Original Research

Identification of microRNAs for the early diagnosis of Parkinson's disease and multiple system atrophy

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MicroRNAs are reportedly involved in the pathogenesis of neurodegenerative diseases, including Parkinson's disease and multiple system atrophy. We previously identified 7 differentially expressed microRNAs in Parkinson's disease patients and control sera (miR-30c, miR-31, miR-141, miR-146b-5p, miR-181c, miR-214, and miR-193a-3p). To investigate the expression levels of the 7 serum microRNAs in Parkinson's disease and multiple system atrophy, 23 early Parkinson's disease patients (who did not take any anti- Parkinson's disease drugs), 23 multiple system atrophy patients, and 24 normal controls were recruited at outpatient visits in this study. The expression levels of the 7 microRNAs in serum were detected using quantitative real-time polymerase chain reaction. A receiver operating characteristic curve was used to evaluate whether microRNAs can differentially diagnose Parkinson's disease and multiple system atrophy. Clinical scales were used to analyze the correlations between serum microRNAs and clinical features. The results indicated that miR-214 could distinguish Parkinson's disease from the controls, and another 3 microRNAs could differentiate multiple system atrophy from the controls (miR-141, miR-193a-3p, and miR-30c). The expression of miR-31, miR-141, miR-181c, miR-193a-3p, and miR-214 were lower in multiple system atrophy than in Parkinson's disease (all $P < 0.05$). Combinations of microRNAs accurately discriminated Parkinson's disease from multiple system atrophy (area under the receiver operating characteristic curve = 0.951). For the correlation analysis, negative correlations were discovered between the expression of miR-214 and the Hamilton Anxiety Scale and Parkinson's Disease Non-Motor Symptom scores (all $P < 0.05$). Our results demonstrate that the distinctive characteristics of microRNAs differentiate Parkinson's disease and multiple system atrophy patients from healthy controls and may be used for the early diagnosis of Parkinson's disease and multiple system atrophy.

Keywords

Parkinson's disease; multiple system atrophy; serum; microRNA; biomarkers; neurodegenerative disease

1. Introduction

Parkinson's disease (PD) is a common chronic progressive neurodegenerative disease in middle-aged and older people. Its main clinical manifestations are bradykinesia, resting tremor, myotonia and abnormal posture, and gait (Lee et al., 1993). About 1% of people over 65 years old suffer from PD. The main pathological features are selective and progressive degeneration of dopamine (DA) neurons in the substantia nigra compacta of the midbrain, a significant decrease of DA content in the striatum, and eosinophilic inclusion bodies--Lewy body in the residual neurons, which are mainly composed of α-synuclein (α-syn) (Bentwich et al., 2005). After decades of research, the diagnosis and treatment of PD are still not ideal (Kalia and Lang, 2015). An autopsy is a gold standard with the patients grouped according to pre-death clinical information. At present, the clinical diagnosis of PD is mainly through the evaluation of medical history, physical examination, imaging examination, and evaluation of the response of levodopa and other drugs, but symptoms and signs occur only when up to 80% of dopamine deficiency occur in the pars compacta of the substantia nigra (Emamzadeh and Surguchov, 2018; Lotankar et al., 2017). Only when the symptoms are fully developed can specific imaging abnormalities be observed (Ghaemi, 2002). Therefore, searching for biomarkers that can distinguish PD from other diseases with higher sensitivity and specificity is necessary, and fully tracking the progression of PD is preferable. Only in this way can we diagnose PD early.

Multiple system atrophy (MSA) is a sporadic, adult-onset, and progressive neurodegenerative disease (Fanciulli et al., 2019). The onset age is about 60 years old, but it also occurs in both young

Table 1. Patient group characteristics

Variables	NC (n = 24)	PD (n = 23)	MSA (n = 23)	*P*-value[a]
Age, years	62.8 ± 5.7	59.3 ± 8.8	58.7 ± 9.3	$P = 0.11^b$
Sex, n				$P = 0.08^c$
Male	12 (50.0%)	10 (43.5%)	9 (39.1%)	
Female	12 (50.0%)	13 (56.5%)	14 (60.9%)	
UPDRS-II score	NA	9.1 ± 5.8	NA	
UPDRS-III score	NA	23.8 ± 16.0	NA	
UMSARS-II score	NA	NA	24.8 ± 14.1	
HAMD score	NA	9.5 ± 9.7	NA	
HAMA score	NA	8.3 ± 9.1	NA	
PDSS score	NA	127.9 ± 21.5	NA	
PDNMS score	NA	7.7 ± 5.8	NA	
MOCA score	NA	21.0 ± 5.6	NA	
MMSE score	NA	25.4 ± 4.3	NA	
H-Y stage	NA	2.1 ± 0.8	NA	

Values are expressed as the mean ± standard deviation.

n number of samples, *PD* Parkinson's disease, *MSA* multiple system atrophy, *NA* not applicable, *UPDRS* Unified Parkinson's Disease Rating Scale, *UMSARS* Unified Multiple System Atrophy Rating Scale, *HAMD* Hamilton Depression Scale, *HAMA* Hamilton Anxiety Scale, *PDSS* Parkinson's Disease Sleep Scale, *PDNMS* Parkinson's Disease Non-Motor Symptom, *MOCA* Montreal Cognitive Assessment, *MMSE* Mini-Mental State Examination, *H-Y stage* Hoehn-Yahr stage.

[a] Student's *t*-test.
[b] PD vs. NC.
[c] MSA vs. NC.

and old age (Batla et al., 2018; Wenning et al., 1997). The clinical manifestation of MSA is complex, which is the combination of autonomic nerve failure, Parkinson's disease, and ataxia. The pathological feature of MSA is the formation of cytoplasmic inclusion bodies composed of misfolded α-syn in glial cells (Gilman et al., 1998). MSA is currently divided into 2 clinical subtypes, including Parkinson's syndrome as a prominent clinical subtype called the MSA-P type and cerebellar ataxia for more severe cases called the MSA-C type (Fanciulli et al., 2019; Gilman et al., 1998).

Both MSA and PD are caused by pathological aggregation of α-syn (Yamasaki et al., 2019). Although clinical criteria for MSA have been established, prenatal diagnosis is still difficult. Previous autopsy studies showed that the diagnostic accuracy was 29%-86% (Hughes et al., 2002; Osaki et al., 2002; Wenning et al., 1997). Clinical diagnosis may also be problematic when MSA co-exists with other neurodegenerative diseases with the same clinical characteristics, such as Alzheimer's disease, Louis-related disease (Ozawa, 2004), or tauopathy (Uchikado et al., 2006). Early in the disease, the MSA-P type can be difficult to distinguish from PD (Litvan et al., 1997; Osaki et al., 2002; Seppi et al., 2005a). Data show that, clinically, approximately 10%-30% of MSA in the early stage are misdiagnosed as PD (Poewe and Wenning, 2002). The prognosis and treatment response of MSA and PD are different. Patients with MSA-P have higher morbidity and mortality than PD patients. They usually respond poorly to levodopa and develop faster dyskinesia (Muller et al., 2000; Quinn and Marsden, 1993; Seppi et al., 2005b). Early diagnosis can avoid mistaking MSA-P patients into PD pharmacological and neurosurgical tests (Lang and Lozano, 1998a,b). Therefore, it is particularly important to study the biomarkers that can accurately distinguish and diagnose MSA and PD in the early stage of the disease.

MiRNAs are a small cluster of endogenous non-coding single-stranded RNA regulatory molecules with a length of about 20-22 nucleotides, which are encoded by endogenous genes and cannot be transcribed into proteins. They inhibit the translation of target mRNAs or promote mRNAs degradation by binding to the 3'-untranslated region (3'-UTR) of target mRNAs, thus inhibiting the expression of target genes at the post-transcriptional level (Vivekanantham et al., 2015). MiRNAs are highly conservative. Their expression is sequential and tissue-specific. They participate in almost all pathological and physiological processes of mammals and play an important role in the occurrence and development of many diseases (Cao and Zhen, 2018; Hu et al., 2019; Junn et al., 2009; Vishnoi and Rani, 2017). They also play a crucial role in regulating cell proliferation, differentiation, growth, metabolism, stress response, apoptosis, and heterochromatin formation (Kim et al., 2007; Santosh et al., 2009; Tutar, 2015). Post-transcriptional regulation is an important process in the pathogenesis of PD (Dorval et al., 2012; Filipowicz et al., 2008; Hombach and Kretz, 2016; Patop et al., 2019). The role of miRNAs in the pathogenesis of PD has attracted increasing attention from researchers (Chang et al., 2017; Kim et al., 2019; Leggio et al., 2017).

MiRNA expression was monitored by qPCR in PD patients and healthy controls. Eight of 224 pre-miRNAs are highly expressed in the midbrain (Kim et al., 2007). Among them, pre-miR-133b was the most downregulated in the PD group. However, these miRNAs found in human brain tissue cannot be directly used as biomarkers for clinical use, and using brain tissue samples from PD patients to diagnose PD is impossible. Early diagnosis requires not only the identification of disease specificity but also minimally invasive biomarkers.

The low stability of RNA molecules limits their ability to be used as biomarkers. However, serum miRNAs are very stable (Jin et al., 2013). In addition to their high sensitivity and specificity, serum miRNAs are also convenient and inexpensive and have other advantages as biomarkers (Zhou et al., 2012). Geekiyanage et al. (2012) found that some serum miRNAs have similar changes in the brain. Cogswell et al. (2008) found the presence of AD-specific miRNAs in cerebrospinal fluid (CSF), suggesting that some miRNAs produced by the diseased tissue and cells can enter the CSF and then enter the peripheral blood through the circulatory system. The results of these studies provide a basis for serum miRNAs as PD biological markers.

We previously identified 7 differentially expressed miRNAs in PD patient and control sera (miR-30c, miR-31, miR-141, miR-146b-5p, miR-181c, miR-214 and miR-193a-3p), and the levels of these 7 miRNAs in serum were significantly lower in the PD patient group than in the control group (Dong et al., 2016). In this paper, we compare the difference in the expression of these serum miRNAs between 23 de novo PD cases and 23 MSA patients in order to analyze the clinical application value of the serum miRNAs in the diagnosis of PD and MSA.

2. Subjects and methods

2.1 Research object and scale assessment

The ethics committee approved the study of the Affiliated Brain Hospital of Nanjing Medical University, and it was completed based on the ethical standards established by the 1964 Declaration of Helsinki and its later amendments. All participants gave written informed consent.

PD Group: This group included 23 de novo PD outpatients from the Department of Neurology, Affiliated Brain Hospital, Nanjing Medical University. The incidence of PD was within 1 year, and patients did not take anti-PD drugs. All patients were followed for 2-3 years to confirm PD, and the diagnosis was in line with the British Brain Bank PD diagnostic criteria (Table 1).

MSA group: This group included 23 MSA outpatients from the Department of Neurology, Affiliated Brain Hospital, Nanjing Medical University. The duration of MSA was 1-3 years, and the diagnostic criteria were in line with the 1999 MSA diagnostic criteria proposed by Gilman (Gilman et al., 1998).

NC group: For this group, 24 physically healthy cases (with no serious chronic physical illness for the elderly patients) were from Affiliated Brain Hospital, Nanjing Medical University. Informed consent was obtained. No noticeable difference in sex or age between the PD group, MSA group, and control group was observed.

The data, history, and treatment of the 3 groups were obtained by means of history collection and assessment scales. All PD patients underwent the questionnaires and scales administered by 1-2 neurologists in 2 hours in a quiet environment.

2.2 Serum preparation and RNA isolation

Venous blood samples were collected from all controls and patients, and blood was separated by centrifugation at a speed of 800 $\times$ g for 15 min. The supernatant was collected and stored at -80 °C.

TRIzol reagent (Invitrogen, USA) was used to extract total RNA according to the manufacturer's instructions for qRT-PCR detection. The RNA was preserved at -80 °C for further analysis.

2.3 qRT-PCR analysis

TaqMan probe-based real-time PCRs were performed using a TaqMan miRNA PCR kit (Applied Biosystems, USA). The expression of miRNAs (miR-30c, miR-31, miR-141, miR-146b-5p, miR-181c, miR-214, and miR-193a-3p) were normalized to the level of external reference (TIANGEN Biotech Co., Ltd., P. R. China). cDNA was synthesized from the total RNA with the stem-loop RT primer (Applied Biosystems, USA) and AMV reverse transcriptase (TaKaRa, P. R. China). A TaqMan miRNA probe was used to perform real-time PCR on the Applied Biological System 7300 Sequence Detection System (Applied Biosystems, USA). The relative miRNA expression levels were calculated by the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2002).

2.4 Statistical analysis

Statistical analysis was performed with SPSS 18.0 software. Data were reported as the means $\pm$ standard errors of the means (SD). Comparisons were made using the 2 independent samples test. The receiver operating characteristic (ROC) curves of miRNAs were drawn by SPSS software. The level of significance was set at 0.05.

3. Results

3.1 Comparison of the expression levels of 7 miRNAs in patients with PD and controls

Our preliminary results identified 7 differentially expressed miRNAs between patients and controls (n = 93) (Dong et al., 2016). Our present study enrolled 23 PD patients and 23 MSA patients. The quantitative real-time polymerase chain reaction (qRT-PCR) results showed that of the 7 miRNAs (Table S1), the expression of miR-214 in serum was significantly higher compared with the control group ($P < 0.05$, Fig. 1A). However, the serum miR-214 levels of 18 PD patients tended to decrease with age (Fig. 1D). The data were reported as the mean $\pm$ SD.

The differences in miRNAs expression between 23 MSA patients and 24 healthy controls indicated that the relative expression of 3 (miR-141, miR-193a-3p, and miR-30c) of the 7 miRNAs was significantly lower in the MSA group than in the control group ($P < 0.05$, Fig. 1B).

The differences in the expression of miRNAs between 23 PD patients and 23 MSA patients demonstrated significant differences in the relative expression of 5 (miR-31, miR-141, miR-214, miR-181c, and miR-193a-3p) of the 7 miRNAs ($P < 0.05$, Fig. 1C); moreover, their expression levels in MSA were lower than that in PD.

3.2 ROC curve analysis of serum microRNAs for the diagnosis of PD or MSA

The area expressed the diagnostic approach of the accuracy evaluation index under the ROC curve (AUC). Through the analysis and comparison of diagnostic tests for unified disease, these indicators can help identify the best diagnostic methods. Our results showed that the accuracy of AUC was moderate for all indexes to distinguish PD or MSA from the control group.

A binary logistic regression analysis was used to determine whether combinations of miRNAs could meliorate their usage as biomarkers. Compared with a single miRNA, the combination of miRNAs resulted in enhanced discrimination of the MSA from the control group (Fig. 2B). The model included miR-141, miR-193a-
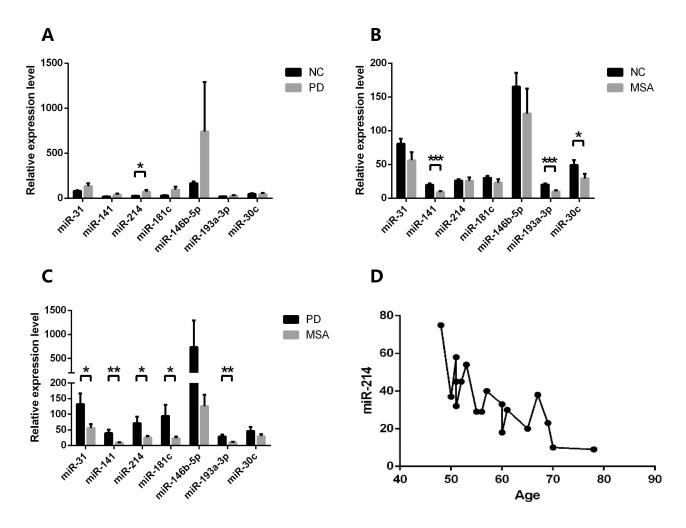
Fig. 1. Comparison of the expression levels of 7 miRNAs in PD patients, MSA patients, and controls. (A) PD versus NC. (B) MSA versus NC. (C) PD versus MSA. (D) The serum miR-214 level of 18 PD patients tended to decrease with age. Data were reported as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

3p, and miR-30c. The AUC increased to 0.895 (P < 0.001, 95% CI = 0.807~983), indicating that the diagnostic accuracy could be improved through the combined application of these miRNAs. A model that differentiated PD from MSA by executing a similar analysis was generated (Fig. 2B), and it included miR-31, miR-141, miR-214, miR-181c, and miR-193a-3p. The diagnostic accuracy was improved to AUC of 0.951 with this model (P < 0.001, 95% CI = 0.870~1.000).

3.3 Correlation analysis of the expression of 7 miRNAs in the serum of PD patients and clinical symptoms

We used Spearman's correlation analysis to estimate the correlation between miRNA expression in PD patients and the patient's clinical information and found the following (Table S2): HAMD was negatively correlated with miR-31; HAMA was negatively correlated with all of the microRNAs except miR-193a-3p; PDNMS was negatively correlated with miR-214 and miR-30c; and UPDRS II was negatively correlated with miR-181c, miR-30c, and miR-193a-3p.

3.4 Bioinformatics predicts PD- and MSA-related target genes for each microRNA

To further investigate the possible roles of 4 miRNAs in the development of PD and MSA, we used miRNA target prediction database sites, including TargetScan, miRanda, and PicTar, for the bioinformatics prediction analysis. The results show that some PD- and MSA-related genes are potential target genes for these 7 miRNAs (Table 2). Interestingly, some of these genes may be simultaneously regulated by 2 or more miRNAs in our study. Additionally, the miRNAs coregulate target genes of PD and MSA.

4. Discussion

Neural miRNAs may be stably packaged in microvesicles and conveyed to blood and other peripheral biofluids (urine, saliva, breast milk) (Alexander et al., 2015; Cai et al., 2018; Haqqani et al., 2013; Moldovan et al., 2013; Valadi et al., 2007). Some miRNAs have similar changes in serum and brain tissue, suggesting that changes in serum miRNAs can be used to predict changes in miRNAs in the central nervous system (CNS). For example, miR-153 and miR-223 are significantly downregulated in both nerves and circulation (Cressatti et al., 2019). MiR-9 has also been re-
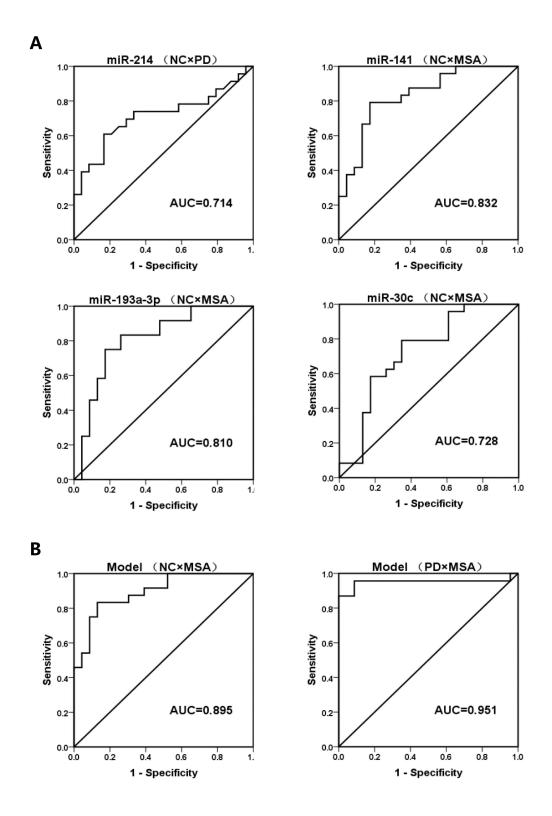
A

B

Fig. 2. The ROC curve was used to analyze the sensitivity and specificity of serum miRNAs in the diagnosis and differential diagnosis of PD and MSA and to evaluate the clinical value of miRNAs. Sensitivity is the ordinate, and 1-specificity is the abscissa. (A) The ROC curves of miRNAs that were significantly dissimilar between the patient groups. The compared patient groups were shown in parentheses. The area under the curve (AUC) values ranged from 0.714 to 0.832. (B) The ROC curves of models created from the binary logistic regression to enhance the distinction between the groups. To differentiate MSA from NC, a model including miR-141, miR-193a-3p, and miR-30c was built and resulted in an AUC of 0.895. For the model of PD versus MSA, miR-31, miR-141, miR-181c, miR-193a-3p, and miR-214 were included, and it showed an AUC of 0.951.

Table 2. Target genes linked to PD or MSA for each miRNA

microRNA	Target genes linked to PD or MSA
miR-31	*PARK2, GIGYF2*
miR-141	*LRRK2, SNCA, PARK2*
miR-214	*SNCA, GIGYF2, PRKAG2, UCHL1*
miR-30c	*LRRK2, GIGYF2, UCHL1, SQSTM1*
miR-181c	*LRRK2, PARK2, MAPT, PRKAG2*
miR-146b-5p	*PARK2, SLC1A4*
miR-193a-3p	*LRRK2, GIGYF2, SNCA*

SNCA synuclein alpha, *PARK2* parkin E3 ubiquitin-protein ligase, *GIGYF2* GRB10-interacting GYF protein 2, *LRRK2* leucine-rich repeat kinase 2, *PRKAG2* protein kinase AMP-activated gamma 2 noncatalytic subunit, *UCHL1* ubiquitin-C-terminal hydrolase L1, *SQSTM1* sequestosome 1, *MAPT* microtubule-associated protein tau, *SLC1A4* solute carrier family 1 member 4.

ported to be decreased in both blood and brain tissue (Jin et al., 2013). Distinguishing PD from MSA by detecting the content and types of miRNAs in serum is of great significance. Previous studies have almost always used external parameters, and increasing evidence now exists that internal references are unstable in testing serum miRNAs. In contrast, we used a short-chain RNA that was not found in humans and animals and did not affect human serum as an external reference.

Our previous work confirmed that 7 miRNAs levels (miR-30c, miR-31, miR-141, miR-146b-5p, miR-214, miR-181c, and miR-193a-3p) in the serum of patients with PD were lower compared with the controls (Dong et al., 2016). Based on the achievements of the previous work, we designed a way to further test the expression levels of the 7 miRNAs in early PD, MSA, and control groups. The results showed that the levels of miR-214 in the serum of PD patients were distinctly higher than those in the serum of the healthy control group (Fig. 1A). These results are different from our previous results that the levels of miR-214 in the serum of PD patients were reduced compared with the normal controls (Dong et al., 2016). The reason may be that the patients in the previous study had a few years of onset (the average was 6 years), but the de novo patients in this study had less than 1 year of onset. The serum miRNA levels may change dynamically in patients with early-onset and late-onset. The miR-214 levels were upregulated at the beginning of PD. This may be due to the existence of compensation in the body. We speculate that the levels decreased again as patients became older and more ill (Fig. 1D). This result provides a clinical basis for miR-214 to become an early biomarker of PD.

MiR-214 was downregulated in the mesencephalon of PD mice and MPP$^+$-treated SH-SY5Y neuroblastoma cells (Wang et al., 2015). This result is in line with our previous results. In the sera of patients with PD who had PD for several years, miR-214 was significantly downregulated (Dong et al., 2016). In summary, miR-214 may be upregulated in the early PD stage (incidence within 1 year) (Fig. 1A). As the disease progresses, the level of miR-214 may decrease again (Fig. 1D). These speculations need further verification. The study demonstrated that miR-214 could restrain autophagy and the apoptosis of hippocampal neurons as a functional miRNA (Zhang et al., 2016). MiR-214 is related to the nervous system and may affect the pathogenesis of PD.

Other miRNAs in 7 miRNAs were also reported to have altered levels in autopsy brains of PD patients compared with controls. The decreased expression levels of miRNA-30 and miRNA-193 were verified in autopsy samples of PD patients (Briggs et al., 2015). MiR-181 was identified to be associated with PD from the brain and blood samples (Chatterjee and Roy, 2017). However, how these miRNAs change in MSA is still unknown. We further compared the expression differences of 7 serum miRNAs between MSA patients and healthy controls. The relative expression of 3 (miR-141, miR-193a-3p and miR-30c) of the 7 miRNAs were significantly different from the control group (Fig. 1B). By comparison, miR-141 was most important for identifying normal controls and MSA (Fig. 2A). The difference in the expression levels of 7 serum miRNAs between PD and MSA patients indicated that miR-31, miR-141, miR-181c, miR-193a-3p, and miR-214 were significantly lower in MSA than in PD (Fig. 1C). The specificity and sensitivity of these 5 miRNAs showed that they had diagnostic significance (Fig. 2B).

Our experiments further analyzed the correlations between these miRNAs expression levels and PD clinical scales. The results showed that the expression of miR-214 was negatively correlated with anxiety and PDNMS. The more severe the anxiety and nonmotor symptoms were, the lower the level of miR-214. In our study, we selected patients who did not take anti-PD drugs within 1 year of onset. Their H-Y stage and intelligence damage were relatively low. The increased level of miR-214 may be a compensatory response in the body at the beginning of the disease. With the increase of age, the miR-214 levels decreased gradually. The correlations between miRNA expression and clinical scales need to be further studied in an amplified sample size.

To date, 18 PD-related chromosomal sites have been reported, *PARK1-PARK18*. Some new genes have been proposed, such as *GIGYF2 (Park11)*, *VPS35*, and *Pitx3* (Chen et al., 2013; Lautier et al., 2008; Liu et al., 2012). The genes related to MSA include *SQSTM1* and *SLC1A4*. *SNCA* is a gene related to both MSA and PD.

Other groups have reported that miR-214 can target *SNCA*, a key protein of PD, and reduce its protein expression (Wang et al., 2015). Altered levels of miR-214 may affect *SNCA* protein levels and thus affect disease progression. For the MSA-related genes, *SNCA* was predicted to be the target gene of miR-141 (Table 2). In our work, miR-141 was the most important for identifying normal controls and MSA. These predictions have yet to be validated by more *in vivo* and *in vitro* experiments.

5. Conclusions

We have proved that miR-214 can be used as a molecular marker for PD diagnosis and its early diagnosis. Five microRNAs (miR-31, miR-141, miR-214, miR-181c, and miR-193a-3p) can be used for the antidiastole of PD and MSA.

Author contributions

Study conception, design, and supervision: WGL, HD, YH, and HC; history collection, assessment scales: PH and YC; sample collection: LTL, CYY, and LY; implementation of the experiment: JHY and PH; image drafting and revision: JHY; data anal-

ysis, statistical analysis: JHY and HD; manuscript writing: JHY; manuscript revising: HD, LWG, HZ, ZJZ, HZ, and QHY. All authors consented to the final version of this manuscript.

Ethics approval and consent to participate

The ethics committee approved the study of the Affiliated Brain Hospital of Nanjing Medical University, and it was completed based on the ethical standards established by the 1964 Declaration of Helsinki and its later amendments. All participants provided informed consent.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://jin.imrpress.com/EN/10.31083/j.jin.2020.03.163.

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