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Association of *SNCA* variants with α-synuclein of gastric and colonic mucosa in Parkinson's disease

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ABSTRACT

Background: Alpha-synuclein (α-Syn) immunostaining in the enteric nervous system (ENS) has been investigated to determine the role of diagnostic biomarker of Parkinson's disease (PD). However, determining factors for alpha-synuclein (α-Syn) deposition in the ENS of humans are still unclear. We aimed to investigate a possible association between *SNCA* variants and the presence of α-Syn immunostaining in the ENS in patients with PD and healthy individuals.

Methods: The study subjects consisted of 38 patients with PD and 46 healthy individuals. α-Syn immunohistochemistry was performed for gastric and colonic mucosal tissues of patients with PD and controls. Mucosal biopsy tissues of the ENS were obtained using standard biopsy forceps by endoscopic gastroduodenoscopy or colonoscopy. Two variants within the *SNCA* gene (the single nucleotide polymorphism [SNP] rs11931074 and the microsatellite REP1) were genotyped.

Results: In patients with PD, the rs11931074 (G allele) was significantly associated with the presence of α-Syn immunostaining in the ENS (OR = 5.96, 95% CI = 1.70–20.97, $P = 0.01$). In an interaction analysis, SNP rs11931074–PD status interaction was significantly associated with positive α-Syn immunostaining in the ENS (OR = 7.33, 95% CI = 1.58–33.88, $P = 0.01$). Longer *SNCA* REP1 alleles were not associated with positive α-Syn immunostaining in the ENS.

Conclusion: This exploratory study demonstrated that α-Syn deposition in the ENS may be associated with *SNCA* variants in patients with PD.

1. Introduction

Alpha-synclein (α-Syn) is the major component of Lewy bodies, which is the main pathological hallmark of Parkinson's disease (PD) [1]. Braak et al. reported a temporal sequence of α-Syn aggregation in the brain of patients with PD beginning in the olfactory bulb and dorsal motor nucleus of the vagus nerve in the medullar oblongata [2,3]. α-Syn aggregation was also detected in the Meissner's and Auerbach's plexus of the gastrointestinal tract in cases staged for PD-related brain pathology [4].

Based on these characteristics of α-Syn pathology, α-Syn immunostaining in the enteric nervous system (ENS) has received much

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attention as a potential biomarker because patients with PD display Lewy body pathology within their ENS [5–8]. However, the presence of α-Syn immunoreactivity in the ENS was detected in a similar manner in both patients with PD and healthy individuals, suggesting a limited role of enteric mucosal α-SYN as a diagnostic biomarker for PD [9–11]. Of note, the determining factors for a-Syn deposition in the ENS of humans are still unclear.

Point mutations in or multiplication of the *SNCA* gene, which encodes the α-Syn protein, are known causes of monogenic PD, and several *SNCA* variants confer susceptibility to PD, presumably by an overexpression mechanism (www.pdgene.org) [12–15]. In this context, we hypothesized that polymorphisms in the promoter region or 3′ region of *SNCA* are associated with α-Syn immunostaining in the ENS. Therefore, we investigated a possible association between *SNCA* variants and α-Syn immunostaining in the ENS of patients with PD and controls.

2. Methods

2.1. Subjects

We studied 38 patients with PD and 46 controls with adequate tissue samples from our prior study for α-Syn immunostaining of the gastric and colonic mucosa in PD, which reported that α-Syn immunoreactivity in the enteric mucosa was detected to a similar extent in patients with PD and controls [10]. All subjects were born and resided in South Korea. All subjects are unrelated and ethnic Koreans without any foreign family member. Experienced movement disorder specialists (S.J.C., J.K., and Y.J.K.) established a diagnosis of PD according to the clinical diagnostic criteria of the United Kingdom Parkinson's Disease Society Brain Bank criteria [16]. Patients with PD were enrolled from the clinical practice of the Department of Neurology of the Asan Medical Center and underwent the procedure according to the study protocol after having provided informed consent for the study [10]. Control subjects requiring endoscopic gastroduodenoscopy or colonoscopy because of routine medical screening, constipation, abdominal discomfort, and diarrhea were enrolled from the outpatient clinic of the Department of Gastroenterology of the Asan Medical Center [10]. Age-matched control subjects had no neurological or psychological diseases, tremor, and impairment in cognitive function or activities of daily living [10]. Controls subjects were also screened negative for PD or parkinsonism [10]. The Institutional Review Board (IRB) of Asan Medical Center approved the study, and all subjects provided informed consent in accordance with the IRB regulations.

2.2. Genotyping

Genotyping at the *SNCA* locus included a single nucleotide polymorphism (SNP) rs11931074 and REP1, a dinucleotide repeat marker. The SNP rs11931074 was selected for genotyping because this variant showed the most significant association with PD in Korean population [17]. The REP1 variants was genotyped because REP1 allele-length variability was associated with an increased risk of PD in previous studies, including a collaborative study using European and Asain populations [18–20].

Genomic DNA was analyzed using peripheral blood samples. First, the *SNCA* SNP rs11931074 was genotyped by sequencing as follows: A 512-bp fragment including rs11931074 in the region 3′of *SNCA* was amplified by polymerase chain reaction (PCR) with the following primers: forward: ATCTATTCCGCCCATCCTGT, reverse: ACACACAAACC ACCAAGCAAAC. PCR products were Sanger sequenced on an automated sequencing machine (ABI-3130 Genetic Analyzer, Applied Biosystems (Carlsbad, CA, USA)) and genotypes at rs11931074 were determined using Mutation Surveyor software (SoftGenetics, State College, PA, USA) and visual inspection of the electropherograms. The REP1 genotype, a dinucleotide repeat sequence, was established by fragment length analysis of a PCR product containing REP1 by using published primers (Farrer et al. 2001). Fragments were separated according to size on the ABI-3130 Genetic Analyzer. A size standard was included to enable correct determination of the fragment length. Analysis was conducted manually.

2.3. α-Syn immunostaining of the gastric and colonic mucosa

Methods of stomach and colon biopsies, α-Syn immunohistochemistry, and tissue assessment procedures are described in detail in our previous study [10]. In brief, endoscopic gastroduodenoscopy or colonoscopy was performed, and mucosal biopsies were obtained using standard biopsy forceps in 38 patients with PD and 53 controls. Primary antibodies for α-Syn (1:200, EP1536Y; ABCAM, Cambridge, UK) and S100 protein (1:200, 18-0046; Zymed; South San Francisco, CA, USA) with Benchmark autostainers (Ventana Medical Systems, Tuscan, AZ, USA) were used. Evaluation for α-Syn immunoreactivity was performed using the immune scoring system according to the density of S-100 positive nerve fibers and the presence of α-Syn-positive nerve fibers [10]. Biopsy specimens were assessed to be adequate for α-Syn immunostaining when a sufficient number of sections were available for analysis and immune-scoring classification was at least 1 (low to moderate density of neural tissue) with the presence of muscularis mucosa. Therefore, 38 patients with PD and 46 controls with adequate tissue samples were analyzed in this study with the primary outcome defined as the presence of α-Syn immunostaining of the stomach or colon.

2.4. Statistical analysis

The REP1 dinucleotide repeat sequence was recorded using two methods: 1) the number of alleles longer than the 259-bp allele, 2) the number of alleles longer than the 261-bp allele. Accordingly, individuals were scored 0, 1, or 2. To investigate the role of REP1 in more detail, REP1 dinucleotide repeat sequence in each individual was also coded as the sum of two allele scores, with each 259-bp allele contributing 0 points, each 261-bp allele contributing 1 point, and each copy of a 263-bp allele contributing 2 points, giving a total score (sum of the two allele scores) ranging from 0 to 4. Therefore, REP1 genotypes were coded and categorized as shorter REP1 alleles (score of 0–2) vs. longer REP1 alleles (score of 3–4) [18,21].

Baseline continuous variables were described as mean ± standard deviation (SD) and compared descriptively using the Student *t*-test or Mann-Whitney *U* test.

No deviation from Hardy-Weinberg equilibrium was detected at the *SNCA* SNP rs11931074 in controls (P from exact test > 0.01).

Separately within PD patients and controls, we tested for association between the presence of α-Syn immunostaining of the ENS and *SNCA* SNP rs11931074 and REP1 variants in logistic regressions using additive coding scheme. In addition, for illustrative purposes, we predicted the presence of α-Syn immunostaining of ENS in PD patients and controls simultaneously using independent variables of SNP rs11931074, PD status, and their interaction. Odds ratios (OR), 95% confidence interval (CI), and two-tailed *P* values are reported. The sensitivity and specificity of the genotype of *SNCA* variants for the presence of α-Syn immunostaining in the ENS were also determined.

The SAS statistical package (version 9.1.3; SAS Institute Inc., Cary, NC, USA) and PLINK (version 1.07) were used for analyses. *P* values < 0.05 were considered significant, and *P* values were corrected for the testing of two variants, using Bonferroni correction. We hypothesized more notable associations with PD patients than within controls, so that within one genetic variant, we employed a sequential testing strategy and tested for significance within controls only if there was a significant association within PD patients.

Table 1
Clinical characteristics of the study subjects.

	PD	Controls	*P* value
Sample, n	38	46	
Women, n (%)	15 (39.5%)	24 (52.2%)	0.245
Age at study entry (year), mean ± SD	66.31 ± 8.25	64.11 ± 7.39	0.200
Age at onset (year), mean ± SD	58.45 ± 7.99		
Duration of disease (years), mean ± SD	7.68 ± 4.68		
Levodopa dose (mg/day)	567.76 ± 248.57		
Levodopa equivalent dose (mg/day)	820.35 ± 333.20		
Hoehn and Yahr stage	2.5 ± 0.61		
UPDRS of PD			
Total	41.39 ± 16.55		
Part I	2.82 ± 2.31		
Part II	11.92 ± 5.54		
Part III	23.42 ± 9.35		
Part IV	3.24 ± 2.97		

PD, Parkinson's disease; SD, standard deviation; UPDRS, Unified Parkinson's Disease Rating Scale.
#Descriptive *P* values are from Fisher's exact test and *t*-test, respectively.

3. Results

3.1. Demographic features and α-Syn immunostaining in the ENS

Clinical characteristics of 38 patients with PD and 46 controls are summarized in Table 1. There was no difference in the mean age at study between patients with PD and controls. The mean duration of disease in patients with PD was 7.7 years.

Using routine endoscopic gastric and colonic biopsies, the presence of α-Syn immunostaining in the ENS was detected in a similar manner in 38 patients with PD (N = 12 [31.6%] for stomach and N = 4 [10.4%] for colon) and 46 controls (N = 15 [32.6%] for stomach and N = 8 [17.4%] for colon), which are described in detail in our prior study [10]. α-Syn immunostaining was detected in both stomach and colon in three patients with PD and two controls.

3.2. Association of SNP rs11931074 and REP1 variants and the presence of α-Syn immunostaining in the ENS

Genotypes of SNP rs11931074 according to the presence of α-Syn immunostaining in the ENS in patients with PD and controls are illustrated in Fig. 1. In patients with PD, rs11931074 (G allele) was significantly associated with the presence of α-Syn immunostaining in the ENS (OR = 5.96, 95% CI = 1.70–20.97, $P_{corrected}$ = 0.01; Table 2). In controls, there was no significant association between SNP rs11931074 and the presence of α-Syn immunostaining in the ENS.

The longer *SNCA* REP1 allele variant (REP1 score of 3–4) was not significantly associated with the presence of α-Syn immunostaining in the ENS in patients with PD and controls (Table 2). Other coding schemes of *SNCA* REP1 allele variants did not show any association with the presence of α-Syn immunostaining in the ENS.

3.3. Interaction effect of SNP rs11931074, REP1 variants, and PD status on the presence of α-Syn immunostaining in the ENS

As expected from the stratified analysis above, PD status-rs11931074 (G allele) interaction was associated with the presence of α-Syn immunostaining in the ENS (OR = 7.33, 95% CI = 1.58–33.88, illustrative *P* = 0.01; Table 3). In contrast, there was no association of longer *SNCA* REP1 allele variants, PD status, and their interaction for the presence of α-Syn immunostaining in the ENS in all coding schemes for REP1 (Table S1). In analyses using dichotomized scoring of two REP1 alleles, there was also no significant association between longer *SNCA* REP1 allele variants and the presence of α-Syn immunostaining in the ENS (Table S2).

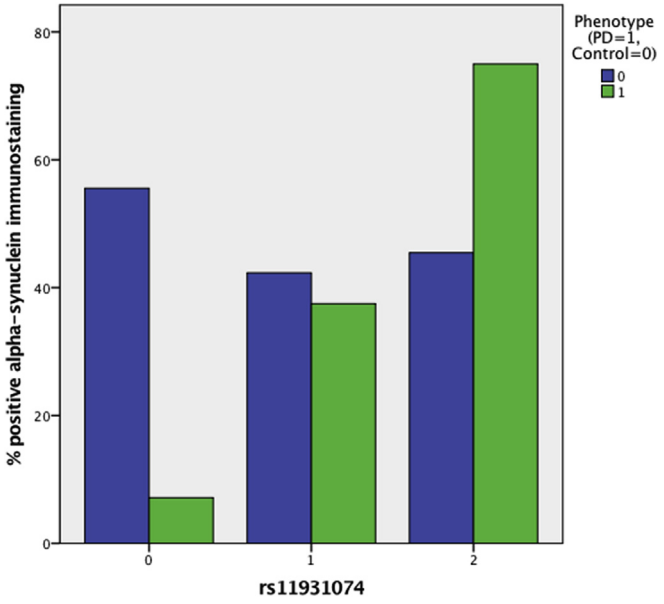

Fig. 1. Genotypes of *SNCA* single nucleotide polymorphism rs11931074 according to the presence of α-synuclein immunostaining in the enteric nervous system in patients with Parkinson's disease (PD) and controls. In the logistic regression model including interaction, there is a main effect for phenotype (PD vs control, *P* = 0.01) and for the interaction between phenotype and rs11931074 (*P* = 0.01).

3.4. Predictive values of SNCA rs11931074 for the presence of α-Syn immunostaining in the ENS

To estimate the diagnostic utility of the *SNCA* rs11931074 genotype for the presence of α-Syn immunostaining in the ENS, the sensitivity and specificity were determined and are summarized in Table 4. In patients with PD, the sensitivity of the G allele of *SNCA* rs11931074 to predict the presence of α-Syn immunostaining in the ENS was relatively high (92.3%, 95% CI = 77.8–100.0) and the specificity was modest (52.0%, 95% CI = 32.4–71.6). In controls, estimated values of sensitivity and specificity were low (Table 4).

4. Discussion

Our exploratory study revealed that *SNCA* SNP rs11931074 was significantly associated with the presence of α-Syn immunoreactivity in the ENS of patients with PD, although the longer *SNCA* REP1 allele was not associated with α-Syn immunoreactivity in the ENS. These findings suggest that specific genetic variants in *SNCA* may be associated with α-Syn immunoreactivity in the ENS in patients with PD.

α-Syn, encoded by *SNCA*, is a small (14kD) presynaptic nerve terminal protein that is abundantly present in the brain; it has physiological roles in neuronal homeostasis, including facilitating SNARE complex assembly at the synapse and controlling the dynamics of neurotransmitter release from synaptic vesicles [22,23]. Similar patterns of α-Syn immunoreactivity in the ENS between patients with PD and healthy individuals may be accounted for by the fact that conventional immunohistochemistry cannot differentiate pathological α-Syn protein from normal physiological α-Syn protein.

The SNP rs11931074 is located about 6 kb downstream of the *SNCA* gene and has repeatedly been demonstrated to be associated with PD risk (OR = 1.34, 95% CI = 1.26–1.41, $P = 4.46 \times 10^{-25}$ based on meta-analysis across all available studies [www.pdgene.org]). Further, it has been suggested that this association is possibly caused by *cis-*

Table 2
Association of *SNCA* genetic variants with α-synuclein (α-Syn) immunoreactivity in the stomach and colon mucosa of patients with Parkinson's disease (PD) and controls.

Variants	Position[a]	Region	Allele (major/minor)	MAF (case/control)	Additive model (with adjustment[f])	
					OR (95% CI)	$P_{corrected}$[g]
PD/α-Syn(+)[b] (n = 13) vs. PD/α-Syn(-)[c] (n = 25)						
rs11931074	90639515	downstream	T/G	0.692/0.280	5.96 (1.70, 20.97)	0.01
REP1	–	upstream	–	–	2.27 (0.27, 19.43)	0.91
Control/α-Syn(+)[d] (n = 21) vs. Control/α-Syn(-)[e] (n = 25)						
rs11931074	90639515	downstream	T/G	0.500/0.540	0.85 (0.34, 2.09)	1.00
REP1	–	upstream	–	–	4.87 (1.05, 22.66)	0.09

MAF, minor allele frequency; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.
[a] NCBI build 36 of the human genome.
[b] PD/α-Syn(+) means PD patients who had positive α-Syn immunoreactivity in the enteric nervous system (ENS).
[c] PD/α-Syn(-) means PD patients who had not positive α-Syn immunoreactivity in ENS.
[d] Control/α-Syn(+) means controls who had positive α-Syn immunoreactivity in ENS.
[e] Control/α-Syn(-) means controls who had not positive α-Syn immunoreactivity in ENS.
[f] Analysis was performed with adjustment for age at study entry and sex.
[g] $P_{corrected}$ is the *P* value that was calculated using Bonferroni correction.

regulation of the *SNCA* gene expression [15,17,24,25]. REP1, a polymorphic dinucleotide complex repeat sequence at approximately 10 kb upstream of the *SNCA* transcription start site, has also been associated with PD risk via the regulation of *SNCA* transcription [15,26]. In this study, the G allele at rs11931074 was significantly associated with α-Syn immunoreactivity in the ENS in patients with PD, not only in a main analysis for each subject group of patients with PD and controls, but also in an interaction analysis. Genotypes of SNP rs11931074 in relation to the presence of α-Syn immunostaining in the ENS in patients with PD and controls are illustrated in Fig. 1 that shows the percentage of individuals with a specific genotype at the rs11931074 being positive for α-Syn. This illustrates that within patients with PD (green), the percentage increases with varying genotypes from < 10% to almost 40% in heterozygotes and > 70% in the homozygotes. In contrast, the differences in percentages of positive α-Syn are much smaller in control subjects (blue), ranging from < 60%, to just above 40% for both heterozygotes and homozygotes.

Accordingly, Table 2 shows that in patients with PD, the rs11931074 (G allele) was significantly associated with the presence of α-Syn immunostaining in the ENS (OR = 5.96, 95% CI = 1.70–20.97, $P_{corrected}$ = 0.01; Table 2). In controls, there was no significant association between SNP rs11931074 and the presence of α-Syn immunostaining in the ENS. These findings suggest that α-Syn immunoreactivity assessed by conventional immunohistochemistry in the ENS may be associated with *SNCA* genotypes that regulate *SNCA* expression in patients with PD. The association of rs11931074 with α-Syn immunoreactivity in the ENS was not evident among healthy individuals. Although the presence of α-Syn immunoreactivity in the ENS is detected in a similar manner in both patients with PD and healthy individuals, *SNCA* variants that are well-known to increase PD risk were associated with α-Syn in ENS only in patients with PD, not in controls. These observations may suggest that enteric α-Syn deposition in patients with PD is a distinct α-Syn strain compared with the α-Syn strain of enteric α-Syn deposition in normal healthy individuals [27].

Table 4
The sensitivity and specificity of G allele of *SNCA rs11931074* for the presence of alpha-synclein (α-Syn) immunostaining in the enteric nervous system (ENS) of 38 patients with Parkinson's disease (PD) and 46 controls.

	Sensitivity, % (95% CI)	Specificity, % (95% CI)
Parkinson's disease	92.3 (77.8–100.0)	52.0 (32.4–71.6)
Controls	76.2 (58.0–94.4)	16.0 (1.6–30.4)

In the present study, the sensitivity of the G allele of *SNCA* SNP rs11931074 for the presence of α-Syn immunoreactivity in the ENS of patients with PD were relatively high (92.3%), although the specificity was modest. The potential predictive roles of *SNCA* SNP rs11931074 for the presence of α-Syn immunoreactivity in the ENS need to be further evaluated in larger samples.

Our study also has several limitations. First, the sample size of this association study was small, although based on a comparatively large-scale study investigating enteric α-Syn in living humans. Given this small sample size and small effect size of the two common variants in the *SNCA* tested, an association of *SNCA* variants with susceptibility to PD could not be evaluated because of low statistical power. Second, mRNA or quantitative α-Syn protein measurements were not performed. However, this study investigated α-Syn immunoreactivity in detail using the immune-scoring system according to density of S-100 and α-Syn positive nerve fibers, which indirectly reflects *SNCA* expression levels. Third, this study focused on two common, well established risk variants in *SNCA*. Future well-designed studies are needed to evaluate other causal or risk genes of PD and their interactions with *SNCA* to further clarify the association of genetic modifiers with enteric α-Syn deposition.

Disclosures

1) All coauthors have seen and agree with the contents of the

Table 3
Interaction analysis using a logistic regression model to predict alpha-synclein (α-Syn) immunostaining in the enteric nervous system (ENS) of 38 patients with Parkinson's disease (PD) and 46 controls with adequate stomach and colon mucosa samples.

	Regression coefficient	Standard error	OR (95% CI)	*P* value
Phenotype (PD vs control)	−2.42	0.96	0.09 (0.01, 0.58)	0.01
SNCA SNP rs11931074	−0.19	0.45	0.83 (0.34, 2.01)	0.68
Interaction between PD phenotype and rs11931074	1.99	0.78	7.33 (1.58, 33.88)	0.01
Constant	0.02	0.55		

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

manuscript, the ICMJE requirements for authorship have been met, and that each author believes that the manuscript represents honest work.
2) The data contained in this manuscript being submitted have not been previously published, have not been submitted elsewhere for publication, and will not be submitted elsewhere while under consideration by *Parkinsonism & Related Disorders*.
3) There are no other submissions or previous reports that might be regarded as redundant publication of the same or very similar work of this study.
4) All authors have no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within 3 years of beginning the work submitted that could inappropriately influence (bias) our work.
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Relevant conflicts of interest statement

All authors have no actual or potential conflicts of interest.

Financial disclosures of all authors

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2018.10.028.

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