Intestinal flora imbalance affects the prognosis of Parkinson's disease by regulating the expression of fecal microRNA-146a and GDNF: a preliminary study

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Abstract

Introduction: There is mounting evidence showing the association between the gut microbiome and Parkinson's disease (PD). In this study, we aimed to explore the role of intestinal flora imbalance in the progression of PD and the underlying molecular mechanism involving the regulatory relationship between miR-146a and glial cell line-derived neurotrophic factor (GDNF).
Material and methods: A total of 42 PD patients were recruited to this study and grouped according to their prognosis. Real-time PCR, computational analysis and luciferase assay were carried out to explore the role of miR-146a and GDNF in PD patients as well as the regulatory relationship between the expression of miR-146a and GDNF. A rat model was used to study the effect of intestinal flora imbalance by treating the rats with *Lactobacillus rhamnosus* NCDC17.
Results: A lower count of total *Lactobacillus* and *Bacteroides fragilis* may predict the progression of PD. GDNF was identified as a virtual target gene of miR-146a, whose expression in fecal, plasma and cerebral tissue samples was significantly reduced in PD patients. In addition, the presence of miR-146a precursors and GDNF siRNA significantly reduced the mRNA and protein levels of GDNF in SH-SY5Y and U251 cells, and the inhibitory effect of miR-146a precursors on GDNF expression was similar to that of GDNF siRNA.
Conclusions: Taken together, the findings of this study demonstrated that GDNF is a key factor involved in the prognosis of PD. We suggest that the administration of *Lactobacillus rhamnosus* NCDC17 promoted the pathogenesis of PD by regulating the expression of miR-146a and GDNF in the brain.

Key words: Parkinson's disease, miR-146a, intestinal flora, GDNF, prognosis.

Introduction

Parkinson's disease is characterized by selectively degenerating dopaminergic neurons in the substantia nigra and the production of fibrillar cytoplasmic Lewy bodies that contain α-synuclein and ubiquitin [1]. Rare familial Parkinson's disease (PD) cases have been associated with mutations in parkin or α-synuclein, though the genetic mechanisms of PD remain uncertain [2–4].

The microflora in the human intestine contains a dynamically changing mixture of microbes [5]. Patients with sepsis, inflammatory bowel disease (IBD), and chronic liver disease frequently exhibit disturbances

in the gut flora [6, 7]. Fecal samples from liver cirrhosis patients show significantly increased counts of viable Gram-negative and Gram-positive aerobic bacterial species along with significantly lowered numbers of obligate anaerobes, including *Bacteroides* and *Bifidobacterium* [8]. Changes in the components of the intestinal flora show a pathophysiological relationship with Parkinson's disease (PD) [9]. More significantly, the potential cause-effect correlation between PD pathogenesis and gut flora has been confirmed in animal models of PD. In recent studies, it has been indicated that probiotics are beneficial for the symptoms of constipation in PD patients [9]. Moreover, in mice, it was recently shown that fecal microbiota transplantation (FMT) had a protective effect against the neurotoxicity triggered by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Probiotics and FMT can be used in effective therapies for PD.

MicroRNAs (miRNAs) are non-coding RNAs with a short length of around 22 nucleotides and can negatively regulate post-transcriptional expression of their target mRNAs [10]. Perturbations in miRNA expression were found in many nervous system disorders, such as PD, indicating that miRNA dysregulation is related to the pathogenesis of these diseases [11]. Host fecal miRNAs can directly regulate microbial gene expression and growth. When bacteria are cultured with miRNAs, the host miRNAs can enter the bacteria and specifically regulate bacterial gene expression and growth [12]. In a previous study, it was confirmed that miRNAs such as miR-155 and miR-1224 were present in the feces and gut lumen of humans and mice [12]. Also, the gut microbiota can alter tumor cells in colorectal cancer patients through their metabolites, while several miRNAs including miR-34a were proved to serve as mediators of these metabolites [13]. By affecting the expression of these miRNAs, the metabolites produced by microbiota can alter the signaling pathways in tumor cells [13]. Moreover, the expression of miR-181 was also demonstrated to influence gut dysbiosis and contribute to the development of obesity and insulin resistance [14]. In this study, we selected several candidate miRNAs, including miR-486-3p [15], miR-96 [16], miR-342-3p [17], miR-155 [18], miR-146a [19, 20], miR-34a [21], miR-182 [22], miR-183 [22], miR-505 [23] and miR-221 [24], and studied their possible role in intestinal flora imbalance during the progression of PD.

As an effective neurotrophic factor that increases the viability of dopaminergic neurons of the midbrain, the glial cell line-derived neurotrophic factor (GDNF) was separated from the B49 glial cell line of rats [25]. In recent studies, it has been indicated that this molecule shows various biological activities and affects the peripheral and central nervous systems. It has been shown that GDNF has restorative and protective properties *in vivo*, especially with respect to the neurons in dopaminergic nerves in the substantia nigra [26–28]. In *in vitro* studies, it has been shown that GDNF can decrease cell death induced by oxidative stress, and evidence shows that a single injection with GDNF increases the activity of catalase, glutathione peroxidase, and superoxide dismutase in rodents [29–31]. Since oxidative stress is enhanced in PD patients, GDNF can be beneficial to the motor function of PD patients [32–35].

GDNF has been reported as a key factor in the prognosis of PD [36]. By searching an online miRNA database, we found that GDNF contains a miR-146a binding site in its 3'-UTR. In this study, we recruited PD patients and followed them up for one year to investigate the effect of intestinal flora imbalance on the expression of PD-related miRNAs, as well as to investigate the effect of these miRNAs on the expression of GDNF in the brain.

Material and methods

Human subjects

We started our study in 2013. All studies were performed after signed informed consent forms were obtained from all subjects. 42 PD patients were followed up for about 20 months. The inclusion criteria are: (1) Subjects diagnosed with PD; (2) Unified Parkinson's Disease Rating Scale (UPDRS) score of 1 or 2; (3) Records of disease progression are available. The exclusion criteria are: (1) Those who are diagnosed with other neurological disorders such as stroke or glioma; (2) Those who are diagnosed with cardiac, renal or liver dysfunction; (3) Those who are diagnosed with intestinal diseases such as Crohn's disease, intestinal malfunction or malignancy.

The patients were then divided into two groups based on their prognosis indicated by the severity of PD in year 1 compared with the severity of PD in year 0. Accordingly, patients who received a UPDRS score of 1 or 2 in year 1 were allocated to the stable group (N = 24) and patients who received a UPDRS score of 3 or 4 in year 1 were allocated to the deteriorated group (N = 18). The mean age of the 42 PD patients was about 70 years (ranging 40–81 years). In years 0 and 1, fecal specimens were collected from the 42 patients for analysis. All studies were approved by the Ethics Committee of the Yantai Yuhuangding Hospital Affiliated to Qingdao University in accordance with the last version of the Declaration of Helsinki.

Clinical assessment

The severity of PD was assessed in years 0 and 1 using parts I–IV of the Unified Parkinson's Dis-

ease Rating Scale (UPDRS), the bedside Hoehn and Yahr (HY) scale, the Frontal Assessment Battery (FAB) [25], and the Mini Mental State Examination (MMSE). As only outpatients were included, HY and UPDRS III were assessed 2–3 hours after treatment and evaluated in the ON phase. The volume of anti-parkinsonian agent was evaluated using the L-dopa-equivalent dose (LED). The authors carried out a survey with a questionnaire to measure the frequency of milk consumption, and stool samples were taken per week to assess the status of constipation.

Fecal sampling and microbiota examination

The fecal samples were collected from PD patients and their microbiota profiles were examined according to a previously published method [37]. In brief, each patient used a fecal container to collect their fecal samples every morning. The containers were transferred on ice and stored at –80°C prior to testing. Total fecal DNA was extracted from the samples using a QIAamp DNA Stool Mini Kit (Qiagen GmbH, Germany) following the manual provided by the manufacture. The concentration of genomic DNA in each sample was quantified using a Nano-Drop 2000 spectrophotometer. The V3–V4 regions of the 16S ribosomal RNA were amplified by PCR and pyro-sequenced for taxonomic assignment following the in-house protocol targeting the V4–V5 regions of the bacterial 16S rRNA gene according to previously published methods [38].

Animals and treatment

All 24 adult male Wistar rats used in the present study were obtained from the experimental animal center at our institute. In the experiment, the rats were housed at 22 ±2°C and a density of four per cage under a 12/12 hours light/dark cycle. Before the beginning of the experiment, all rats were adapted for at least seven days and were fed with a normal chow diet. Then, these rats were assigned to three groups: a control group (rats fed on a normal chow diet, $n = 8$), a SHAM group (rats treated with milk and PBS, $n = 8$), and a treatment group (rats fed with milk fermented with *Lactobacillus rhamnosus* NCDC17, $n = 8$). After 12 weeks, all rats were decapitated following deep anesthetization by IP injection of 90 mg/kg pentobarbital. Their brains were snap frozen in liquid nitrogen to collect striatal tissues. Similarly, peripheral blood samples and feces samples were collected from each rat and stored in liquid nitrogen for functional analysis. All protocols of the animal experiment were approved by the Animal Ethics Committee of the Yantai Yuhuangding Hospital Affiliated to Qingdao University in accordance with the Guide for the Care and Use of Laboratory Animals.

Immunohistochemistry

Brain samples collected from the rat experimental groups were fixed with pre-cooled paraformaldehyde at 25°C for 30 minutes and then treated for 15 minutes with PBS containing 0.2% Triton X-100. The sections were then blocked with 5% normal goat sera at room temperature for 30 minutes and then incubated with primary anti-GDNF antibodies at 37°C for 60 minutes. Subsequently, the sections were developed with appropriate FITC conjugated secondary antibodies (1:1000 dilution; cat. no. ab6785; Abcam, Cambridge, MA) or TRITC-conjugated secondary antibodies (1:1000; cat. no. ab6725; Abcam, Cambridge, MA), counter-stained with DAPI (Vector Laboratories, Burlingame, CA), and mounted on cover slips. The expression of GDNF was visualized using an Olympus IX71 microscope.

RNA isolation and real-time PCR

Total RNA was isolated from fecal and plasma samples of PD patients and rat models and converted to cDNA via reverse transcription. Subsequently, the relative expression of miR-486-3p, miR-96, miR-342-3p, miR-155, miR-146a, miR-34a, miR-182, miR-183, miR-505 and miR-221 in the samples was measured using real-time PCR and SYBR Green Mix following a standard procedure. Similarly, the expression of fecal, plasma and cerebral miR-146a and GDNF was compared among different groups of PD patients. The relative expression of investigated genes was determined by the $2^{-\Delta\Delta Cq}$ method [39], and U6 and β-actin were used as internal controls for miRNAs and GDNF respectively. The sequences of primers used for real-time PCR are presented in Supplementary Table SI.

Analysis of fecal levels of acetate, butyrate and propionate

Hydrochloric acid was added to 20 mg of fecal samples, followed by hydrazinoquinoline derivatization and protein precipitation. The organic phase was diluted by the mobile phase prior to injection for mass spectrometry analysis, and the LC-MS/MS method was applied to measure the fecal levels of acetate, butyrate and propionate in the samples according to previously published methods [40].

Analysis of plasma levels of acetate, butyrate and propionate

The plasma levels of acetate, butyrate and propionate were analyzed following a previously published method [41]. For the extraction of acetate, butyrate and propionate, polypropylene hollow

fibers were immersed into diluted plasma samples. Subsequently, acetate, butyrate and propionate were injected into a fused-silica capillary column for gas chromatography analysis.

Cell culture and transfection

SH-SY5Y and U251 cells were cultured in DMEM + 10% FBS + appropriate antibiotics. When the cells were 90% confluent, they were assigned to three groups, i.e., a scramble control group (transfected with a scramble control miRNA), a miR-146a group (transfected with miR-146a precursors), and a GDNF siRNA group (transfected with GDNF siRNA). The transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The expression of GDNF mRNA/protein and miR-146a in transfected cells was measured at 48 h post transfection using Western blot and real-time PCR assays.

Vector construction, mutagenesis and luciferase assay

The presence of a miR-146a binding site in the 3'-UTR of GDNF was confirmed using TargetScan (www.targetscan.org) and the miRBase database (www.mirbase.org). Then, GDNF 3'-UTR was inserted into a psiCHECK2 vector (Promega, Madison, WI) termed wild-type GDNF 3'-UTR. In addition, the miR-146a binding site in the 3'-UTR of GDNF was subjected to site-directed mutagenesis to produce mutant GDNF 3'-UTR. Subsequently, SH-SY5Y and U251 cells were co-transfected with wild type/mutant GDNF 3'-UTR and miR-146a using Lipofectamine 2000. At 48 after transfection, the luciferase activity of transfected cells was measured using a luciferase assay (Promega, Madison, WI).

Western blot analysis

Proteins were extracted from clinical and cultured samples by lysis and centrifugation, resolved using 10% SDS-PAGE, blotted onto a PVDF membrane, blocked with 5% milk, and then incubated with primary anti-GDNF antibodies (1:5000 dilution; cat. no. ab176564; Abcam, Cambridge, MA). The membrane was washed with TBST three times and then incubated with horseradish peroxidase-labelled secondary antibodies (1:2000 dilution; cat. no. ab6721; Abcam, Cambridge, MA). The expression of GDNF protein was detected using enhanced chemiluminescence solutions (Bio-Rad, Hercules, CA) on a Kodak medical X-ray machine. β-actin was utilized as the internal control to quantify the relative protein expression of GDNF, and Western blots were quantified by ImageJ software (version 1.41; National Institutes of Health).

Statistical analysis

Each experiment was done in triplicate under each condition. All data were expressed as means ± standard deviation (SD). SPSS 19.0 was used for statistical analysis. Statistical differences were calculated using an unpaired two-tailed Student's *t*-test or multivariate analysis (inter-group comparisons) and one-way ANOVA followed by Tukey's test (multi-group comparisons). A p value of < 0.05 was deemed statistically significant.

Results

Comparison of bacterial counts between stable and deteriorated groups

As shown in Table I, the basic characteristics including demographic parameters and habitual consumption of milk showed no obvious difference between the stable and deteriorated PD groups. 19 bacterial species, including 6 anaerobic species predominating in the intestine, 5 potential pathogens and 8 *Lactobacilli,* were detected in year 0 and year 1 in stable and deteriorated groups. Student's *t* test was performed to compare the bacterial counts between the 2 groups in year 0 and year 1. As shown in Table II, there was no obvious difference in total fecal bacterial counts between year 0 and year 1 in the deteriorated group. In contrast, the stable group showed

Table I. Comparison of demographic and clinicopathological characteristics between stable and deteriorated Parkinson's disease (PD) groups

Characteristics	Stable group (*N* = 24)	Deteriorated group (*N* = 18)	*P*-value
Age [years]	70.32 ±15.32	69.65 ±14.19	0.672
Sex (male)	18 (75.00)	13 (72.22)	0.712
BMI	23.11 ±4.32	24.31 ±5.16	0.845
MMSE	28.53 ± 2.12	26.83 ±3.88	0.076
Habitual consumption of milk	16 (66.67)	12 (66.67)	0.558
Stool frequency [/week]	3.8 ±1.3	3.1 ±1.7	0.138

BMI – body mass index, MMSE – Mini Mental Sate Examination

Table II. Comparison of bacterial counts between years 0 and 1 in stable and deteriorated Parkinson's disease (PD) groups

Characteristics	Stable group (*N* = 24)			Deteriorated group (*N* = 18)		
	Year 0	Year 1	*P*-value	Year 0	Year 1	*P*-value
Prevotella	7.5 ±0.8	7.3 ±0.5	0.3056	7.4 ±0.6	7.6 ±0.7	0.3641
Enterococcus	7.3 ±0.9	7.2 ±0.4	0.6224	7.1 ±0.6	7.2 ±0.8	0.6743
Bifidobacterium	9.5 ±0.3	9.4 ±0.3	0.2542	9.6 ±0.5	9.5 ±0.7	0.6255
Total fecal bacterial count	10.5 ±0.3	10.3 ±0.5	0.1013	10.8 ±0.4	10.7 ±0.3	0.4026
B. fragilis group	9.2 ±0.4	7.6 ±0.4	< 0.0001	9.4 ±0.4	9.3 ±0.7	0.6030
C. coccoides group	9.4 ±0.4	9.2 ±0.3	0.0567	9.6 ±0.8	9.7 ±0.3	0.6247
Total *Lactobacillus*	7.8 ±0.5	6.2 ±0.5	< 0.0001	7.9 ±0.9	7.8 ±0.4	0.6706
Enterobacteriaceae	7.2 ±0.6	7.4 ±0.8	0.3328	7.0 ±0.8	6.8 ±0.7	0.4304
C. leptum subgroup	9.6 ±0.7	9.6 ±1.2	1.0000	9.5 ±0.7	9.6 ±0.5	0.6255
Staphylococcus	4.8 ±0.6	4.6 ±0.3	0.1536	4.6 ±0.9	4.5 ±0.6	0.6978
Atopobium cluster	9.6 ±0.9	9.5 ±0.7	0.6696	9.6 ±0.6	9.5 ±0.4	0.5609

lower counts of total *Lactobacillus* and *Bacteroides fragilis* in year 1 than in year 0, suggesting that lower counts of total *Lactobacillus* and *B. fragilis* in year 1 may be predictive for the progression of PD.

MiR-146a was associated with progression of PD

Real-time PCR was used to compare the expression of miR-486-3p (Figures 1 A and 2 A), miR-96 (Figures 1 B and 2 B), miR-342-3p (Figures 1 C and 2 C), miR-155 (Figures 1 D and 2 D), miR-146a (Figures 1 E and 2 E), miR-34a (Figures 1 F and 2 F), miR-182 (Figures 1 G and 2 G), miR-183 (Figures 1 H and 2 H), miR-505 (Figures 1 I and 2 I) and miR-221 (Figures 1 J and 2 J) in fecal (Figure 1) and plasma (Figure 2) samples between the stable and deteriorated groups. The results showed that only the expression of miR-146a was reduced in the stable group, while the expression of other miRNAs was comparable between the two groups. Moreover, the levels of acetate (Figures 1 K and 2 K), butyrate (Figures 1 L and 2 L) and propionate (Figures 1 M and 2 M) in fecal (Figure 1) and plasma (Figure 2) samples were comparable between the stable and deteriorated groups. Meanwhile, the level of plasma GDNF in the deteriorated group was evidently reduced compared with the stable group (Figure 2 N), and the correlation analysis for plasma GDNF did not find it to be significantly correlated with the plasma expression of acetate (Figure 2 O), butyrate (Figure 2 P) or propionate (Figure 2 Q).

GDNF was a virtual target gene of miR-146a

To further study the potential role of miR-146a in the progression of PD, a public online miRNA database was utilized to search the target genes of miR-146a. As shown in Figure 3 A, a complementary binding site of miR-146a was found in GDNF 3'-UTR. To clarify whether miR-146a directly mediates GDNF expression via binding to GDNF 3'-UTR, we conducted luciferase assays by co-transfecting SH-SY5Y and U251 cells with wild-type and mutant GDNF 3'-UTR and miR-146a mimics. As shown in Figure 3 B, only the SH-SY5Y and U251 cells co-transfected with miR-146a mimics and wild type GDNF 3'-UTR exhibited lower luciferase activity compared with the NC group, while the luciferase activity of SH-SY5Y and U251 cells co-transfected with miR-146a mimics and mutant GDNF 3'-UTR showed no obvious difference, suggesting that miR-146a directly targeted GDNF to reduce its expression.

Differential expression of miR-146a and GDNF among various groups

The expression of miR-146a and GDNF mRNA/protein in fecal, plasma and cerebral samples of rats was investigated. As shown in Figure 4, miR-146a expression in the fecal (Figure 4 A), plasma (Figure 4 B) and cerebral (Figure 4 C) samples of the treatment group was much higher than that in the untreated and sham groups, while the mRNA (Figure 5 A) and protein (Figure 5 B) expression of GDNF showed an opposite trend. Also, according to the immunohistochemistry results of GDNF shown in Figure 6, GDNF level was evidently reduced in the cerebral samples of the treatment group.

Effect of miR-146a on expression of GDNF

To further confirm whether miR-146a negatively regulates GDNF expression, we measured the mRNA and protein expression of GDNF in SH-SY5Y (Figure 7) and U251 (Figure 8) cells transfected with miR-146a precursors or GDNF siRNA. The ex-

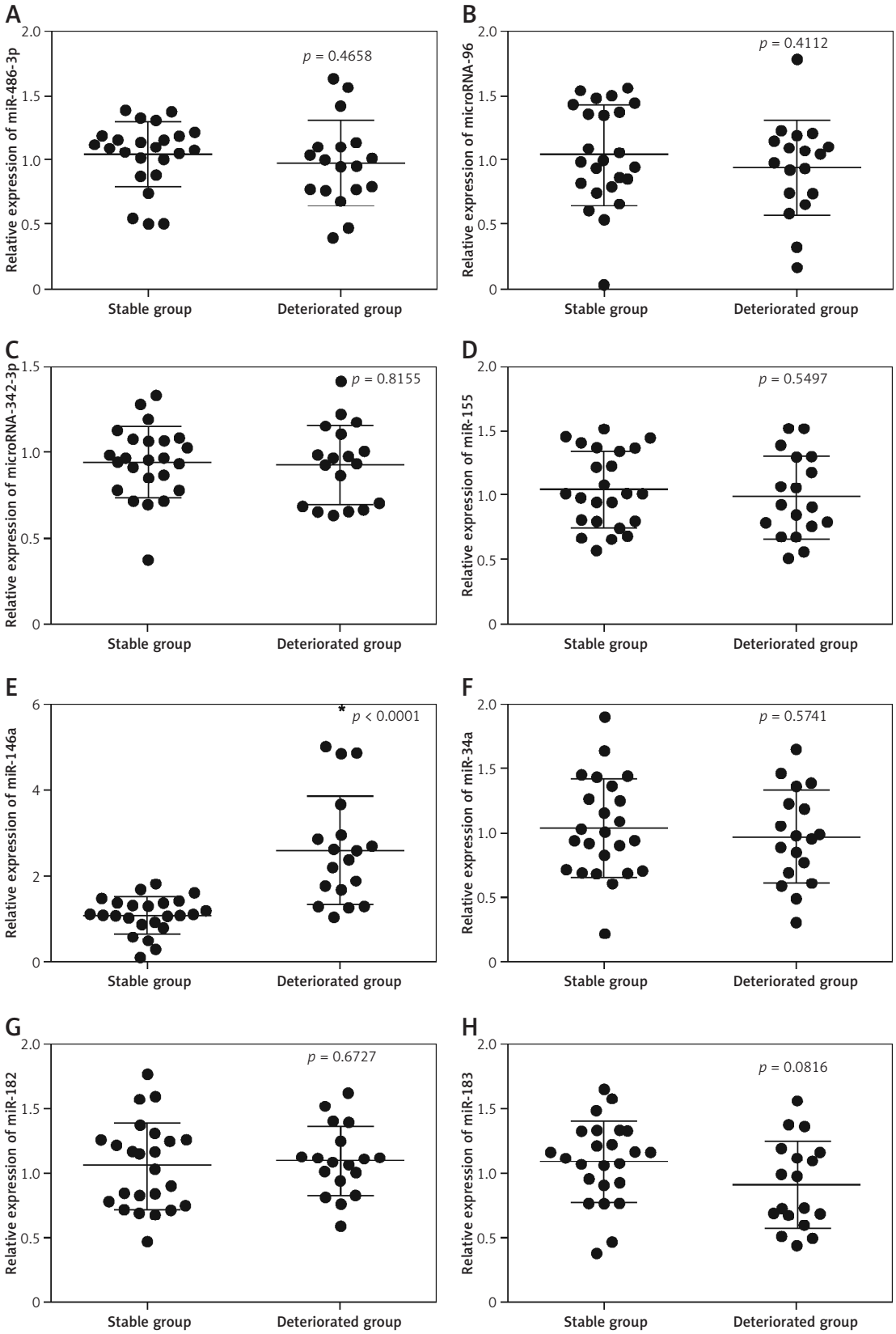

Figure 1. Fecal miR-146a expression was associated with progression of PD, and miR-146a (**E**, $p < 0.001$) expression in the stable group was much lower than that in the deteriorated group. Expression levels of miR-486-3p (**A**, $p = 0.4658$), miR-96 (**B**, $p = 0.4112$), miR-342-3p (**C**, $p = 0.8155$), miR-155 (**D**, $p = 0.5497$), miR-34a (**F**, $p = 0.5741$), miR-182 (**G**, $p = 0.6727$), miR-183 (**H**, $p = 0.0816$) were comparable between the stable and deteriorated groups (*$p < 0.05$ compared with the stable group)

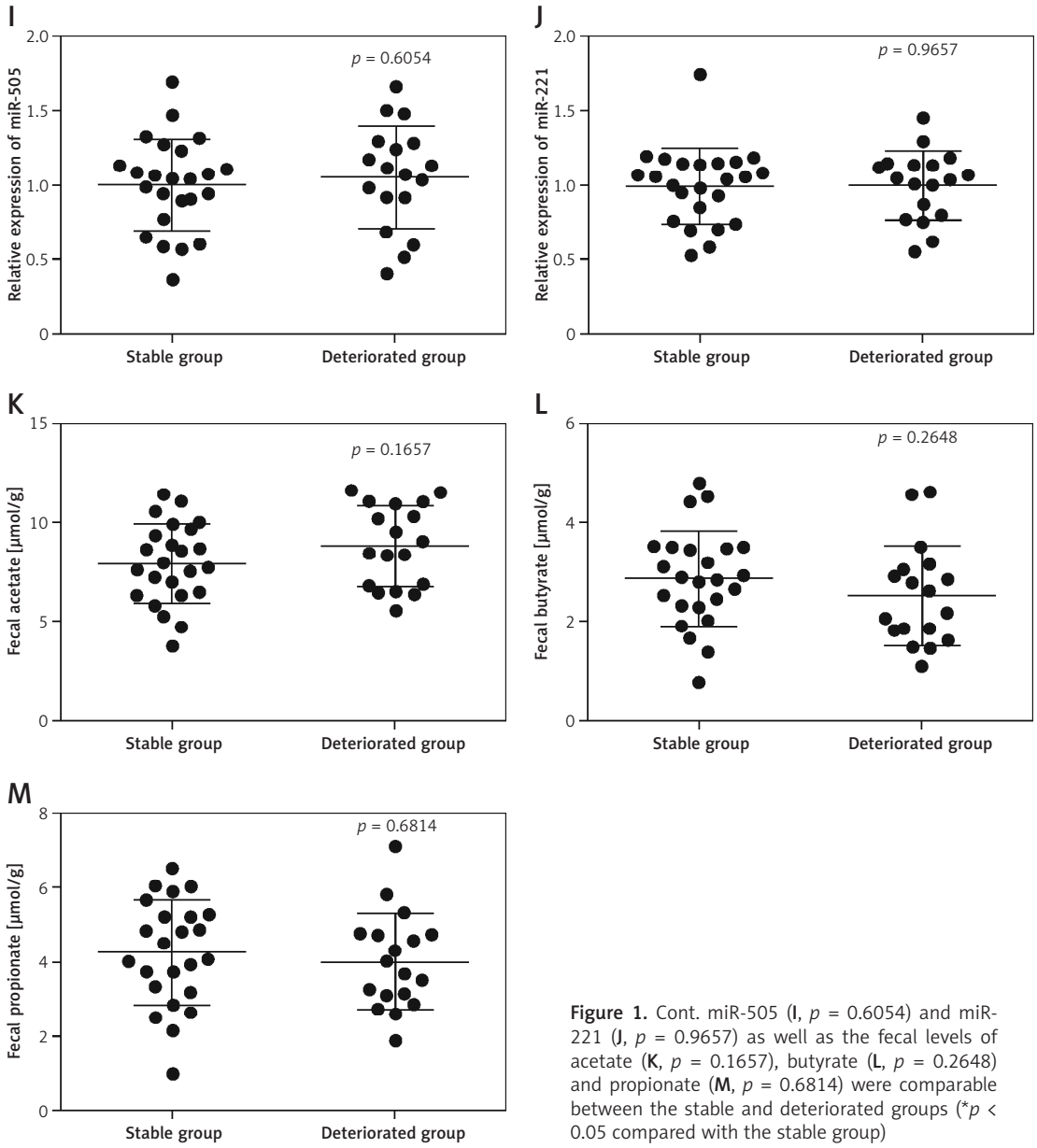

Figure 1. Cont. miR-505 (**I**, $p = 0.6054$) and miR-221 (**J**, $p = 0.9657$) as well as the fecal levels of acetate (**K**, $p = 0.1657$), butyrate (**L**, $p = 0.2648$) and propionate (**M**, $p = 0.6814$) were comparable between the stable and deteriorated groups (*$p < 0.05$ compared with the stable group)

pression of GDNF mRNA (Figures 7 A and 8 A) and protein (Figures 7 B and 8 B) in SH-SY5Y (Figure 7) and U251 (Figure 8) cells transfected with miR-146a precursors or GDNF siRNA was significantly reduced.

Discussion

In this study, we enrolled 42 PD patients and performed real-time PCR to compare the expression of miR-486-3p, miR-96, miR-342-3p, miR-155, miR-146a, miR-34a, miR-182, miR-183, miR-505 and miR-221 in fecal and plasma samples between patients in the stable and deteriorated groups. The results showed that the activity of *Lactobacillus* and *B. fragilis* was respectively up-regulated and down-regulated in the deteriorated group. In addition, miR-146a expression was higher in the deteriorated group compared with that in the stable group.

As one of the most common chronic diseases causing neurodegeneration, PD is primarily characterized by significant loss of nigrostriatal dopaminergic neurons. Many genes have been found to be associated with rare forms of familial PD [42, 43]. Nevertheless, most PD cases are sporadic and not associated with a known mutation [44]. In addition, irritable bowel syndrome (IBS) was observed in about 24.3% of PD patients [45], who showed disrupted gut microbiota and a decreased abundance of *Prevotella* species in feces [45]. Our study identified different bacteria involved in the progression of PD, and such a discrepancy can be attributed to the different PD patient populations in the two studies. It was also found that patients

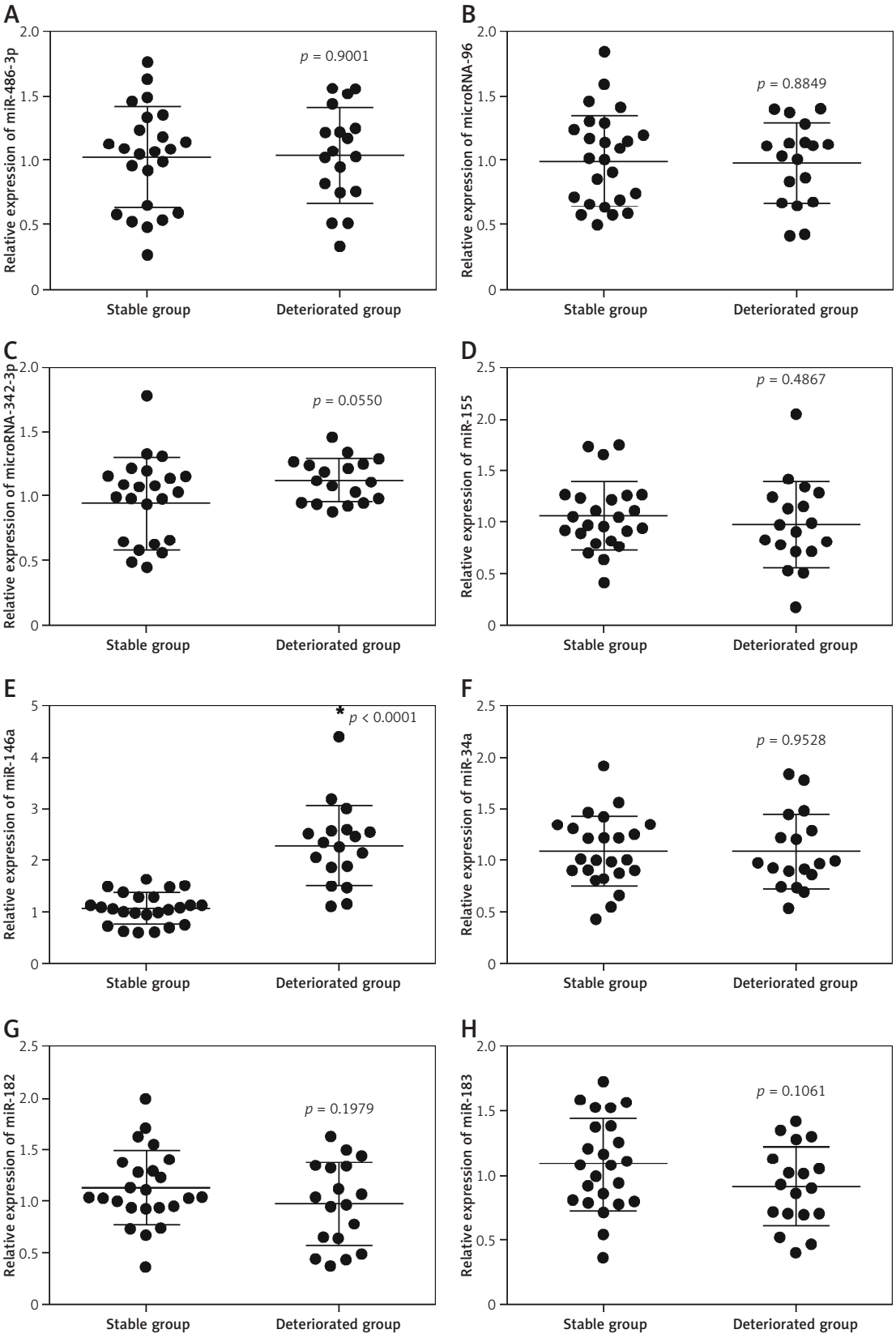

Figure 2. Plasma miR-146a expression was associated with progression of PD, and miR-146a (**E**, $p < 0.001$) expression in the stable group was much lower than that in the deteriorated group. Expression levels of miR-486-3p (**A**, $p = 0.9001$), miR-96 (**B**, $p = 0.8849$), miR-342-3p (**C**, $p = 0.0550$), miR-155 (**D**, $p = 0.4867$), miR-34a (**F**, $p = 0.9528$), miR-182 (**G**, $p = 0.1979$), miR-183 (**H**, $p = 0.1061$), were comparable between the stable and deteriorated groups. (*$p < 0.05$ compared with the stable group)

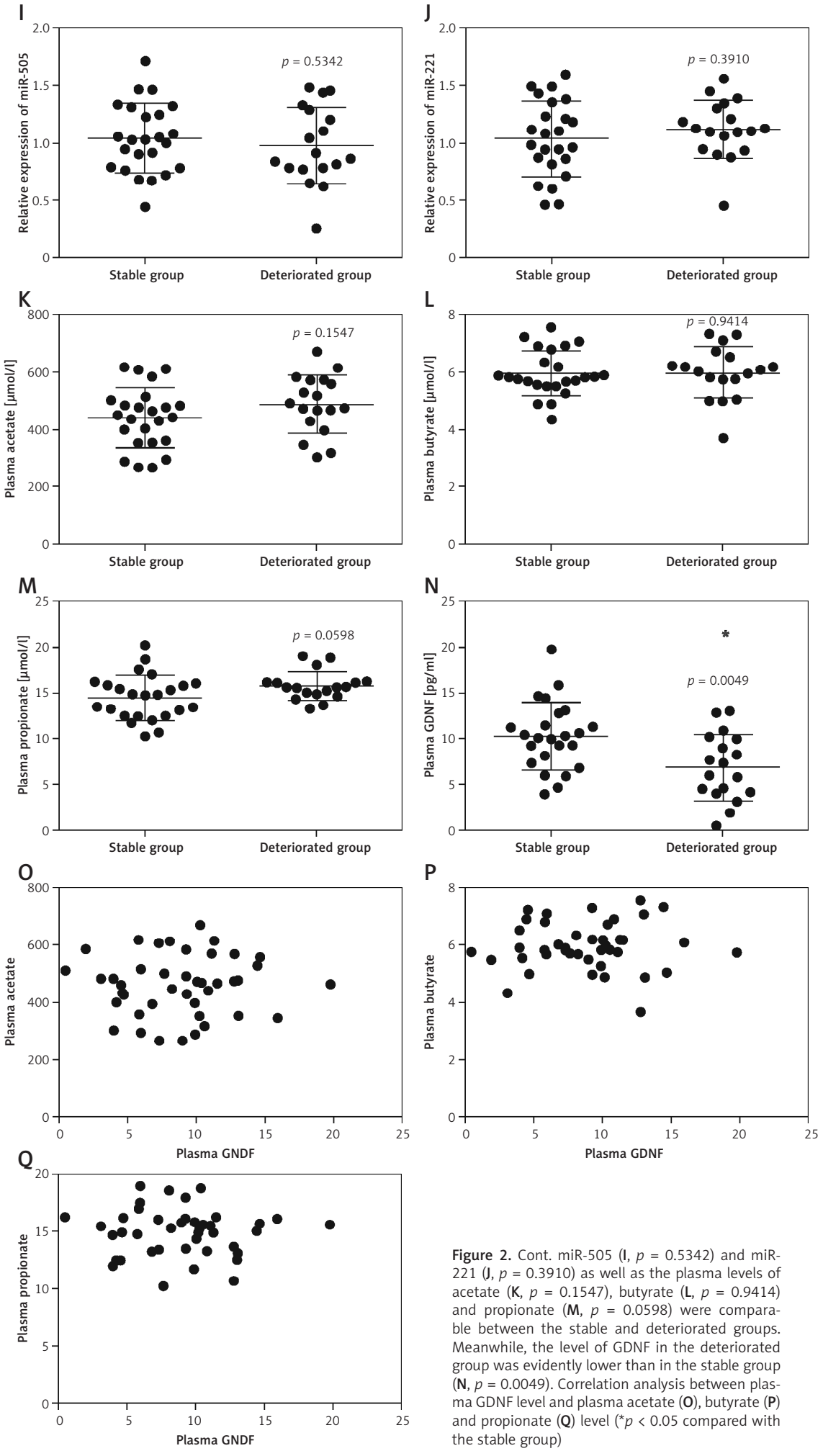

Figure 2. Cont. miR-505 (**I**, $p = 0.5342$) and miR-221 (**J**, $p = 0.3910$) as well as the plasma levels of acetate (**K**, $p = 0.1547$), butyrate (**L**, $p = 0.9414$) and propionate (**M**, $p = 0.0598$) were comparable between the stable and deteriorated groups. Meanwhile, the level of GDNF in the deteriorated group was evidently lower than in the stable group (**N**, $p = 0.0049$). Correlation analysis between plasma GDNF level and plasma acetate (**O**), butyrate (**P**) and propionate (**Q**) level (*$p < 0.05$ compared with the stable group)

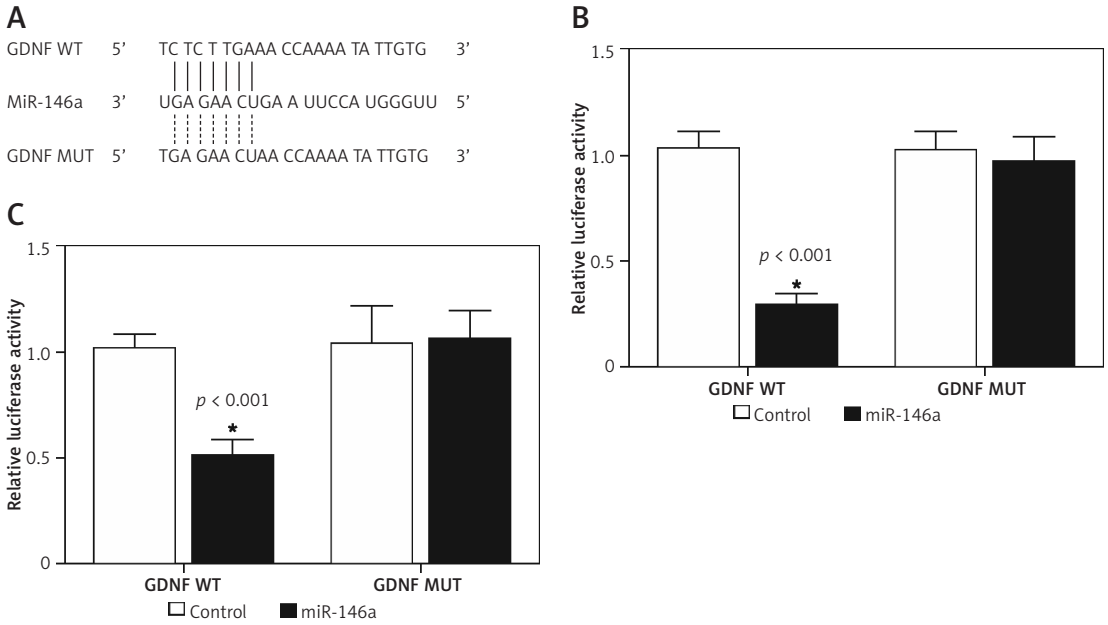

Figure 3. Computational analysis showed the presence of a miR-146a seed sequence in the 3'-UTR of GDNF (**A**), and the luciferase assay showed that the luciferase activity of SH-SY5Y cells co-transfected with miR-146a and wild-type GDNF 3'-UTR was much lower than that in the scramble control group, while the luciferase activity of the cells co-transfected with miR-146a and mutant GDNF 3'-UTR showed no difference compared with that in the scramble control group (**B**, $p < 0.001$). Similar results were obtained in U251 cells (**C**, $p < 0.001$), which confirmed GDNF as a virtual target gene of miR-146a (*$p < 0.05$ compared with the control group)

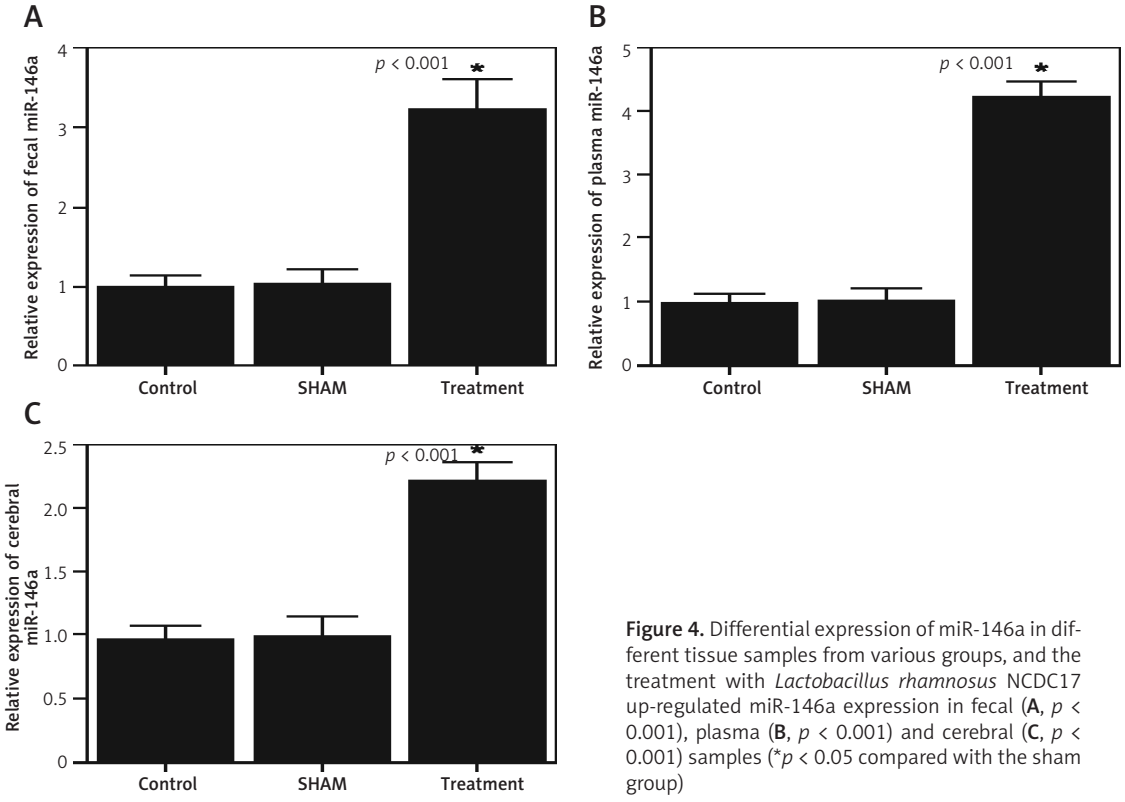

Figure 4. Differential expression of miR-146a in different tissue samples from various groups, and the treatment with *Lactobacillus rhamnosus* NCDC17 up-regulated miR-146a expression in fecal (**A**, $p < 0.001$), plasma (**B**, $p < 0.001$) and cerebral (**C**, $p < 0.001$) samples (*$p < 0.05$ compared with the sham group)

with progressive PD showed a lower count of *Bifidobacterium* species in their fecal specimens [46], and constipation and stool consistency are associated with intestinal flora diversity and abundance of different bacteria species [47]. For example, patients with functional constipation showed decreased lactobacilli and bifidobacteria counts. In addition, probiotics could improve ab-

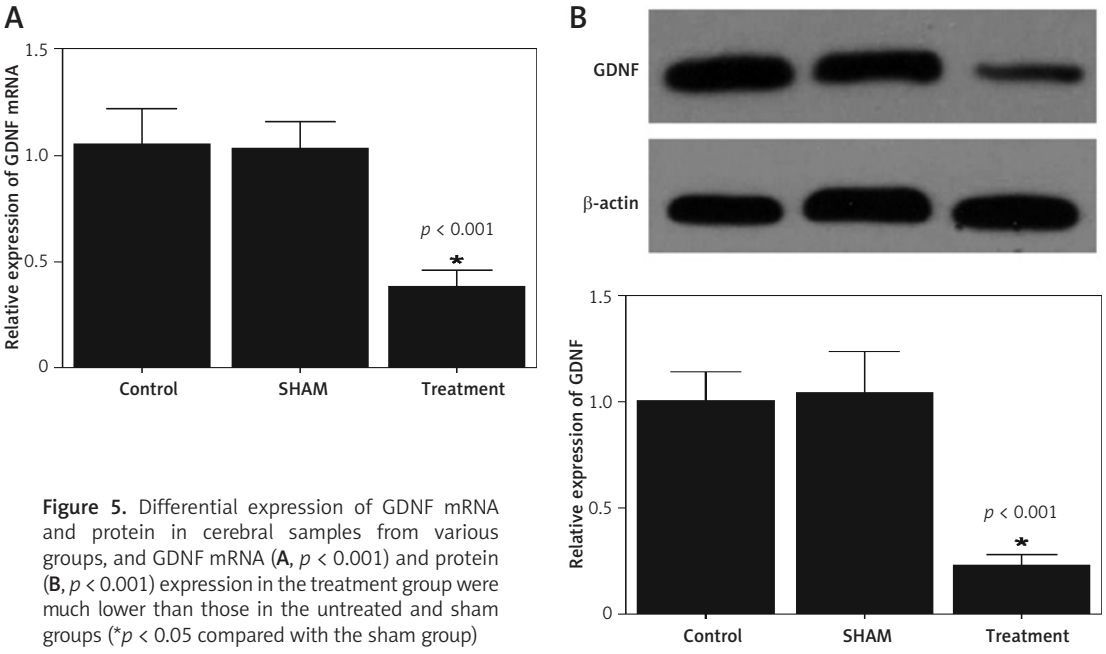

Figure 5. Differential expression of GDNF mRNA and protein in cerebral samples from various groups, and GDNF mRNA (**A**, $p < 0.001$) and protein (**B**, $p < 0.001$) expression in the treatment group were much lower than those in the untreated and sham groups (*$p < 0.05$ compared with the sham group)

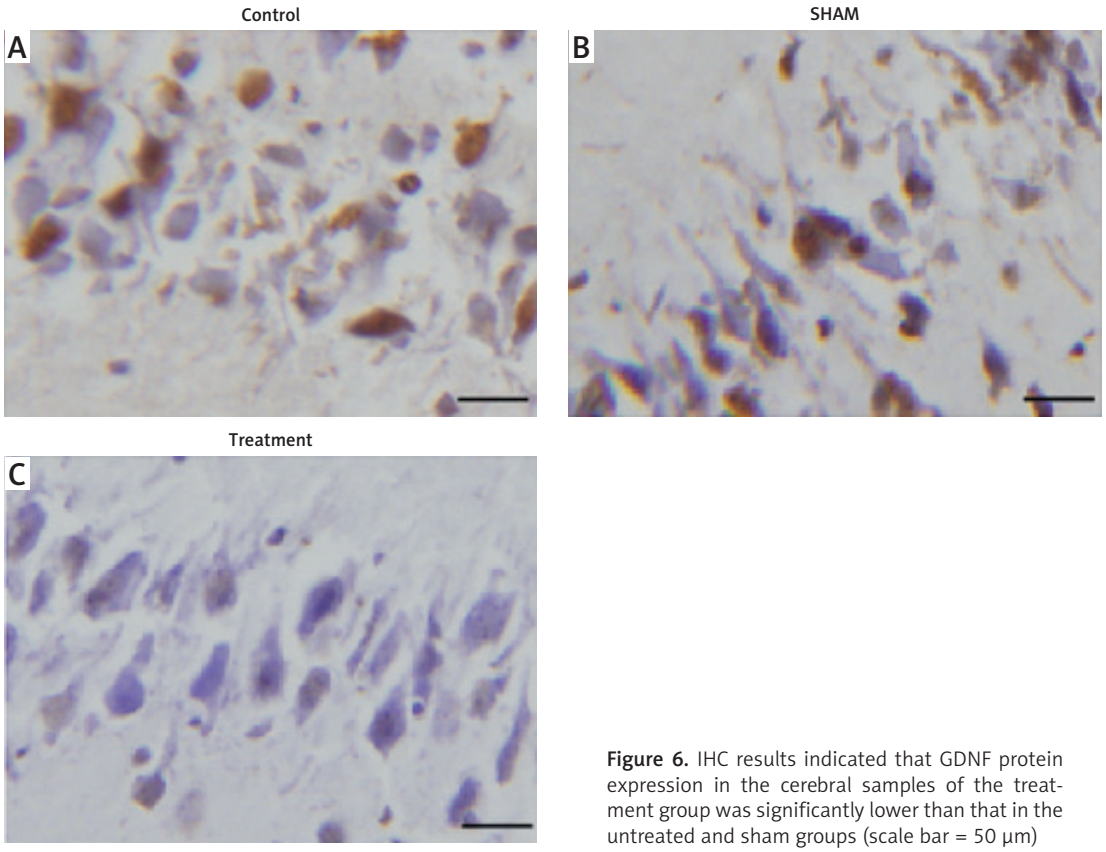

Figure 6. IHC results indicated that GDNF protein expression in the cerebral samples of the treatment group was significantly lower than that in the untreated and sham groups (scale bar = 50 μm)

normal gut motility and constipation in the general population [48].

As an abundant bacterial genus beneficial to animals and humans, *Lactobacillus* has been applied in the food and medicine industries for years. *Lactobacillus* enrichment may have different effects on adults and infants, although *Lactobacillus* is generally beneficial to the gastrointestinal tract and enhances the resistance of the gut to pathogens. *Bacteroides* species are Gram-negative, anaerobic, non-spore-forming bacilli and normal inhabitants in the intestinal tract, vagina and mouth [49]. However, it has been found that infection by this type of bacilli is associated with

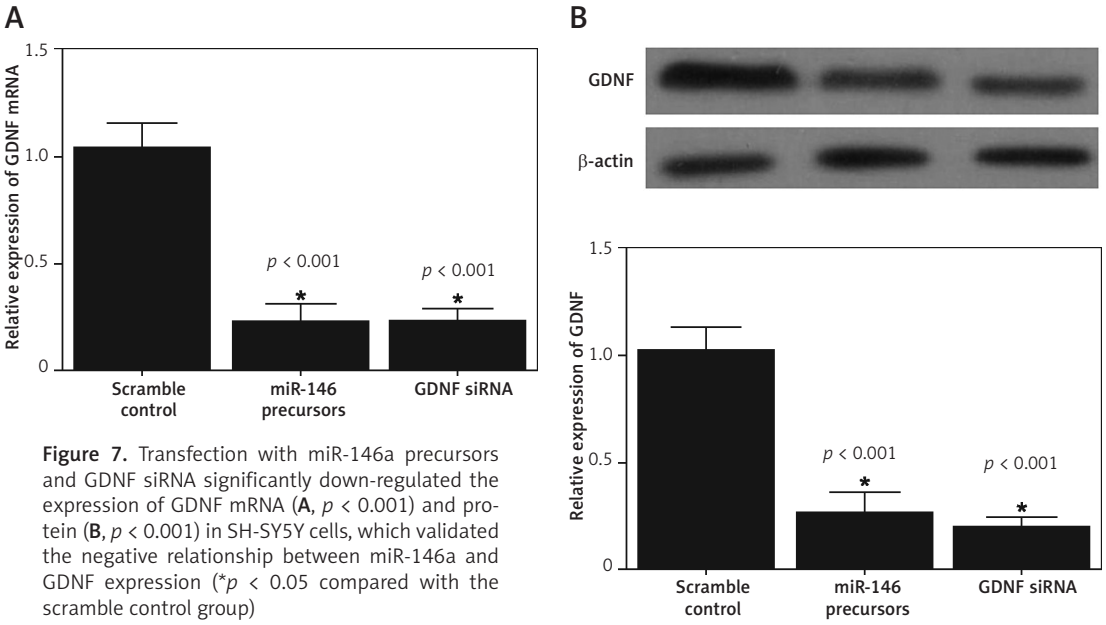

Figure 7. Transfection with miR-146a precursors and GDNF siRNA significantly down-regulated the expression of GDNF mRNA (**A**, $p < 0.001$) and protein (**B**, $p < 0.001$) in SH-SY5Y cells, which validated the negative relationship between miR-146a and GDNF expression (*$p < 0.05$ compared with the scramble control group)

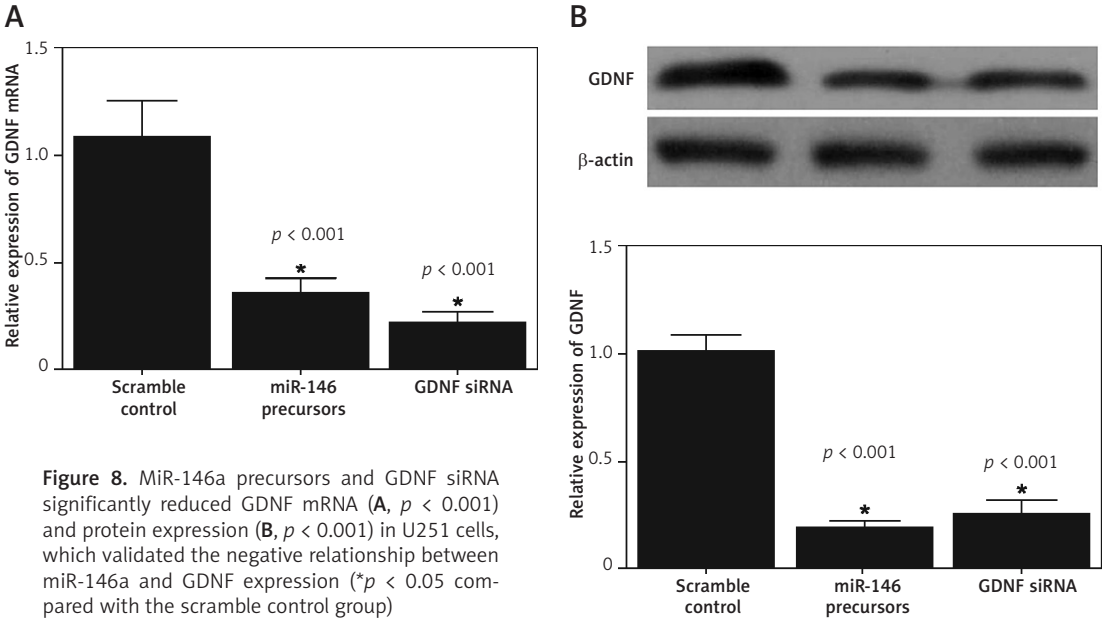

Figure 8. MiR-146a precursors and GDNF siRNA significantly reduced GDNF mRNA (**A**, $p < 0.001$) and protein expression (**B**, $p < 0.001$) in U251 cells, which validated the negative relationship between miR-146a and GDNF expression (*$p < 0.05$ compared with the scramble control group)

pulmonary emboli, pelvic abscesses, and diverticulitis [50–52]. Moreover, bacterial enzymes can invade the bloodstream through septic lesions [53], and intestinal flora imbalance was observed to potentially influence the expression level of miR-146a [54].

MicroRNAs (miRNAs) are short noncoding RNAs known to affect the functions of specific mRNAs in cancer progression. MiRNAs can also decrease the expression level of many targeted mRNAs genes by degrading or suppressing their translation [55, 56]. In a number of recent studies, fecal miRNAs have been shown to be relatively stable, and a number of fecal miRNAs, such as miR-106a, miR-92a, miR-144* and miRNA-21 (miR-21), show lower expression in healthy volunteers than in CRC patients [57–60].

In our study, we observed up-regulated expression of miR-146a in PD patients with worse prognosis. However, a previous study investigated several candidate miRNAs including miR-132, miR-146a, miR-26a and miR-155 in PBMCs of controls and Parkinson's patients treated with L-dopa, and found that the miR-155-5p expression was usually increased while miR-146a expression was suppressed in PD patients [17, 19, 61]. This contradictory result may be due to the difference in the cohorts of the two studies. Furthermore, Revishchin et al. showed that miR-146a can negatively regulate the pro-inflammatory responses

of monocytes in a constitutive manner, while decreasing the expression of presenilin 2 (PS2) at the same time [62]. In addition, PS2 mutations were implicated in autosomal dominant Alzheimer disease [63]. In this study, we carried out computational analyses and luciferase assays to explore the potential role of miR-146a in PD. The results showed that miR-146a directly targeted GDNF to reduce its expression. In addition, we carried out real-time PCR and Western blot analysis to evaluate the effect of miR-146a on GDNF expression. The results showed that miR-146a inhibited GDNF expression.

Originally purified from a rat glioma cell line, GDNF can promote survival and differentiation of dopamine neurons in the midbrain of rats while increasing dopamine uptake and the outgrowth of neurites *in vitro* [64]. Furthermore, in dopamine neurons, the formation of new axon terminals is stimulated by GDNF [65]. The GDNF gene of rodents contains three exons while the GDNF gene of humans contains six exons [66, 67]. GDNF functions as a homodimer and is stabilized through a disulfide bond [68]. In this study, we showed that the treatment of rats with NCDC17 significantly increased the expression of miR-146a while reducing the expression of GDNF. Currently, GDNF is among the most promising agents to treat PD [69, 70], and can significantly enhance the differentiation and survival of neurons in dopaminergic nerves [71]. In rodent models of pharmacologically triggered PD, nigral dopaminergic neurons are protected by sustained administration of GDNF [72, 73]. GDNF delivered using the lentiviral vector system completely blocks nigrostriatal degeneration and reverses the functional deficits in two MPTP-induced PD models of primates [74]. Furthermore, during a phase I clinical trial, it was observed that various PD symptoms in five PD patients were significantly improved by chronic intra-putamen infusion of GDNF [75].

Nevertheless, the results of this study are limited due to the small sample size. In our subsequent study, a larger sample size should be considered.

In summary, the findings of this study demonstrated that GDNF is a key factor involved in the prognosis of PD. By searching an online miRNA database, we identified miR-146a as a regulator of GDNF expression. In this study, we also recruited PD patients and followed them up for one year to investigate the effect of intestinal flora imbalance on the expression of miRNAs related to PD as well as the effect of miR-146a on the expression of GDNF in the brain.

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Conflict of interest

The authors declare no conflict of interests.

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