Hypomethylation of SNCA in blood of patients with sporadic Parkinson’s disease

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Abstract

SNCA is a pathogenic gene identified in rare familial PD, and over-expression of SNCA was suggested in the pathogenesis of familial and sporadic PD. Rep1 polymorphism of SNCA was associated with susceptibility to sporadic PD and SNCA expression in vitro and in vivo. Hypomethylation in SNCA intron-1 was associated with increased SNCA expression and was observed in postmortem brains of patients with sporadic PD. We studied the methylation status of SNCA intron-1, SNCA mRNA levels and Rep1 genotypes in PBMCs of 100 sporadic PD patients and 95 controls and explored the relationship between DNA methylation, mRNA expression and Rep1 genotypes. Hypomethylation of SNCA intron-1 was detected in PBMCs of PD patients, and DNA methylation levels were associated with Rep1 polymorphism. The shorter allele was associated with higher level of SNCA intron-1 methylation, and genotypes carrying the shorter allele showed significantly higher methylation level of SNCA intron-1 than genotypes carrying the longer allele. However, SNCA mRNA levels were not associated with disease status, Rep1 polymorphism or DNA methylation of SNCA intron-1 in our study.

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1. Introduction

Parkinson’s disease (PD), the second most frequent neurodegenerative disorder, is pathologically characterized by the progressive loss of dopaminergic neurons in the substantia nigra. Several genes have been identified in rare familial PD cases with Mendelian inheritance, but the etiology of sporadic PD remains obscure [1].

SNCA is a causative gene of rare familial PD, and its encoding protein (alpha-synuclein) is a major component of Lewy bodies, a pathological hallmark of PD [1]. Triplication and duplication of SNCA were identified in rare familial PD cases, and increased expression of wild-type SNCA was reported in postmortem brain tissues of patients with SNCA multiplications, suggesting that the pathogenic effects of SNCA multiplication is possibly mediated by SNCA over-expression [2–5]. SNCA over-expression was also reported in brain tissues of patients with sporadic PD [6–8], suggesting that SNCA over-expression may also contribute to the pathogenesis of sporadic PD.

Rep1, a polymorphic dinucleotide repeat located ~10 kb upstream of the translational start site of SNCA, has been associated with PD by multiple studies [9–12]. Studies showed that the longer allele of SNCA-Rep1 conferred increased risk to develop PD, while the shorter allele is associated with reduced risk for PD. Similar to the effect of SNCA multiplication, Rep1 variants were reported to have an effect on SNCA-mRNA levels in brains of patients with sporadic PD. Homogenous genotype of the shorter allele was associated with lower levels of SNCA-mRNA compared with genotypes carrying longer alleles [13]. Studies in cell cultures and animal models revealed that Rep1 had an effect on transcriptional activities of SNCA and the longer allele was associated with higher levels of SNCA expression relative to the shorter allele [14,15]. All together, these findings suggest that Rep1 allele length variability modulates the susceptibility to PD possibly via regulation of SNCA expression.

DNA methylation is a key epigenetic mechanism involved in the regulation of gene transcription. Methylation refers to the transfer of a methyl group from S-adenosyl methionine to cytosine residues at the CG dinucleotides on the DNA [16]. Recently, hypomethylation of SNCA intron-1 was observed in postmortem brain tissues from patients with sporadic PD and hypomethylation of this region was associated with increased expression of SNCA in vitro [17,18].

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In the present work, we investigated DNA methylation status of SNCA intron-1, Rep1 genotypes and SNCA-mRNA expression in peripheral blood mononuclear cells (PBMCs) of patients with sporadic PD.

2. Materials and methods

2.1. Subjects

The study was approved by the Medical Ethics Committee of Xiangya Hospital, Central South University and written consents were obtained from every participant. 100 sporadic PD patients (mean ± SD age = 62.1 ± 9.6 years, female = 45, male = 55) and 95 controls (mean ± SD age = 61.4 ± 9.8 years, female = 45, male = 50) were enrolled in this study. PD patients were collected from the Department of Neurology, Xiangya Hospital, Central South University; National Laboratory of Medical Genetics of China and Neurodegenerative Disorders Research Center, Central South University. All patients underwent a standardized neurological examination by two movement disorder specialists. The clinical diagnosis of PD was established according to a standardized neurological examination by two movement disorder specialists. The clinical diagnosis of PD was established according to the United Kingdom PD Brain Bank Criteria [19]. All of the patients were of Chinese Han ethnicity from China. PD clinical stages were evaluated according to the classification of Hoehn and Yahr (H-Y) [20]. 69 patients had received L-dopa treatment before the study. The control group was composed of age-, gender-, and origin-matched healthy individuals from Health Examine Center of Second Xiangya Hospital. Subjects were excluded if they had major organ dysfunction, neurological disease, or family history of movement disorders.

2.2. Isolation of PBMCs

A 30 ml sample of venous peripheral blood was drawn from each subject into ethylenediamine tetra-acetate (EDTA) vacutainer tubes. All blood samples from these subjects were drawn in the morning after an overnight fast. PBMCs were isolated by Ficoll–Hypaque density gradient centrifugation (Shanghai Hengxin Chemical Reagent Co., Shanghai, China).

2.3. Nucleotide extraction

Genomic DNA was extracted from PBMCs using the TIANamp Genomic DNA blood kit (Tiangen Biotech, Beijing, China). Total RNA was isolated by standard Trizol method (Qiagen). DNA and RNA samples were stored in −80 °C until experiments.

2.4. Bisulfite sequencing

A 451 bp SNCA intron-1 fragment (−926 to −476) containing 23 CpG sites was analyzed by bisulfite sequencing (Fig. 1A). Bisulfite conversion of genomic DNA was carried out with Epitect Bisulfite Kit (Qiagen). The 451 bp SNCA intron-1 fragment was amplified by chain reaction (PCR) and cloned into the pGEM-T easy vector (Promega, Madison, WI, U.S.A.). At least 10 independent clones were sequenced for each subject. Primer sequences for PCR were described previously [17]. Quality control was performed by BiQ analyzer (quality control software for DNA methylation data from bisulfite sequencing). The level of DNA methylation was determined as percentage of methylated CpG. For each subject, methylation level (methylated CpG/total CpG) at individual CpG site and the mean methylation level of all the 23 CpG sites were calculated.

2.5. Genotyping of Rep1

The region of SNCA containing Rep1 was amplified by PCR from genomic DNA isolated from PBMCs. SNCA Rep1 alleles were sized on a high-resolution capillary electrophoresis platform using ABI 3730XL automated sequencer (Applied Biosystems, Inc., Foster City, CA) and allelic sizes were analyzed using GeneScan Version 4.0 software (Applied Biosystems, Inc., Foster City, CA). The Rep1 alleles (with a size difference of two nucleotides) were defined according to the length of the PCR product. Due to ethnic differences, Rep1 calling may be inconsistent across studies [10,12,21]. We termed the alleles according to the rules determined by Izumi et al.: allele 0 = 265 bp, allele 1 = 265 bp, allele 2 = 267 bp, allele 1 = 269 bp, and allele 2 = 271 bp. The primers were described previously [21].

2.6. cDNA synthesis and real-time quantitative PCR (RT-PCR)

cDNA was synthesized with an input of 2 μg of total RNA using the RevertAid™ First Strand CDNA synthesis Kit (Fermentas, Burlington, Canada). RT-PCR was performed on a ABI 7900 HT Fast Real-time PCR system (Applied Biosystems, Inc., Foster City, CA), using the SYBR Premix Ex Taq™ real-time PCR Kit (Takara Biotech, Co., Dalian, China). All reactions were run for two times, and duplicate each time. β-Actin was used as the internal control.
as an internal control. Primer sequences for RT-PCR were described previously [22]. The mRNA level of SNCA relative to β-actin was determined by the comparative CT method [23].

2.7. Statistical analyses

All statistical analysis was performed using Statistical Package for Social Sciences (SPSS, version 17.0). All data was expressed as mean ± SD. Chi-square test was used to analyze data as frequencies and percentages. Linear regression analysis was performed to assess the association of mRNA or DNA methylation levels with primary variables (disease status and Rep1 genotypes) as well as secondary variables (gender and age) that may affect DNA methylation and mRNA expression levels [13,16,22]. For post hoc multiple comparisons between different genotypes, Dunnett T3 test was applied. Pearson correlation was performed for correlation between DNA methylation and mRNA levels. For all analyses, p < 0.05 was considered statistically significant.

3. Results

3.1. Comparison of methylation between patients and controls

Demographic data was summarized in Table 1. There was no significant difference in gender or age distribution between PD patients and controls.

First, we compared the DNA methylation levels of SNCA intron-1 between PD patients and controls by linear regression analysis adjusting for Rep1 genotypes, age, and gender. As shown in Fig. 2A and Table 1, the mean methylation level of SNCA intron-1 was lower in the PD patients compared to controls (p = 0.033). A detailed comparison of DNA methylation levels at individual CpG site between PD and controls showed that PD patients’ DNA was significantly hypomethylated at CpG sites 6, 8 and 9 (p = 0.036, 0.012, 0.029, Fig. 2B).

3.2. Effect of Rep1 genotypes on DNA methylation

Rep1 allele distribution and frequencies were listed in Table 2. There was no significant difference in allele distribution between PD and controls. Since alleles −1 and −2 were rare in both groups, they were not included in the following analysis.

In order to determine the potential effect of Rep1 polymorphism on DNA methylation of SNCA intron-1, we compared DNA methylation levels among different Rep1 genotypes by linear regression analysis adjusting for disease status, gender, and age. Significant difference of DNA methylation levels was observed between different Rep1 genotypes (p = 0.001, Fig. 3A), and genotypes carrying allele 0 (0/0, 0/1 and 0/2) showed significantly higher methylation levels than genotype 1/2 (p = 0.044, 0.038, 0.031) and genotype 2/2 (p = 0.009, 0.007, 0.006, Table 3). Genotype 1/1 did not show significant difference of DNA methylation level compared with any of the other genotypes (p > 0.05). We further compared the DNA methylation levels between different allele carrier statuses of alleles 0, 1 and 2. Significant difference of methylation levels was observed between non-carriers of 0 and carriers of 0 (p = 0.000, Fig. 3B), but not between non-carriers of 2 and carriers of 2 (p = 0.442) or between non-carriers of 1 and carriers of 1 (p = 0.984).

3.3. mRNA levels between patients and control

We then compared SNCA-mRNA levels between PD patients and controls by linear regression analysis adjusting for gender, age and Rep1 genotypes. No significant difference of mRNA levels was found between the two groups (p = 0.844, Fig. 4A).

We evaluated the effects of gender, age, onset age, disease duration, H-Y stage and medication (L-dopa) on methylation and mRNA levels, and none of these factors was associated with methylation levels or mRNA levels (data not shown).

3.4. Effect of Rep1 genotype on mRNA level

We then examined the association of SNCA-mRNA levels with Rep1 genotypes. SNCA mRNA levels did not differ between different Rep1 genotypes (p = 0.136, Fig. 4B). And no correlation was observed

Table 1
Summary of demographic data and levels of SNCA methylation and mRNA of PD patients and controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>PD (n = 100)</th>
<th>Control (n = 95)</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>55/45</td>
<td>50/45</td>
<td>0.740</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62.1 ± 9.6</td>
<td>61.4 ± 9.8</td>
<td>0.575</td>
</tr>
<tr>
<td>SNCA methylation level&lt;sup&gt;b&lt;/sup&gt; (%)</td>
<td>14.22 ± 8.54</td>
<td>16.90 ± 8.66</td>
<td>0.013</td>
</tr>
<tr>
<td>SNCA mRNA level</td>
<td>0.0013 ± 0.0007</td>
<td>0.0013 ± 0.0008</td>
<td>0.844</td>
</tr>
</tbody>
</table>

<sup>a</sup> p value of Chi-square test.

<sup>b</sup> p values were presented by linear regression analysis.

Table 2
Comparison of the allele frequencies of Rep1 in PD patients and controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>Rep1 allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−2*</td>
</tr>
<tr>
<td>Control (n=190)</td>
<td>1 (0.5%)</td>
</tr>
<tr>
<td>PD (n=200)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>p value&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.487</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rep1 nomenclature: −2 = 263 bp, −1 = 265 bp, 0 = 267 bp, 1 = 269 bp, and 2 = 271 bp.

<sup>b</sup> n = 2 × case numbers.

<sup>c</sup> p value of Chi-square test.
between SNCA-mRNA and DNA methylation levels (p = 0.263, Pearson correlation).

4. Discussion

PD is a progressive neurodegenerative disease and the major symptoms of PD arise from the degeneration of dopaminergic neurons in substantia nigra pars compacta. Because of difficult access to brain tissues, PD related biomarkers in accessible tissues have been the focus of researchers. We chose to use PBMCs for studies because some PD-specific biochemical alterations observed in postmortem brains, including mitochondrial complex deficiency [24], decreased proteasome activity and increased caspase activity [25], and altered content of dopamine and dopamine transporters [26–28], were also detected in PBMCs of PD patients, suggesting that PBMCs may reflect some disease-related pathogenesis. Furthermore, decreased mRNA levels of NURR-1, a gene involved in dopaminergic neurogenesis and pathogenesis of PD were observed in PBMCs of patients with sporadic PD [29,30]. Increased SNCA mRNA was detected in PBMCs of patients with sporadic PD [22]. Consistent with the association between SNCA expression in brains and Rep1 variants [13], SNCA protein (alpha-synuclein) levels in PBMCs were also associated with Rep1 polymorphisms [31]. A recent study identified concordant methylation alterations in brain tissues and PBMCs of patients with PD, further lending support to the feasibility of using PBMCs for studies [32].

In the present work, we found that SNCA intron-1 was hypomethylated in PBMCs of patients with sporadic PD compared with controls. This result is consistent with previous studies which showed hypomethylation of the same region in postmortem brains of patients with sporadic PD [17,18]. Admittedly, such a small difference of methylation level (~3%) may not be a useful biomarker for PD diagnosis, but the previous study in postmortem brains showed similarly small difference of methylation level between PD patients and controls (~5%) [17]. Thus, this study can be taken as an indication that alterations of the methylation level of SNCA intron-1 in PBMCs may reflect the changes of methylation in brains of patients with sporadic PD. Furthermore, studies in cell cultures showed that 40% (Hela cells) or 20% (293 cells) decrease of methylation levels of SNCA intron-1 could induce increased SNCA-mRNA expressions [17,18]. Given the low baseline methylation levels of SNCA intron-1 [16.9% in controls], the ~3% difference of methylation amounts to a 16% decrease of methylation in PD patients compared with controls. Thus the modest hypomethylation observed in PD patients may have an effect on the regulation of SNCA expression according to these researches. In addition, nearly all CpG sites were located within the predicted TF binding sites (Fig. 1B), suggesting that DNA methylation of this region, especially CpG sites 6, 8, 9 which were found to be significantly hypomethylated in PD patients, may have an effect on SNCA transcription.

Genetic and environmental factors may be involved in the alterations of DNA methylation. In this study, we evaluated the effects of

![Fig. 3. Comparison of mean methylation level of SNCA intron-1 between different Rep1 genotypes. A. Comparison of methylation level between 6 genotypes of Rep1. Genotypes 0/0, 0/1 and 0/2 were significantly hypermethylated than genotypes 1/2 and 2/2 (p < 0.05). B. Comparison of methylation level between different allele 0 carrier statuses. Allele 0 carrier status was significantly hypermethylated than allele 0 non-carrier status (p = 0.000). ** indicates significant difference (p < 0.01).](image3)

![Fig. 4. A. SNCA-mRNA levels in PBMCs from PD patients and controls. No significant difference was observed (p = 0.844). B. Comparison of SNCA-mRNA levels between different genotypes of Rep1. No significant difference was observed (p = 0.136).](image4)

### Table 3
Comparison of SNCA methylation levels between different Rep1 genotypes.

<table>
<thead>
<tr>
<th>Rep1 genotype</th>
<th>Control</th>
<th>PD</th>
<th>SNCA methylation level (%)</th>
<th>p value(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/0 (n = 30)</td>
<td>12</td>
<td>18</td>
<td>17.6 ± 8.5</td>
<td>0.001</td>
</tr>
<tr>
<td>0/1 (n = 38)</td>
<td>23</td>
<td>15</td>
<td>17.6 ± 9.4</td>
<td></td>
</tr>
<tr>
<td>0/2 (n = 37)</td>
<td>18</td>
<td>19</td>
<td>18.4 ± 10.8</td>
<td></td>
</tr>
<tr>
<td>1/1 (n = 24)</td>
<td>12</td>
<td>12</td>
<td>15.6 ± 8.4</td>
<td></td>
</tr>
<tr>
<td>1/2 (n = 43)</td>
<td>18</td>
<td>25</td>
<td>12.1 ± 5.6</td>
<td></td>
</tr>
<tr>
<td>2/2 (n = 20)</td>
<td>11</td>
<td>9</td>
<td>10.6 ± 4.8</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) SNCA methylation level represents mean methylation level of SNCA intron-1.

\(^b\) p value presented was derived from the comparison of DNA methylation between the six genotypes above by linear regression analysis, adjusting for disease status, gender and age.

\(^c\) No significant difference of genotype distribution between patients and controls (p > 0.05).
Rep1 polymorphism and medication (l-dopa) on the methylation levels of SNCA intron-1. We did not observe an effect of l-dopa on the methylation levels of SNCA intron-1. Rep1 genotypes were demonstrated to be associated with methylation levels of SNCA intron-1. Genotypes carrying allele 0 were significantly hypermethylated compared to genotypes 1/2 and 2/2. Allele 0 carrier status was associated with higher level of methylation compared with 0 non-carrier status. Together, this study showed that the shorter allele was associated with higher methylation level of SNCA intron-1, although the Rep1 is distant from the CpG islands in SNCA intron-1. Recent studies showed that DNA methylation might be regulated by genetic variants through unknown mechanisms [33–35]. For example, a genome-wide association study by Zhang et al. showed that, DNA methylation was frequently heritable and thousands of SNPs were associated with methylation of specific CpG sites. The regulation of methylation could be cis-acting or trans-acting, and the associated SNPs could be distant (thousands of base pairs) from the CpG locus. In fact, most (87.9%) associated SNPs were more than 2 kb away from the CpG positions [34]. The most compelling evidence of the association between SNPs and DNA methylation is the IGF2/H19 locus, the methylation of which is associated with SNPs in cis [33]. Thus, our data is concordant with previous studies and further studies exploring the mechanism underlying the association between Rep1 polymorphism and methylation of SNCA intron-1 are warranted. The association of Rep1 with sporadic PD was reported in large-scale collaborative analysis [12], but as reviewed by Farrer et al. [9], there was disparity in association findings between small studies with potential biases. In this study, we failed to determine any allele frequency discrepancy between PD patients and controls, possibly due to the relatively small study samples included.

We compared the SNCA mRNA expression in PD patients and controls, and no significant difference was observed. Previous studies on SNCA mRNA levels in PD patients and controls showed conflicting results [22,23]. Experimental confounding factors, especially contamination of red blood cells and platelet, may explain the different results among studies [37,38]. Since Rep1 variants were reported to be associated with SNCA-mRNA levels, the evenly distributed Rep1 alleles between PD patients and controls in our study may also be a reason for the negative result. Furthermore, we did not observe any association between SNCA mRNA expression in PBMCs and Rep1 polymorphism or methylation of SNCA intron-1. This finding was not consistent with the previous studies which indicated the association between SNCA mRNA levels and Rep1 polymorphism in brain tissues and cell cultures [13,17], and the association between SNCA mRNA levels and methylation of SNCA intron-1 in vitro [14]. Tissue specific regulation of SNCA transcription may explain the conflicting results and other mechanisms may be involved in the regulation of SNCA mRNA level in PBMCs. Taken together, our results revealed hypomethylation of SNCA intron-1 in PBMCs of patients with PD and confirmed the effect of Rep1 on DNA methylation of SNCA. These findings may contribute to better understanding of the mechanisms underlying the associations between methylation and PD.

Declaration of interest
No conflict of interest exits in the submission of this manuscript.

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