Original Article

Dopamine Agonists Exert Nurr1-inducing Effect in Peripheral Blood Mononuclear Cells of Patients with Parkinson's Disease

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Abstract

Background: Nurr1 plays an essential role in the development, survival, and function maintenance of midbrain dopaminergic (DA) neurons, and it is a potential target for Parkinson's disease (PD). Nurr1 mRNA can be detected in peripheral blood mononuclear cells (PBMCs), but whether there is any association of altered Nurr1 expression in PBMC with the disease and DA drug treatments remains elusive. This study aimed to measure the Nurr1 mRNA level in PBMC and evaluate the effect of Nurr1 expression by DA agents *in vivo* and *in vitro*.
Methods: The mRNA levels of Nurr1 in PBMC of four subgroups of 362 PD patients and 193 healthy controls (HCs) using real-time polymerase chain reaction were measured. The nonparametric Mann-Whitney U-test and Kruskal-Wallis test were performed to evaluate the differences between PD and HC, as well as the subgroups of PD. Multivariate linear regression analysis was used to evaluate the independent association of Nurr1 expression with Hoehn and Yahr scale, age, and drug treatments. Besides, the Nurr1 expression in cultured PBMC was measured to determine whether DA agonist pramipexole affects its mRNA level.
Results: The relative Nurr1 mRNA levels in DA agonists treated subgroup were significant higher than those in recent-onset cases without any anti-PD treatments (*de novo*) ($P < 0.001$) and HC groups ($P < 0.010$), respectively. Furthermore, the increase in Nurr1 mRNA expression was seen in DA agonist and L-dopa group. Multivariate linear regression showed DA agonists, L-dopa, and DA agonists were independent predictors correlated with Nurr1 mRNA expression level in PBMC. *In vitro*, in the cultured PBMC treated with 10 μmol/L pramipexole, the Nurr1 mRNA levels were significantly increased by 99.61%, 71.75%, 73.16% in 2, 4, and 8 h, respectively ($P < 0.001$).
Conclusions: DA agonists can induce Nurr1 expression in PBMC, and such effect may contribute to DA agonists-mediated neuroprotection on DA neurons.

Key words: Dopamine Agonists; Nurr1; Parkinson's Disease

Introduction

Nurr1 also known as NR4A2, belongs to the nuclear receptor superfamily of transcription factors, is highly expressed in midbrain dopaminergic (DA) neurons, and plays an essential role in the development, survival and function maintenance of midbrain DA neurons.[1-4] Nurr1 is a potential target for Parkinson's disease (PD), which is the second common neurodegenerative disease and both genetic and environmental neurotoxin are important causes.[5,6] Nurr1 can activate the transcription of tyrosine hydroxylase and enhance the expression of DA transporter.[7-9] It was reported that homozygous Nurr1 knockout mice failed to develop DA neurons, and heterozygotes reduced their brain DA and developed age-dependent locomotor deficits including impaired horizontal and vertical movement, difficultly performance on rotarod test compared to age-matched wild-type mice.[3,10-14] Reduction of Nurr1 in the adult brain may increase the vulnerability of DA

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neurons to stress and participate in the pathogenesis of PD.[11]

Several polymorphisms of *Nurr1* gene including −291Tdel and −245T-->G have been reported to result in a marked decrease of Nurr1 mRNA levels in transfected cell lines and lymphocytes of affected individuals.[15] Furthermore, Le *et al*.[16] reported a decreased *Nurr1* gene expression in peripheral blood mononuclear cells (PBMCs) of PD patients, which anticipated Nurr1 as a potential biomarker for PD diagnosis. But to date, little has been done to investigate the association among Nurr1 expression in PBMC, disease progression of PD, and DA agents. This study was to measure the Nurr1 mRNA level in PBMC and evaluate the effect of Nurr1 expression by DA agents *in vivo* and *in vitro*.

Methods

Study population

The 362 PD patients at Department of Neurology, the First Affiliated Hospital of Sun Yet-Sen University between January 2012 and August 2014 were recruited. The diagnosis was made in accordance with the United Kingdom PD Society Brain Bank Criteria.[17] Besides recent-onset PD patients without any anti-PD treatments (*de novo*), each patient reported to have taken anti-PD medicines at least 2 years and all patients were assessed according to the Hoehn and Yahr (H and Y) scale.[18,19] They participated voluntarily and provided written informed consents before enrollment. Meantime, 193 healthy controls (HCs) were enrolled either from Physical Examination Center of Sun Yat-Sen University or the spouses of the patients. Exclusion criteria for HC were based on our previous published criteria.[20] The study was approved by the Ethics Committee of Sun Yat-Sen University.

Preparation of peripheral blood mononuclear cell both from Parkinson's disease patients and healthy controls

Peripheral blood samples (5 ml) of PD patients and HCs were drawn from antecubital venous and put into natrium citricum-containing tubes. PBMC from venous blood of PD patients and HCs were prepared by Ficoll-Hypaque density gradient centrifugation. After centrifugation at 2200 r/min for 20 min, buffy coats were collected and washed three times with phosphate-buffered saline (PBS), then centrifuged at 1800 r/min for 10 min to collect PBMC cells. Then PBMC were lysed by adding 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and stored at −80°C until used.

In order to further explore the pattern of Nurr1 expression, an *in vitro* analysis on cultured human PBMC was conducted. PBMC from venous blood of 10 HCs was prepared by Ficoll-Hypaque density gradient centrifugation through centrifuging at 2200 r/min for 20 min. The buffy coats were collected and washed twice with PBS, then washed once with complete RPMI 1640 medium (Gibco, German) containing 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum and suspended at a concentration of 2×10^6 cells/ml with complete RPMI 1640 medium. PBMC was delivered in 3.5 cm diameter dishes with 2×10^6 cells separately with complete RPMI 1640 medium. After 12 h cultured, the cells were treated with 10 μmol/L pramipexole (Sigma, St. Louis, MO, USA) for 2, 4, 8, 12 and 24 h and cultured at 37°C under the humidified 5% CO_2 atmosphere. All the PBMC from 10 HCs were treated similarly. At each selected time, the PBMC cells were collected for RNA extraction.

Reverse transcription polymerase chain reaction analysis of Nurr1 expression on peripheral blood mononuclear cell

Then total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. The 1 μg of total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using a reverse transcription (RT) kit (Takara, Japan) according to the manufacturer's protocol in a final volume of 20 μl. For analysis, on Nurr1 expression with quantitative real-time RT-polymerase chain reaction (RT-PCR), human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control, and the sequences of primers as follows: Nurr1 forward: 5'-TCCAACGAGGGGCTGTGCG-3'; Nurr1 reverse: 5'-CACTGTGCGCTTAAAGAAGC-3'; and GAPDH forward: 5'-GAAGGTGAAGG TCGGAGTC-3'; GAPDH reverse: 5'-GAAGATGGTGATGGGATTTC-3'. The 2 μl of the synthesized cDNA were used in all real-time PCR together with the SYBR Green I Master Mix (SYBR® Premix Ex Taq™, Takara, Japan) on MJ Research Opticon2 real-time thermocycler (Bio-Rad, Hercules, CA, USA). Fluorescent reading from real-time PCR reaction was quantitatively analyzed by determining the difference of Ct (delta Ct) between Ct of Nurr1 and its internal GAPDH, and the *Nurr1* gene expression was determined by the formation of $2^{-\text{delta Ct}}$. In addition, for all the analyzed samples, only triplicates with a standard deviation of the Ct < 0.20 were accepted.

Statistical analysis

Quantitative data (age, H and Y scale, the relative Nurr1 mRNA relative level) were shown as mean ± standard error (SE) or median and interquartile range, depending on the distribution of the data. Categorical variables (gender) were shown as a percentage. The Chi-square test was used to analyze the differences of gender between PD and HCs or among the subgroups of PD. The nonparametric Mann-Whitney U-test and Kruskal-Wallis test were performed to evaluate the differences between PD and HC or among the subgroups of PD. The multivariate linear regression models were used to simultaneously evaluate the cross-sectional association of dependent variables (the Nurr1 mRNA relative level) and independent variables (H and Y scale, age, and the different drug treatments). After adjustment of H and Y scale and age, the covariance analysis was utilized to test the differences of Nurr1 mRNA relative level among different drug treatments. One-way analysis of variance was used to analyze the difference of Nurr1 mRNA level among the different hours treated with pramipexole.

Data were analyzed with statistical software SPSS 21.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ (two sides) was considered as statistically significant.

Results

Clinical characteristics of the subjects

The 362 PD patients (209 males, 153 females) were enrolled in this study. Among these 362 patients, 136 patients were recent-onset cases and did not receive any anti-PD treatment before blood drawing (*de novo*); 83 patients were administrated with L-dopa, 38 patients received D2/D3 agonists (piribedil or pramipexole), 105 patients took both L-dopa and DA agonists. These patients were divided into four subgroups according to the different drug treatments: *De novo*, L-dopa, DA agonist, and L-dopa and DA agonist. The demographic and clinical characteristics of PD patients and HCs are summarized in Table 1, showing no significant differences ($P = 0.683$) in gender between PD patients and HCs and among the four subgroups of PD patients. The average age of PD patients in L-dopa and L-dopa and DA agonist subgroups was older than that of *de novo* subgroup or HCs ($P < 0.05$). Furthermore, the median scale of PD was 2.0 of H and Y stage in all three subgroups (L-dopa, DA agonists and L-dopa and DA agonist), which was significantly higher than *de novo* subgroup ($P < 0.05$).

The association of dependent variables (the Nurr1 mRNA relative level) and independent variables (Hoehn and Yahr, age, and the different drug treatments)

Generalized estimating equations in linear regression models were performed on the data from all subjects. H and Y scale, age and different drug treatments of PD patients were identified to be independently associated with Nurr1 mRNA relative level. In both the stepwise model and inclusive model, the independent predictors of Nurr1 mRNA relative level were DA agonists, L-dopa, and DA agonists. The formulae for Nurr1 mRNA relative level were created based on the regression coefficients (β): In stepwise model, for DA agonists, $\beta = 4.551$ (95% confidence interval [*CI*], 2.200–6.903; $P < 0.001$); for L-dopa and DA agonists, $\beta = 4.398$ (95% *CI*, 2.883–5.912; $P < 0.001$); and in inclusive model, for age, $\beta = -0.024$ (95% *CI*, −0.069–0.022; $P = 0.311$); for H and Y scale, $\beta = -0.120$ (95% *CI*, −1.092–0.852; $P = 0.809$); for *de novo*, $\beta = -0.633$ (95% *CI*, −2.739–1.473; $P = 0.555$); for L-dopa, $\beta = -0.672$ (95% *CI*, −2.058–3.402; $P = 0.629$); for DA agonists, $\beta = 4.657$ (95% *CI*, 1.552–7.762; $P = 0.003$); for L-dopa and DA agonists, $\beta = 4.561$ (95% *CI*, 1.973–7.147; $P = 0.001$). The Nurr1 mRNA relative level was made using the following formulas: Nurr1 mRNA level = 2.973 + 4.551 × DA agonists + 4.398 × L-dopa and DA agonists, or Nurr1 mRNA level = 4.545 + 4.657 × DA agonists + 4.561 × L-dopa and DA agonists.

Nurr1 gene expression in subgroups of Parkinson's disease and health controls

According to different drug treatments, the PD patients were divided into 4 subgroups. As shown in Figure 1, the relative levels of Nurr1 mRNA in *de novo* subgroup and HCs were 2.38 ± 0.42 versus 3.18 ± 0.42, respectively ($P = 0.307$), a slight decrease but no statistical significance in *de*

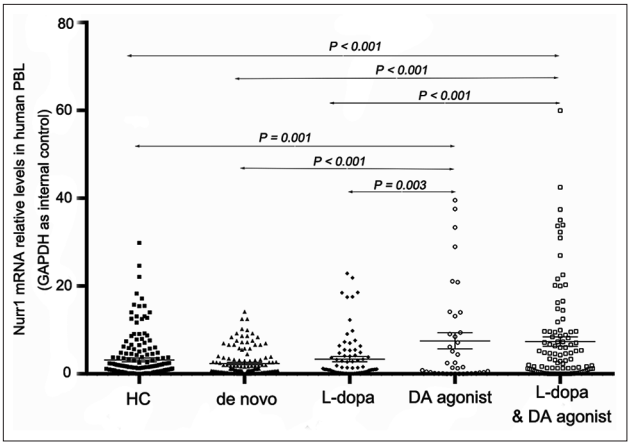

Figure 1: Scatter plots of Nurr1 mRNA relative levels in peripheral blood mononuclear cells of subgroups of PD patients. Fluorescent reading from real-time polymerase chain reaction was quantitatively analyzed by determining the difference of Ct (delta Ct) between Ct of Nurr1 and internal control. Glyceraldehyde-3-phosphate dehydrogenase and the *Nurr1* gene expression was determined by the formation of $2^{-\text{delta Ct}}$. Horizontal bars represented mean ± standard error. HC: Healthy control; PD: Parkinson's disease; *de novo*: PD without any anti-PD treatment; DA agonists: PD treated with DA agonists; L-dopa: PD treated with L-dopa; L-dopa and DA agonists: PD treated with L-dopa and DA agonists; DA: Dopaminergic.

Table 1: Demographic and clinical characteristics of the subjects

Groups	Number	Gender (*n*)		Age of onset (years)			Hoehn and Yahr scale		
		Male	Female	Median	Minimum	Maximum	Median	Minimum	Maximum
HC group	193	113	80	60	19	83	0	0	0
PD group	362	209	153	61	24	89	2.0	1	5
De novo	136	79	57	58	24	86	1.5	1	3
L-dopa	83	54	29	64*,†	31	82	2.0*	1	5
DA agonists	38	20	18	65	27	80	2.0*	1	4
L-dopa and DA agonists	105	56	49	62*	35	89	2.0*	1	5

DA: Dopaminergic; HC: Healthy control; PD: Parkinson's disease; *De novo*: PD without any anti-PD treatment; DA agonists: PD treated with DA agonists; L-dopa: PD treated with L-dopa; L-dopa and DA agonists: PD treated with L-dopa and DA agonists. *P<0.05 compared with *de novo*; †P<0.05 compared with HC.

novo subgroup without any DA agonists, L-dopa or other anti-PD drugs. The relative level of Nurr1 mRNA in L-dopa PD group was 3.37 ± 0.57 versus 3.18 ± 0.42 in HC ($P = 0.841$), which showed no statistical significance. However, the relative Nurr1 mRNA level in DA agonist subgroup was 7.52 ± 1.82, significant higher than those both in *de novo* group ($P < 0.001$) and HC ($P = 0.001$). The Nurr1 mRNA level in DA agonist and L-dopa subgroup was up to 7.37 ± 1.07, which was significantly increased versus *de novo* subgroup or L-dopa subgroup ($P < 0.001$). There were no significant differences of Nurr1 mRNA level between DA agonist and DA agonist and L-dopa subgroups.

The Nurr1 mRNA level in *de novo* subgroup before and after treatments

In 136 recent-onset cases, 25 were treated with L-dopa and 19 were treated with DA agonists. Their Nurr1 mRNA relative levels pre and posttreatment were detected [Figure 2]. The Nurr1 mRNA level in 25 patients was 7.87 ± 1.70 before L-dopa treatment, and 7.59 ± 1.59 after L-dopa taken, but no significant difference ($P = 0.905$). The Nurr1 mRNA levels in 19 patients before and after DA agonist treatment were 5.92 ± 1.42 and 8.47 ± 2.52, respectively, indicating a slight but nonsignificant increase after DA agonist treatment ($P = 0.066$).

The Nurr1 mRNA level in peripheral blood mononuclear cell treated with pramipexole

To further explore whether DA agonists have effect on Nurr1 expression, we measured the Nurr1 mRNA level in PBMC from 10 healthy volunteers with 10 μmol/L pramipexole [Figure 3]. Compared with control, the Nurr1 mRNA relative levels in PBMC by DA agonist were significantly increased to 199.61 ± 13.17%, 171.75 ± 11.33%, 173.16 ± 11.42% in 2, 4 and 8 h, respectively ($P < 0.001$). *In vitro* experiment, the Nurr1 mRNA levels of PBMC treated by DA agonist for 12 h and 24 h were 95.30 ± 6.26% and 104.60 ± 4.32%, respectively, and there were no significant differences compared with control ($P > 0.05$), which was in accord with the half-life of 8–12 h.

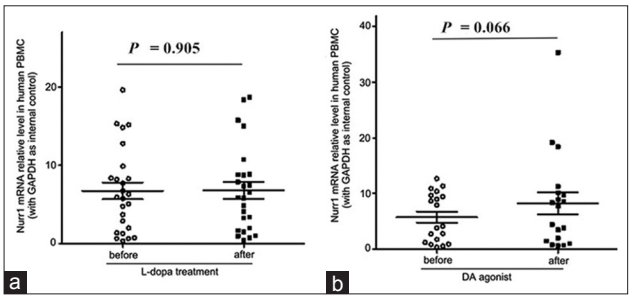

Figure 2: Scatter plots of Nurr1 mRNA relative levels in peripheral blood mononuclear cells before and after treatment of L-dopa (a) ($n = 25$) or DA agonist (b) ($n = 19$) in *de novo* subgroup. There were no obvious changes before and after L-dopa treatment neither for the whole group nor for each individual, while there were three individuals showed obvious increase of Nurr1 mRNA levels though there was no significance between before and after DA agonist treatment as a whole group. DA: Dopaminergic.

Discussion

Nurr1 is highly expressed in the developing and adult ventral midbrain and required for the acquisition and maintenance of the DA phenotype in nigrostriatal neurons.[1-4] It has been reported that besides central nervous system, Nurr1 is also an expression in many tissues including bone, endothelial cells, and PBMC.[21-23] The key point for clinical evacuation of Nurr1 is whether the alteration of Nurr1 expression is secondary to anti-PD drug effects. Using RT-PCR, we analyzed the Nurr1 mRNA level in PBMC from 362 sporadic PD patients and 193 HCs from Southern China, and found that the Nurr1 expression level in *de novo* subgroup with a tendency of nonsignificant decrease compared to HCs, but a statistically significant increase in both DA agonist or DA agonist and L-dopa subgroups, which indicated that DA agonists may exert an up-regulation effect on the expression of Nurr1 in PBMC.

As a transcription factors, the expression of Nurr1 can be affected by many factors including growth factors, neurotransmitters, and drugs.[24-26] For example, injection of 6-hydroxydopamine into the striatum produced an increase in the number of cells expressing Nurr1 in both substantia nigra compacta (SNc) and substantia nigra reticulate.[27] Similar to our data, a series of evidence indicated that DA receptor agonists may play an active role on the Nurr1 expression. For instance, ropinirole has been documented to prevent the progression of PD in Nurr1 deficient mouse, and Pan *et al*.[28] reported that SH-SY5Y cells treated with D3 receptor agonist pramipexole enhanced the expression of Nurr1 mRNA and protein *in vitro*.

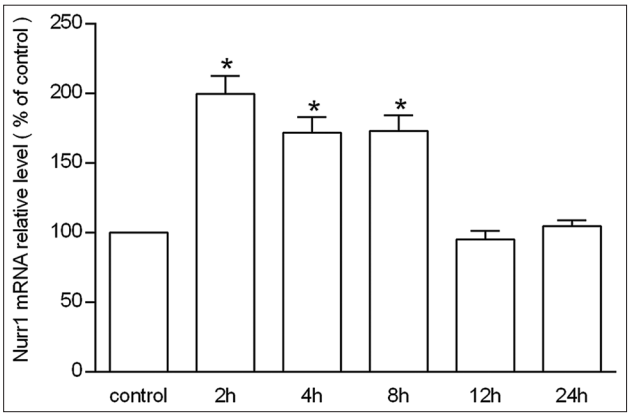

Figure 3: Nurr1 mRNA relative levels *in vitro* measured by real-time polymerase chain reaction treated with pramipexole. Peripheral blood mononuclear cells from health volunteers were treated with 10 μmol/L pramipexole for 0–24 h. Glyceraldehyde-3-phosphate dehydrogenase was monitored as internal control. Nurr1 mRNA level was compared with control. All experiments were performed at least three independent times (*$P < 0.001$).

Studies on postmortem brains have been found that an age-related decline of DA phenotypic markers was associated with down-regulation of Nurr1 expression in human SN.[29] Chu *et al*.[30] reported the optical density of Nurr1 immunofluorescence was significantly decreased in nigral neurons containing α-synuclein-immunoreactive inclusions in PD patients. In addition, recent studies showed a significant decreased of Nurr1 mRNA in SNc of D2 dopamine receptor −/− mice ($D2R^{-/-}$).[31] However, our data showed that the expression of Nurr1 mRNA in PBMC was affected by drugs like DA agonist, but was no significant correlation with the disease severity (H and Y scale) or age of onset of PD. For further exploring the pattern of Nurr1 expression *in vitro*, we found that Nurr1 mRNA relative levels in PBMC increased significantly after treated with 10 μmol/L pramipexole for 2, 4, and 8 h, which in accordance with our clinical investigation from blood cells of PD patients, demonstrating DA agonist plays an active effect on *Nurr1* gene in PBMC. Similarly, Pan *et al*.[28] reported that levels of Nurr1 mRNA and protein in SH-SY5Y increased after pramipexole treatment, indicating that the biological pattern of Nurr1 expression in PBMC might be different from the changes in degeneration of DA neurons in midbrain.

In summary, we observed that Nurr1 mRNA level in PBMC from our PD patients was significantly influenced by antiparkinsonism drugs DA agonists but not L-dopa, and such effect may contribute to DA agonists-mediated neuroprotection on DA neurons. Furthermore, we found a slight insignificant reduction of Nurr1 level in our *de novo* PD patients, which might present a correlation with the disease. A larger sample of *de novo* PD patients may be needed to evaluate whether reduced expression of Nurr1 in PBMC can be used as a biomarker for early PD.

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