ORIGINAL RESEARCH

The Screening of Piwi-Interacting RNA Biomarkers in Sporadic Parkinson's Disease

Yuan Gao, MD; Lei Zhang, MD; Qiang Meng, MD

ABSTRACT

Background • piRNAs play key roles in various diseases. However, the role of piRNAs in sporadic Parkinson's disease (PD) remains unclear. This study was conducted to explore key piRNAs that can be used as biomarkers for sporadic Parkinson's disease.

Methods • Differentially expressed piRNAs (DEPs) and their interaction were investigated using bioinformatics analysis, while the diagnostic value and expression of the selected piRNAs were detected.

Results • 42 DEPs were screened between PD and controls. Moreover, most of the physiological piRNA-piRNA interactions and linkages in normal samples had been altered in the sporadic PD samples. 14 overlapping piRNAs were selected, and six key piRNA biomarkers were screened. The different expressions of piR-hsa-327831, piR-hsa-1968818, piR-hsa-3770447, piR-hsa-1325354, and piR-hsa-2524778 had high efficiency and sensitivity in the diagnosis of PD.

Conclusion • PiR-hsa-327831, piR-hsa-1968818, piR-hsa-3770447, piR-hsa-1325354, piR-hsa-758566 and piR-hsa-2524778 could be biomarkers of PD. (*Altern Ther Health Med.* [E-pub ahead of print.])

Lei Zhang, MD; **Qiang Meng,** MD, Department of Neurology; The First People's Hospital of Yunnan Province; The Affiliated Hospital of Kunming University of Science and Technology; Kunming, Yunnan; China. **Yuan Gao,** MD, Department of Neurology; The First People's Hospital of Yunnan Province; The Affiliated Hospital of Kunming University of Science and Technology; Medical School; Kunming University of Science and Technology; Kunming, Yunnan; China.

Corresponding author: Qiang Meng, MD
E-mail: mengqiang301@sina.com

INTRODUCTION

Parkinson's disease (PD) is a complex neurodegenerative disease that is rare in people under the age of 40.[1] The incidence of PD ranges from 5 to >35 new cases per 100 000 individuals yearly in worldwide.[1,2] In addition to treating PD as a motor disorder, it is clear that PD has many non-motor features, such as cognitive impairment, autonomic dysfunction, disorders of sleep, depression, and hyposmia (impaired smell), which are part of the disease and greatly increase the overall burden.[3] The diagnosis of PD mainly depends on the history, clinical symptoms, and signs, and there is no abnormal change in general auxiliary examination. Due to the diversity of clinical manifestations of PD, its diagnosis is difficult, with a high rate of misdiagnosis.[4] In the future, diagnostic algorithms will need to incorporate additional tests and biomarkers to further enhance diagnostic accuracy and sensitivity of early and precursor disease stages.

Non-coding RNAs (ncRNAs) are a group of regulatory molecules which can perform their biological functions at the RNA level without coding to proteins.[5] PIWI-interacting RNA (piRNA) is a type of non-coding small RNA with a length of ~30 nt,[6] which has been reported to regulate chromatin modifications and further silence the expression of transposable elements (TEs) of eukaryotic genomes in germline cells.[7] More recently, the roles of piRNAs have also been revealed in somatic cells, including post-mitotic neurons. piRNAs and piRNA-like molecules were detected in the cerebral cortex of rhesus macaque and cultured mouse hippocampal neurons.[8,9] Based on a large number of animal experiments, the Piwi/piRNA complex was proved to regulate genomic heterogeneity in neurons by controlling TEs transposition, affecting the neurogenesis and synaptic plasticity and inducing behavioral deficits.[10] Importantly, piRNAs have recently been described as differentially regulated in sporadic PD brain tissue, connecting them to the process of neurodegeneration.[11] The expression of piRNAs derived from short interspersed nuclear elements (SINEs) and long interspersed nuclear elements (LINEs) is significantly downregulated in fibroblasts, induced pluripotent stem cells (iPSCs), and differentiated neuronal cells from patients with sporadic PD.[12] The potential involvement of piRNAs in the alterations of the epigenome of sporadic PD, such as DNA

methylation and gene silencing, is also present as different expression levels of piRNAs in the patients' peripheral blood.[13]

In this study, we screened the differentially expressed piRNAs (DEPs) in peripheral blood samples of sporadic PD patients and controls. Based on bioinformatics and biological tools, we performed preliminary analysis**.** Then we used PCR validation to detect the expression of relative piRNA, which exhibited that piR-hsa-372831, piR-hsa-1968818, piR-hsa-3770447, piR-hsa-1325354, piR-hsa-758566, and piR-hsa-2524778 can be used as biomarkers in sporadic PD. These pioneering studies on disease-associated piRNAs will contribute to improving our understanding of the pathogenesis of sporadic PD and the development of novel therapeutic strategies.

MATERIALS AND METHODS

Sample collection and RNA extraction

In this study, 8 patients diagnosed with sporadic PD in the Department of Neurology of the First People's Hospital of Yunnan Province were selected as the disease group, and all patients had not received relevant treatment before collecting peripheral blood. Based on the criteria suggested by Movement Disorder Society (MDS),[14] all patients underwent the clinical and neuropsychological examination, brain magnetic resonance imaging (MRI), and thyroid function tests. In addition, 8 healthy volunteers without related diseases were randomly selected as the control group according to the age and gender of the patients. The sample information is shown in Supplementary Table 1. Informed consent was obtained from all participants.

Moreover, this study was approved by the Ethics committee of the First People's Hospital of Yunnan Province (KHLL2021-KY071, September 2021). After admission, 2.5 ml of blood was collected on an empty stomach in the morning, and the first 1 ml of blood was discarded to reduce the influence of some epithelial cells on the blood samples. Peripheral blood samples were stored at -80 °C for reserve. Then, total RNA was severally extracted from all samples using TRIzol Reagent (Ambion, USA) for a follow-up test.

Library construction and high throughput sequencing

NEB's Purified mRNA or rRNA Depleted RNA and NEBNext® Ultra™ II RNA Library Prep Kit for Illumina® were used for the construction of sequencing libraries. Briefly, poly-A tail containing mRNA was isolated using oligo-dT attached magnetic beads. The mRNA was fragmented and transformed into ~ 200bp fragments using divalent cations at high temperatures. Then, the first strand cDNA synthesis, the second strand cDNA synthesis, linker connection, and the separation and amplification of cDNA fragments were carried out. Finally, each constructed sample library was quantified. The cDNA size distribution was determined using a bioanalyzer, the final library was assembled at an equal mole ratio, and qPCR amplification was evaluated using a real-time PCR system according to the manufacturer's instructions. After preprocessing and library construction, high-throughput sequencing was performed on the Illumina HiSeq X Ten platform. Finally, each constructed sample library was quantified. The cDNA size distribution was determined using a biological analyzer, the final library was assembled at an equal molar ratio, and the qPCR amplification was evaluated using a real-time PCR system according to the manufacturer's instructions.

piRNA quantification and gene expression analysis

Firstly, quality control and filtering of sequencing data were carried out. Tbtools_jre1.6. jar was used to predict and remove the connector automatically. Then, the sequence after quality control was compared with the reference piRNA sequence by Bowtie software, while human piRNA V2.0 data as reference piRNA sequences were downloaded from piRBase database (http://www. regulatoryrna. org/database /piRNA/ download.html). According to the comparison results, the piRNA expression count matrix of piRNAs was obtained, and the count value was normalized to the CPM (count-per-million) value for subsequent analysis. The Limma package was used for differential analysis to screen out differentially expressed piRNAs between patients and controls.

Construction of piRNA-targeted mRNA/piRNA-targeted lncRNA regulatory network

Because of the similarities in patterns of degradation/regulation between piRNAs and miRNAs, a famous miRNA-target prediction software program, "miRanda," was used for piRNA–target prediction. GrCH38. p13 genome-wide coding sequence was downloaded from NCBI for targeted mRNA prediction (https://ftp.ncbi.nlm.nih.gov/genomes/refseq/vertebrate mammalian/Homo sapiens/Latest_assembly_versions/). Human lncRNA sequences were downloaded from the LNCipedia database (https://lncipedia.org/) for lncRNA prediction. Moreover, the top 100 interaction pairs between piRNAs and lncRNA/mRNA were used to construct the piRNA-targeted lncRNA/piRNA-targeted mRNA network.

Identification and validation of the diagnostic biomarkers

The LASSO method was used to identify the diagnostic biomarkers based on mRNA prediction results. Then Wilcox. Test was used to compare the expression levels between the diseased and normal groups to verify the effectiveness of the piRNAs. Finally, the "survival ROC" package. A time-dependent ROC curve analysis was conducted using the "survival ROC" package to evaluate the model's predictive ability.

Quantitative real-time PCR

The peripheral blood of patients and control samples from our hospital were collected for qRT-PCR validation, and the total RNA was extracted using TRIzol Reagent (Ambion, USA). Then the RNA was reversed, and the cDNA was synthesized using sweScript RT I First strand cDNA SynthesisAll-in-OneTM First-Strand cDNA Synthesis Kit (Servicebio, china). Then, RT-PCR was conducted using 2xUniversal Blue SYBR Green qPCR Master Mix (Servicebio,

china). GAPDH was taken as the internal control. The relative mRNA expression was normalized and calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

RESULTS

Screening and co-expression analysis of DEPs

To improve the accuracy of gene differential expression analysis, we analyzed the gene expression in different samples to eliminate the aberrant samples. As a result, the abnormal gene expression was found in LR21F21FC1 and LR21F21FC6 (Figure 1A). Moreover, 42 DEPs, including 25 upregulated and 17 downregulated piRNAs, were screened between 8 PD and 8 control samples (Figure 1B). The expression levels of top100 DEPs between patients and control samples are shown in Figure 1C. What's more, of the up-regulated piRNAs, including piR-hsa-1968818, piR-hsa-3770447, piR-hsa-758566, piR-hsa-397627 and piR-hsa-4471643, were significantly related with sporadic PD. Besides, the expression of piR-hsa-2825592, piR-hsa-3763427, and piR-hsa-8345782 in normal controls' peripheral blood was higher than in PD patients.

Then, to explore the interaction of different piRNAs in the patients and control samples, Spearman's correlation analysis was used to calculate the correlation of DEPs. The correlation of DEPs was visualized with the co-expression network in Figure 1D and E. There were more connections among piRNAs in control samples, suggesting that most of the physiological piRNA–piRNA interactions and linkages in normal samples had been altered in the sporadic PD samples.

The prediction of piRNA-targeted mRNAs and lncRNAs

According to the NCBI and LNCipedia database, 2081 mRNAs and 3014 lncRNAs were found to be the target genes of 14 piRNAs and 25 piRNAs, respectively. Then, the 14 overlapping piRNAs were ultimately selected as the target piRNA (Figure 2A). The top 100 relationship pairs were chosen to construct the regulatory network, as shown in Figure 2B. A lot of mRNAs and lncRNAs were associated with piR-hsa-4468162, while piR-hsa-2524778 was associated with more lncRNAs and piR-hsa-2825592 only associated with lnc-GOPC-1. It was worth noting that 6 mRNAs were simultaneously regulated by piR-hsa-4468162 and piR-hsa-1968818. Moreover, piRNA-targeted mRNA SKOR2 maintained a significant correlation with piR-hsa-367290, piR-hsa-4468162, and piR-hsa-3770447.

The identification and confirmation of biomarkers

Firstly, LASSO regression analysis was used to construct a potential diagnostic biomarker with 6 piRNAs, including piR-hsa-327831, piR-hsa-1968818, piR-hsa-3770447, piR-hsa-1325354, piR-hsa-758566 and piR-hsa-2524778 (Figure 3A, B). As shown in Figure 3C, the expression levels of key piRNAs between the patients and control samples were significantly different. Compared to control samples, the expression of piR-hsa-327831, piR-hsa-758566, piR-hsa-1968818, and piR-hsa-3770447 were higher, while the lower expression levels piR-hsa-1325354 and piR-hsa-2524778

Figure 1. Screening and co-expression analysis of DEPs. A. Distribution of gene expression value of all samples; B. Volcano plot of DEPs between the PD and control samples. The red dots indicate up-regulated differential expression, the green dots indicate down-regulated differential expression and the gray dots indicate no significant difference; C. The expression levels of the top 10042 DEPs between the PD and control samples; D: The co-expression network of DEPs in control samples; E: The co-expression network of DEPs in PD samples.

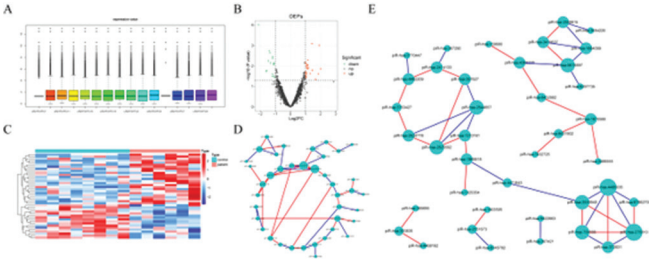


Figure 2. The prediction of piRNA-targeted mRNAs and lncRNAs. A. Venn plots of piRNA-mRNA and piRNA-lncRNA; B. Regulatory network of piRNA-lncRNA/piRNA-mRNA (top100 relationship pairs).

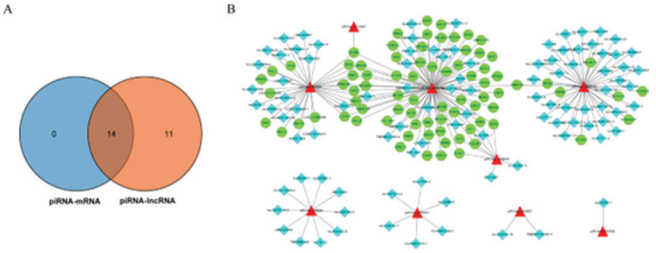


Figure 3. Identification and confirmation of biomarkers. A. Least absolute shrinkage and selection operator (LASSO) coefficient profiles of the key piRNAs; B. Tuning parameter selection by tenfold cross-validation in the LASSO model; C. The expression levels of key piRNAs between the PD and control samples. * P <.05, ** P < .01, *** P < .001, ns means no significant difference; D.ROC curve analysis of key piRNAs.

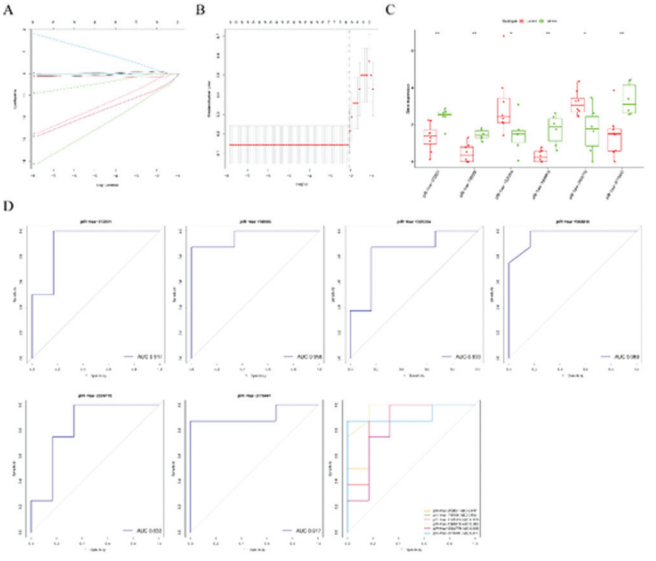


Figure 4. Functional analysis of piRNAs-targeted mRNA. A. GO enrichment analysis for all piRNA-targeted mRNAs; B. KEGG enrichment analysis for all piRNA-targeted mRNAs. C. GO enrichment analysis for key piRNA-targeted mRNAs. D: KEGG enrichment analysis for key piRNA-targeted mRNAs.

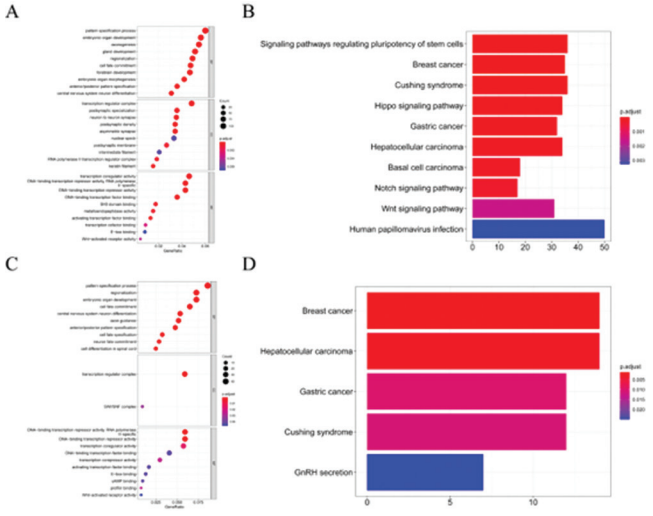


Figure 5. Regulatory network of key piRNA-lncRNA/key piRNA-mRNA (top 100 relationship pairs).

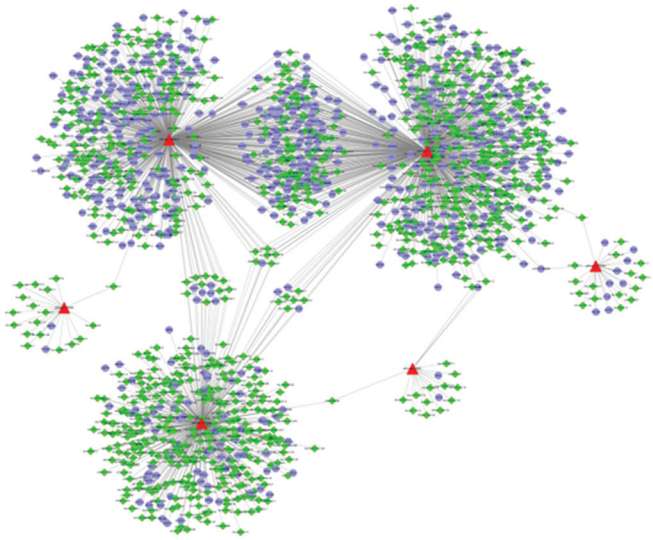
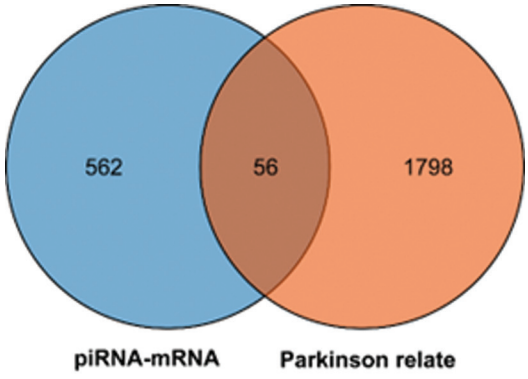


Figure 6. Venn plots of key piRNA-targeted mRNAs and key piRNA-targeted Parkinson-related genes.



were discovered in PD samples. What's more, the diagnostic ability of the key six biomarkers in discriminating sporadic PD from the control samples demonstrated a favorable diagnostic value with an AUC greater than 0.8 (Figure 3D).

The functional analysis of piRNAs-targeted mRNA

To explore the potential function of piRNA-targeted mRNAs in sporadic PD, functional enrichment analysis was performed for all and key targeted mRNAs separately. For all 2081 piRNA-targeted mRNAs, a total of 575 GO terms were enriched, which were mainly enriched in biological processes such as embryo development, axon formation, cell fate, forebrain development, and neuron differentiation of central nervous system, enriching in transcriptional regulatory complexes, synapse related cell components and transcriptional regulatory activators, DNA binding transcriptional inhibitors and other molecular functions (Figure 4A). KEGG analysis showed that there were 10 pathways enriched, including the totipotency signaling pathway and various cancerous pathways (Figure 4B).

Then, a separate functional enrichment analysis was performed on six key piRNAs-targeted mRNAs. There were 618 targeted mRNAs for key piRNAs, and a total of 154 GO terms were enriched, 92% of which were biological processes (BP), including embryonic development, axon orientation, cell fate, differentiation of spinal cord cells, neuronal differentiation of central nervous system, etc. Consisted with all 2081 piRNA-targeted mRNAs, the key piRNA-targeted mRNAs were also enriched in the same cell components and molecular functions. Similar enrichment results proved the validity and reliability of biomarkers.

Construction of key piRNA-targeted mRNA/ piRNA-targeted lncRNA regulatory network

To analyze the regulatory relationship between key piRNAs and their targeted mRNAs or lncRNAs, we constructed a regulatory network. As shown in Figure 5, the red triangle represented the 6 key piRNAs, the green diamond was the targeted lncRNAs and the purple circle meant the targeted mRNA. piR-hsa-1968818, piR-hsa-3770447, and piR-hsa-2524778 were found that they could regulate more targeted mRNAs and lncRNAs, one mRNA SCAF1 and 6 lncRNAs (ZMIZ1-AS13, ZMIZ1-AS10, WDFY3-A, DFY3-AS27, lnc-TACR1, and lnc-PRL-2) simultaneously associated with this three piRNAs. Meanwhile, the same piRNA-targeted lncRNA lnc-PAQR3-1 was regulated by piR-hsa-758566 and piR-hsa-1968818, while LINC006590 was regulated together by piR-hsa-2524778 and piR-hsa-372831.

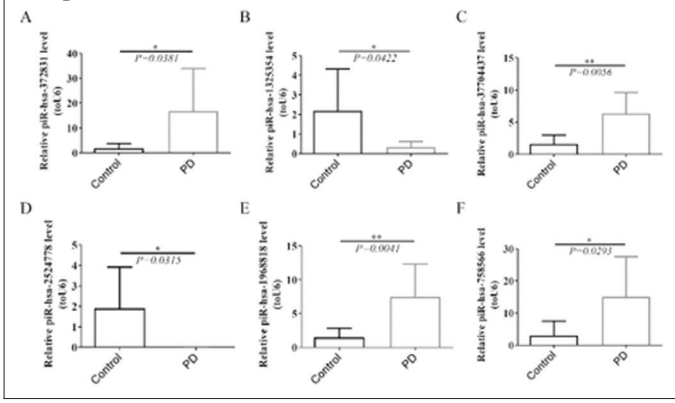
The relationship of key piRNA-targeted mRNAs and sporadic PD

1854 PD-associated genes from the DisGeNET database and 56 genes intersected with the key piRNA-targeted mRNAs were obtained, which exhibited that we have high confidence with key piRNAs piR-hsa-327831, piR-hsa-1968818, piR-hsa-3770447, piR-hsa-1325354 and piR-hsa-2524778 (Figure 6).

The expression level of 6 piRNAs biomarkers

To further verify the efficiency of the biomarkers, qRT-PCR was utilized to compare the expression levels of 6 piRNAs in normal and patient samples. The results displayed that there was a significant up-regulation for the expression of piR-hsa-327831, piR-hsa-758566, piR-hsa-1968818, and piR-hsa-3770447, while the expression of piR-hsa-1325354 and piR-hsa-2524778 were obviously downregulated (Figure 7).

Figure 7. qRT-PCR analysis of key piRNAs in PD and control samples.



DISCUSSION

Sporadic PD is a neurodegenerative disease, and its pathogenesis is still unclear. The neurodegeneration begins decades before the appearance of clinical symptoms, and the increasing attention has turned to the preclinical diagnosis.[15] Brain imaging of the nigrostriatal dopamine system has been used to diagnose at an early stage, but the cost is extremely high, with a high ratio of false negatives.[16] Cerebrospinal fluid analysis of α-synuclein is invasive, and both sensitivity and specificity of which are relatively poor and vary across studies.[17]

Some piRNAs have been regarded as biomarkers in a wide variety of cancers, such as colorectal, prostate, gastric, bladder, breast, lung, and hepatic cancers, as well as malignant melanoma.[18] PiRNA pathways are shown to be associated with several processes that occur in cancer, so the aberrant piRNA expression in patients might be introduced as a particular signature of cancer.[19] Likewise, a variety of studies have indicated the likelihood of piRNA involvement in the epigenetic regulation of genes associated with neuronal activity, neurogenesis, and neuronal plasticity. Moreover, some piRNAs might have a correlation with the aseptic inflammatory response in neurons.[9] Drawing on the experience of cancer diagnosis, we supposed that some piRNAs involved in neurodegeneration could be considered as less costly and relatively non-invasive biomarkers of sporadic PD.

In the current study, the potential function of DEPs and key piRNAs in sporadic PD was investigated by functional enrichment analysis. Although the correlation between piRNAs and the nervous system was not obvious via KEGG analysis, the piRNAs were considered to chiefly influence the stability of neurons and synaptic transmissionwas obtained from GO analysis (Figure 4A; Figure 4B). The main pathological features of PD are the loss of dopaminergic neurons with subsequent depigmentation of the substantia nigra pars compacta (SNpc), which is caused by the intracellular accumulation of Lewy bodies (LBs).[20] LBs are composed of misfolded α-synuclein and ubiquitin, producing direct or indirect toxic effects on the function of mitochondria and proteasome, damaging the biological membranes and cytoskeleton, and altering the synaptic function.[21] A sequential model of the formation of LBs and degeneration of neurons spreads from SNpc to the brain stem and cortex in a prion-like manner, which could be influenced by the gut microbiome.[22] More and more evidence prove that growth factors, neurotrophins and cytokines are also involved in embryo neural development and final neural degeneration at the molecular level.[23] Genes related to these factors are silenced at birth and can be activated by inflammatory products in the intestinal system and chronic physiological stimulation of the innate immune system.[24] This occurs in the brain decades later, can destroy the embryo neural structures, and lead to age-related neurodegeneration.[25] Our analysis showed that these GEPs were intimately associated with the functions of embryo development, axon formation, cell fate, forebrain development, and neuron differentiation of the central nervous system, which was the same as the analysis of key piRNAs. PiRNAs play crucial roles in gene regulation, epigenetics, TEs silencing, and genome rearrangement, as well as in cell self-renewal and embryogenesis.[26] PiRNAs also have mitochondrial regulation, which is associated with NADH-ubiquinone oxidoreductase chain 4 L (ND4L), Cyclooxygenase 2 (COX2), and NADH-ubiquinone oxidoreductase chain 5 (ND5).[27] The mitochondrial (mt)-piRNAs are involved in cell responses to oxidative stress, which is a vitally important reason for aggregations of LBs.[28] Besides, as we mentioned before, dysregulated piRNA features are likely due to the impact of the pathophysiology of sporadic PD itself, by comparing transcriptomic and epigenomic analysis using RNA-seq between fibroblast, iPSCs and differentiated neurons in sporadic PD and controls.[12] Both GO and KEGG analysis as well as pathophysiological studies demonstrated that piRNAs would be biomarkers with high validity and reliability.

The diagnostic value of the key six biomarkers was verified in this study. Moreover, a regulatory network to analyze the regulatory relationship between key piRNAs and their targeted mRNAs or lncRNAs was constructed. What' s more, SCAF1 and 6 lncRNAs (ZMIZ1-AS13, ZMIZ1-AS10, WDFY3-A, DFY3-AS27, lnc-TACR1 and lnc-PRL-2) were proved to be the target gene of piR-hsa-1968818, piR-hsa-3770447 and piR-hsa-2524778. Meanwhile, pir-hsa-758566 and pir-hsa-1968818 regulate lncRNA lnc-paqr3-1 targeted by the same piRNA, while linc006590 is jointly regulated by pir-hsa-2524778 and pir-hsa-372831. Each piRNA was the simultaneously associated with PD-related genes except piR-hsa-758566. LncRNAs play a role in the pathophysiology of

sporadic PD via the "sponge effect of lncRNA". That is, one lncRNA can bind a large set of miRNAs and alter their activities.[29] Various proteins obtained from postmortem tissues and patient-derived dopaminergic cells have been analyzed in parallel to reveal the network of lncRNAs, mRNAs, and PD-related genes.[30] SCAF1 and PRL-2 have been detected in many human malignancies, qualifying as a potential biomarker,[31] and the role of which in PD is still unclear. ZMIZ1 is a coactivator of several transcription factors, including p53, the androgen receptor, and NOTCH1, which is highly expressed in the brain to regulate the neural development.[32] WDFY3 in mitochondrial homeostasis may be involved in neuron differentiation, neural development, and age-dependent neurodegeneration.[33] The overexpression of TACR1 can mediate pro-proliferation effect in neuroblastoma cells.[34] PAQR3-1 was originally discovered as a potential tumor suppressor factor, which plays an important role in neuroprotection against oxygen-glucose deprivation/reperfusion injury.[35] However, DFY3-AS27 and LINC006590 are almost absent in sporadic PD. Chen et al. have found that lncRNA rich nuclear abundant transcript 1 (NEAT1) lncRNA rich nuclear transcript 1 (NEAT1) can up-regulate the expression of phosphodiesterase 4B (PDE4B) by adsorbing mirna-124-3p, thereby accelerating the progress of PD.[29] Another lncRNA, named growth arrest-specific 5 (GAS5), has demonstrated the pro-inflammatory effect on rotenone-induced PD mice and lipopolysaccharide (LPS)-treated microglial cells by competitively binding to miR-223-3p.[36] Cascade reactions between piRNAs and their targeted mRNAs or lncRNAs in PD span multiple pathways, our results provide the basis for continuous functional exploration.

Several promising objective biomarkers for sporadic PD have emerged, and the majority of these are identified in the clinically diagnosed PD population. The discovery of novel lncRNAs with diverse biological functions within different diseases makes lncRNAs serve as diagnostic biomarkers, while MicroRNAs (miRNAs) are the most promising therapeutic targets of PD.[37] The evidence for the use of piRNAs as a diagnostic marker is increasing at this stage. Fortunately, piRNAs are capable of passing through the cell membrane, even the blood-brain barrier (BBB), and remain stable in human samples even after frequent freeze-thaw cycles or long-term incubation at room temperature.[38] piRNAs are present in exosomes or as free-circulating in serum, plasma, saliva, and stool,[39] which can be detected using microarray, sequencing and qRT-PCR.[40] In this study, qRT-PCR was employed to compare the expression levels of 6 piRNAs in normal and patient samples, which exhibited that the expression of piR-hsa-327831, piR-hsa-758566, piR-hsa-1968818, and piR-hsa-3770447 were significantly up-regulated while of piR-hsa-1325354 and piR-hsa-2524778 were obviously down-regulated in PD patients. The abnormal expression of circulating piRNAs might be good biomarkers with higher specificity and sensitivity compared to circulating miRNAs and lncRNA-based biomarkers.[40] Recently, Zhang et al. have identified novel piRNAs as biomarkers for their abnormal expression in different PD subtypes (PD and Parkinson's disease dementia (PDD)) and stages (premotor and motor).[6] This study highlighted the role of the piRNAs and promoted the development of diagnostic markers for sporadic PD.

CONCLUSIONS

In conclusion, our results suggested that these 6 piRNAs could effectively distinguish between samples from sporadic patients and controls, thus providing a useful diagnostic tool. The bioinformatic analysis was likely to provide us with a better understanding of the pathogenic processes leading to PD.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

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Supplementary Table 1.

	Gender	Age		Gender	Age
PD1	Male	78	HC1	Male	76
PD2	Male	77	HC2	Male	76
PD3	Male	75	HC3	Male	75
PD4	Male	78	HC4	Male	78
PD5	Female	68	HC5	Female	69
PD6	Female	69	HC6	Female	70
PD7	Female	75	HC7	Female	77
PD8	Female	78	HC8	Female	79