most likely scenario is that circRNAs are either a common driving mechanism or by- or end-product of oncogenesis.

However, circRNAs may still be valuable as cancer biomarkers, just not for a particular type of cancer. In clinical settings, diagnosing specific cancer depends on clinical phenotypes and other data to analyze a particular malignancy. According to this, circRNAs could benefit in diagnosing several malignancies if combined with other factors or biomarkers.

In conclusion, circRNAs are a type of regulatory RNA that are highly expressed and functionally involved in cellular processes, suggesting that they may have a role in the emergence of several disorders, including cancer. Due to their ectopic expression in cancer, the current research has established that these circRNAs are essential in designing the approaches to investigate them in diagnosis and anticancer therapies. In this paper, we highlight and urge the design of advanced techniques to evaluate the critical roles of circRNAs in modulating cellular processes such as cell proliferation, migration, invasion, apoptosis, and autophagy for a better picture of their involvement in cancer development. Considering the critical applications of circRNAs and the potential to provide the template for designing anticancer drugs, we anticipate that using circRNAs in clinical sittings will shortly lead to a breakthrough in cancer therapy.

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Conflict of interest

All authors declare that they have no conflicts of interest.

Author contributions

Conceptualization: Faiz Ali Khan and Shaoping Ji
Supervision: Weijuan Zhang and Wenqiang Wei
Visualization: Faiz Ali Khan
Writing – original draft: Faiz Ali Khan
Writing – review & editing: Faiz Ali Khan, Bernard Nsengimana, Nazeer Hussain Khan, Jingjing Huang, Haojie Guo, Usman Ayub Awan, Weijuan Zhang, and Wenqiang Wei

All authors made substantial, direct, and intellectual contributions to the work and approved the paper for publication.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors consent to publication.

Availability of data

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REVIEW ARTICLE

Network biology: Recent advances and challenges

Pei Wang*

School of Mathematics and Statistics, Henan University, Kaifeng, 475004, P.R. China

Abstract

Biological networks have garnered widespread attention. The development of biological networks has spawned the birth of a new interdisciplinary field – network biology. Network biology involves the exploration of complex biological systems through biological networks for better understanding of biological functions. This paper reviews some of the recent development of network biology. On the one hand, various approaches to constructing different types of biological networks are reviewed, and the pros and cons of each approach are discussed; on the other hand, the recent advances of information mining in biological networks are reviewed. The principles of guilt-by-association and guilt-by-rewiring in network biology and their applications are discussed. Although great advances have been achieved in the field of network biology over the past decades, there are still many challenging issues. First, efficient and reliable network inference algorithms for high-dimensional and highly noisy omics data are still in great demand. Second, the research focus will be on multilayer biological network theory. This plays a critical role in the exploration of the multi-scale or dynamical characteristics of complex biomolecular networks by integrating multi-source heterogeneous omics data. Third, a close cooperation among biologists, medical workers, and researchers from network science is still a prerequisite in the applications of network biology. The rapid development of network biology will undoubtedly raise important clues for understanding complex phenotypes in biological systems.

Keywords: Biological network; Network construction; Omics data; Informative gene identification; Multilayer network

***Corresponding author:**
Pei Wang
(wangpei@henu.edu.cn)

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1. Introduction

With the development of the complex network theory, complex networks have become effective tools to model and investigate various complex systems[1-4]. In biological systems, biomolecules rarely work alone. The interactions among various biomolecules consist of different types of biological networks[3,4]. Typical biological networks include gene regulatory network (GRN)[5-7], protein-protein interaction (PPI) network[8-13], transcriptional regulatory network (TRN)[14-16], metabolic network[17,18], signaling transduction network[19-21], gene co-expression network (GCN)[22-26], disease network[27,28], and drug-target network[29]. Different types of biological networks describe the biological systems at different scales and perspectives[30]. The rapid advances of biological networks have given birth to a new interdisciplinary field – network biology[3,4,31,32].

Network biology uses the complex network theory to model and explore biological systems and help people better understand, predict, and control complex biological

systems. There are many issues in network biology. An important issue is how to construct biological networks, which is a reverse problem. This construction of biological networks is also known as network inference, network construction, topological identification, and so on. In fact, network construction is the first step in network biology[4,32-42]. Reliable biological networks guarantee the accuracy of results from network analysis and the subsequent real-world applications. The second issue is how to effectively explore bioinformatics in biological networks. This is known as biological network analysis or topological/structural analysis of biological networks[4,32,43-53]. The third issue is the applications of network biology. In fact, biological networks are essential for understanding the cellular mechanisms of various phenotypes; they also have wide applications in exploring relationships among human diseases, discovering new drug targets, guiding drug repositioning, and controlling biological systems[27,28,31]. The applications of network biology rely on reliable network construction and efficient modeling and analysis of related biological networks[4,28,29,44-46,53].

The rapid development and wide applications of network biology encouraged us to conduct this review. This review focuses on the aforementioned issues of network biology, aiming at introducing some recent advances and challenges of several basic research topics, principles, and applications in network biology. The rest of the paper is organized as follows: Section 2 briefly introduces the complex network theory; Section 3 reviews some recent advances of network biology, including the recent progresses of network construction, network-based identification of important genes/proteins, and their related applications; Section 4 puts forward some of the challenges in network biology; and the final section comprises the conclusion remarks.

2. Complex network theory

A complex network consists of nodes and edges[1]. Nodes represent the concerned entities in the system, while edges denote the relationships among nodes. As to biological networks, nodes may be genes, ribonucleic acids (RNAs), microRNAs (miRNAs), proteins, metabolites, or other molecules[4]. Edges indicate that there are physical or chemical interactions, chemical reactions, or co-expression relationships among biomolecules. A weight can be assigned to each edge to represent the strength of the interaction or co-expression between two nodes. Depending on the types of nodes and the meaning of edges, a biological network can be directed or undirected and weighted or unweighted (Figure 1A). Different types of networks can be modeled and explored through different methods.

Mathematically, a complex network can be described by its adjacency matrix, edge list, or node-edge matrix (Figure 1A). $A=(a_{ij})_{n\times n}$ is often denoted as the adjacency matrix of a complex network. If $a_{ij} > 0$, there is an edge between nodes i and j in which a_{ij} represents the weight of the edge. If $a_{ij} = 0$, there is no edge between nodes i and j. For an undirected network, the adjacency matrix A is symmetrical, otherwise not. For an unweighted network, a_{ij} only takes 1 or 0. Different types of networks encompass different measures to describe their structural features.

Taking undirected and unweighted networks as examples, some basic topological features of complex networks are introduced[1,2,4]. Some of the commonly used measures to evaluate the topological structure of complex networks include average degree and degree distribution, average clustering coefficient, average path length (APL), disassortativity, and so on[1,2,4]. Average degree is defined as the average neighbors of each node in the network. The clustering coefficient is used to describe the degree to which the adjacent points of a node are connected to each other. APL is defined as the average number of steps along the shortest paths for all possible pairs of nodes in the network. The assortativity coefficient is the Pearson correlation coefficient (PCC) of degree between pairs of linked nodes. If PCC > 0, the network is assortative; but if PCC < 0, the network is disassortative. In a disassortative network, nodes with high degrees tend to connect with low-degree nodes. This is a typical feature of biological networks[4]. The disassortative features of biological networks are different from those of social networks. In social networks, high-degree nodes tend to connect with high-degree ones. In addition to the aforementioned features, there are many other statistical indices to describe a network. For more details, several references can be referred to Barabási, Chen *et al.*, and Lü *et al.*[1,2,4]

Extensive centrality measures such as degree centrality, clustering coefficient centrality[1,2,54], betweenness centrality[55], k-shell[56], semi-local centrality[57], PageRank[58], LeaderRank[59], adaptive LeaderRank[60], SpectralRank[61], and so on have been developed to measure the importance of nodes in a complex network. These measures are all based on the characteristics of the node and edge in the network. Different measures evaluate the importance of a node from different aspects. For example, degree centrality measures how many neighbors a node has; betweenness centrality evaluates how many shortest paths that path through a node, and whether a node can act as a bottleneck in the network[54]; PageRank and LeaderRank are all based on random walks on the networks; the recently proposed SpectralRank is based on the dominant eigenvector of the augmented network of the originally considered network[61]. The augmented network is obtained by adding a leader node that is bidirectionally connected with all the nodes in the network. It shows that SpectralRank can well

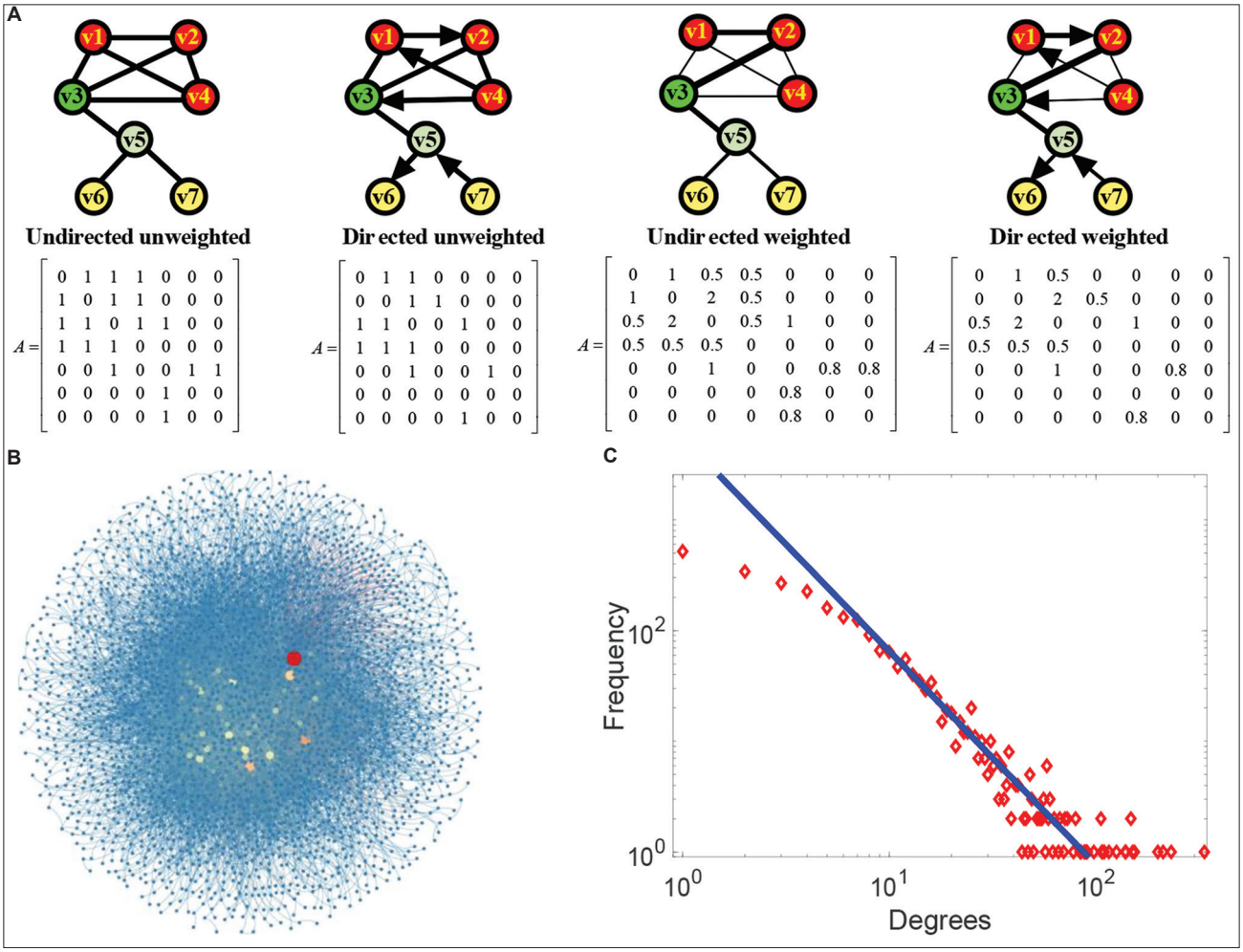

Figure 1. Networks with adjacency matrices or degree distribution. (A) Examples of different kinds of networks and its adjacency matrices. (B) A human PPI network with 2549 disease genes (OMIM) as nodes. (C) Degree distribution of human PPI network in log-log coordinates indicates a power-law distribution.

identify actually influential spreaders in various types of networks, including bipartite, undirected or directed, and weighted or unweighted networks. It has been reported that SpectralRank can also identify functional important global regulator and command interneurons in transcriptional networks and neuron networks, respectively.

Extensive investigations have reported that many real-world biological networks are sparse, scale-free, disassortative, small-world, and with modularity structures[4,13,62-64]. Although real-world networks contain tens to tens of thousands of nodes and hundreds to millions of edges, their connection densities are very low, which indicate that real-world biological networks are sparse. A network is considered small-world if it has high clustering coefficient and short APL[63], whereas a network is considered scale-free if its degree distribution follows a power-law distribution in log-log coordinates[64]. It has been reported that many biological networks adhere to the power-law degree distribution[4], and their power-law exponents roughly locates in [-3, -2]. For example, the *Yeast* PPI network has been reported to have a power-law exponent of −2.5[65,66]. Another typical feature of biological network is modularity. Since the expression of many genes show tissue-specific features, the corresponding biological networks often show apparent modular features[4].

Figure 1B shows the above-mentioned features in a human PPI network, which has been constructed from the BioGRID database[67]. The network consists of 2549 nodes and 11,438 undirected edges. The 2549 nodes correspond to disease genes that are reported in the Online Mendelian Inheritance in Man (OMIM) database[13,68]. The degree distribution of the constructed PPI network follows a power-law distribution (Figure 1C), with power-law exponent around −1.9280; the average degree of the

network is 8.9745; the APL is 3.5550; the graph density is 0.0040, which is a very sparse network; the PCC is −0.1222; and the average clustering coefficient of the network is 0.1830. The PPI network shown in Figure 1B has typical scale-free, small-world, disassortative, and sparse characteristics.

3. Recent advances of network biology

Some recent advances of network biology are reviewed in this section. We mainly consider three aspects: Network construction, important node identification in biological networks, and applications of biological networks.

3.1. Biological network construction

Network construction is the first step in network biology. Biological networks can be determined either from experimental detection or data-driven/model-driven inference. Experimental detection is generally costly and inefficient, but it is more reliable than model inference. To experimentally determine whether there are interactions among pairs of biomolecules, researchers have developed many experimental methods, such as ChIP-Seq, CLIP-Seq, yeast-two-hybrid[69] and yeast-three-hybrid (Y3H), rec-YnH, phage display technology, surface plasmon resonance (SPR), fluorescence resonance energy transfer (FRET), coimmunoprecipitation, glutathione S-transferase (GST) pull-down, and so on[4,69-71]. It has been reported that the recently developed rec-YnH can simultaneously detect putative, multi-domain direct protein-protein, and multi-protein-RNA interactions under physiological conditions[71].

There are four approaches to constructing biological networks (Figure 2 and Table 1). First, and most conveniently, existing network data can be downloaded from online databases, such as TRANSFAC, OPHID, MIPS, DIP, MINT, STRING, BioGRID, HPRD, KEGG, BBID, Reactome, and BIGG[4,67,72,73]. These online databases collect both experimentally determined and literature-curated interaction data among biomolecules, which provide timely and updated valuable resources for biological networks. However, the databases only collect interaction data for some model organisms or organisms that have been investigated by researchers. The data for many organisms are still in a state of uncertainty. Second, it is feasible to artificially construct biomolecular networks through computer algorithms. Classical algorithms are based on the duplication-divergence (DD) model[66,74-83]. In the DD model, duplication processes of biomolecules are mimicked by node duplication, while divergence processes are expressed as random node deletion, edge deletion, dimerization, and so on. By appropriately tuning parameters in the DD model, artificial biological networks that have similar topological features with real-world biological networks can be generated. Based on the artificial DD model, the evolution characteristics of biological networks can be explored[66,77,82]. However, the artificial models can only be used to theoretically explore possible features of biological networks. There are still some gaps between real-world and artificial biological networks, which limit the real-world applications of artificial biological networks. Third, by having insights on part of the network topology and node dynamics, the unknown topological connections can be predicted by dynamical complex network theory[84-89]. However, this approach requires node dynamics, which is generally difficult to be applied to infer biological networks. The last, but the most intriguing is data-driven approaches, which have been the focus in the field of network inference. Based on experimentally collected biological data, researchers have developed various mathematical and statistical models to infer biological networks, such as those methods based on correlation analysis[22-26,90-94], information theory[95,96], regression[36,37,39,42,88], Granger causality[40,97], Bayesian inference[98-101], and Gaussian graphical model[33-35]. Popular software packages for biological network construction include WGCNA[22], iDirect[102], and many others[3,4,93,103]. Data-driven approaches are efficient in inferring biological networks from data; however, for organisms without known biological network information, it is difficult to determine whether the inferred networks coincide with real-world ones; furthermore, determining the cutoff threshold values for some methods is also a problem[22-26,90-94].

Other than traditional biological networks, there are also studies on differential co-expression networks[49,103-111]. Differential co-expression networks reveal the differential co-expression patterns among genes when comparing between treatments and controls, which are effective tools for exploring omics data with different experimental settings. The tools or algorithms that can be used to perform differential co-expression analysis include DiffCoEx[102], CoXpress[103], DINGO[104], and many others. Both co-expression and differential co-expression analyses are application orientated; the edges in the co-expression or differential co-expression networks do not necessarily indicate physical interactions between two genes; instead, they only reflect the similarity between their expression profiles. Differential co-expression networks facilitate researchers to effectively explore bioinformatics from omics data, which have important applications in network biology.

3.2. Recent advances in the explorations and applications of network biology

In network biology, guilt-by-association and guilt-by-rewiring are two frequently used principles to explore

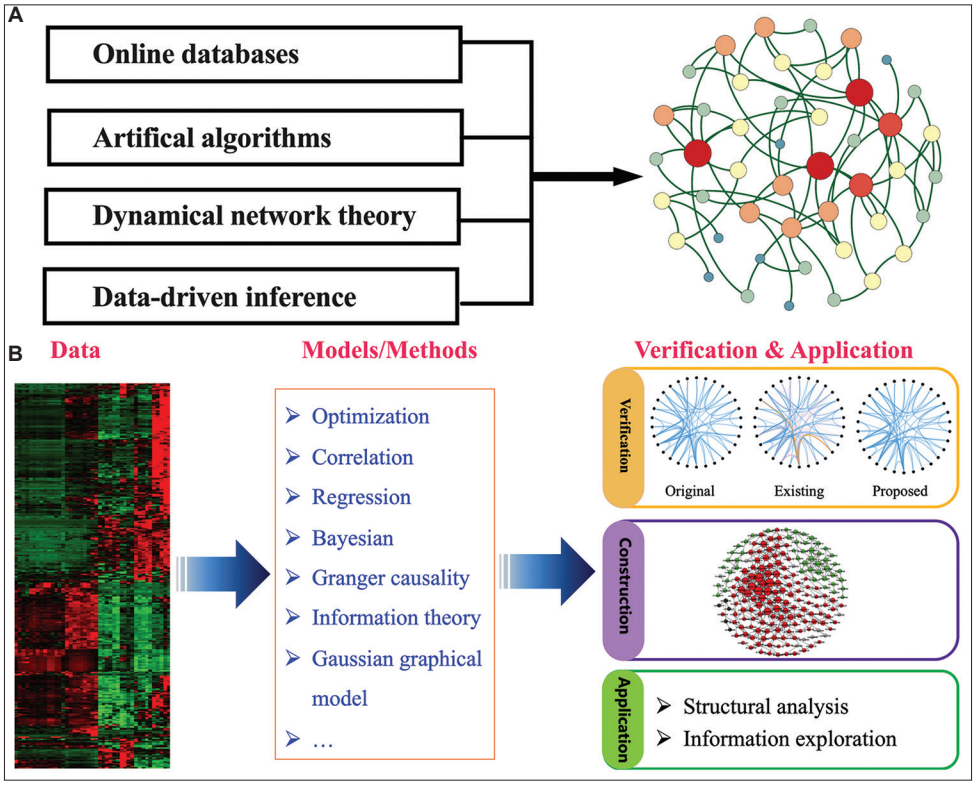

Figure 2. Biological network construction. (A) Approaches to construct biological networks. (B) Flowchart of data-driven biological network construction and applications. Based on biological data, various mathematical and statistical models can be developed to realize network construction; the network construction method should be further verified by datasets with known network structures, and subsequently, real-world networks can be constructed for various applications based on experimentally detected datasets.

Table 1. Advantages and disadvantages of the four network construction approaches.

Approach	Advantages	Disadvantages
Online databases	Easily obtained.	Only applicable for limited organisms; some databases are not timely updated.
Artificial algorithms	Easily generated; evolutions of network features can be explored.	Only limited to theoretical investigations; there are still gaps in real-world biological networks.
Dynamical network theory	Based on the theory of dynamical systems; potentially applicable for real-world control.	Requires node dynamics and dynamical system theories; difficult to be used in biological networks.
Data-driven approaches	Various mathematical and statistical models can be developed; explainable by data.	Difficult to determine the correctness for organisms without any known network information; discrepancy among different methods; difficult to determine the cutoff threshold values for certain methods.

bioinformatics[44,52,107,112,113] (Figure 3A). The guilt-by-association principle assumes that genes/proteins that highly connected with disease genes tend to be disease ones[44,112,113], whereas a basic assumption of the guilt-by-rewiring principle is that genes/proteins that altered their co-expression/interaction relationships under treatment are closely related to the causal phenotypes[52,107]. The two principles have been widely applied to identify important genes/proteins, predict the functions of genes/proteins, and explore the molecular mechanisms behind certain phenotypes.

3.2.1. Identifying informative genes/proteins based on biological network analysis

Important node identification is a foundational topic in complex networks[4,56-61,114,115]. Nodes in complex networks are heterogeneous, and different nodes generally play different roles; it is significant and interesting to identify important nodes[114]. Depending on the types of networks and the concerned questions, important nodes have different definitions. In biological contexts, important nodes largely represent informative genes/proteins in the

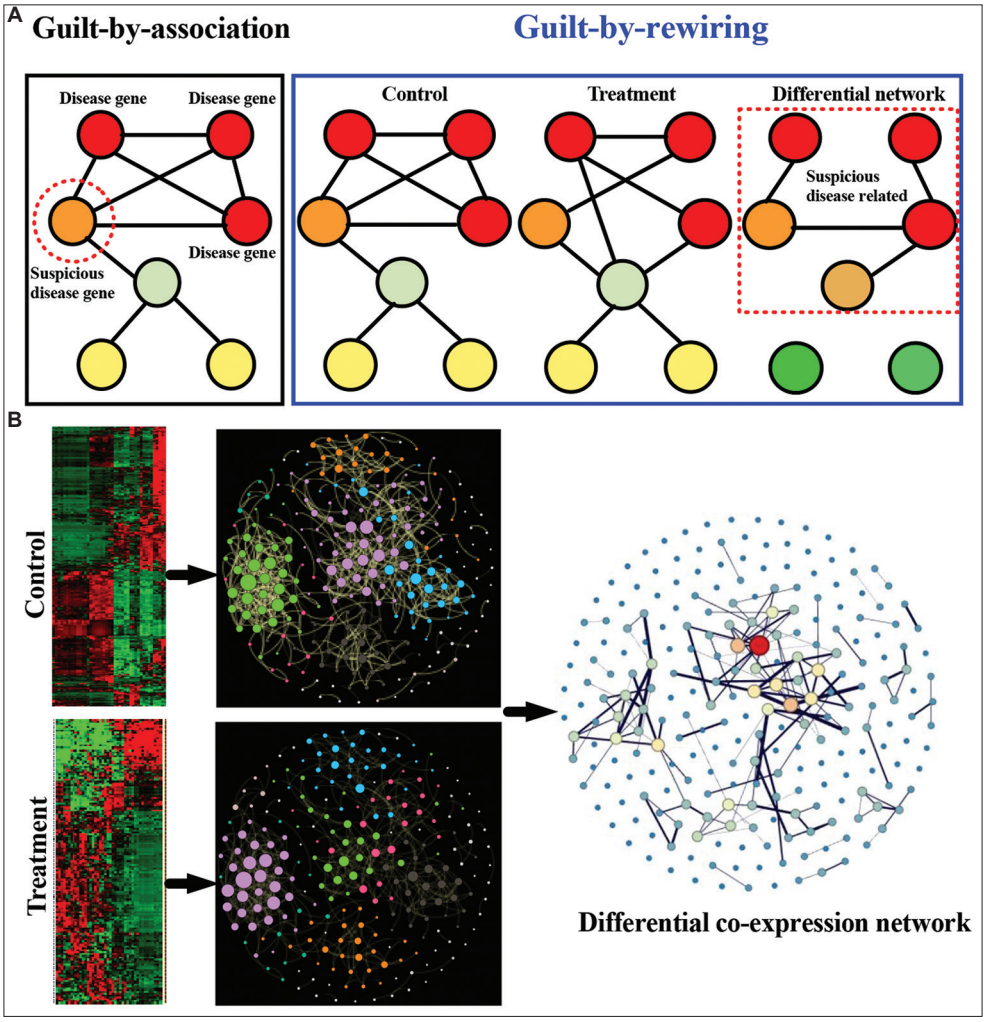

Figure 3. Schematic diagrams illustrating the principles of guilt-by-association and guilt-by-rewiring in network biology. (A) According to the guilt-by-association principle, genes that are connected with disease genes are suspicious disease genes, but according to the guilt-by-rewiring principle, genes that altered their interactions after treatment are deemed suspicious to disease. (B) Differential co-expression network uses the guilt-by-rewiring principle, which reflects the differences in the co-expression networks between control and treatment. Nodes with high degrees in the differential co-expression network are related to the phenotypic differences between treated and control samples.

considered biological systems. In fact, many fundamental problems in biological systems can be reduced to the identification of informative genes/proteins; for instance, drug targets are informative nodes in network medicine, and genes/proteins that control certain phenotypes are important nodes in biological systems. Traditional methods in molecular biology mainly rely on experiments to determine the functional roles of a gene/protein, whereas network biology provides a more cost-effective approach to predicting functionally important genes/proteins.

Based on the guilt-by-association principle, and given a biological network, researchers have developed various centrality measures, such as degree, betweenness, closeness, semi-local centrality, k-shell, h-index[116], eigenvector centrality, and motif centralities[47,117], to evaluate the importance of genes/proteins in a complex biological network. In 2001, Jeong *et al.* proposed the centrality-lethality rule in PPI network for *Saccharomyces cerevisiae*; they reported that the most highly connected proteins in the cell are the most important for its survival[43]. Other researchers have also discussed the possibility of discovering disease genes through topological features of human PPI network[13,44,45]. It has been found that the hereditary disease genes ascertained from OMIM in the PPI network tend to interact with other disease genes, share more common neighbors, as well as have larger degrees and quick communication with each other[44]. In 2008, based on the constructed biological networks and a well-established regression model, Wu *et al.*[45] proposed a computational framework to unravel the complex relationships between

phenotypes and genotypes. A tool called CIPHER, which integrates human PPIs, disease phenotype similarities, and known gene-phenotype associations, was developed to predict and prioritize disease genes; CIPHER has been shown to be applicable to genetically uncharacterized phenotypes and effective in genome-wide scans of disease genes. In 2016, based on BioGRID[67], HPRD[73], and literatures[118], we constructed a large-scale human PPI network and explored the topological features of essential genes, viable genes, conserved genes, disease genes, house-keeping genes, and tissue-specific genes[13]. It was found that the lethal, conserved, house-keeping, and tissue-specific genes had hallmark graphical features. With regard to degree, k-shell, eigenvector centrality, closeness, and other centrality measures, essential genes can be distinguished from viable ones with an accuracy as high as approximately 70%. Closeness, semi-local, and eigenvector centralities can distinguish house-keeping genes from tissue-specific ones with an accuracy of about 82%. Based on topological properties of disease genes in the PPI network, an improved random forest classifier has been proposed to detect disease-related genes[119].

Besides the traditional centrality measures for complex networks, some motif-based methods have also been developed. It has been reported that biological networks consist of functional building blocks, known as network motifs[5,120,121]. Theoretical and experimental perspectives have proven that network motifs have critical biological functions[5]. Koschützki *et al.* developed a motif-based centrality based on network motifs[117]. For a given motif in a biological network, they tallied the frequency of each node involved in motifs, matching the given motif, and they ranked the nodes according to their frequency. The proposed motif-based method was applied to the GRN of *Escherichia coli*, yielding interesting results about key regulators. In 2014, we also proposed a motif centrality[47]. Different from the motif-based centrality in another study[117], we considered the frequency of each node involved in the 2-node, 3-node, and 4-node motifs in a biological network and developed a novel index based on principal component analysis. The motif centrality was applied to the neural network for *Caenorhabditis elegans*, TRNs for *E. coli* and *Yeast*, the *Drosophila* developmental transcriptional network, as well as the human signal transduction network. The results revealed that the proposed motif centrality can effectively identify command interneurons and key transcriptional factors in the neural network and TRNs, respectively. By integrating various known centrality measures, and based on principal component analysis, an integrative measure to identify structurally dominant proteins in PPI networks has been proposed[82].

In addition to the above works, based on the guilt-by-rewiring principle, many other methods have been developed to explore crucial genes/proteins in biological systems, such as those based on differential co-expression analysis (Figure 3B)[49,103-111] or hidden Markov random field (HMRF) models[52]. For instance, dynamical network biomarker (DNB)[49] and single sample DNB[109] have been developed to detect critical gene/protein sets that dominant the development of complex diseases. The basic idea of DNBs is hinged on the guilt-by-rewiring principle, where genes/proteins that extensively altered their co-expression patterns between treated and control samples are closely related to the causal phenotypes (Figure 3B). By incorporating differential rewiring signals from the genome-wide association study (GWAS) and the HMRF model, Hou *et al.* developed a method to prioritize disease genes[52]. We have recently proposed a new framework to construct a gene differential co-expression network (GDCN) based on several RNA-Seq data[107]. Further basing on the topological structures of the constructed GDCN, three measures have been designed to explore important genes that are closely related to phenotypic changes between treatments and controls. The proposed GDCN-based approach, which integrates the guilt-by-association and guilt-by-rewiring principles, provides alternative tools for omics data analysis and network biology.

3.2.2. Applications of network biology

Network biology is widely applied in various scientific fields, including molecular biology, systems biology, ecology, and network medicine (Figure 4). Some of its applications are discussed in this section.

Network biology can be used to predict functions of genes/proteins. Conventionally, functions of gene/protein can be inferred by sequence alignment analysis[122] or biological experiments[123]. Two genes/proteins with high sequence similarity tend to have similar biological functions; therefore, the functions of genes/proteins in one species can be predicted through sequence alignment with genes/proteins in model organisms. Different from traditional approaches, network biology, based on guilt-by-association and guilt-by-rewiring principles, provides an

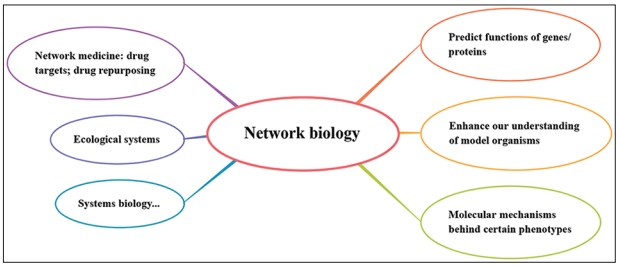

Figure 4. Applications of network biology.

alternative, cost-effective choice in predicting the functions of genes/proteins. On the one hand, based on the guilt-by-association principle, the considered genes in a biological network tend to have similar functions if the neighbored genes have certain biological functions. On the other hand, since biological networks have modularity structures, genes in the same module of a biological network tend to have similar functions[124,125]. In fact, many of the reviewed works in Section 3.2.1. follow this rule, where the identified important genes tend to have critical biological functions. For example, based on GDCN and the guilt-by-rewiring principle, our recent work predicted the functions of several unannotated genes in *Brassica napus*[107], including *BnaC03g00220D* and *BnaC05g02200D*. Other than that, several researchers have attempted to predict the function of miRNA through the identification of miRNA targets; if the targets of a given miRNA are enriched in a biological process or pathway, then it is reasonable to infer that the regulating miRNA is involved in that process[48].

Network biology enhances our understanding of many model organisms. For example, in *Saccharomyces cerevisiae*, researchers have detected pairwise genetic interactions among ~90% genes[6]. It is reported that essential genes of *Saccharomyces cerevisiae* are network hubs, displaying 5 times as many interactions as non-essential genes. The set of genetic interactions or the genetic interaction profile for a gene provides a quantitative measure of its function. A global genetic interaction network underlines the functional organization of a cell and provides a resource for predicting gene and pathway function. It is predicted that six poorly characterized genes – MTC2, MTC4, MTC6, CSF1, DLT1, and YPR153W – may function as novel functional modules that are important for the growth of *Saccharomyces cerevisiae* in high-pressure and cold environments[6].

Network biology enables the clarification of molecular mechanisms behind certain phenotypes and the control of biological systems[126-134]. On the one hand, following the identification of important genes and the prediction of their functions, KEGG pathway analysis or GO enrichment analysis can be performed to analyze the functions of gene clusters[130,135]. In particular, it is feasible to identify the genes involved in crucial pathways that dominate the related biological phenotypes[107]. There are many tools for enrichment analysis, including GSEA[135,136], DAVID[137], ClusterProfiler[138], GOEAST[139], and so on. On the other hand, based on the structural controllability theory of complex networks, the identified functional genes can be investigated from the perspective of control[126-134]. Research has revealed that about 21% of proteins in human PPI network are indispensable. These indispensable proteins are primary targets of disease-causing mutations, human viruses, and drugs[127]. There are also studies that have discussed the application of network control theory in identifying driver genes in cancer[133,134]. Interestingly, researchers have successfully predicted the functions of neurons in *C. elegans* connectome based on structural controllability[131], thus providing a direct experimental proof of the validity of these widely used control principles.

Network biology has important applications in network medicine[140-148]. On the one hand, network biology provides effective tools for identifying drug targets[130,141-144]. In 2007, Yıldırım *et al.*[29] built a bipartite graph composed of the United States Food and Drug Administration-approved drugs and proteins linked by drug-target associations; the topological analyses of the constructed network revealed that many drugs target already targeted proteins. By including drugs currently under investigation, they identified a trend towards more functionally diverse targets improving polypharmacology. The associated investigation is able to guide rational drug design. Besides that, from the perspective of structural controllability, Vinayagam *et al.*[130] reported that the structural controllability theory can be used to identify disease genes and drug targets. The copy number alterations data of 1547 cancer patients were analyzed, and they found 56 indispensable genes, which are frequently amplified or deleted in nine different cancers. However, 46 out of the 56 genes have not been previously reported to be associated with cancer. Recently, Valle *et al.*[142] developed a network medicine framework to show that the proximity of polyphenol targets and disease proteins can predict the therapeutic effects of polyphenols. The work revealed the predictive power of network biology. On the other hand, network biology has potential implications in drug combinations and drug repurposing[145-148]. It has been widely reckoned that drug development for complex diseases is seeing a shift from targeting individual proteins or genes to system-based attacks targeting dynamic network states[140,143,144]. Based on the theoretical tools developed in network biology, Cheng *et al.*[146] proposed a network-based approach to identify clinically efficacious drug combinations for specific diseases. The proposed method facilitated the identification and verification of antihypertensive combinations, offering a powerful tool to identify efficacious combination therapies in drug development[146]. Drug repurposing can effectively promote the processes in drug development. It is a cost-effective approach to preventing human diseases, especially major epidemic diseases. For drug repurposing, Cheng *et al.*[145] have identified hundreds of new drug-disease associations for over 900 FDA-approved drugs by quantifying the network proximity of disease genes and drug targets in the human PPI network. Most recently, several researchers have looked into drug repurposing for COVID-19[147,148].

Gysi *et al.*[148] developed a network framework to explore drug-repurposing opportunities for COVID-19. Among the six drugs that reduced viral infection, four drugs could be directly repurposed to treat COVID-19, thus proposing novel treatments for COVID-19.

In addition to the above discussions, there are many other research works and applications of network biology in systems biology[2-5] and ecology[150]. Systems biology investigates the composition of complex biological systems and the interactions among genes, mRNAs, proteins, and all other biomacromolecules. Hence, network biology is pivotal for systems biology. Network biology and systems biology share many common topics. Due to knowledge limitations, we will not delve in further on other applications.

4. Challenges of network biology

Although great advances have been achieved in network biology during the past few decades, there are still some challenges. First, it is still difficult to predict the causal relationships among genes for many organisms; network construction remains a problem to be further studied[37,71,102]. Second, the currently investigated biological networks are still far from the whole connectome and are always noisy; the currently investigated PPI networks for *yeast* cover approximately 90% genes of the whole genome[6]; and the considered human PPI networks encompass about 17,000 genes out of 25,000 genes[13]. There are still uncertainties whether the obtained knowledge from sub-networks still holds in the whole connectome. For example, it has been reported that the subnets of scale-free networks are in fact not scale-free[149]. Therefore, it would be interesting to further explore whether the current results about network biology still hold in the whole connectome. False-positive edges in existing biological networks may also affect the obtained results. It is also intriguing to develop effective tools to exclude false-positive edges in large-scale biological networks. Third, real-world biological systems are far more complex; they may involve different scales of interactions, including gene, RNA, protein, and metabolic levels. Most studies tend to focus only on certain levels of interactions among the same type of biomolecules. With the development of high-throughput technologies and the accumulation of various omics data, it would be interesting to perform network biology analysis by integrating multi-omics data[140-152]. Fourth, most of the investigated biology networks are static. Real-world biological networks may dynamically change their structures over time[153]. Moreover, some genes may be only expressed in specific tissues and at some given time points; biological networks may display temporal, dynamical, and modularity features[17,79,154,155]. The time-varying and modularity features of biological networks are in need of the development of the multilayer network theory[156-162]. In fact, multilayer biological networks have been extensively researched on in recent years[134,158-162]. However, publicly available multilayer biological network datasets are still lacking. With the accumulation of various omics data and the development of network inference techniques, it is possibly to develop efficient data-driven frameworks to construct multilayer biological networks. Finally, the cooperation of scientists from different fields is essential for the applications of network biology. Network biology provides effective tools for exploring bioinformatics, and both, biologists and medical workers are prerequisites for the applications of the theoretical results.

5. Conclusion

This paper reviews some of the recent progresses and challenges of network biology. We discuss two aspects of network biology, biological network construction, and their applications and summarize four approaches to constructing biological networks: Online database, artificial algorithms, topological identification based on dynamical network theory, and data-driven approaches. Among the four approaches, the data-driven approach is currently a research focus in network biology, which has wide applications in biological systems. We also briefly introduce their applications in identifying important genes/proteins, predicting the functions of genes/proteins, exploring the molecular mechanisms behind complex phenotypes and biological network control, identifying drug targets, and exploring drug combinations and drug repurposing. We declare that most of the applications of network biology rely on the guilt-by-association and guilt-by-rewiring principles. Some challenges of network biology are also discussed in this paper, reflecting our future research direction. Network biology is a promising interdisciplinary field, which will undoubtedly provide important clues for understanding complex phenotypes in biological systems.

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Conflict of interest

The author declares no conflict of interest.

Author contributions

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

REVIEW ARTICLE

A review of current diagnostic and treatment methods for key aldosterone-related diseases

Sujuan Wang[1,2], Qiaohui Zhao[2], and Tianyun Wang[1]*

[1]Henan International Joint Laboratory of Recombinant Pharmaceutical Protein Expression System, Xinxiang Medical University, Xinxiang 453003, Henan, China

[2]Zhengzhou Immuno Biotech Co., Ltd., Henan, China

Abstract

Aldosterone is a steroid hormone secreted from the adrenal cortex and metabolized primarily in the kidneys. It promotes sodium retention and potassium excretion. Most plasma aldosterone exists in free form, with a rapid turnover rate. The increased in aldosterone in the body may lead to various metabolic diseases, such as primary aldosteronism, diabetes, and chronic kidney disease. The clinical detection methods for aldosterone include radioimmunoassay, chemiluminescence immunoassay, and liquid chromatography tandem mass spectrometry (LC-MS/MS). In addition to addressing the issue of false negatives and false positives from cross-reaction in immunoassays, the advantages of high-throughput detection are reflected through the use of LC-MS/MS. Furthermore, there is also new progress in the development of a related mineralocorticoid receptor (MR) antagonist, from spironolactone to eplerenone, and to a third-generation MR antagonist, and finerenone, which has been approved by the United States Food and Drug Administration in 2021. The side effects of spironolactone and eplerenone can be overcome by finerenone, and the third-generation antagonist has shown significant effect in the treatment of chronic kidney disease associated with Type 2 diabetes. In this paper, aldosterone-related diseases, the clinical detection methods, and the corresponding treatment methods are discussed.

Keywords: Aldosterone; Primary aldosteronism; Liquid chromatography tandem mass spectrometry; Mineralocorticoid receptor antagonist

***Corresponding author:**
Tianyun Wang
(wtianyuncn@126.com)

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1. Introduction

Aldosterone (ALD), which was first discovered in the 1950s, is a mineralocorticoid secreted by the adrenal cortex in the face of physiological stimuli, such as angiotensin II. It can increase potassium ion (K^+) levels and decrease sodium ion (Na^+) levels in the plasma[1]. In human kidneys, aldosterone is metabolized to dihydro and tetrahydro derivatives, and subsequently glucuronidated to form aldosterone 18β-glucuronide, which plays an important role in daily aldosterone clearance[2]. Aldosterone not only regulates sodium-potassium balance in the body, but also blood pressure in the extracellular space as well as in secondary hypertension, atherosclerosis, diabetes, cardiovascular diseases, and other diseases. Aldosterone has an independent pro-inflammatory effect, leading to varying degrees of damage to target organs, such as

the heart, brain, and kidney, through inflammatory responses[3]. It is activated in a mineralocorticoid receptor (MR)-dependent and -independent manner through both, genomic and non-genomic pathways, but its effects are mediated primarily through the MR. On binding to MR, the aldosterone-MR complex is translocated to the nucleus to regulate gene expression (genomic pathways)[4]. Non-genomic effects, which may be mediated by MR or other receptors (e.g., G protein-coupled estrogen receptor I or angiotensin receptor Type I), are exerted within minutes, independent of transcription or translation. Whether through aldosterone itself or other receptors, aldosterone can affect vascular smooth muscle cells and endothelial function without involving MR[5].

Therefore, the early detection and regulation of aldosterone levels are crucial for the prevention of chronic metabolic diseases, such as hypertension, diabetes, and atherosclerosis[6]. Considering the importance of early detection of aldosterone levels, this review focuses on aldosterone-related diseases, the clinical detection methods, and the corresponding treatment methods.

2. Relationship between aldosterone and diseases

2.1. Relationship between aldosterone and hypertension

Hypertension is a chronic disease that is prevalent worldwide. There are about 245 million hypertensive patients in China, and the prevalence is still rising. Hypertension can be divided into primary hypertension and secondary hypertension. The causes of primary hypertension are unknown, and there is no cure for it. Secondary hypertension is a condition that occurs during the onset of certain diseases and resolves when the primary disease is cured[7]. Among them, primary aldosteronism (PA), which is related to abnormal aldosterone secretion, is one of the common causes of secondary hypertension. Its clinical manifestations include hypertension, hypokalemia, and decreased renin secretion.

The synthesis of physiological aldosterone is mainly regulated by potassium ions and the renin-angiotensin-aldosterone system (RAAS); it also has an acute response to adrenocorticotropic hormone[8]. RAAS is usually activated in the context of intravascular volume depletion or decreased renal artery perfusion. It increases intravascular volume by promoting sodium and water reabsorption in distal renal tubules and collecting ducts. The hyperactivation of the renal MR by aldosterone leads to intravascular volume expansion, which increases the risk of hypertension with or without hypokalemia[9]. Sodium and aldosterone together, in excess, promote inflammation, fibrosis, and vascular remodeling in various target organs. Therefore, compared with essential hypertension (EH), an excess of aldosterone caused by PA is more likely to induce cardiovascular complications, including coronary artery disease, myocardial infarction, stroke, transient ischemic attack, atrial fibrillation, heart failure, and other diseases[10].

2.2. Relationship between aldosterone and diabetes

Diabetes is a hyperglycemic metabolic disease caused by insulin deficiency or insulin insensitivity. The number of diabetic patients is gradually increasing with the improvement of the population's standard of living[11]. As of 2021, statistics from the International Diabetes Federation have shown that there are about 537 million diabetic patients worldwide, of which the number of adult diabetic patients in China is about 140 million; moreover, the trend has been increasing year after year with the average age of patients decreasing. Although the specific pathogenesis of diabetes is still unclear, islet B cell damage has become a recognized pathogenesis and basis for the disease. According to several reports, excess aldosterone may easily lead to decreased responsiveness of peripheral target organs such as liver and skeletal muscle to insulin. In addition, increased aldosterone levels not only affect the function and survival of islet B cells, but also lead to oxidative stress in islet B cells, which may, in turn, result in functional impairment and apoptosis of islet B cells[12,13]. There are many reasons for islet B cell damage: High glucose and lipids, inflammatory factors, and common mineralocorticoids and glucocorticoids, among which aldosterone is an indispensable mineralocorticoid, involved in physiological regulation and the regulatory system of RAAS[14]. On RAAS activation, the level of aldosterone increases, which leads to metabolic diseases, such as diabetes, hypertension, and obesity. According to epidemiological data, there is a gradual increase in plasma aldosterone level in diabetic patients[12].

In a study of safflower ginger leaf aqueous extracts in diabetes mellitus, streptozotocin-induced mice were divided into a control group (treated with distilled water), HC group (*Hedychium coronarium* and ginger lily extract), and SO group (ginger lily, a supplement that aids blood sugar regulation and contains two substances: Red yeast rice [RYR, 15%] and HC [7.2%], called SugarOut). After dissolving the two test substances in distilled water, the mice were gasserized in accordance with the dosage standards in FDA guideline. Fasting glucose and oral glucose tolerance tests as well as aldosterone and insulin tests were performed. The results showed that in the detection of fasting blood glucose and glucose tolerance, the rate of weight gain of mice after gastric HC and SO was slower than that of the

control group, and the fasting blood glucose and glucose tolerance of the mice in the HC group were significantly reduced. Furthermore, the insulin levels of the mice in both the HC and SO groups were significantly higher than those in the control group. Significant reductions in aldosterone were also found in both the HC group and the SO group compared to the control group. The increase in aldosterone may lead to impaired glucose tolerance, decreased islet ß-cell function, decreased insulin sensitivity to tissues, and thus increase the risk of diabetes. The study also confirmed that aldosterone levels are closely related to the action of insulin and RAAS plays a key role in diabetes[15].

2.3. Relationship between aldosterone and atherosclerosis

PA increases the risk of cardiovascular disease (CVD) and independent of blood pressure. Animal models have suggested that aldosterone accelerates atherosclerosis through pro-inflammatory changes in innate immune cells[16]. According to the study, patients with PA are more likely to develop atherosclerotic cardiovascular events compared to essential hypertension. This suggests that chronic exposure to high aldosterone levels, which is increasingly prevalent, has adverse effects on the cardiovascular system. A growing number of clinical studies have also emphasized that excessive activation of RAAS not only leads to hypertension, but also causes atherosclerosis, congestive heart failure, and other diseases[17]. The activation of MR in endothelial cells promotes infiltration of inflammatory cells, followed by inflammation and fibrosis. The infiltrating macrophages engulf oxidized low-density lipoprotein and become foam cells, thereby promoting atherosclerosis[9].

2.4. Relationship between aldosterone and chronic kidney disease

Chronic kidney disease (CKD) is also a significant public health concern. Based on evidence, the excessive activation of RAAS is one of the key pathogeneses in its complex pathophysiology. RAAS is distributed throughout the blood vessel walls, heart, kidneys, and other organs of the body, but the kidneys, which secrete renin, are the initial organs of the RAAS[9]. Therefore, when RAAS is overactivated, renin secretion increases, which, in turn, stimulates angiotensin I receptors, leading to vascular endothelial cell growth, oxidative stress, and vascular inflammation. This exacerbates arterial stiffness and accelerates vascular aging. The increased angiotensin II and aldosterone levels may promote cardiomyocyte inflammation, fibrosis, and hypertrophy, leading to cardiovascular remodeling and dysfunction[17].

2.5. Relationship between aldosterone and obesity

It is still unclear whether elevated aldosterone levels in the body lead to obesity or whether obesity leads to elevated aldosterone levels. Studies have yet to determine which factor is the cause and which is the effect. In a study, obese patients who had excess aldosterone before losing weight continued to have lower plasma aldosterone levels after losing weight[18]. There are also studies that have investigated the correlation between fat tissue and aldosterone in obese men and women with normal blood pressure. The findings revealed that only the aldosterone levels in women were significantly positively correlated with adipose tissue and body mass index (BMI) independently of renin. The study raised a possibility for investigating the sex-specific effect of adipose tissue on aldosterone[19].

In obese patients, the level of leptin is significantly increased. Leptin can increase the aldosterone level through RAAS and adrenal spherical cells[20]. Studies have shown that leptin stimulates sympathetic activity by releasing renin through β-adrenaline receptors and the sympathetic nervous system activates RAAS by elevating angiotensinogen in nerve tissue[21]. Renin can also act with adrenal globular band cells to enhance the expression of CYP11B2 (cytochrome P450, subfamily XIB, and polypeptide 2 gene) by relying on the signaling pathway of calcineurin, thereby promoting elevated aldosterone levels[22]. In addition, since aldosterone impairs the insulin signaling pathway only at concentrations that may activate glucocorticoid receptors, elevated aldosterone levels may also reduce insulin sensitivity in fat cells. However, the direct relationship between the two is still being studied[23,24].

3. Diagnostic methods for aldosterone-related diseases

Aldosterone is a steroid hormone. Since the levels of steroid hormones in the body are extremely low, the general detection level is in nmol/L or pmol/L, which increases the difficulty of clinical diagnostic testing. At present, PA is considered one of the most common causes of hypertension. Hiramatsu *et al.* first introduced the aldosterone-renin ratio (ARR) as an indicator of PA screening[25]. Although ARR is now an important screening method for the diagnosis of PA, the high false-positive rates exhibited by ARR methods are a concern. Therefore, each measurement of aldosterone and renin as well as the calculated ARR value is all extremely important[26]. According to another piece of evidence, low renin activity or concentrations are seen in the majority of hypertensive individuals, especially when renin levels are undetectably low, and aldosterone concentrations are also significantly lower than normal[27].

Therefore, to improve the accuracy of PA screening, new diagnostic methods need to be explored.

3.1. Radioimmunoassay

At present, radioimmunoassay (RIA) is used to detect plasma aldosterone concentration (PAC) and plasma renin activity (PRA). PRA is reflected by measuring the rate of conversion of angiotensinogen to angiotensin I (Ang I) per unit time. However, its downside is that it cannot directly reflect the active concentration of renin[28]. RIA detection of PAC is based on the principle of homogeneous competition, in which the content of aldosterone in samples is detected by radioiodine labeling[29]. RIA is one of the commonly used methods for clinical testing internationally. Although RIA has an ideal and mature technical process, it has several limitations: (1) Its detection of PRA is affected by the activity of renin itself and the interaction with its own substrate, which reduces the repeatability of the experimental results; additionally, its performance is poor, and the detection differences between each product are inconsistent; (2) plasma aldosterone and plasma renin activities are detected separately, and its operation process is cumbersome and time-consuming; and (3) it has certain radioactivity, with higher requirements for the experimental environment and experimental operators[27].

3.2. Chemiluminescence immunoassay (CLIA)

Although RIA is commonly used in China, the sensitivity of renin detection cannot be overlooked. Hence, the novel active/direct renin concentration (ARC/DRC) measurement method has become a new trend. CLIA is an emerging method that enables automated simultaneous measurement of renin and aldosterone concentrations[30]. CLIA is a method often used in biochemical immunoassays, usually by means of using chemiluminescence reagents that label antigens or antibodies and react with antibodies or antigens coated on the sample/magnetic particles. The antigen or antibody, which is bound or separated, will be labeled with the luminescent agent in its free state to evaluate the content of the target quantitatively or qualitatively in the sample to be tested[27-29]. CLIA can directly measure plasma renin concentration. Studies have successively proven that the effect of using this method for screening PA is not only equivalent to that of the RIA method, but that it can also overcome the shortcomings of RIA. Therefore, in 2008, this method was included in the European Endocrine Society Clinical Practice Guidelines. However, since most of the fully automatic equipment used in China is imported, it requires imported reagents. Considering the cost and the equipment itself, this method has yet to be widely employed in clinical practice in China[24].

3.3. Liquid chromatography tandem mass spectrometry

RIA and CLIA are two commonly used methods based on immune technology in China. However, they are prone to cross-reactions with other steroid hormones or metabolites, resulting in false positives or false negatives[31,32]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a detection method that combines chromatographic separation and mass spectrometry analysis. LC-MS/MS is predominantly used for small molecule detection and newborn screening, prenatal care, *etc*. Having several advantages, including high sensitivity, high specificity, and high throughput, LC-MS/MS is taking the lead in the detection field[33]. However, this technology uses liquid-liquid extraction, which takes a long time for chromatographic analysis and requires a substantial amount of samples to address the issue of sensitivity. In addition to its high requirements for the laboratory environment and operators, its equipment cost is also high. Therefore, it has not been widely used in local clinical practice. In the screening of PA, although the RIA method has several disadvantages, it is still one of the commonly used methods due to its mature technology and high consistency with CLIA results. LC-MS/MS, as a novel approach in the detection field, has not gained sufficient market recognition in China. Therefore, hospitals and laboratories have not conducted enough research to collect clinical data, thus making it impossible to establish a definitive correlation with RIA and CLIA in the detection of ARR and other methods[31]. While promoting LC-MS/MS, it would be beneficial to compare the detection performance of immunoassay and mass spectrometry through experimental data, determine the correlation and difference, as well as identify the best entry point between them, so as to improve the diagnostic accuracy[34].

4. Drugs related to the treatment of aldosterone-related diseases

Aldosterone is a mineralocorticoid secreted by the adrenal cortex, and it is the last active substance of the RAAS. Aldosterone is named as such due to the oxidation of methyl by cholesterol to an aldehyde group at position C18 following a series of enzymatic reactions[2,3]. Aldosterone promotes potassium excretion and sodium reabsorption in the distal convoluted tubules and cortical collecting ducts, and it is a key hormone that regulates sodium-potassium balance and maintains homeostasis in the body. A large number of data have proven that aldosterone is related to various target organ damage diseases, which can be effectively treated by inhibiting the excessive activation of aldosterone on the MR. Studies have shown that MR

antagonists (MRAs) can competitively bind to MR with ALD, thus inhibiting the effect of ALD, and subsequently improving blood pressure[35]. At present, steroidal MRAs (spironolactone and eplerenone) are widely available, and the third-generation non-steroidal MRA, which will benefit patients with hypertension and aldosteronism, is now also in the clinical stage.

4.1. First-generation MRA: spironolactone

When sodium ion content in the body decreases or potassium ion content increases, the secretion of aldosterone will be stimulated. However, the sensitivity to potassium ion content is higher compared to sodium ions. The increase of serum potassium by 0.1 mmol/L can stimulate the secretion of aldosterone[36]. Spironolactone is the first-generation of synthetic steroid-based MRA, and its chemical structure is similar to that of aldosterone. It binds to MRA non-selectively, so that the K^+ and Na^+ exchange across the membrane of the distal convoluted tubule and collecting duct is blocked, and Na^+ excretion is increased, thus lowering blood pressure[35,37]. Other than binding to MRs, spironolactone can also bind to glucocorticoid receptors. Hence, there are certain side effects with its use, such as male impotence, sexual dysfunction, and masculinization caused by the decrease of progesterone, testosterone, and other sex hormones. However, these side effects will resolve on withdrawal[38,39].

4.2. Second-generation MRA: eplerenone

A new generation of selective aldosterone antagonist, eplerenone, was developed by Pfizer-Pharmacia under the trade name Inspra and was first marketed in the United States in 2002[38]. Eplerenone is a selective MRA with a long half-life and good tolerance. It only binds to MRs and has low affinity for receptors such as androgen and progesterone; thus, it can reduce the side effects caused by the first-generation MRA and be used in the treatment of heart failure and hypertension following myocardial infarction[35,36]. According to a study, the combined use of eplerenone and standard treatment drugs reduced the total mortality rate of the treatment group with heart failure following acute myocardial infarction by 15% ($P = 0.008$)[35].

4.3. Third-generation of MRA: finerenone

Finerenone is an oral non-steroidal MRA, discovered by Bayer in Germany. It is based on the previous two generations. Finerenone can block the adverse effects caused by excessive activation of MRs caused by aldosterone. It has shown a significant curative effect on chronic heart failure and chronic kidney disease[40,41]. Its mechanism of action involves the maintenance of sodium and potassium excretion in the epithelial cells of the distal nephron, thereby maintaining the stability of the electrolyte homeostasis. In this case, it plays a key role in maintaining a constant extracellular volume and regulating blood pressure. Studies have shown that it also has a significant effect on cardiovascular disease, but its mechanism of action needs to be further studied[42]. As a new generation of non-steroidal antagonists, its steroidal structure endows it with high selectivity and affinity for MR, allowing it to overcome the adverse effects of hyperkalemia associated with spironolactone and eplerenone (Table 1) as well as achieve maximum cardiovascular activity. The findings from animal experiments have revealed that finerenone can effectively protect the heart and kidney.

In terms of clinical research, the results of clinical Phase I trials have shown that MR antagonists are well absorbed *in vivo*. Pharmacokinetic studies have found that finerenone has an effect when its plasma concentration reaches 5–20 ng/mL. It has high oral availability, reaching about 40%, with a half-life of 15 h. Its average plasma concentration reaches a peak after 1-3 h following oral administration. In the clinical Phase II trial, 764 patients with diabetic nephropathy were observed for 60 days, and it was found that there was no difference in adverse effects between the 10 mg/day finerenone group and the placebo group compared with the control group. In the 2015 Annual Report of the European Society of Cardiology, it was reported that finerenone plays a role in improving heart failure caused by Type 2 diabetes or chronic kidney disease to a certain extent. Phase III clinical trials have shown that finerenone is safe and effective and can effectively reduce the incidence of hyperkalemia; it helps maintain a good hemodynamic effect and contributes to the prognosis of patients[39-42].

5. Summary and prospect

Aldosterone is an important steroid hormone among mineralocorticoids. It is difficult to detect aldosterone accurately because its content in blood ranges in units as low as pg/mL. CLIA addresses the limitations of radioimmunoassay and enables the simultaneous detection of renin and aldosterone concentrations; nevertheless, it is unable to solve the cross-reaction problem. In addition, reducing costs and improving the research and application of equipment and imported reagents are also major issues to be solved. The stability of the test data of the emerging liquid chromatography technology in clinical diagnosis and its correlation with RIA and CLIA should also be ascertained to determine the best entry point for the detection and diagnosis of this method.

In recent years, the number of patients with metabolic diseases caused by abnormal aldosterone

Table 1. Comparison of three generations of MRAs

Drug type	First generation	Second generation	Third generation
Representative drug name	Spironolactone	Eplerenone	Finerenone
Molecular structure	Steroidal	Steroidal	Non-steroidal
Selectivity for MR	Non-selective	Non-selective	Selective
Half-life (h)	1.4	4–6	1.7–2.8
IC50 (nM)	24	990	17.8
Usual dosage (mg/d)	25–50 (RHTN)	50–100 (RHTN)	10–20 (hypertension)
Status	Listed	Listed	Listed
Adverse reactions	Gynecomastia, irregular menstruation in women, renal insufficiency, and hyperkalemia	Renal insufficiency and hyperkalemia	Increased levels of creatine kinase and blood glucose levels, headache, dizziness

IC_{50}, drug concentration required to inhibit 50% of receptor activation; RHTN: Refractory hypertension

levels is still increasing and it is difficult to effectively treat the complications associated with these diseases. Spironolactone, as a non-selective competitive antagonist with the longest clinical application, can regulate the balance of salt and water in the body as well as improve the adverse cardiovascular effects caused by abnormal ALD levels. However, it is challenging to overcome the hormonal disorders caused by the long-term use of spironolactone, such as male-female emulsification and irregular menstruation in women. Therefore, if the aforementioned adverse reactions occur during treatment, another antagonist known as eplerenone will be used in place of spironolactone. Eplerenone is a selective antagonist that can effectively overcome the adverse reactions caused by the long-term use of spironolactone. However, it is imperative to address the adverse reactions including hyperkalemia caused by spironolactone and eplerenone. Finerenone has shown absolute advantage in lowering blood pressure and serum potassium, with some improvement in diabetes, chronic kidney disease, and other diseases. Therefore, finerenone, a third-generation antagonist that has been approved for marketing by the FDA, not only plays a role in the treatment of chronic kidney disease with Type 2 diabetes, but also has great potential in the treatment of cardiovascular disease.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization: Sujuan Wang

Writing – original draft: Sujuan Wang

Writing – review & editing: Sujuan Wang, Qiaohui Zhao, Tianyun Wang

Ethics approval and consent to participate

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Consent for publication

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REVIEW ARTICLE

Recent insights into USP7: Construct, pathophysiology, and inhibitors

Yuanming He[1], Yueya Zhong[1], Yiqian Wang[2], and Xinliang Mao[1]*

[1]Guangzhou and Guangdong Key Laboratory of Protein Modification and Degradation, School of Basic Medical Sciences and State Key Laboratory of Respiratory Diseases, Guangzhou Medical University, Panyu District, Guangzhou, 511436, China

[2]School of Biological Sciences, Guangzhou Medical University, Panyu District, Guangzhou, 511436, China

Abstract

The ubiquitin-proteasome pathway (UPP) is essential for proteostasis and cellular homeostasis. Most of the human proteins are degraded through the UPP in which proteins should be tagged with a specific polyubiquitin chain in a sequential cascade of E1 ubiquitin (Ub)-activating enzymes, namely, E2 Ub-conjugating enzymes and E3 Ub ligases. Meanwhile, the ubiquitination process can be reversed by deubiquitinating enzymes (DUBs), which protect the target proteins from ubiquitination, and so far, around 100 DUBs have been reported to present in human cells. Ubiquitin-specific protease 7 (USP7) is a member of the DUBs family, which has been reported to play crucial role in the development of human tumors and diseases; however, the molecular mechanisms of disease and malignant tumor progression mediated by USP7 has not been fully elucidated. In addition, the therapeutic potential of USP7 in cancer treatment remains to be further explored. Therefore, this review begins with a review of the structure and function of USP7, and then focuses on the development of USP7 inhibitors and their potential applications in various human diseases.

Keywords: Degradation; Ubiquitin-proteasome pathway; Deubiquitinating enzymes; Ubiquitin specific protease 7; Molecule inhibitors

***Corresponding author:**
Xinliang Mao
(xinliangmao@gzhmu.edu.cn)

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1. Introduction

Ubiquitination is an essential and important post-translational modification process for most of the proteins, through which the proteins are covalently tagged with ubiquitin (Ub) molecules. Ub molecule, which is composed of 76 amino acids, is widely expressed, and the structure of the molecules is highly conserved across different species[1]. At the consumption of ATP, Ub molecules are covalently conjugated to a certain lysine residue of the target protein, which is further catalyzed by the E1 Ub-activating enzymes (for activation), E2 Ub-conjugating enzymes (for conjugation), and E3 Ub ligases (for ligation)[2]. It has been reported that there are around 2 Ub-activating enzymes (E1s), at least 38 Ub-conjugating enzymes (E2s), and more than 600 Ub ligases (E3s) in human, and the specific combinations of these enzymes determine the specificity of the protein ubiquitination process. Interestingly, Ub molecules contain seven lysine residues, namely K6, K11, K27, K29, K33, K48, and K63, which could be conjugated to other Ub molecules, providing diversity, and specificity for the ubiquitination

process of the substrate proteins[3]. On the other hand, ubiquitination is a reversible and dynamic process, where Ub molecule chains can be cleaved away from the target proteins by certain deubiquitinating enzymes (DUBs), protecting the target protein from degradation[4]. In brief, DUBs are a type of ubiquitin-specific proteases (USPs), which can induce the hydrolysis of Ub chains from the specific substrate proteins, leading to recycling of the Ub molecules, processing of the Ub precursors, and reversing of the Ub conjugation[5,6]. In terms of biological functions, DUBs can regulate several cellular processes in humans, such as protein stabilization[7], gene transcription[7], signal transduction[8,9], cell cycle progression, protein localization[10], DNA damage response, kinase activation[11,12], and many other functions.

Further, there are around 100 DUBs in human cells, which are able to reverse the ubiquitination reaction by removing the covalently attached Ub molecules from the substrates. Based on their structure and functional characteristics, DUBs can be divided into five subfamilies: (1) USPs, the largest subfamily; (2) Ub C-terminal hydrolase; (3) ovarian tumor; (4) Machado-Josephin disease protein; and (5) Jab1/Mov34/Mpr1-Pad1N-terminal+(JAMM)[13]. USP7 is a cysteine protease, which belongs to the USP subfamily. It has been widely reported that USP7 interacts with the substrates to prevent ubiquitination and degradation of the target proteins[14]. In this review, the recent research advances about USP7 in terms of its structure, pathological processes, and development as specific inhibitors are discussed.

2. The structure of the USP7

USP7, also known as herpes virus-associated ubiquitin-specific protease (HAUSP), has been demonstrated to participate in various pathological processes and diseases, including neurological disorders, metabolic disorders, immune dysfunction, and particularly carcinogenesis. In addition, USP7 is highly expressed in different types of cancers, including breast cancer[15], medulloblastoma cancer[16], T-cell leukemia[17], ovarian cancer[18], lung cancer[19], and multiple myeloma (MM)[20]. Furthermore, USP7 is closely related to malignant progression through its deubiquitinating activity in stabilizing the oncogene proteins, such as c-Maf and MafB transcription factors[20], E3 Ub ligase human homolog of double minute 2 (HDM2) protein[21], oncoprotein c-Myc, and enhancer of zeste homolog 2 (EZH2) protein[22,23]. Interestingly, USP7 also has been shown to stabilize several tumor suppressor proteins, including p53 and phosphatase and tensin homolog (PTEN)[10,24]. Due to the diversity of its substrates, it is difficult to determine whether USP7 functions as an oncogene or as a tumor suppressor; therefore, it is essential to identify the specific substrate proteins, to understand better the roles of USP7 in specific clinical setting. Thus, the structural domains of USP7 and their effects on the USP7 activity, and the correlation between USP7 and intracellular signaling pathways, as well as the molecular mechanisms underlying the effects of USP7 on cancer and other diseases are further discussed in this article. Finally, the application of USP7-specific inhibitors and their potential applications in human diseases are included in this article as well.

2.1. The tumor necrosis factor-receptor associated factor (TRAF) domain

USP7 belongs to the UBP subfamily featured with an N-terminal TRAF-like domain[25]. The TRAF domain consists of 160–165 amino acid residues, which can mediate substrate recognition and interaction[26]. It is well-known that TRAF domain binds to both p53 and HDM2, thereby mediating their deubiquitination[26,27]. However, the deletion of the N-terminal TRAF-like domain does not prevent the deubiquitination of p53 and HDM2 by USP7, suggesting the presence of a secondary binding site in USP7 with the substrate proteins. Later, this secondary binding motif in USP7 is believed to be the ubiquitin-like (UBL) domain[27], indicating that there are different binding modes between USP7 and the substrates.

2.2. The UBL domain

In addition to the TRAF domain, UBL domain is another common structural domain to UBPs. Most of the proteins within the UBP subfamily have one or more UBL domains, including USP4, USP6, USP7, USP9X/Y, USP11, USP14, USP15, USP19, USP24, USP31, USP32, USP34, USP43, USP47, USP48, and USP52[28]. Of note, the UBL domain exhibits different regulatory functions. For example, the UBL domain in USP4 has dual functions; it modulates USP4 localization to the proteasome to enhance its catalytic activity, and at the same time, the UBL domain can mimic Ub molecule and competes for Ub binding, subsequently causing a reduction in the ubiquitination of the substrate[29]. Interestingly, USP7 has five UBL domains (UBL1/2/3/4/5) at the C-terminus spanning from 560 to 1050 amino acids, and they are organized in a UBL1/2-UBL3-UBL4/5 sequence. The last two of the UBL domains are found to modulate the catalytic activity of USP7, specifically, by promoting a conformational change of USP7 through interacting with the catalytic domain (CD), leading to binding and reshaping of the catalytic center[25,29,30]. In contrast, in the absence of these UBL domains, USP7 loses its catalytic activity about 100-fold[31], meanwhile the UBL4/5 domains can activate

USP7 through binding and rearranging the switching loop in the CD, leading to about 100-fold increase in USP7 enzymatic activity[30]. In addition, this process can be increased with the help of allosteric activator guanosine 5'-monophosphate synthase (GMPS)[32], where GMPS can binds to UBL1/2/3, subsequently increases the interaction between CDs and UBL4/5, thereby promoting the USP7 activity[32]. This process suggests that the UBL domains are indispensable for USP7 to perform its deubiquitinating functions.

2.3. The CD

The CD, which is essential for the cleaving process of the polyubiquitin chains, in USP7 is composed of 208–560 amino acids. It should be noted that the proteolytic activity of USP7 is highly diverse and varies depending on the specific substrate proteins[25]. The catalytic triad contains three types of amino acids, including cystine (Cys), histidine (His), and asparagine (Asn)/aspartic acid (Asp) residues[33], of which the Asn/Asp residue is important for polarization of His residue, which in return stabilizes the catalytic activity of DUBs[28]. Moreover, the triad is rearranged in the presence of a covalently bound Ub aldehyde to generate a catalytically competent state of the CD[34]. It is important to note that, in the absence of the UBL domains, the CD will lose almost all of its activity.

3. USP7 regulates diverse types of ubiquitination

DUBs can hydrolyze the polyubiquitin chain of target proteins, thereby protecting them from degradation or modulating their function or cellular localization, depending on the types of ubiquitination process[35]. As discussed above, ubiquitination is a process that covalently attaches a Ub molecule to the specific lysine (K) residue on a substrate protein. In a Ub molecule, there is a highly conserved region, which is composed of the seven lysine residues, namely, K6, K11, K27, K29, K23, K48, and K63, which may lead to the diverse forms of Ub modification. Based on the Ub chain, Ub modifications could be classified into monoubiquitination (substrate protein tagged with a single Ub molecule) and polyubiquitination (substrate protein tagged with a multiple Ub molecule), and according to the Ub binding sites, ubiquitination are accordingly named K6-, K11-, K27-, K29-, K23-, K48-, and K63-linked ubiquitination[36]. Importantly, USP7 can hydrolyze almost all types of Ub through monoubiquitination as well as K48- and K63-linked polyubiquitination[10,37]. Furthermore, USP7 has been shown to markedly reduce the polyubiquitination levels of the substrate proteins, thereby exerting diverse functions[20,38,39].

3.1. Targeting monoubiquitination by USP7

In monoubiquitination, a single Ub molecule attaches to substrate proteins. PTEN protein, one of the best-known tumor suppressors which is involved in a plethora of cancer development, is a typical protein that is subject to monoubiquitination. PTEN can be ubiquitinated in various ways, including monoubiquitination, K27-, K48-, and K63- linked polyubiquitination, to modulate its subcellular localization, aggregation, stability, and functionality[40-42]. It is known that nuclear localization of PTEN is associated with monoubiquitination of PTEN at K289, which is indispensable for its nuclear import and tumor suppression[43]. In addition, USP7 hydrolyzes monoubiquitination of PTEN, which leads to PTEN nuclear exclusion and loss of phosphatase activity, eventually promoting prostate cancer tumorigenesis. Another example is mothers against decapentaplegic homolog 3 (SMAD3) protein. The biological activity of SMAD3 depends on the monoubiquitination modification. USP7 interacts with SMAD3 and subsequently deubiquitinates its monoubiquitination level, thereby repressing cancer progression in p53-deficient lung cancer. Furthermore, USP7 also deubiquitinates monoubiquitination of other proteins including forkhead box protein O4, proliferating cell nuclear antigen, histone H2B, and others[44-46].

3.2. Hydrolyzing K48-linked polyubiquitination by USP7

Compared with monoubiquitination, polyubiquitination is more common in protein modification, and there are seven types of polyubiquitination on thousands of proteins. At present, USP7 is believed to have the largest number of substrate proteins in terms of deubiquitination as shown in Table 1. It is known that intratumoral hypoxia can induce hypoxia-inducible factor 1-alpha (HIF-1α) and subsequently promote tumorigenesis, metastasis, and treatment resistance. A previous study has revealed that USP7 can remove the K48-linked polyubiquitination by physically interacts with HIF-1α, thereby increasing the protein stability and resulting in tumor cell epithelial-mesenchymal transition, metastasis, and tumor progression[47].

In addition, ADP-ribosylation factor 4 (ARF4) is a small guanine nucleotide-binding protein, which belongs to Ras superfamily of small G proteins. Previous studies have demonstrated that ARF4 is an anti-apoptotic protein, which acts as a BAX inhibitor, and its function is indispensable in glioblastoma tumorgenesis[52,115]. Interestingly, USP7 has been shown to be significantly upregulated in glioblastoma patient samples. Further study revealed that USP7 directly binds to ARF4, catalyzes the removal of the K48-linked

Table 1. List of identified USP7 substrates

Substrates	Ref	Substrates	Ref	Substrates	Ref	Substrates	Ref
AR	[48]	GATA1	[49]	NF-κB	[50]	SIRT1	[51]
ARF4	[52]	GMPS	[32]	NHE3	[53]	SOCS1	[54]
ARMC5	[55]	HDM2	[56]	NLRP3	[57]	So×9	[58]
BCR-ABL	[38]	HIF-α	[47]	N-Myc	[59]	SPRTN	[60]
Bmi1	[61]	Histone H2B	[32]	NOTCH1	[62]	SUMO	[63]
Bub3	[64]	Histone H3	[65]	Nrf1	[66]	Tip60	[67]
Cdc25A	[68]	HLTF	[69]	p53	[24]	TPP1	[70]
CDK1	[71]	ICP0	[72]	PHF8	[15]	TRAF6	[73]
Chk1	[74]	IKKγ	[75]	PLK1	[76]	TRIM27	[77]
Clapsin	[76]	IRSs	[78]	Polymerase η	[45]	UbE2E1	[79]
c-MYC	[80]	JMJD3	[81]	POT1	[82]	UHRF1	[83]
CSB	[84]	KDM6B	[85]	PPARγ	[86]	UL35	[87]
DAF16	[88]	LANA	[89]	PRC1	[90]	UVSSA	[91]
DLC1	[92]	LSD1	[93]	PTEN	[10]	vIRF1	[94]
DNMT1	[95]	MAF	[20]	Rb	[96]	vIRF4	[97]
E1B	[98]	MCL1	[99]	RECQL4	[100]	WDR5	[101]
EBNA1	[102]	MDMX	[103]	RING1B	[104]	XPC	[105]
ERα	[106]	MLL2	[101]	RIP1	[98]	YAP	[107]
EZH2	[108]	Mule/ARF-BP1	[109]	RNF168	[110]	ZNF638	[111]
FOXO4	[44]	NEK2	[112]	RUNX2	[113]	β-catenin	[114]

polyubiquitinated chain from ARF4, and promotes malignant progression of glioblastoma. In addition, *in vivo* experiments revealed that treatment of USP7 specific inhibitor, P5091, can significantly suppress the growth of tumor models through ARF4 degradation[52].

3.3. Hydrolyzing other polyubiquitination by USP7

The Maf proteins are critical factors in myelomagenesis, and it is an independent biomarker indicating poor prognosis in MM, a malignant cancer that is derived from plasma cells. In our previous work, wefound that Maf proteins, including c-Maf and MafB, are substrates of USP7, indicated by affinity-purification couple tandem mass spectrometry analysis of c-Maf and MafB interactomes[20]. In addition, USP7 can interact with Maf proteins through both TRAF and UBL domains, thereby preventing c-Maf and MafB from polyubiquitination and increasing their stability by prolonging their half-lives. Further, knockdown of USP7 or treatment with a specific small molecule inhibitor against USP7 leads to Maf protein degradation, followed by MM cell apoptosis[20].

In addition, we also identified the fusion protein BCR-ABL, the fundamental non-receptor tyrosine kinase in chronic myelogenous leukemia (CML), which acts as a substrate of USP7[38]. Cell-based experiments demonstrated that USP7 specifically binds to BCR-ABL protein, subsequently protects them from proteasome degradation, resulting in CML cell proliferation. Further, overexpression of USP7 increases the protein stabilization of BCR-ABL and induces CML cell survival; in contrast, knockdown of UPS7 decreases the protein level of BCR-ABL and its downstream target protein, phosphorylated STAT5, resulting in the inhibition of CML cell growth[38].

4. The broad substrate spectrum of USP7

The synthesis and degradation of proteins are a dynamic equilibrium process, which maintains the homeostasis of the cells. In addition, protein degradation is an essential process, which is regulated through the UPP pathway, and this process can be reversed by the DUB. USP7 is a DUB with a long list of substrate proteins (oncoproteins or tumor suppressor proteins); therefore, USP7 is known to be widely involved in the development and progression of various cancers. Abnormal activation of an oncogene or dysfunction of a tumor suppressor gene in cells may result in malignancy and induction of a series of abnormal gene expression.

TP53, also known as p53, is a protein encoded by the *TP53* gene in human and plays an important role in tumor

suppression. The tumor suppressive role of p53 protein has been widely reported, and as a transcriptional factor, p53 regulates numerous physiology processes, such as cell cycle, DNA damage repair, cell apoptosis, antitumor activity, and cell stress response[116]. In contrast, mutations and dysregulation of p53 have been reported to occur in more than 50% of all malignancies, leading to the loss of its antitumor activity[117]. The stability of p53 is regulated through the UPP and its polyubiquitination, and mediated by HDM2, an E3 Ub ligase[118]. Interestingly, HDM2 is also degraded through the UPP in an autoubiquitination pattern, at the same time p53 and HDM2 interacts with each other by forming a feedback loop[119]. Interestingly, USP7 can regulate both p53 and mouse double minute 2 homolog (MDM2). USP7 interacts with p53 through its TRAF-like domain and stabilizes the p53 protein level by decreasing its ubiquitination level, even in the presence of HDM2[24]. Meanwhile, USP7 also binds with HDM2 and inhibits its polyubiquitination[21]. Thus, these three proteins form a complex, as evidenced by their crystal structure analyses, where both HDM2 and p53 interacts with the TRAF-like domain of USP7, further, the interaction of HDM2/USP7 is closer than p53/USP7[120,121]. The previous studies have shown that partial knockdown of USP7 induces the degradation of p53, while the complete inhibition of USP7 expression leads to the stabilization of p53, and this phenomenon is related to HDM2[21,122]. Another study reported that USP7 stabilizes both p53 and HDM2 protein *in vivo*. However, it is believed that USP7 maintains HDM2 to a certain protein level, to regulate the polyubiquitination degradation of p53.

In addition, UPS7 has been shown to have other substrate proteins, including PTEN, c-Maf, MafB, trans-activator of transcription, NAD-dependent deacetylase sirtuin-1, BCR-ABL, GATA-binding factor 1 (also known as GATA1), and ring finger protein 168[10,20,21,24,38,49,51,110,123]. Many of these proteins are associated with cell cycle, DNA damage, regulation of transcription, and tumorigenesis. In addition, some of the substrate proteins have been shown to function as a mediator in certain signaling pathways, indicating the complexity of the substrate proteins function in human cells, thus, more studies are required to explore and understand its functions.

5. USP7 modulates signaling transduction

It has been reported that USP7 is involved in multiple cellular signaling pathways, such as PI3K/PTEN/AKT signaling[10,124], Wnt/β-catenin signaling[39], Hippo pathway[107], NF-κB signaling[125], type I IFN signaling[126], and DNA damage signaling[110] (Table 2). These pathways have different effects on cells, although their activities are regulated by USP7. For example, in multiple cellular systems, USP7 directly interacts with and stabilizes Axin protein by decreasing its polyubiquitination and inhibiting the Wnt/β-catenin pathway thereby suppressing Wnt-induced osteoblast differentiation[39]. In addition, Hippo pathway is essential to the organ development, and its deregulation has been identified in a wide variety of tumors. Further, a transcription coactivator Yorkie has been demonstrated to be stabilized by USP7 in hepatocellular carcinoma (HCC) cells; interestingly, this protein is the upstream of the Hippo signaling[107]. Taken together, USP7, which regulates Hippo pathway, could bea potential therapeutic target for HCC. In addition, the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) signal transduction pathway has been described previously to participate in the development and dysfunction of the immune system. Most of the proteins in this pathway are known to regulate biological processes, such as innate and adaptive immunity, inflammation, and stress responses. USP7 stabilizes NF-κB signaling and increases its transcription, leading to the expression of downstream genes in response to the immune system activation[127].

Table 2. Signaling pathway regulated by USP7

Signaling pathway	Ref	Signaling pathway	Ref
p300-p53/p21 pathway	[128]	BCR-ABL signaling	[38]
EZH2-CCF-cGAS signaling	[129]	MDM2/MDMX-p53 pathway	[130]
FBP1-DNMT1 pathway	[131]	NEDD4L-SMAD pathway	[132]
Cell cycle and EMT pathway	[133]	JMJD3/CLU signaling	[81]
p53/TfR1 pathway	[134]	Wnt/β-catenin signaling	[39]
PI3K/Akt pathway	[124]	Hippo pathway	[107]
Akt/ERK signaling	[135]	NF-κB signaling	[136]
AMPK pathway	[124]	Shh pathway	[16]
NOX4/NLRP3 pathway	[137]	Glucose Metabolism signaling	[138]
So×9-PTHrP-PTH1R axis	[58]	Insulin/IGF-1-like signaling	[88]
HIF-1α signaling pathway	[139]	Hedgehog signaling	[140]
Shoc2-ERK1/2 pathway	[141]	p53-MDM2 pathway	[21]

6. USP7 and diseases

6.1. The roles of USP7 in viral infection and inflammation

Interferon (IFN) is a low molecular glycoprotein induced by virus, bacteria, and IFN inducers acting on cells. IFN is not toxic to the cell itself, but it inhibits the replication of a variety of viruses and possesses antitumor activity[142].

In general, IFN can be divided into three types: (1) type I IFN, including IFN-α (20 subtypes) and IFN-β (1 subtype), which are produced mainly by white blood cells, fibroblasts, and virus-infected tissue cells; (2) type II IFN, including IFN-γ, and it has only one subtype, which is produced by activated T cells and NK cells; and (3) type III IFN, including IFN-γ1, IFN-γ2, and IFN-γ3. The main functions of type I IFN are inhibiting viral replication, providing resistance to parasitic infection, inhibiting cell proliferation, stimulating immune cells, eliciting antitumor activity, and regulating immunomodulatory system. Meanwhile, type II IFN possesses antiviral and anti-proliferation activities, and its main function is in immune regulation[143,144]. For example, in response to virus invasion, the body produces IFN, and the process is mediated by several factors. USP7 has been reported previously as an important negative modulator of virus-induced signaling, where USP7 interacts with the E3 Ub ligase tripartite motif 27 (TRIM27), and their interaction is further enhanced after virus invasion. In addition, it has been reported that TNF receptor-associated factor family member-associated NF-κB binding kinase (TBK)-1, which phosphorylates IFN regulatory factor (IRF)-3 and IRF-7, promotes the nuclear translocation and induces the expression of antiviral products[145,146]. Interestingly, TBK-1 is degraded through the UPP, which is mediated by TRIM27. More remarkably, USP7 decreases TBK-1 expression and inhibits IFN antiviral efficacy by stabilizing TRIM27 protein[126]. In another example, the suppressor of cytokine signaling 1 is a negative regulator of IFN-induced gene expression, which inhibits IFN antiviral efficacy by interacting with USP7 through its deubiquitinase activity[54,147,148]. In addition, the p53 protein has been identified as a positive regulator in response to virus infection[149]. Kaposoi's sarcoma-associated herpesvirus (KSHV)-encoded viral interferon regulatory factor 4 (vIRF4), is one of the KSHV protein which interacts with USP7 and suppresses its enzyme activity, resulting in p53 degradation. Further studies have identified that vIRF4 stabilizes HDM2 by inhibiting its autoubiquitination process, and destabilizes p53[97,150]. The above findings suggest the crucial role of USP7 in antiviral response; therefore, USP7 can be treated as a potential target for antiviral therapy. However, USP7 also plays an important role in homeostasis of normal cells; therefore, these factors should be considered when using USP7 inhibitors.

6.2. USP7 in diabetic foot

Diabetes mellitus (DM) is a common disease that happens worldwide. Blood vessel disorders are frequently seen in patients with DM, and it is commonly associated with diabetic foot, one of the most common complications of a diabetic patients. However, at present, there is no effective treatment for diabetic foot. USP7 expression is found to be upregulated in human umbilical vein endothelial cells (HUVECs), when advanced glycation end products are used to establish the diabetic cell model[151]. Inhibition of USP7 was shown to attenuate HUVECs cell cycle arrest and cell senescence. It has been further noted that the mechanism is regulated by USP7, which promotes cycle arrest and senescence of HUVECs cells by regulating the level of p53 polyubiquitination. The downregulation of p53 can reverse USP7-mediated HUVECs cell cycle arrest and cell senescence. Treatment of diabetic rat models with USP7 inhibitors can relieve the symptoms of diabetic foot. In conclusion, inhibition of USP7 induces p53 protein degradation and alleviates the symptoms of diabetic foot. These results showed that USP7 is a potential target for the treatment of diabetic foot ulcers.

6.3. USP7 in pulmonary hypertension

Pulmonary hypertension is a hemodynamic and pathophysiological condition in which the pulmonary artery pressure increases beyond a certain threshold. Pulmonary hypertension can be a group of independent diseases, complications, or syndromes. Pulmonary hypertension can be caused by the changes in the pulmonary artery itself, while others factors which can contribute to the development of this disease include genetic factors, drugs, toxins, and other diseases, such as congenital heart disease[152,153].

Recent work has uncovered the role of USP7 in the development of pulmonary hypertension[154]. Abnormal hyperplasia of pulmonary smooth muscle cells (PASMCs) is an important pathological feature of pulmonary hypertension, and platelet-derived growth factor (PDGF)-induced PASMCs proliferation is a critical factor in the development of pulmonary hypertension. Studies have revealed that PDGF increases the protein expression of USP7 and its downstream MDM2 and cyclinD1, resulting in the induction of PASMCs proliferation. In addition, downregulation of USP7 can attenuate PDGF-induced MDM2, cyclinD1 stabilization, and cell proliferation. On the other hand, MG132 treatment can also abolish PDGF-induced cyclinD1 elevation and cell proliferation. In short, inhibition of USP7 activity might be a potential therapeutic strategy for the treatment of pulmonary hypertension. Notably, USP7 inhibits tumor progression in p53-deficient lung cancer cells, and USP7 maintains homeostasis in normal cells. Therefore, other factors should be considered when USP7 inhibitors are used for pulmonary hypertension.

6.4. USP7 in cancers

Among the diseases, cancers are the most intensively investigated research area in terms of USP7. USP7 is highly overexpressed in most tumors, such as breast cancer, medulloblastoma, T-cell leukemia, ovarian cancer, HCC, MM, prostate cancer, and CML[15-17,20,23,38,107], suggesting the role of USP7 in tumor promotion. Relative protein expression in Figure 1 shows USP7 expression in several cancers[155].

USP7 is overexpressed in human breast cancer, and its expression is positively correlated with its substrate protein, PHF8. USP7 decreases the polyubiquitination of PHF8 and increases the expression of cyclin A2 protein, which is an essential and important protein for the development of breast cancer[15]. Estrogen receptor α (ERα) is reported to be overexpressed in about 70% breast tumors. Studies have identified the positive correlation between ERα and USP7, depending on the deubiquitinase activity of USP7 and their interactions, where USP7 silencing led to cell growth inhibition and delayed tumor growth[106]. In addition, USP7 has also been proven to be associated with poor prognosis of breast cancer, suggesting USP7 as a potential target for clinical treatment. Further, USP7 acts as a DUB of p53; therefore, the negative effects of p53 protein degradation should be considered when using USP7 inhibitors for breast cancer treatment.

Prostate cancer is a form of cancer that develops in the prostate, and is considered a slow-growing tumor. The incidence of prostate cancer is increasing by 2–3% every year, with more than 10% of death in male[156]. As a histone methylase, EZH2 plays an important role in the development of diverse tumor, including prostate cancer. Studies identified that the deubiquitinase USP7 is highly expressed in prostate cancer, and more importantly, USP7 interacts with EZH2 and increases its protein stabilization through its deubiquitinating activity. In contrast, a reduction in USP7 expression fails to rescue the degradation of EZH2 through the UPP, resulting in the inhibition of cell growth and the suppression of tumor progression[23]. Consistent with previous reports, USP7-specific inhibitors in combination with PARP inhibitor can produce a more lethal effect on prostate cancer cells, suggesting USP7 as a promising therapeutic target for prostate cancer.

HCC is one of the most common malignancies in China, and its incidence is ranked the third highest across the country[157]. Studies have identified that USP7 is frequently overexpressed in HCC tissues, and positively correlated with poor prognosis, indicating UPP's role in the development of HCC[158]. In addition, recent studies have demonstrated that USP7 promotes the proliferation, invasion, and migration of HCC cells through activating the bcl-2-like protein 4 (Bax), which subsequently

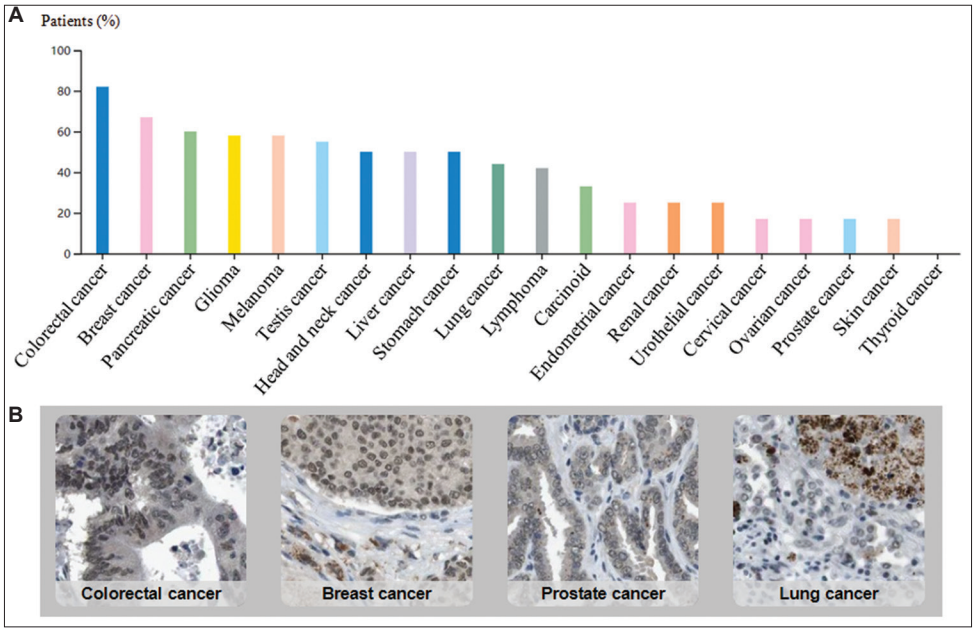


Figure 1. USP7 is highly expressed in cancer. (A) USP7 expression analysis of TCGA samples from The Human Protein Atlas database. Image reproduced from USP7 Protein Expression Summary; Malignant cells showed weak to moderate nuclear and cytoplasmic immunoreactivity (antibody CAB008108); available from https://www.proteinatlas.org/ENSG00000187555-USP7/pathology (image credit: The Human Protein Atlas). (B) Immunohistochemical staining of USP7 in representative tumors via the Human Protein Atlas (HPA) database. USP7 is highly expressed in a variety of tumors, including colorectal cancer, breast cancer, prostate cancer, and lung cancer. Image reproduced from USP7 Protein Expression; available from https://www.proteinatlas.org/ENSG00000187555-USP7/pathology (image credit: The Human Protein Atlas).

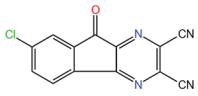
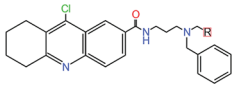
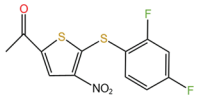
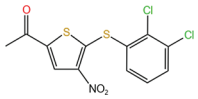
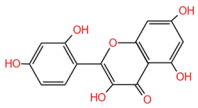
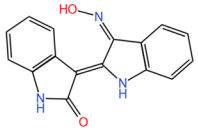
induces tumorigenesis and chemoresistance[159]. On the other hand, USP7 stabilizes thyroid hormone receptor-interacting protein 12, which subsequently induces the polyubiquitination of p14(ARF), inactivate p14(ARF), and lastly promotes HCC progression[160]. The above studies corroborate USP7 as the novel potential therapeutic target for HCC treatment.

Hematological malignancies are usually caused by uncontrolled cell proliferation. MM is a cancer of blood plasma cells with high incidence and mortality rates, and currently there is no effective treatment strategy. MafB/c-Maf are highly expressed in several myeloma cell lines, such as RPMI-8226 and LP1, and they are critical transcription factors in myelomagenesis, promoting the cell growth, proliferation, metastasis, and cell cycle progression[161]. USP7 has been shown to participate in MM progression by increasing the stabilization of MafB/c-Maf proteins. In addition, CML is a clonal malignancy of hematopoietic stem cells, featured with fusion protein kinase BCR-ABL. It has been demonstrated that deubiquitinase USP7 binds to BCR-ABL and reduces its polyubiquitination, thereby protecting it from degradation and inducing CML cell proliferation[38]. Therefore, inhibition of USP7 kinase activity could serve as a potential clinical treatment strategy for hematological disease.

7. Development of USP7-specific inhibitors

Given the diverse roles of USP7 in various diseases, USP7 has been widely believed to be an ideal therapeutic target, and many inhibitors against USP7 have been developed. The structural characteristic of USP7 has been described earlier in this article. The TRAF-like domain of USP7 is important for substrate binding. According to the role of TRAF domain in USP7, researchers have found the first inhibitor of USP7 through high-throughput screening (Table 3). Studies have revealed that HBX41108 disrupts the interaction between USP7 and HDM2, which inhibits USP7 deubiquitinating activity and fails to stabilize HDM2 protein, thereby inducing p53 accumulation and resulting in cell cycle arrest and apoptosis in tumor cells[162]. Since USP7 belongs to the Cys protease family, which are highly conserved in terms of the structural composition, such as USP5, USP8, USP10 and CYLD, it has been demonstrated that both of their activity can be inhibited by HBX41108[163,164], suggesting that the inhibition effect of HBX41108 on USP7 activity is not specific enough and further research is needed.

Table 3. The chemical structure of USP7 inhibitors, their validated targets, their effect on cell level and tumor model growth

Name	Structural formula	Validated targets	Functional consequence	*In vitro* cell suppression	Ref
HBX 41108		↓MDM2↑p53; p21	Cell cycle arrest Apotosis	Colon cancer cells	[162]
HBX 19818		↓MDM2↑p53; p21	Cell cycle arrest Apoptosis Growth inhibition	Colon cancer cells	[164]
P22077		↓MDM2; claspin; Pchk1; DDB1; TIP60↑p53; p21; Cle-Caspase-3	Growth inhibition Apotosis	Colon cancer cells; Neuroblastoma cells; Hepatocellular cells; Lung cancer cells	[67,159,165-167]
P5091		↓MDM2; MDMX; MafB; c-Maf; BCR-ABL; EZH2↑p53; p21	Cell cycle arrest Apotosis Autophagy growth inhibition	Colon cancer cells; Neuroblastoma cells; Hepatocellular cells; Glioblastoma cells; Prostate cancer cells; Ovarian cancer cells Multiple myeloma cells	[18,20,52,161,168]
Morin		↓ Prickle1, mTORC2	Cell migration inhibition	Rheumatoid arthritis cells	[169]
I3MO		↓ NEK2, PA28γ, PA200 NF-κB signaling	Apotosis DNA damage Cell cycle arrest	Multiple myeloma cells	[170]

In addition, CD plays a key role in cleaving polyubiquitin chains, especially the Cys223 residue, which is essential for the deubiquitinase activity of USP7. Using biochemical assays and activity-based protein profiling in living systems, HBX19818 and HBX28258 were found to be catalytic activity inhibitors of USP7, which can selectively inhibit the USP7 deubiquitinase activity by binding to its catalytic active site[165] (Table 3). In addition, these two compounds have a higher selectivity to USP7, and have been shown to decrease its downstream proteins, leading to cell cycle arrest and growth inhibition in several tumor cells[164].

Two new compounds, P22077 and P5091, which were discovered through a high-throughput screen, have been shown to selectively inhibit USP7 activity[161,165]. P22077 can activate p53 and its downstream protein p21 in human colon carcinoma cells. Further studies found that P22077 stabilizes p53 by inducing the degradation of HDM2[57].

P5091, the most recognized inhibitor against USP7 activity, has been identified to induce MM cell apoptosis and inhibit xenograft tumor growth. Mechanistically, P5091 leads to the decrease of HDM2, and subsequently stabilizes p53 and p21 in tumor cells by inhibiting USP7 activity. Interestingly, when MM cell line KMS11 was treated with P5091, there was no obvious increase of p53, suggesting that p53 is dispensable for the cytotoxicity of P5091[57]. It's worth pointing out that P5091 shows high specificity without affecting other protease activity, except for USP7[161]. These findings suggest that the USP7 inhibitors have potential value in clinical therapy.

Recent studies have found that Morin (3, 5, 7, 2', 4'-pentahydroxyflavone), an anti-arthritis compound that is present in foods of plant origin, suppresses the pathological migration of fibroblast-like synoviocytes, and prevents focal adhesion turnover[169]. A molecular operating environment software has been used to show that Morin could bind closely to His461, Met292, and Phe409 amino acid residues in the CD of USP7 through hydrogen bonds, thus hindering the rotation of the Phe409 side chain and subsequently inhibiting the binding of USP7 and substrate Ub. Further, USP7 protects Prickle1 from degradation, and this process can be reversed by Morin to block FLS migration.

Furthermore, indirubin-3'-monoxime (I3MO), is one of the derivatives of indirubin. The previous study has reported that the anti-MM activity of I3MO in both drug-sensitive and drug-resistant MM cells by sensitizing MM cells to bortezomib-induced apoptosis[170]. In return, I3MO suppresses the growth of MM cells via down-regulating the USP7 expression, inducing NEK2 degradation, and suppressing NF-κB signaling in MM.

In summary, exploring the potential inhibitors against USP7 can provide a novel therapeutic strategy in tumor treatment.

8. Conclusions

Ubiquitination and deubiquitination are two opposite biological processes, which work together to regulate the post-translational modification of proteins. Various biological functions of USP7 have been discovered, including its role as a vital regulator of transcription[20], nuclear export[10], inflammatory responses[57], DNA damage repair[11], antiviral responses[54], and induction of cell apoptosis[20,171]. Furthermore, USP7 is considered a cancer-promoting protein in a variety of tumors, including lung cancer, prostate cancer, HCC, as well MM. The progression of all the above diseases can be related to the substrate proteins of USP7. USP7 binds to its substrates and stabilizes their proteins or maintains their activity, thereby inducing abnormal cell proliferation and tumor development. Moreover, studies have uncovered that USP7 interacts with HDM2, which is a critical negative regulator of p53, to stabilize its protein level[24]. Contrary to the oncogenic role of USP7, several studies have found that it has dual function in cancer development. For example, in colon carcinoma, USP7 inhibits cell proliferation *in vitro* and tumor growth *in vivo* in the presence of stress due to the constitutive expression level of p53 protein[172]. Therefore, further studies are required to better understand the role of USP7 in tumors. More remarkably, in recent years, accumulating research has focused on designing small molecule inhibitors of USP7, mainly based on its physical structure or enzyme activity, to provide potential strategies for cancer treatment. However, the premise is we need to fully understand the role or mechanism of USP7 in tumor development, since USP7 stabilizes p53 and its function is unclear in the presence of stress or radiation damage[172]. The diversity of USP7 substrates determines the complexity of its function in different diseases and tumors, and the complications of diseases bring new challenges for USP7 as a target in cancer therapy. Therefore, further investigation is required to evaluate the benefits and adverse effects of inhibiting the enzymatic activity of USP7. In addition, with the emergence of new molecular tools and in-depth exploration of USP7 substrates and different strategies to modulate USP7 activity, the therapeutic potential of USP7 in diseases will be further evaluated.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Xinliang Mao
Supervision: Xinliang Mao
Writing – original draft: Yuanming He, Yueya Zhong
Writing – review & editing: Yuanming He, Yiqian Wang, Xinliang Mao

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

REVIEW ARTICLE

Exosomes: A bridge of periodontitis and systemic diseases

Jing Xu[1,2†], Xin Chang[1,2†], Huixin Zhang[1,2], Mengying Si[1,2], Huiying Su[1,2], Lilan Cao[1,2], Yingying Li[3], and Yuankun Zhai[1,2,4*]

[1]Department of Oral Biology, School of Stomatology, Henan University, Kaifeng, Henan, 475000, China

[2]Kaifeng Key Laboratory of Periodontal Tissue Engineering, Kaifeng, Henan, 475000, China

[3]Luoyang Orthopedic Hospital of Henan Province (Orthopedic Hospital of Henan Province), Zhengzhou, Henan, 450000, China

[4]Henan International Joint Laboratory for Nuclear Protein Regulation, Kaifeng, Henan, 475000, China

Abstract

Periodontitis, a common oral disease, is featured with complex etiology, progressive and prognosis varies according to the severity of periodontitis. Exosomes belong a kind of cystic vesicles with biological activity, which widely exist in human body fluids. Exosomes play an irreparable role in signal transmission and material exchange between cells, maintaining cell functions, and regulating body immunity and homeostasis. Exosomes are closely related to periodontitis, and recent study of exosomes has provided new directions and ideas for the diagnosis and treatment of periodontitis. Similarly, as extracellular vesicles, exosomes play a bridging role between periodontitis and some systemic diseases. In this process, exosomes participate in and regulate the process of systemic diseases by carrying nucleic acids, proteins, lipids, etc., and exhibit different bioactive effects according to the different substances carried in exosomes. In this paper, we summarize the latest research progress of exosomes, especially in the periodontitis and some systemic diseases, and review the potential value of exosomes in periodontitis diagnosis and treatments.

Keywords: Periodontitis; Exosomes; Periodontal regeneration; Osteoporosis; Kidney disease

[†]These authors contribute equally to this work.

***Corresponding author:**
Yuankun Zhai
(zhaiyuankun@henu.edu.cn)

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1. Introduction

Periodontitis is an infectious disease that occurs in the supporting tissues of teeth and can result in pathological loss of periodontal ligament and absorption of alveolar bone[1,2]. The occurrence and development of periodontitis are quite complex, and the pathogenic mechanisms vary from patient to patient. The pathogenesis of periodontitis is now mainly recognized as deregulated inflammatory interactions between periodontal pathogens and host immune system, involving both innate and adaptive responses, which lead to a chronic inflammation in periodontal tissues[3,4].

In recent years, the connections between periodontal diseases and systemic conditions have been explored, revealing that periodontal diseases can influence the

risk of systemic conditions, including osteoporosis[5,6], kidney diseases[7,8], Alzheimer's disease (AD)[9,10], stroke[11], and cardiovascular diseases[12,13]. The periodontitis insult and/or the associated pro-inflammatory cascade could contribute to the pathogenesis of these systemic diseases.

Chronic inflammation is driven by various mediators, of which an important part is attributed to the interactions within cytokine networks. Among pro-inflammatory cytokines, interleukin (IL)-1α, IL-1β, tumor necrosis factor alpha (TNF-α), IL-6 and IL-17 accelerate acute and chronic inflammation, and tissue injuries; however, other cytokines have antagonist effects which can restrain inflammation reactions, such as IL-10[14].

Extracellular vesicles are membranous vesicles that can naturally be released by most cells, which are induced by differentiation, activation, senescence, transformation, etc.[15] Extracellular vesicles can be divided into exosomes and ectosomes categories based on their sizes. The former is 50–150 nm and the latter is 100–500 nm, and they also have different methods of assembly, composition, and release as well as different regulation mechanisms[16,17]. Exosomes act as communication mediators between cells, and according to recent studies, they can even be used as biomarkers for diagnosis and prognosis of diseases[18-20].

Local injection of gingival mesenchymal stem cells (GMSC)-derived exosomes significantly reduced periodontal bone resorption[21]. Li *et al.* revealed that exosomal miR-207 alleviated symptoms of depression in stressed mice by targeting Tril to inhibit nuclear factor kappa B (NF-κB) signaling in astrocytes[22]. Fotuhi *et al.* compared the levels of long non-coding RNA BACE1-AS levels in plasma and plasma-derived exosomes between AD and healthy people, and found that plasma BACE1-AS level may serve as a potent blood-based biomarker for AD[23]. In this paper, we mainly review the production, composition, and biological function of exosomes, and summarize the research of exosomes in periodontitis and its bridging role between periodontitis and systemic diseases[24].

2. Pathogenesis of periodontitis

Periodontitis is a kind of chronic inflammation in periodontal tissue, mainly manifested as gingival redness and swelling, periodontal pocket abscess and tooth loosening[25]. The occurrence of periodontitis can be induced by multiple factors, including formation of dental plaque biofilm, defect in neutrophil defense, defect in phagocyte function, immune deficiency, and high level of inflammatory factors[26] (Figure 1).

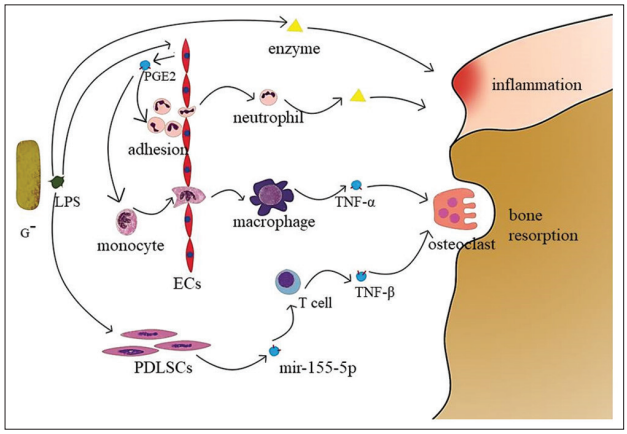

Figure 1. Pathogenesis of periodontitis. In the progression of periodontitis, pathogens can directly or indirectly cause periodontal inflammation by producing bacterial enzymes or lipopolysaccharide (LPS), respectively. LPS can stimulate epithelial cells (ECs) to produce exosomes containing prostaglandin E2 (PGE2), resulting in chemotaxis, aggregation and transformation of inflammatory cells, subsequently exacerbating inflammation. Meanwhile, LPS can also act on periodontal cells to produce exosomes containing special signals, which can activate and regulate the signal pathway of immune cells to generate new cytokines and aggravate the development of periodontal inflammation. Abbreviations in the image: ECs: epithelial cells; G⁻: Gram-negative bacteria; LPS: lipopolysaccharide; miR-155-5p: microRNA-155-5p; PDLSCs: periodontal ligament stem cell; PGE2: prostaglandin E2; TNF-α: tumor necrosis factor alpha; TNF-β: tumor necrosis factor beta.

2.1. Dental plaque is the initial factor of periodontitis

As the initial factor of periodontitis, plaque biofilm can cause tissue damage and lesions, which is not only related to the virulence and quantity of bacteria, but also the host's defense ability[27]. The pathogenic microorganisms of chronic periodontitis mainly including *Porphyromonas gingivalis*, *Prevotella nigrescens*, *Treponema denticola*, *Prevotella intermedia*, *Fusobacterium nucleatum*, etc.[28] Their pathogenicity is mainly related to lipopolysaccharide (LPS), bacterial enzymes, capsule, cilia and also extracellular vesicles. Recent studies have shown that endotoxin contents are positively correlated to clinical symptoms and alveolar bone absorption[29]. For example, the main pathogenicity of *P. gingivalis* is related to gingival protease, LPS, indole and organic acids. LPS, a component of cell wall, can directly activate host's innate immune system and induce a series of inflammatory reactions, which contribute to the damage of periodontal tissue. It has been demonstrated that the periodontal inflammatory response mediated by *P. gingivalis* is one of the dominant mechanisms of chronic periodontitis[30,31].

2.2. Different cells involved in the occurrence of periodontitis

The immune reactions and inflammatory responses induced by dental plaque plays an important role during

the occurrence and development of periodontitis. When bacteria invade in periodontal tissue, epithelial cells (ECs) can act as a physical barrier against them, and elicit innate and acquired immune responses[32]. Neutrophils will be recruited and release lysosomal enzymes to kill bacteria, but they also can damage the surrounding normal periodontal tissue when they are hyperactivated[33,34]. Macrophages can perform chemotaxis in order to phagocytize foreign substances, produce enzymes and many inflammatory factors, such as prostaglandin E2 (PGE2), IL-1β, and TNF-α. These factors play an pivotal role in stimulating the bone resorption activities[35].

When bacteria and their products invade periodontal tissues as antigens, they will induce a kind of specific immune response. Initially, macrophages in dental pulp matrix and dendritic Langerhans cells within epithelium and odontoblast layer can take up microbial antigenic materials and bring them into the lymphoid tissue to activate lymphocytes[36]. Once lymphocytes reach the damage sites, B cells will transform to antibody-producing plasma cells, which can produce corresponding antibodies to fight against bacteria[37]. The amount and activity of antibodies are considered important factors governing the protection effects against chronic periodontitis[38]. When the antigens enter periodontal tissue again, they can combine with IgG and IgM attached to mast cells; this process can neutralize toxins and assist with the phagocytosis of antigens[39]. Similarly, if the multivalent antigens binds to the prebound IgE, mast cells degranulate and release inflammatory factors, such as histamine, chemotactic factor, PGE2 and leukotriene, and trigger type I hypersensitivity[40].

In this case, T cells can be activated by antigen-presenting cells, while it can also be activated by inflammatory factors released by mast cells during the degranulation[41]. T cells might contribute to the cell-mediated immune responses by stimulating various T helper (Th) cell responses: Th1, Th2, and Th17, but their specific mechanism and timing of their role are still unclear[42]. Although all these cells have different roles to play in fighting pathogens, it is worth noting that normal immune response can kill bacteria, excessive immune response will aggravate inflammatory reaction and lead to tissue damages[43].

2.3. Inflammatory factors are the mediators between pathogens and periodontitis

As a bridge between immune cells and pathogens, inflammatory factors also play an irreplaceable role in the occurrence of periodontitis. With the onset of inflammation, the necrotic or apoptotic cells can release numerous inflammatory mediators, causing vasodilation, increasing permeability, and promoting neutrophils, macrophages and other inflammatory cells homing into damaged sites[44]. In the current paper, we introduce several common inflammatory factors. TNF-α, produced by macrophages, lymphocytes, and natural killer cells, is a cytokine with extensive biological effects, can initiate the inflammatory reactions[45]. During the pathogenesis of periodontitis, TNF-α not only promote osteoclast maturation and bone resorption, but also reduce the activity of periodontal ligament fibroblasts, thus inhibiting the differentiation of periodontal ligament fibroblasts into osteoblasts and subsequently resulting in osteoclastic absorption[46]. PGE2 also is an inflammatory factor that is closely related to the loss of periodontal attachment[47]. PGE2 has a variety of functions, such as increasing vascular permeability and chemotactic leukocyte, promoting bone resorption, and leading to pain occurrence[48]. Similarly, IL-1β also induces osteoclasts activation, mediates the synthesis of cyclooxygenase, increases the concentration of PGE2 in osteoclasts, and aggravates bone resorption[49,50]. In addition, IL-1β can also enhance the expression of matrix metalloproteinases (MMPs) and increase the degradation of periodontal extracellular matrix, therefore resulting in the destruction of periodontal tissue[51]. As a polypeptide member of the transforming growth factor-β (TGF-β) superfamily of cytokines, TGF-β1 can mediate chemotaxis of neutrophils and fibroblasts as well as promote the initiation, growth and differentiation of inflammatory cells, which has a high correlation with the occurrence of periodontitis[52].

In addition to those mentioned above, there are also many other inflammatory factors work for the development of periodontitis. It is worth to note that these different inflammatory factors are encapsulated by different extracellular vesicles to secrete and play various physiological roles.

3. Formation and physiological functions of exosomes

3.1. Biogenesis of exosomes

Extracellular vesicles mainly include ectosomes and exosomes, but they are produced via different mechanisms. Ectosomes are generated by cells and released through plasma membrane budding followed by pinching off, and then released into the extracellular space[53]. However, exosomes are generated *de novo* by cells through the endocytosis process and plasma membrane invagination. The invagination of plasma membrane is a cup-shaped structure that includes cell-surface proteins and soluble proteins, lipids, and nucleic acids, which is driven by endosomal sorting complex required for transport[54]. This leads to the formation of an early-sorting endosome (ESE),

which can directly merge with a preexisting ESE. The trans-Golgi network (TGN) and endoplasmic reticulum (ER) also can contribute to the formation and contents of ESE. ESEs can mature into late-sorting endosomes (LSEs), they can become multivesicular bodies (MVBs) due to the LSEs accumulation. This process results in MVBs containing several exosomes[55]. MVBs can fuse with autophagosomes, and the contents are degraded in the lysosomes ultimately, MVBs can also directly fuse with lysosomes for degradation. The degradation products could be recycled by the cells[56]. Besides, MVBs can be transported to plasma membrane and docked on the luminal side of plasma membrane by the help of MVB-docking proteins to release these exosomes[53,57]. Through the above process, cells can package cargoes consisting of selective lipids, proteins, and nucleic acids in exosomes, and such cargoes can be transported to recipient cells, contributing to expressional and functional changes[58] (Figure 2).

3.2. Uptake of exosomes

As a kind of extracellular vesicles, exosomes can be released from a variety of cells including fibroblasts, intestinal ECs, neurons, and adipocytes[59,60]. Exosomes tend to move through the intercellular junctions, leave their initial fluid and move to adjacent areas of tissues, and communicate with neighboring cells through cell-cell connections, which is the primary function of exosomes. Meanwhile, exosomes also enter serum, lymph and cerebrospinal fluid, breast milk, urine, saliva, and other body fluids to play different biological roles[61,62].

After released by parent cells, exosomes can navigate through extracellular fluid for varying times and distances. When exosomes reach the location of particular tissue, they are internalized by recipient cells through receptor-mediated endocytosis, pinocytosis, and phagocytosis, or through the fusion with cell membrane, which results in direct release of contents into cytoplasm[63]. The receptor-mediated endocytosis plays a critical role during the entry of exosomes into target cells. Exosomes can interact with recognized specific target cells and then establish interactions with the surface of recipient cells by binding to their receptors or other appropriate sites, which is followed by exosomes fusion with plasma membrane. After this process, exosomes can discharge the luminal cargoes into the recipient cells, resulting in the physiological change of recipient cells. After that, exosomes components can be reassembled in new vesicles that are recycled by other cells to activate effector networks[16]. Exosomes can also release contents into recipient cells by fusing with cells. Subsequently, exosomes membranes and cargoes can redistribute in the recipient cell, which can then be recycled for MVBs or plasma membrane assembly, respectively. Recycled exosomes are released to the extracellular fluid

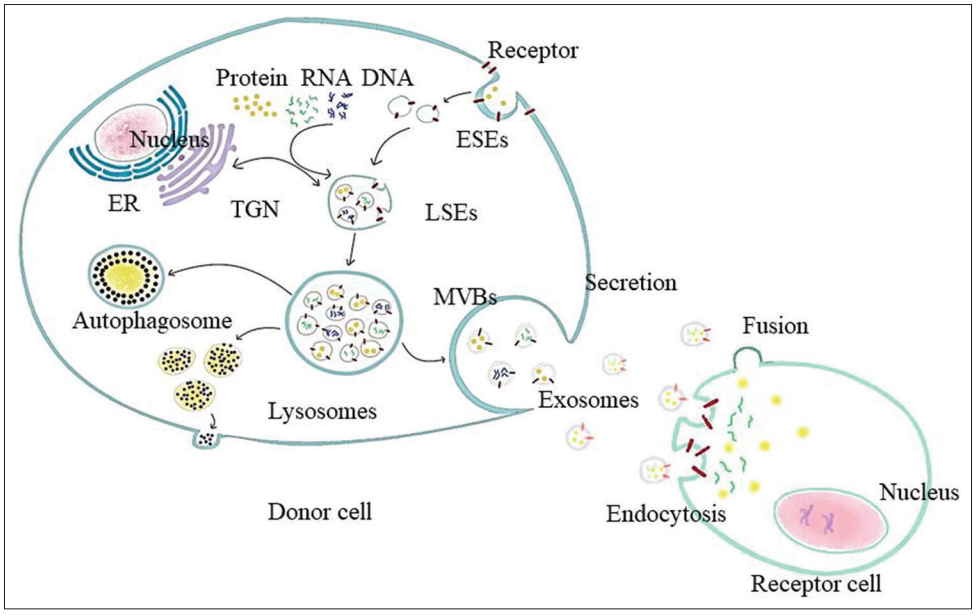

Figure 2. Biogenesis and uptake of exosomes. Exosomes are originated from the invagination of cell membranes containing special contents, which leads to the production of ESEs. Instantly, intracellular proteins and nucleic acids merged with ESEs to form LSEs, ER and TGN also can participate in this process. Eventually, LSEs mature into MVBs, which contain many newly formed exosomes and some of them will be excreted out of the body after being digested by autophagosomes or lysosomes, while others will be secreted out of donor cell as carriers. Along with the transport of body fluids, these exosomes can release contents into the recipient cells by fusion or endocytosis, causing physiological changes in the recipient cells. ESEs: Early-sorting endosomes, ER: Endoplasmic reticulum, TGN: Trans-Golgi network, LSEs: Late-sorting endosomes.

by the previous target cell, which becomes the parent cell eventually[61,64]. Another process that does not occur frequently is that exosomes directly recognize the cell surface receptors, which is not followed by exosome fusion but results in the activation of intracellular signaling[65].

3.3. Composition and main contents in exosomes

The contents of exosomes mirror the composition of donor cells; therefore, they contain various kinds of constituents due to diverse cell origin, including lipids, cytosolic and cell-surface proteins, metabolites, DNA, RNA, and so on[57]. Lipids in exosomes are not only involved in the formation of exosome, but also play an irreplaceable influence in the physiological and pathological processes of cells. As mentioned above, lipids and lipid-metabolizing enzymes are involved in the formation and release of exosomes[66]. On the one hand, exosomes are formed by lipid bilayer membrane, and released by cells after fusion of MVBs with the plasma membrane. Although the molecular mechanism of this process is still unclear, a study has already shown that glycosphingolipids can be involved in the release of exosomes[67]. On the other hand, exosomes contain lots of other lipids, including cholesterol, sphingolipid, sphingomyelin, phosphatidylserine, arachidonic acid, and other fatty acids; prostaglandins and leukotrienes in these fatty acids can be utilized as inflammation biomarkers[18].

Exosomes also include some specific proteins and non-specific proteins. Specific proteins include integrins, tetraspanins, major histocompatibility complex (MHC) Class I, II, and so on. Among these proteins, tetraspanins can assemble into functionally active membrane structures together with other transmembrane proteins[68]. Moreover, CD63 and CD81 are recognized as specific markers of exosomes[69]. A range of fusion and transferring proteins such as Rab2, Rab7, flotillin, and annexin, heat shock proteins (HSP) such as HSP70 and HSP90, cytoskeleton proteins such as actin, myosin and tubulin, and proteins that mediate MVBs formation such as Alix belong to non-specific protein types in exosomes[70]. The nucleic acids contained in exosomes include mRNA, miRNA as well as long non-coding RNA, cyclic DNA, and so on[71]. Some studies have proven that nucleic acids in exosomes are involved in many diseases, such as tumors, cardiovascular diseases, and autoimmune diseases[69,72]. MiRNAs perform negative regulation and confer characteristic changes in the expression levels of target genes[73]. Interestingly, some miRNAs were found to have novel functions when they are in exosomes. Exosomal miR-21 and miR-29a, in addition to the classic role of targeting mRNA, were first discovered to have the capacity to act as ligands that bind to toll-like receptors (TLRs) and activate immune cells[74]. Furthermore, miRNAs in the upstream of mRNA can also affect cell survival, especially changing the levels of components essential for the control of cell migration, development and metastasis[75].

3.4. Physiological and pathophysiological functions of exosomes

The function of exosomes depends on the status of original cells or tissues at the stage of exosome generation[61]. Exosomes can regulate many pathophysiological processes including immune responses, inflammation, tumor growth, and infections[71]. As mediators of cell-cell communication, exosomes play a crucial role in the maintenance of cells, homeostasis and regulation of cellular functions. Exosomes are involved in the recycling of cell surface proteins and signaling molecules to affect cell proliferation, differentiation, and apoptosis through paracrine pathways. Meanwhile, exosomes as cellular garbage bags can expel excess and nonfunctional cellular components to maintain normal cellular functions[61,64]. In the inflammatory responses, exosomal cargos, such as interferon alpha, can suppress the infection effects by limiting viral replication or enhancing antiviral immunity[18]. Similarly, exosomes can regulate the function of immune cells which mediate immune responses. Some studies have shown that exosomes derived from antigen presenting cells, such as dendritic cells, can express MHC Classes I and II molecules and costimulatory signals on the cells surface to present the peptide antigen to specific T cells directly to induce their activation which contributes to the induction of specific immune responses[76,77].

3.5. Different isolation methods of exosomes

There are different methods for separating exosomes based on their size, shape, flotation density, and labeled protein. Exosomes can be isolated by using ultracentrifugation, density gradient centrifugation, ultrafiltration, precipitation, and immunoaffinity capture from conditioned media or body fluids[78]. However, each method has its advantages and disadvantages as shown in Table 1.

4. The role of exosomes in periodontitis

Exosomes are associated with a variety of inflammatory diseases, and they can be treated as vehicles to transfer inclusions from donor cells to target cells and influence their metabolism[18]. Exosomes secreted by human periodontal ligament stem cells (PDLSCs) are a kind vesicle containing lipids, proteins, mRNAs, and non-coding RNAs, all of them are important during the intercellular communications and periodontitis occurrence[85].

While invading periodontal tissues, pathogenic factors stimulate the production of exosomes in periodontal

Table 1. Isolation of exosomes

Methods	Advantages	Disadvantages	References
Ultracentrifugation	(1) Simple operation (2) high purity	(1) Time-consuming (2) Damage or even loss of exosomes	[79,80]
Density gradient centrifugation	(1) High purity	(1) Long centrifugation time (2) High technical difficulty	[81]
Ultrafiltration	(1) Simple operation (2) Minimized damages to exosome components	(1) High cost (2) Low purity and stability of exosomes	[82]
Precipitation	(1) Simple operation (2) No requirement of professional equipment	(1) Low production	[83]
Immunoaffinity capture	(1) High purity	(1) Low production	[84]

cells, which contain a variety of inflammatory factors. When exosomes reach their destination, their contents are released into the receptor cells and then activate TLRs and subordinate signaling pathway, which activates the expression of cytokines and regulatory factors in inflammatory reactions[86]. In addition, the interaction of inflammatory cells and tissue cells can produce a series of immune responses, which subsequently lead to immune system dysregulation and occurrence of periodontitis.

4.1. The role of nucleic acids in exosomes in periodontitis

At present, studies on nucleic acids related to periodontitis in exosomes mainly focus on miRNAs. Exosomes containing miRNAs regulate cells through surface proteins in the form of ligands and receptors, and they have different effects on periodontitis according to the different type of miRNAs[87].

There is an imbalance of Th17/Treg in the peripheral blood of periodontitis patients, characterized by upregulated Th17 or downregulated Treg[88]. Zheng *et al.* found exosomes from PDLSCs in periodontal inflammatory environment induced with LPS have a regulatory effect on the inflammatory microenvironment by regulating Th17/Treg differentiation and homeostasis. They used inhibitors to knock down miR-155-5p in normal PDLSCs and found that the Th17/Treg ratio increased and then lead to an inflammatory status[89].

Among the main bacteria implicated in the pathology of periodontal disease, *Aggregatibacter actinomycetemcomitans* is well known for causing loss of periodontal attachment and systemic diseases[90]. Han *et al.* analyzed the small RNA expression profiles in activated human macrophage-like cells infected with exosomes from *A. actinomycetemcomitans* and the result provided evidence that these cells can harbor small RNAs of bacterial origin, which contribute to the production of TNF-α through TLR-8 and NF-κB signaling pathways[91]. This indicates that exosomes play an irreplaceable role in the occurrence of periodontitis caused by periodontal pathogens. In addition, Yu *et al.* showed that the detected expression levels of salivary exosomal PD-L1 mRNA were significantly higher in periodontitis patients when compared with non-periodontitis patients, and the mRNA level also has significant differences at all stages of periodontitis[92].

4.2. The role of proteins in exosomes in periodontitis

Proteins in exosomes include transmembrane proteins and embedded proteins which play an important role in the occurrence of periodontitis[93]. Tetrapeptide superfamily is a specific protein of transmembrane proteins with various biological activities, such as cell adhesion, motion, migration, growth, signal transduction, differentiation, and sperm-egg fusion[94]. Zhao *et al.* isolated and identified exosomes derived from PDLSCs, and found PDLSC-derived exosomes could express the common surface adhesion molecules CD9, CD63, CD81 and TSG101[95]. This suggests that exosomes secreted by periodontal cells have the potential to act as vesicles for inflammatory factors.

Huang *et al.* extracted salivary exosomes from 11 young patients with severe periodontitis and identified 26 proteins that were relatively specific to normal people, and found obvious immune correlations among these proteins, suggesting that exosomes may be involved in the immune response during the development of periodontitis[93]. Zhao *et al.* established an inflammatory model *in vitro* and isolated exosomes from primary human periodontal ligament fibroblasts (hPDLFs) which were treated with LPS from *P. gingivalis*. The results showed that the levels of total protein and exosome-enriched protein were higher in LPS-treated group than in controls, indicating that exosome secretion was enhanced by LPS. Moreover, they found that the expression of IL-6 and TNF-α was upregulated at both gene and protein levels after treated with LPS, which also has an influence on bone remodeling[96]. These suggest that

exosomes and their protein contents play a significant role in the pathogenesis of periodontitis.

5. Bridging role of exosomes in systemic diseases accompanied by periodontitis

Periodontal pathogenic bacteria invade periodontal tissue and cause local inflammation, and the dynamic imbalance between pathogen and host immune response contributes to the process of periodontitis[34,97]. In the acute stage of chronic periodontitis, inflammatory periodontal tissues produce many inflammatory mediators, such as IL-6, TNF-α, and PGE2, and release them into exosomes. Subsequently, these exosomes can be transported within body fluids to various organs and tissues, thereby giving rise to the corresponding response and increasing the systemic inflammatory load[98]. A growing line of evidence demonstrated that exosomes play an indispensable role in the pathological development of various systemic diseases, such as osteoporosis, kidney disease, AD, stroke, and cardiovascular disease (Figure 3).

5.1. Bone resorption is the main consequence of both osteoporosis and periodontitis

Osteoporosis is a systemic disease that weakens bones due to decreased bone density and bone mass, which mostly affects cancellous bone[99]. Periodontitis involves local inflammatory alveolar ridge loss, following an infectious breach of the alveolar cortical bone, and then may result in tooth loss[100]. Obviously, osteoporosis and periodontitis are both diseases characterized with bone resorption, and many studies also support the hypothesis that systemic osteoporosis relates to local osteoporotic changes in the loss of tooth-supporting tissues, such as alveolar bone[6].

Increasing evidence indicates that local periodontal inflammation can affect the bone remodeling process by releasing exosomes. Zhao *et al.* evaluated the role of exosomes derived from hPDLFs in the progression of periodontitis, and the results showed that inflammatory hPDLFs which were pretreated with LPS can inhibit the osteogenic activity of osteoblasts by secreting exosomes[96]. Sun *et al.* found that the expression of miR-214 from osteoclast-derived exosomes in the serum of osteoporosis patients and mice was significantly higher than that in normal people and mice, the results proved that miR-214 could affect osteoblasts and inhibit their osteogenic activity through exosomes[101]. Proteins in exosomes may also play an important role during the occurrence of osteoporosis. Huo *et al.* extracted exosomes from the serum of osteoporosis patients and found that the expression of 17 proteins, including integrin β3, integrin α2β1, talin 1, and gelsolin, was significantly changed in osteoporosis group and osteopenia group[102]. They are likely to function by working together, suggesting that these proteins in exosomes affect the process of systemic bone changes.

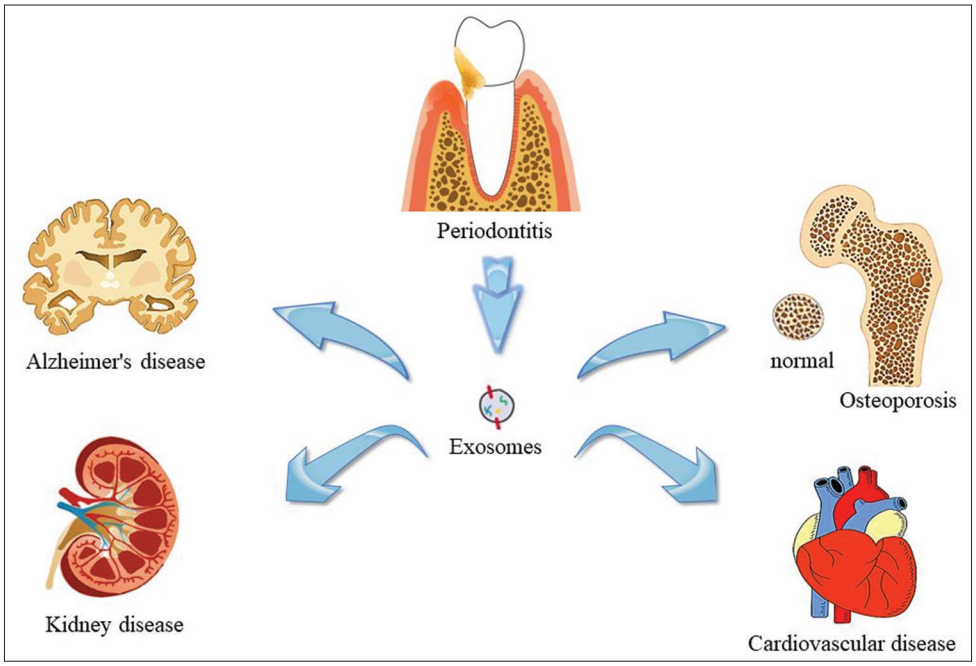

Figure 3. The bridging role of exosomes between periodontitis and systemic diseases. The occurrence of periodontitis renders the body in an inflammatory state, which can promote the secretion of exosomes by a variety of cells. Nucleic acid, protein, and lipid can be transmitted between various cells through the exosomes, which can be used as messengers to mediate multiple cells signaling pathways, and then participate in the pathogenesis of osteoporosis, renal fibrosis, AD, stroke, oral cancer, and cardiovascular disease.

5.2. Periodontitis increases the incidence of chronic kidney disease (CKD)

As a progressive pathological condition, renal fibrosis is the final outcome of a variety of CKDs[103,104]. Renal fibrosis is characterized by excessive accumulation of fibroblasts and cell matrix, which eventually leads to end-stage renal disease[105,106]. On the one hand, various pathogenic factors can stimulate the intrinsic cells of kidney and promote the proliferation of fibroblasts leading to fibrosis[105], and on the other hand, accumulated protein can increase glomerular protein filtration, which contributes to collagen deposition and accumulation, resulting in the gradual hardening of renal parenchyma, the formation of scar, and finally the complete loss of organ functions[107]. The exact pathogenesis of CKD influenced by periodontitis is currently unclear, but it has been reported that the prevalence of chronic periodontitis would lead to an increase incidence of CKD. TGF-β is not only an important cytokine that promotes the development of periodontitis[108], but also a primary factor that drives fibrosis in most forms of CKD[109,110]. Chen *et al.* found that periodontitis caused the downregulation of matrix MMP2 and upregulation of tissue inhibitors of metalloproteinases-1 and TGF-β1 at transcriptional and translational levels by comparing with normal mice; this is a possible mechanism by which periodontitis aggravates kidney damage[111]. Borges *et al.* used kidney injury as a model system and demonstrated that injured ECs produce an increased number of exosomes to activate fibroblasts, which depends on the exosomes delivering *TGF-β1* mRNA[112]. In addition, Sonoda *et al.* used the rat kidney ischemia-reperfusion injury as an acute kidney injury model, and found that miR-16, miR-24, and miR-200c in urine exosomes were increased at the injury stage, and miR-9a, miR-141, miR-200a, miR-200c, and miR-429 were upregulated in the early recovery stage, indicating that these miRNAs are related to renal fibrosis[113].

5.3. Periodontal pathogens lead to the loss of neurons and induce AD

AD is a neurodegenerative disorder characterized by gradual cognitive decline and memory loss, even intellectual loss, or death in some severe cases[114]. AD is characterized by the presence of insoluble plaques and tangles composed of Aβ and hyper-phosphorylated tau (p-tau), respectively[115]. At present, more and more evidence has shown that inflammation plays a key role in the pathophysiological process of AD, and the relationship between periodontitis and AD has also been confirmed by relevant studies[116]. Periodontal pathogens can induce the mutual activation of microglia and inflammatory factors directly or indirectly, as well as the accumulation of amyloid Aβ in brain, and eventually lead to a vicious cycle and the loss of neurons[9,117]. Ide *et al.* assessed the cognition of 60 people with mild-to-moderate AD and took blood samples for screening markers of inflammation and found that the presence of periodontitis was associated with a 6-fold increase in the rate of cognitive decline as assessed. This indicates that periodontitis is associated with worsened cognitive decline in AD, which may be mediated through effects on systemic inflammation[118].

Exosomes are one of the multiple cellular mechanisms that may link amyloid and tau secretion to both toxicity and neurofibrillary lesion spreading in AD[119,120]. In the process of the onset of AD, neurons and neuroglial cells can release exosomes into the extracellular space or transport them to the neighboring cells through blood, and then exosomes can fuse with the membrane and release contents, especially miRNAs, into the intracellular plasma to activate TLRs[121]. TLR7-9 activates the myeloid differentiation factors (MyD88) and then activates nuclear factors and transcription factors activator protein-1, leading to neuroinflammation and neuronal death[122]. Zheng *et al.* isolated exosomes from peripheral plasma and injected them into the hippocampus of an AD mouse model, and found that exosomes can diffuse throughout the brain and clustered around the Aβ plaques, which indicates that exosomes may contribute to the spread of Aβ[123].

5.4. Periodontitis promotes platelet aggregation and cerebral arteriosclerosis which lead to stroke

Stroke is a rapidly progressive ischemic or hemorrhagic encephalopathy, which can be divided into ischemic stroke (IS) and hemorrhagic stroke. Hemorrhagic stroke refers to non-traumatic cerebral parenchymal hemorrhage, also known as cerebral hemorrhage, mainly occurs in patients with hypertension and cerebral arteriosclerosis[124]. IS is mainly caused by thromboembolism of the great arteries supplying blood to brain, which leads to necrosis of brain tissue[125]. Periodontitis is associated with stroke. Sen *et al.* found that the risk of thrombotic stroke in patients with periodontitis is twice the risk in healthy individuals[126]. Periodontitis is associated with elevated levels of C-reactive protein, fibrinogen, and cytokines, and these inflammatory factors can invade vascular endothelial cells, thereby promoting platelet aggregation and atherosclerotic diseases as well as inducing formation of foam cells[127,128]. This suggests that periodontitis may cause cerebral arteriosclerosis and endothelial cell damage, which eventually lead to stroke.

A growing line of evidence demonstrated that exosomes participate in the development of stroke. Some researchers extracted circulating exosomes from the blood of patients

who have acute stroke within 72 h, and found that the expression of miR-223 was higher than that in control group[129]. Li *et al.* divided 55 patients with IS into acute phase group and subacute stage group, and the results showed that plasma exosomal mir-422a and mir-125b-2-3p levels in subacute phase group were significantly lower than those in acute phase group, and the expression level of mir-422a in acute phase group was higher than that in control group[130]. Ji *et al.* detected the alternation in serum exosome concentrations and the levels of serum exosomal miR-9 and miR-124 in 65 patients with acute IS and 66 non-stroke patients, and the results showed that the concentration of serum exosomes was elevated, and the median levels of serum exosomal miR-9 and miR-124 in acute IS patients were significantly higher than those in control group[131].

5.5. Periodontal pathogens increase morbidity and risk of atherosclerosis, which leads to cardiovascular disease

Cardiovascular disease is a common disease that seriously threatens the health of human beings, especially the elder individuals[132]. Cardiovascular disease is a multifactorial disease with complex etiology, and periodontitis may be one of the pathogenic factors. Systemic inflammatory response and vascular endothelial damage caused by periodontitis are related to the development of cardiovascular disease[133]. Eight types of bacteria were identical in both subgingival plaque and atherosclerotic plaque. Among these bacteria, *P. gingivalis* and *Tannerella forsythia* significantly increase morbidity and risk of atherosclerosis. The results strongly correlate periodontal bacterial co-occurrence and periodontal bacterial adhesion factor to atherosclerosis[134]. In addition, there is a certain relationship between periodontal disease and C-reactive protein level, and C-reactive protein may also be involved in the development of cardiovascular disease[135].

The exosomes and their miRNAs may be involved in the pathophysiological process of atherosclerosis as a medium of cell communication[136]. Wang *et al.* have demonstrated that macrophage-derived exosomes can transfer miR-155 to cardiac fibroblasts, thereby inhibiting fibroblast proliferation and enhancing inflammatory response[137]. Widera *et al.* detected the expression of miRNA in plasma exosomes from a large number of patients with acute coronary syndrome, and the results showed that the expression of miR-1, miR-133a, miR-133b, and miR-208b was upregulated[138].

6. Application prospect of exosomes in periodontitis and other systemic diseases

Specific contents are the attributes of exosomes that allow them to send signals to specific recipient cells or tissues. Therefore, exosomes can be used as promising diagnostic biomarker (Table 2) and therapeutic tool for the treatment of periodontitis and other diseases. These roles are attributed to their abilities to transfer RNA, proteins, enzymes, and lipids, thereby affecting physiological and pathological processes in various diseases[61].

Recently, many investigations have shed light on periodontitis treatments using exosomes. Modified exosomes can inhibit inflammation and promote periodontal regeneration by affecting the function of periodontal cells or immune cells. Chew *et al.* observed the effects of mesenchymal stem cells (MSCs) exosomes-loaded collagen sponges on the healing of periodontal defects in immunocompetent rat models, and found that MSC-derived exosomes can increase PDL cells migration and proliferation through CD73-mediated activation of pro-survival AKT and ERK signals to promote periodontal regeneration[164]. Wang *et al.* used osteoblasts stimulate stem cells from human exfoliated deciduous teeth (SHED) to acquired special exosomes, and demonstrated that they have a facilitating effect on the osteogenic differentiation of PDLSCs *in vitro*. Both Wnt/β-catenin and BMP/Smad signaling pathways are involved in this physiological process[98], which indicates that exosomes isolated from SHED may have a great therapeutic potential in the loss of periodontal or alveolar bone. Macrophages are involved in the development of inflammation, and different types of macrophages play various roles in different stages of inflammation. In the early stage of inflammation, M1 cells

Table 2. Exosomes as biomarkers

Diseases	Biomarkers	References
Periodontitis	miRNA-29, miR-207, miR-495, miR-376b-3p, miR-1226	[139-141]
Osteoporosis	miR-324-3p, miR-766-3p, miR-1247-5p, miR-330-5p, miR-3124-5p, miR-214	[142,143]
Kidney disease	miR-29c, miR-21, ceruloplasmin, E-cadherin, vimentin	[144-147]
Alzheimer's disease	miR-342-3p, miR-9-5p, miR-598, miR-128, miR-125b	[148-151]
Stroke	miR-9, miR-124, miR-422a, miR-125b-2-3p, miR-599, miR-451	[130,131,152,153]
Cardiovascular disease	miR-376a-3p, miR-27b, miR-30a-5p, miR-122, miR-126	[154-157]
Tumor	miR-301a, miR-320d, miR-429, miR-25-3p, miR-21, miR-148a	[158-163]

are activated by LPS and secrete pro-inflammatory factors. However, M2 cells are activated by IL-4 produced by Th2 cells, and then release some other cytokines to inhibit inflammatory response and promote tissue repair[165]. Wang *et al.* co-cultured macrophages and exosomes that were isolated from GMSC and stimulated by LPS, and found that the levels of M1 markers, including TNF-α, IL-12, CD86, and IL-1β, were significantly decreased but the level of M2 marker such as IL-10 increased moderately. GMSC-derived exosomes may promote M1 macrophage transformation into M2 macrophages, reducing the pro-inflammatory factors which are produced by M1 macrophages[166]. All of these provide us a new direction for the treatment of periodontitis. As a non-surgical periodontal treatment tool, exosomes showed superior effects on cells, with the additional advantages of safety and easy preparation. They can be applied as a suspension injected locally to the periodontal defects, without additional cost in the preparation of scaffolds[167]. Mohammed *et al.* compared the effects of exosomes and adipose-derived stem cells on the periodontitis mice, and found that the exosomes group showed the most evident proliferation in periodontal tissue with increased cellularity and significant organization, and the alveolar bone was more organized with an osteoid tissue layer[98,168].

In addition to periodontitis, the potential of using exosomes to treat other diseases has also been discovered by more and more researchers. Wei *et al.* found that exosomes from SHED can directly promote the osteogenesis, differentiation and bone formation of bone marrow MSCs. They applied SHED-exosomes to the mouse model with a bone loss, and then found that Runx2 and Smad5, the key transcription factors for osteogenic differentiation, were upregulated and bone loss was restored after injecting of SHED-exosomes[169]. Xie *et al.* reported that MSCs-derived exosomes could promote osteoblast proliferation through inhibiting cell apoptosis, and significantly increase the expression level of GLUT3, which eventually stimulates the osteogenesis differentiation of osteoblasts; this provides a theoretical basis for the clinical treatment of osteoporosis[170]. Recent studies have shown that MSC-derived exosomes therapy improves renal outcomes in several animal models of AKI and CKD, such as ureteral obstruction, ischemia-reperfusion injury, drug/toxin induced nephropathy, and renal vascular disease[171]. The exosomes derived from MSCs can regulate the expression of intercellular adhesion molecule-1 and then inhibit the infiltration of dendritic cells into the kidney. In addition, these exosomes can reduce the production of pro-inflammatory cytokines and inhibit renal fibrosis[172]. Sato *et al.* found that exosomes containing β-secretase 1 (BACE1) siRNA or curcumin reached the brain after peripheral injection and ameliorated AD-like pathology in mice[173]. In addition, exosomes have great potential in the treatment of stroke. Li *et al.* found that injection of enriched plasma exosomes from mice treated with remote ischemic preconditioning could significantly reduce the infarct size in a murine model of cerebral ischemia and improve the neurological function[174]. Inoue *et al.* found that dental pulp stem cells-derived exosomes can induce angiogenesis and neurogenesis, and then reduce the infarct volume[175]. The important value of exosomes in the diagnosis and treatment of cardiovascular diseases cannot be neglected. Preclinical studies have shown that the exosomes play a protective role in ischemic heart disease by reducing myocardial ischemia-reperfusion injury and promoting vascular regeneration[176]. Luo *et al.* used myocardial infarction rats as animal model and injected exosomes enriched with miR-126, and the results showed that the release of inflammatory factors decreased and the infarct size decreased significantly[177].

7. Conclusion

Exosomes, as functional carriers of nucleic acids, lipids, proteins, and other substances, play an important role in the occurrence and progression of periodontitis. Exosomes carrying some specific substances can promote the occurrence of periodontitis, while exosomes carrying other specific substances can play an anti-periodontitis role. This shows that the exosomes from periodontal tissue or cells are pivotal in the occurrence and recovery of periodontitis. In addition, exosomes with different contents playing a bridge role in patients with periodontitis and other systemic diseases. Exosomes can cause periodontal inflammatory mediators to spread to the whole body, thus promoting or antagonizing the occurrence of specific diseases. The current study of exosomes is helpful to explain the pathogenesis of periodontitis and how to regulate the progression of other systemic diseases in patients with periodontitis.

At present, the understanding of exosomes is still limited. Nevertheless, exosomes could serve as an important biomarker of clinical diagnosis, treatment of periodontitis, and intervention of systemic diseases in future.

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Conflict of interest

None.

Author contributions

Data curation: Jing Xu, Xin Chang, Huixin Zhang, Mengying Si, Huiying Su, Lilan Cao
Funding acquisition: Yuankun Zhai
Investigation: Mengying Si
Project administration: Yuankun Zhai
Supervision: Yuankun Zhai
Visualization: Jing Xu, Huixin Zhang
Writing – original draft: Jing Xu, Xin Chang
Writing – review and editing: Yingying Li, Yuankun Zhai

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Gene & Protein in Disease requests that every new submission should be made and accompanied by 3 separate core files, namely manuscript, title page and back matter, and cover letter, whereas resubmission of revision file should be accompanied by 4 separate core files, namely manuscript, title page and back matter, cover letter, and response/rebuttal letter (collectively known as the revision file). Provision of supplementary files and/or confidential accessory files is optional or dependent on the nature of study and findings relevance. The table below briefly summarizes the type of files in a submission, their respective requirements and included items:

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(4) Supplementary files		- Supplementary files should **not exceed 20 MB** in total (15MB individual file limit)	

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(6) Response/rebuttal letter (only applicable to revisions)	DOC or DOCX	- All comments/feedback and responses/rebuttals must be clearly and concisely presented	- Reviewers' comments and feedback - Authors' responses

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Online document with author names:

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Online document without author name:

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Thesis/Dissertation:

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Government report (online):

Department of Health and Ageing, 2008, Ageing and aged care in Australia, viewed November 10, 2008, http://www.health.gov.au/internet/main/publishing.nsf/Content/ageing

No author:

Guide to agricultural meteorological practices, 1981, 2nd ed, Secretariat of the World Meteorological Organization, Geneva, 10–20.

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Investigation: Ali Jackson, Tom Lewis-Hans, Han Xiang

Formal analysis: Han Xiang

Writing – original draft: Ali Jackson

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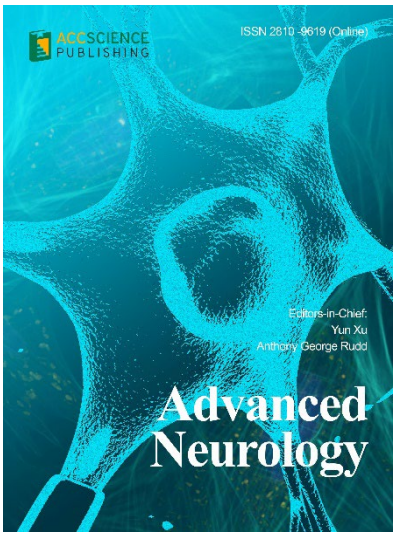

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