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RNA-sequencing of peripheral blood circular RNAs in Parkinson disease

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Abstract

Background: Circular RNAs (circRNAs) play an important role in many neurological diseases and can serve as biomarkers for these diseases. However, the information about circRNAs in Parkinson disease (PD) remained limited. In this study, we aimed to determine the circRNAs expression profile in PD patients and discuss the significance of circRNAs in the diagnosis of PD.

Methods and Results: Using RNA-sequencing in peripheral blood RNAs, we showed that a significant number of mRNAs or circRNAs were differentially expressed between PD patients and normal controls (NCs), which included 273 up-regulated and 493 down-regulated mRNAs, and 129 up-regulated and 282 down-regulated circRNAs, respectively. Functional analysis was performed using the Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway analysis, and the results showed that the second most enriched KEGG pathway was PD. These data suggest that the levels of mRNAs and circRNAs in peripheral blood could be potentially used as biomarkers for PD. In addition, we correlated mRNAs and circRNAs by constructing a competing endogenous RNA (ceRNA) network in PD. The resulted-in ceRNA network included 10 differentially expressed mRNAs from PD pathway, 13 predicted miRNAs, and 10 differentially expressed circRNAs.

Conclusion: Collectively, we first characterized the expression profiles of circRNAs and mRNAs in peripheral blood from PD patients and proposed their possible characters in the pathogenesis of PD. These results provided valuable insights into the clues underlying the pathogenesis of PD.

Abbreviations: circRNAs = circular RNAs, H–Y = Hoehn and Yahr stage, HAMA = the Hamilton Anxiety Scale, HAMD = the Hamilton Depression Scale, KEGG = the Kyoto Encyclopedia of Gene and Genomes pathway, miR-7 = miRNA-7, miRNA = microRNA, MMSE = Mini-Mental State Examination, MoCA = the Montreal Cognitive Assessment, MRC = mitochondrial respiratory chain, ncRNA= non-coding RNA, NCs = normal controls, PD = Parkinson disease, UPDRS = Unified Parkinson Disease Rating Scale.

Keywords: Chinese, circRNAs, Parkinson disease, RNA-sequencing

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FK, ZL, and LW contributed equally to this work.

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Ethics statement: The studies including human participants were reviewed and approved by Affiliated Hospital of Jining Medical University IRB (2017C008) and BGI IRB (BGI-IRB20088). The patients/participants provided their written informed consent to engage in this study.

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Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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1. Introduction

As a common progressive neurodegenerative disorder among elderly people, Parkinson disease (PD) is generally considering to be caused by an interaction of environment and genes.[1] A great deal of efforts has been made to explore the pathogenesis of PD, while the exact etiology of PD remained largely unknown so far. Moreover, the specific and meaningful biomarkers for diagnosis of PD remained limited.

Referring to the fast advances in development of high-throughput sequencing and bioinformatics technologies, circular RNAs (circRNAs) have emerged as a new species of non-coding RNAs (ncRNAs). circRNAs, have been recognized as splicing associated noise initially, are single-stranded RNA molecules produced from pre-mRNAs through a process called back splicing.[2] The discovery of circRNAs in mammalian cells may be an unexpected feature of eukaryotic gene expression and regulation.[3,4] Based on its nature of circular structure, circRNAs are particularly enriched in exosomes, which are defined with extracellular vesicles in diameter of 30 to 100 nm containing various types of signaling molecules such as proteins, lipids, and RNA.[5] To date, the functions of circRNAs have been found as follows: as microRNA (miRNA) sponge, splicing, or transcriptional regulator, and agent interacting with RNA binding proteins, influencing the physiological process of aging.

Recent studies have reported that circRNAs may take part in different disease progressions, such as Alzheimer disease[6] and tumorigenesis.[7] Furthermore, the expressions of circRNAs can be tissue-specific,[8] and some evidence supports the translation of some circRNAs.[9,10] CircRNAs can be found in plasma, cell-free saliva, and exosomes,[11,12] and have the potential to be diagnostic biomarkers or therapeutic targets upon the improved detection and characterization approaches.

A previous study has found that miRNA-7 (miR-7) is associated with PD.[13] CircRNAs can act as a miRNA sponge,[14] indicating that circRNAs may play a role in the pathogenesis of PD. In this study, we aimed to profile expression levels of circRNAs in the peripheral blood from PD patients and provide a conspectus of circRNA as biomarkers of PD.

2. Materials and methods

2.1. Patients and samples

Four PD patients, who were recruited from the Parkinsonism Clinic and Department of Neurology, the Affiliated Hospital of Jining Medical University, Jining, China, were enrolled between October 2018 and March 2019. All patients were diagnosed according to the standards of UK PD Brain Bank Criteria,[15] and those without Parkinsonian syndromes were excluded. To avoid the dropouts and prevent the influence of medicine on our experiment, we collected the peripheral blood samples and the clinical diagnoses data simultaneously before intention-to-treat analyses. Four age- and sex-matched healthy mainland subjects were enrolled from the same geographic areas (Table 1). Exclusion criteria included previous diagnosis of cardiovascular, cerebrovascular, and other systemic diseases. The peripheral blood samples from 4 PD patients and 4 normal controls (NCs) were collected for RNA-sequencing analysis.

2.2. Clinical and neuropsychological assessment

In this study, PD patients underwent a series of demographic and clinical assessment of motor function and neuropsychological investigations. Demographic and clinical assessments of motor function, including Unified Parkinson Disease Rating Scale (UPDRS) motor scores and Hoehn and Yahr (H–Y) stage, and neuropsychological investigations were carried out by trained research staff. Protocols of neuropsychological testing involved the Mini-Mental State Examination (MMSE), the Montreal Cognitive Assessment (MoCA), the Hamilton Depression Scale (HAMD), and the Hamilton Anxiety Scale (HAMA). All tests were recommended by the Movement Disorders (MDS) Task Force.[16]

2.3. Specimen storage

After an 8 to 12 hours of overnight fasting, peripheral blood samples (4 mL) were acquired from each participant. The specimens were gathered in PAXgene Blood RNA Tube (2.5 mL blood and 6.9 mL buffer; BD Company). After each collection, the PAXgene Blood RNA Tubes were left at room temperature for at least 2 hours, transferred into a 4 °C refrigerator, then store at –20 °C for 24 hours and then eventually transferred to a –80 °C refrigerator until RNA extraction.

2.4. RNA extraction and whole transcriptome library construction

The frozen blood samples were returned to room temperature, and total RNAs were extracted according to the instructions of

Table 1

Demographic data of patients with PD and NC.

Case No.	PD1	PD2	PD3	PD4	NC1	NC2	NC3	NC4
Gender	Female	Female	Male	Male	Female	Female	Female	Female
Age, y	63	54	44	69	50	45	62	48
Age of illness,y	2	3	2	2	0	0	0	0
H-Y	2	2	3	2	–	–	–	–
UPDRS-I	6	6	9	2	–	–	–	–
UPDRS-II	6	9	3	0	–	–	–	–
UPDRS-III	40	39	41	18	–	–	–	–
HAMD	6	9	32	4	–	–	–	–
HAMA	4	6	15	8	–	–	–	–
MMSE	11	23	25	28	–	–	–	–

HAMA = Hamilton Anxiety Scale, HAMD = Hamilton Depression Scale, H–Y = Hoehn—Yahr, MMSE = Mini-Mental State Examination, NC = normal control, PD = Parkinson disease, UPDRS = Unified Parkinson Disease Rating Scale.

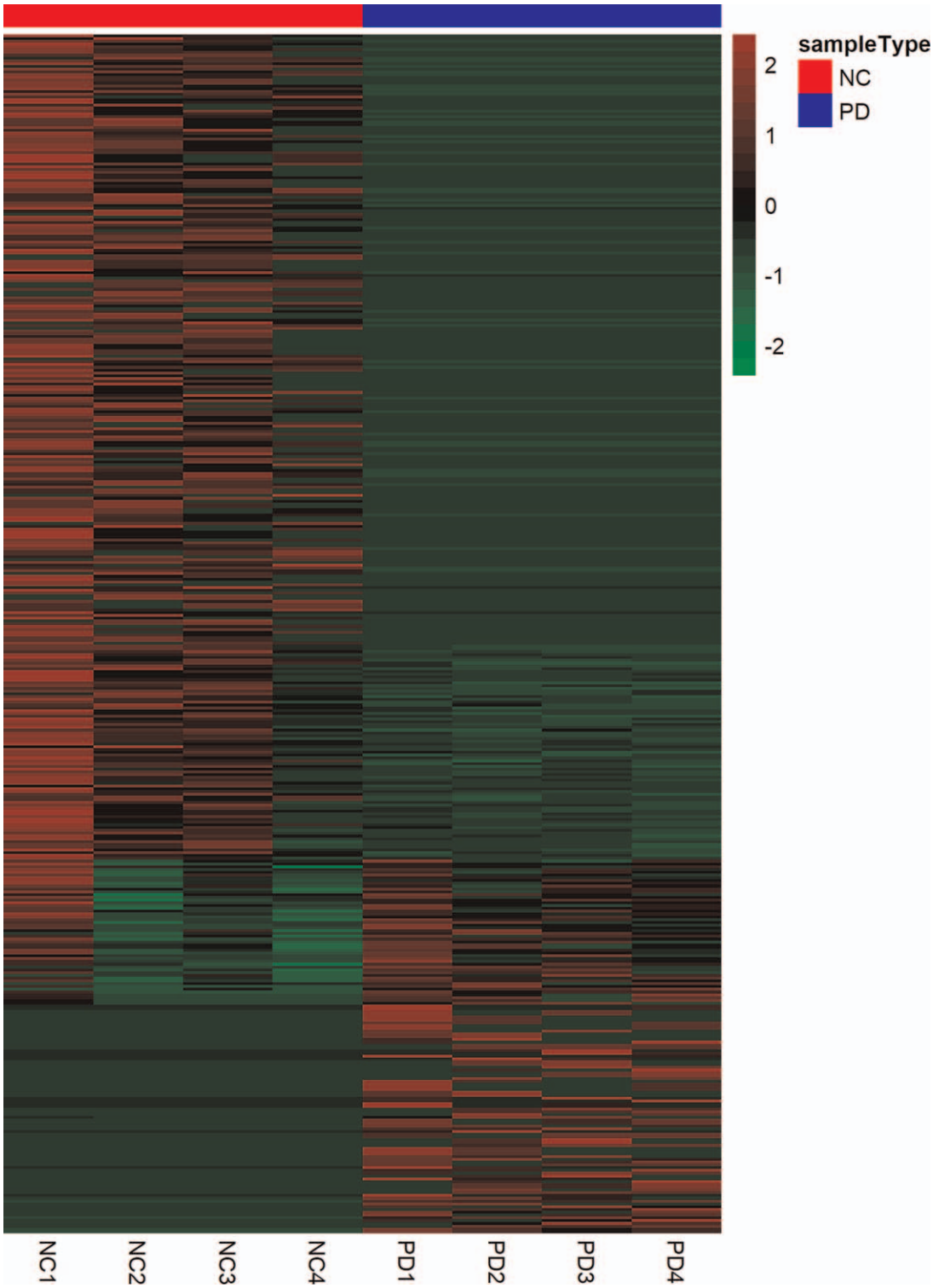

Figure 1. The heatmap of circRNAs from PD patients and NCs. Red means strong intensity, black means medium intensity, and Green means low intensity. circRNAs = circular RNAs, NCs = normal controls, PD = Parkinson disease.

the PAXgene Blood RNA Kit Handbook (QIAGEN, Germany). The RNA concentration was determined using the Nanodrop spectrophotometer (ThermoFisher Scientific, USA). Total RNAs were used to deplete rRNAs by Ribo-Zero Gold (Illumina, USA) to be ready for whole transcriptome libraries and sequencing. NEBNext Ultra RNA Library Prep Kit (NEB, USA) was used for constructing whole transcriptome libraries. BioAnalyzer 2100 system was used for quality control, and qPCR (Bio-RAD, USA) was used for quantifying of the whole transcriptome libraries. Total RNA extraction and whole transcriptome library con-

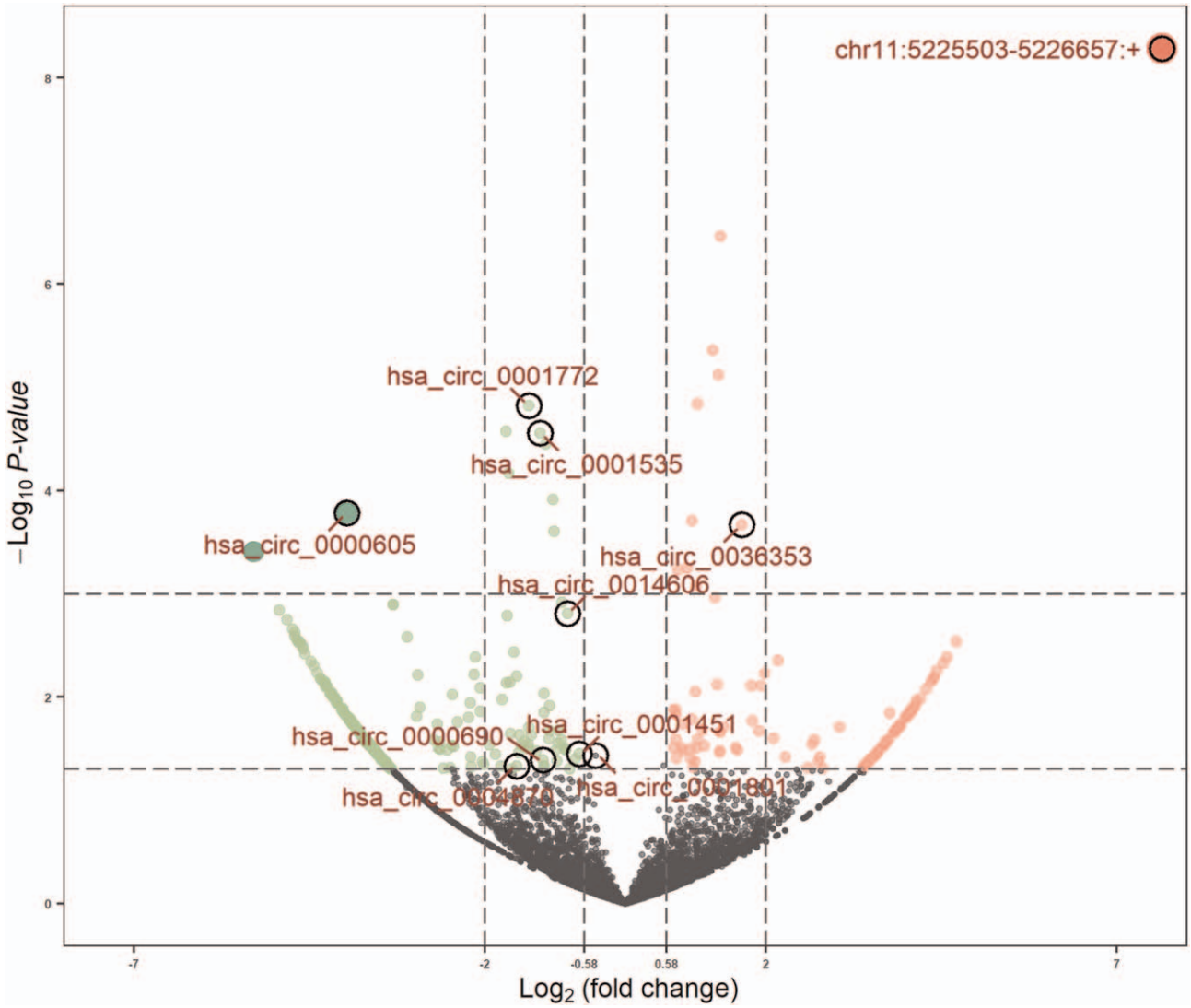

Figure 2. Volcano plots showed circRNAs expression variance in each PD and NC individuals. Green dots indicated circRNAs levels were decreased in PD patients (left), pink dots mean circRNAs levels were increased in PD patients (right) ($P < .05$). circRNAs = circular RNAs, NCs = normal controls, PD = Parkinson disease.

Table 2

The top 2 upregulated and the top 8 downregulated circRNAs from PD.

Name	*P*-value	FC	Chr.	Source gene	Gene name
Upregulated					
chr11:5225503-5226657:+	5.21E-09	7.648	Chr11	ENSG00000244734	*HBB*
hsa_circ_0036353	.000214	1.658	chr15	ENSG00000169375	*SIN3A*
Downregulated					
hsa_circ_0000690	.041	−1.169	Chr16	ENSG00000005844	*ITGAL*
hsa_circ_0001535	2.77E-05	−1.213	Chr5	ENSG00000031003	*FAM13B*
hsa_circ_0001451	.035	−0.654	Chr4	ENSG00000109670	*FBXW7*
hsa_circ_0004870	.047	−1.542	Chr20	ENSG00000131051	*RBM39*
hsa_circ_0000605	.000	−3.966	Chr15	ENSG00000137776	*SLTM*
hsa_circ_0014606	.002	−0.823	Chr1	ENSG00000163374	*YY1AP1*
hsa_circ_0001801	.037	−0.421	Chr8	ENSG00000168300	*PCMTD1*
hsa_circ_0001772	1.50E-05	−1.372	Chr7	ENSG00000184863	*RBM33*

circRNAs = circular RNAs.

Table 3

The source gene of circRNAs enrichment analysis.

	Symbols	Description	Log*P*
Group1	*HBB*, *SIN3A*, *FBXW7*	Response to oxidative stress	−3.14
Group2	*HBB*, *ITGAL*, *SIN3A*	Hemostasis	−2.76

circRNAs = circular RNAs.

struction were performed by the Annoroad Gene Technology Co., Ltd. (Beijing, China). RNA libraries were established and sequenced as 150-bp paired-end reads applying the HiSeq X ten.

2.5. Bioinformatics analysis of the sequencing data

Homo sapiens genome assembly hg38 was used as reference genomes in this study. The reference genomes and gene annotation database were downloaded from ENSEMBL database.[17] Fol-

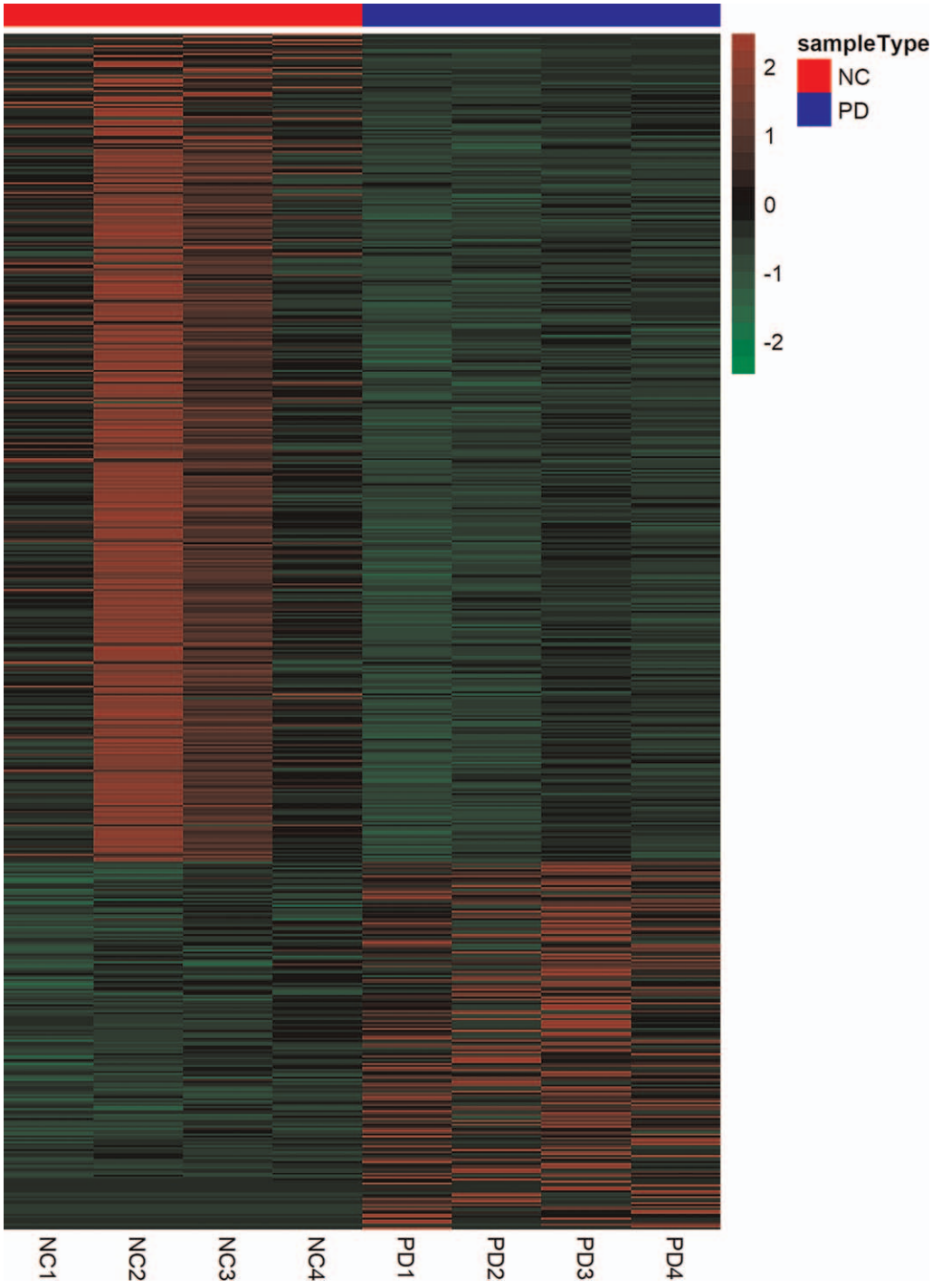

Figure 3. The heatmap of mRNAs from patients with PD and NCs. Red represented strong intensity, black represented medium intensity, and green represented low intensity. NCs = normal controls, PD = Parkinson disease.

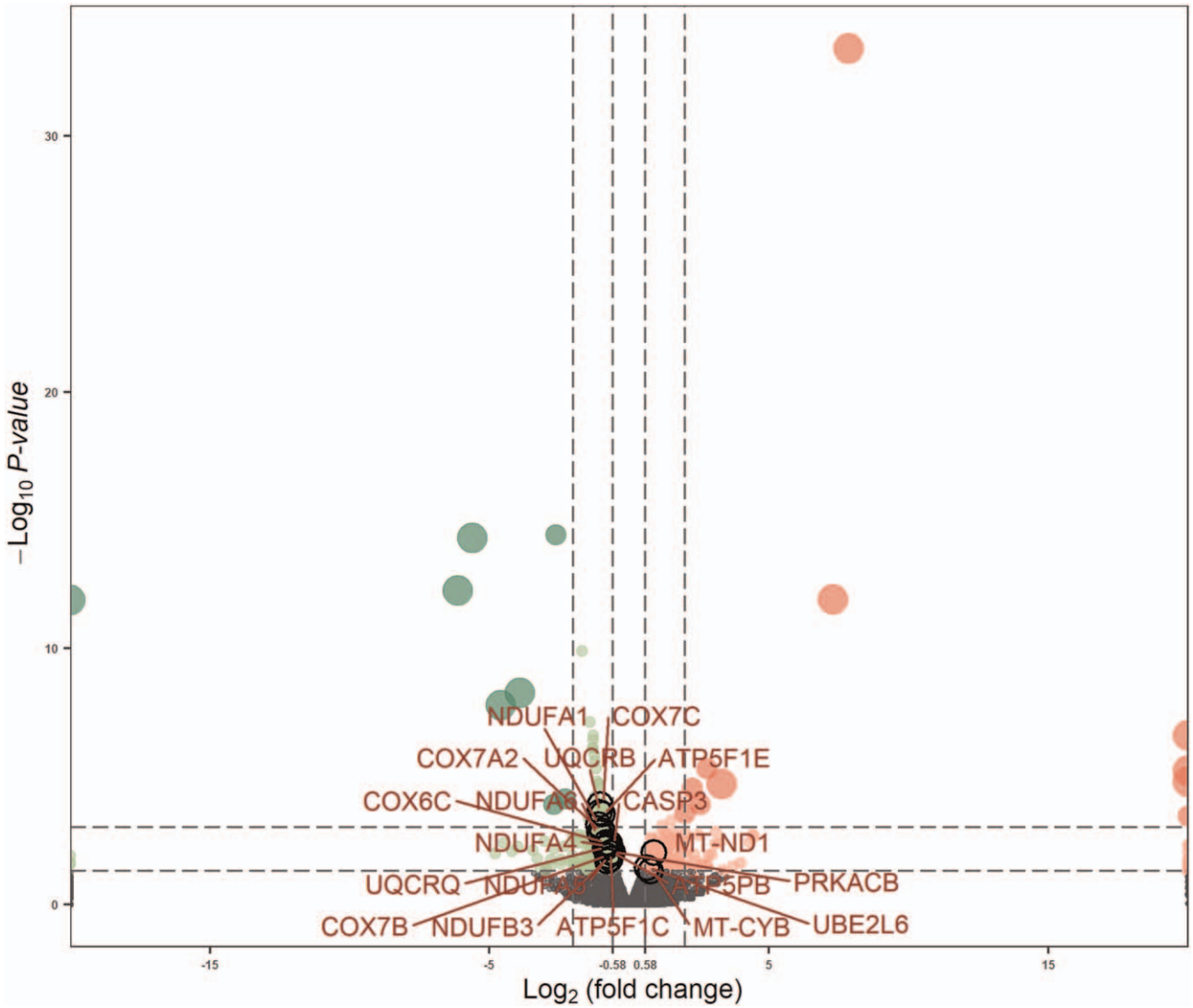

Figure 4. Volcano plots of differentially expressed mRNAs in each PD and NC individuals. Green dots indicated circRNAs levels were decreased in PD patients (left), pink dots mean circRNAs levels were increased in PD patients (right) ($P < .05$). circRNAs = circular RNAs, NCs = normal controls, PD = Parkinson disease.

lowing the instructions provided, the software was used to assemble the transcriptome. In a nutshell, each RNA-sequencing dataset was independently aligned to the reference genomes by the HiSAT2. Subsequently, the transcriptome from each dataset was collected separately using the StringTie program.[18] According to the transcriptome, the profiling differences between the 2 groups were compared using the DESeq2.[19] The source gene of circRNAs enrichment analysis was performed by the webserver Metascape (https://metascape.org/gp/index.html#/-main/step1) using the default settings. The heatmap was analyzed by R software (version v3.1.1). To explore the underlying pathways and biological processes in PD, we conducted the Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway analysis to annotate all differentially expressed mRNAs.

2.6. Construction of the competing endogenous RNA (ceRNA) network

The miRNA–mRNA interactions and the miRNA–circRNA interactions were predicted using the TargetScan and miRTarBase databases, respectively.[20,21] A circRNA-associated ceRNA network was built based on relationships between differentially expressed circRNAs, mRNAs, and miRNAs, and visualized with Cytoscape (version 3.6.0).

2.7. Statistic analysis

Data were expressed as means ± standard deviations or proportionality. The profiling differences between these 2 groups were compared using the "Fold Change" (FC) between PD patients and NCs. A *P*-value was assigned to each gene and adjusted by Benjaminiand Hochberg. Genes with $P < .05$ and $|\log_2 FC| \geq 0.58$ were considered as differentially expressed genes.

3. Results

3.1. CircRNA expression profiling in PD patients

We performed circRNAs analysis of peripheral blood from age- and sex- matched 4 PD patients and 4 NCs. A total of 411 circRNAs were identified as differentially expressed circRNAs.

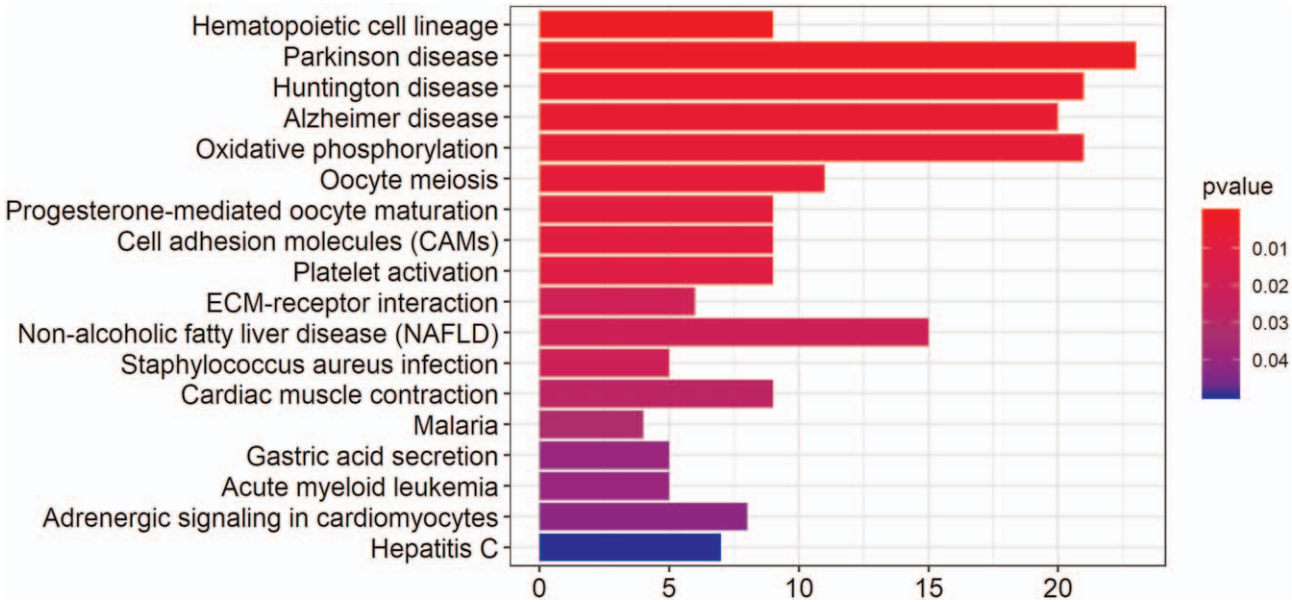

Figure 5. PD signaling pathway. KEGG enrichment analysis identified that genes that were significantly down-regulated in PD patients compared with NC were enriched in the PD signaling pathway. The red boxes indicate the main components in the PD signaling pathway. KEGG = the Kyoto Encyclopedia of Gene and Genomes pathway, NCs = normal controls, PD = Parkinson disease.

Compared with the NCs, 129 circRNAs were up-regulated, while 282 circRNAs were down-regulated in PD patients. The heatmap and volcano map revealed evident circRNAs expression variations between the 2 groups (Figs. 1 and 2). Top 11 of 411 differentially expressed circRNAs were listed in Table 2. Among the 10 circRNAs, there were 9 down-regulated circRNAs, including hsa_circ_0000690, hsa_circ_0000605, hsa_circ_0014606, hsa_circ_0004870, hsa_circ_0001451, hsa_circ_0001535, hsa_circ_0001772; hsa_circ_0036353 and a circRNA not identified previously was up-regulated in PD patients. These circRNA were encoded by *ITGAL*, *SLTM*, *YY1AP1*, *RBM39*, *FBXW7*, *FAM13E*, *RBM33*, *SIN3A*, and *HBB* gene respectively. *FBXW7* was involved in responding to oxidative stress; and *ITGAL* functioned in hemostasis; moreover, *HBB* and *SIN3A* both have functions of oxidative stress and hemostasis (Table 3). Thus, these differentially expressed circRNAs might mediate PD pathogenesis through oxidative response and hemostasis pathways.

3.2. mRNAs expression profiling in PD patients

The heatmap and volcano plots exposed significant variations between 2 groups in terms of expressions of mRNAs (Figs. 3 and 4). mRNAs with $|\log_2 FC| \geq 2.0$ and P-values $< .05$ were considered as significantly differentially expressed. A total of 766 mRNAs were significantly differentially expressed. Compared with the NCs, 273 mRNAs were up-regulated, while 493 mRNAs were down-regulated in PD patients.

3.3. Gene enrichment analysis of circRNAs parental genes in PD patients

To clarify the biological processes related to PD, KEGG analysis was performed on all 273 up-regulated and 493 down-regulated genes in the PD group (Fig. 5). The top 5 KEGG pathways included "Hematopoietic cell lineage," "Parkinson disease," "Huntington disease," "Alzheimer disease," and "Oxidative phosphorylation," where PD ranked the second (Fig. 6). The first pathway was related to hematopoiesis, which was reasonable, because the samples were obtained from whole blood. The fifth position was oxidative phosphorylation, which might be related to neural metabolism.

3.4. CircRNA-targeted miRNA-mRNA network prediction and annotation

In order to determine possible modulatory mechanisms of circRNAs, we studied the potential miRNAs which were predictably bonded with circRNAs ($|\log_2 FC| > 2$, $P < .05$) upon miRTarBase and TargetScan databases, and the gene co-expression network was constructed accordingly. Finally, a total of 10 differentially expressed mRNAs were involved in the ceRNA network, along with 10 circRNAs and 13 miRNAs (Fig. 7). Within the network, approximately 1 to 3 circRNAs targeted a single mRNA.

In all, we first reported the circRNA profile of PD patients, 10 circRNAs expressed differentially in PD patients. circRNAs and mRNA profiles implies that PD pathogenesis might be relative to oxidative response and hemostasis pathways.

4. Discussion

Although circRNAs are poorly studied in PD, miR-7 can directly inhibit gene expression of α-synuclein,[22] and miR-7-triggered down-regulation of α-synuclein protects cells from oxidative stress.[13] Transfection with miR-7 is a more effective way to inhibit α-synuclein in cell lines that do not express ciRS-7, suggesting that ciRS-7 plays a role in regulating α-synuclein through an miR-7-dependent pathway.[14] These results suggest a possible sponge effect between ciRS-7 and miR-7 in vitro.

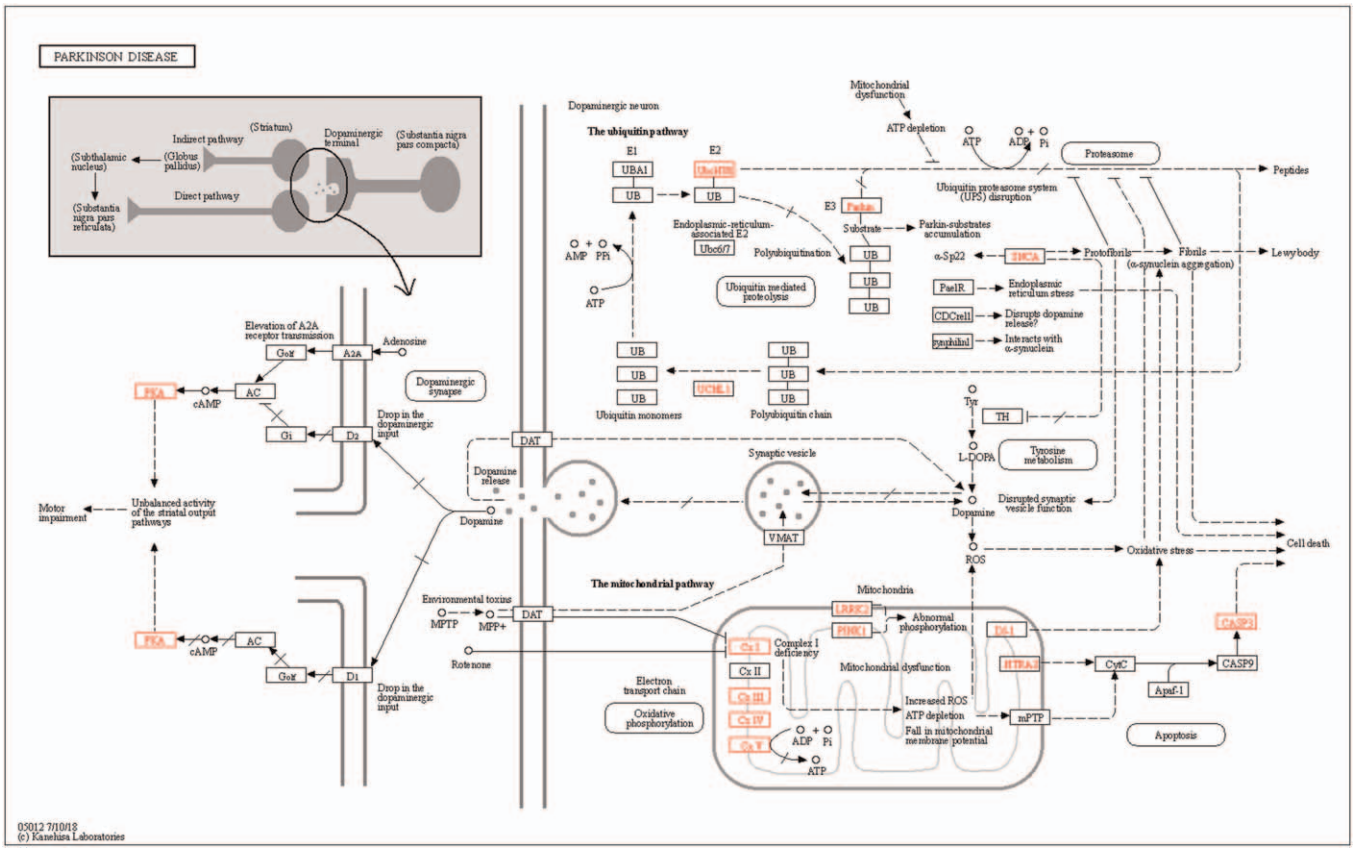

Figure 6. Elevated genes enriched in pathways of Parkinson Disease. The red box represents 18 elevated genes in PD. The pathway information of PD is obtained from KEGG database. KEGG = the Kyoto Encyclopedia of Gene and Genomes pathway, PD = Parkinson disease.

Ramaswamy et al[23] has indicated that miRNAs hold tremendous promise as a putative biomarker in PD, which was consistent with our current research.

We found that the expressions of several mRNAs and circRNAs in PD patients associated with mitochondrial defects and abnormal respiratory chains, as described in previous studies.[24] Respiratory chain impairment is a key feature in sporadic PD patients.[25] Cohen and Kesler[26] have found that mitochondrial dysfunctions, encompassing complexes I–IV of the electron transport chain, are characteristics of PD. *MT-CYB*, encodes the core subunit of complex III, plays a critical role in promoting progression and development of liver cancer,[27] and is an essential part of the oxidative phosphorylation system, which arouses cellular homeostasis and energy production.[28] The expression of MT-ND1 is reduced in Parkinson disease with dementia (PDD).[29] However, the expression of MT-ND1 was up-regulated in our study. *UQCRB* (ubiquinol cytochrome c reductase binding protein) gene plays an important role in hypoxia-induced angiogenesis through mitochondrial ROS-mediated signaling.[30] Inhibition of *UQCRB* blocks mitochondrial ROS-mediated vascular endothelial growth factor receptor type 2 (VEGFR2) signaling pathway in MRC. *NDUFB3* gene encodes mitochondrial complex I, which is the first identified enzyme in the electron transport chain of mitochondria.[31]*UQCRQ* (ubiquinol-cytochrome c reductase complex III subunit VII) gene, encodes a subunit of ubiquinol-cytochrome c reductase complex III.[32,33]*COX6C* (cytochrome c oxidase subunit 6C, K02268) gene is highly prevalent in the plasma of melanoma patients, as well as in patients with ovarian and breast cancers.[34] CASP3 (caspase 3, K02187) gene encodes cysteine-aspartic acid protease, which plays a pivotal role in the execution-phase of cell apoptosis. Interestingly, dopaminergic neurons are programmed to apoptosis, which may be mediated by CASP3.[35] Hartmann et al[36] reported that in dopaminergic cell groups of mesencephalon, the neuronal loss degree in PD patients was positively correlated with the proportion of caspase-3-positive neurons of the normal individuals. Besides, CASP3 modulates synaptic plasticity in the PINK1 mouse model of PD.[37]

The expression of 2 circRNAs, hsa_circ_0001439 and hsa_circ_0014606, also were changed in Alzheimer disease.[38] The expression of hsa_circ_0001439 was up-regulated in Brodmann Area 10 (BM10) of patients who suffered from Alzheimer disease. Furthermore, the expression of hsa_circ_0014606 was increased in many cortial regions, such as parietal cortex tissue, inferior frontal gyrus tissue, frontal pole, superior temporal gyrus, parahippocampal gyrus, it highly correlates with dementia, and neuropathology of Alzheimer disease. The 2 circRNAs' expression was also changed in the plasma of PD patients, however, its expression was decreased but not increased because of the small sample size possibly. Still, our results imply that the 2 circRNAs have the possibility of being related to neurodegenerative diseases.

There were several limitations in this study. Firstly, the specimen size is relatively small so that power analysis was failed

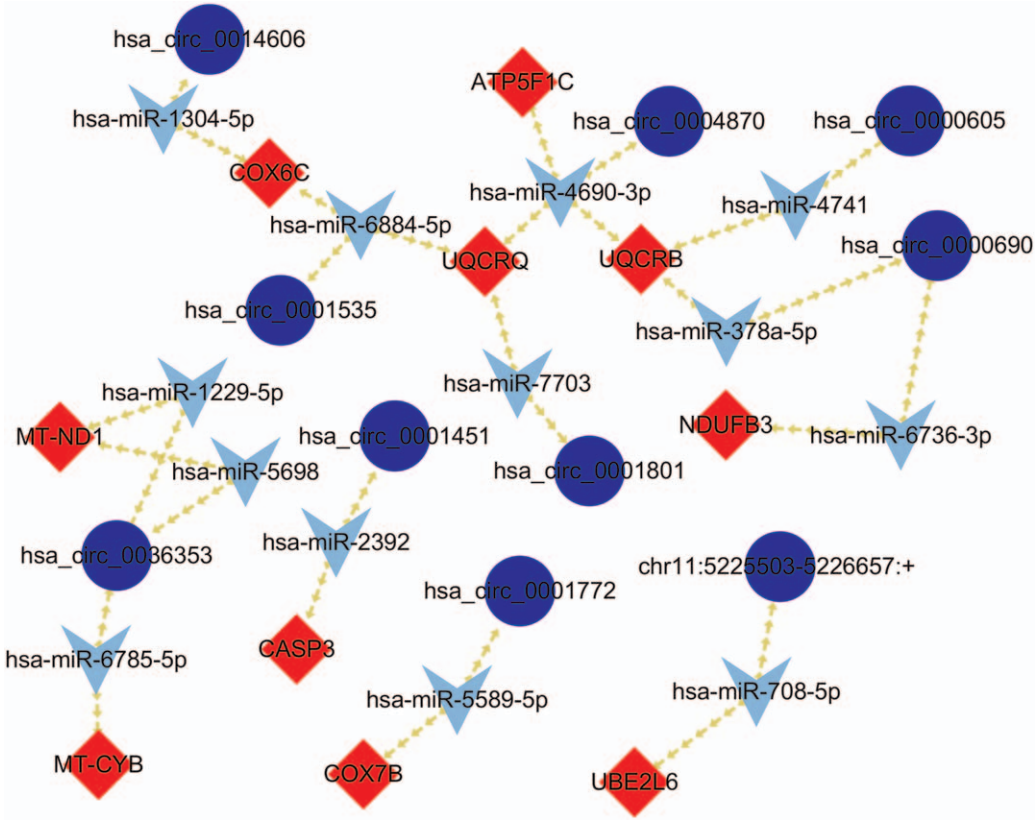

Figure 7. The ceRNA interaction network of circRNAs–miRNA–mRNA in PD. Sky blue circle standed for circRNAs, while red diamond was indicative of the mRNAs, light blue arrow represent the miRNAs, and the small yellow arrow indicates the circRNA-miRNA-mRNA interactions. circRNAs = circular RNAs, PD = Parkinson disease.

to be examined this time, thus large-sized and independent studies are necessary to verify the result. Besides, if circRNAs could be applied as appropriate biomarkers for PD diagnosis should be determined, and their ability to effectively differentiate PD from other neurodegenerative diseases should be further evaluated. Finally, part of the results in this study was obtained through bioinformatics analysis, and further experimental studies are required to verify these results.

Collectively, this is the first report of profiling and analyzing the expression profiles of circRNAs in Chinese PD patients. Our findings provided insights into the pathogenesis of PD and might offer a novel and promising method for investigations of molecular pathogenesis of PD. (http://links.lww.com/MD2/A145).

Author contributions

Conceptualization: Yan Yang, Zhanyun Lv, Chao Nie, Fancong Kong, Lifang Wang, Kui Zhang, Ying Cai, Qingqing Ding, Zuzhen Sun, Hefu Zhen, Fengjuan Jiao, Qianqian Ma, Chao Nie.
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Methodology: Fancong Kong, Hefu Zhen, Fengjuan Jiao, Zhanyun Lv, Qingqing Ding.
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Validation: Zhanyun Lv.
Writing – original draft: Fancong Kong, Lifang Wang, Yan Yang.
Writing – review & editing: Fancong Kong, Lifang Wang, Zhanyun Lv, Yan Yang.

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