

ARTICLE

# Variants in the 3'UTR of *SNCA* do not affect miRNA-433 binding and alpha-synuclein expression

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Alpha-synuclein (*SNCA*) is a major risk gene for Parkinson's disease (PD) and increased *SNCA* gene dosage results in a parkinsonian syndrome in affected families. Regulatory regions relevant for *SNCA* expression include the 3' untranslated region (UTR), which among other regulatory elements contains several micro-RNA-binding sites. Interestingly, variants located in the 3' region of *SNCA* have been associated with PD in two genome-wide association studies. To test whether private mutations in this region contribute to PD, we sequenced the 3'UTR of *SNCA* in 1285 PD patients and 1120 age/sex-matched healthy controls. We found two rare variants, the one corresponding to the single nucleotide polymorphism rs145304567 and the novel variant c.\*1004\_1008delTTTT. Although rs145304567 affects the putative-binding site of microRNA (miRNA) -433, the allele distribution was similar in PD patients and controls, and the expression of *SNCA* mRNA was not related to the genotype. Furthermore, a regulatory effect of miRNA-433 on *SNCA* expression levels was not detected.

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## INTRODUCTION

Alpha-synuclein ( $\alpha$ -synuclein, *SNCA*) is a key component of Lewy-bodies found in Parkinson's disease (PD) patients.<sup>1,2</sup> Mutations in *SNCA* cause rare familial parkinsonian syndromes (PS) with high penetrance. In addition, a dosage effect of *SNCA* duplications and triplication has been identified in familial PS.<sup>3</sup> In idiopathic PD, several genetic association studies have suggested that the *SNCA* gene harbors significant risk haplotypes.<sup>4</sup>

Recent genome-wide association studies in PD pointed to the 3' end of *SNCA*, which had already been implicated in the regulation of *SNCA* expression.<sup>5–7</sup> The 3' untranslated region (UTR) of *SNCA* carries various putative microRNA (miRNA) -binding sites that could have a role in *SNCA* expression and thus contribute to the susceptibility to develop PD.<sup>8,9</sup> We hypothesized that variants located within the 3'UTR might influence the expression of *SNCA* by modulating translation or mRNA stability. For our screening, we identified the evolutionary most conserved part of the 3'UTR as the region in which most probably regulatory variants reside and sequenced the highly conserved region adjacent to the stop codon.

## PATIENTS AND METHODS

### Patients and controls

After obtaining informed consent, all patients underwent a standardized neurological examination by a movement disorder specialist. The diagnosis of PD was based on the UK Brain Bank diagnostic criteria (with the exception that positive family history was not regarded an exclusion criterion).<sup>10</sup> Family history was regarded positive if parkinsonism was reported for one first- or second-degree relative (Table 1). A total of 1285 PD patients were collected at

movement disorders centers in Bonn, Cologne, Luebeck and Belgrade, and represent consecutive series, not originating in a geographic isolate. The initial case-control sample was collected in Bonn and Cologne. A total of 1120 sex- and ethnic-matched healthy persons were included in this study as control sample. PD was rated using the Unified Parkinson Disease Rating Scale (UPDRS) III. The study was approved by the local university ethics committees.

### DNA isolation and genotyping

Screening of patients and controls was performed on genomic DNA isolated from peripheral blood monocytes (pbm) using a standardized protocol. We sought to analyze the most conserved region of the 3'UTR of *SNCA*. Using the publicly available genomic databases (<http://genome.ucsc.edu/>), we defined a sequences segment of around 650 bp located directly after the stop codon (position 399–1056). This segment was selected for analysis because its genomic conservation across species was similar to *SNCA* exons, thereby implicating a putative functional relevance. This fragment was amplified and directly sequenced using the forward primer 5'-TCAAGACTACGAACCTGA AGC-3' and the reverse primer 5'-TTCATGTTGCTTATAAGCATG-3'. For polymorphism numbering, we followed the nomenclature developed by the Human Genome Variation Society (HGVS). Thus, numbering of polymorphisms and primer position was based on the cDNA sequence of the GenBank entry NM\_000345.3, where position +1 corresponds to the A of the ATG translation initiation codon. We searched for putative miRNA-binding sites within the 3'UTR of *SNCA* using the public database microRNA.org ([www.microRNA.org](http://www.microRNA.org)).

### Association analysis

Genotyping of rs356165 (located on the 3'UTR of *SNCA*) was performed using KASP-On-Demand Standard turnaround (KBioscience, Hertfordshire, UK) following the manufacturer's instruction.

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**Table 1 Demographics of the samples analyzed in this study and sequencing results**

	Stage 1, Discovery			Stage 2, Replication			Stage 1 + Stage 2 <sup>a</sup>			Stage 1 + Stage 2 <sup>b</sup>				
	PD patients	Controls	OR	95% CI	P-value <sup>c</sup>	PD Patients	PD Patients	Controls	OR	95% CI	P-value <sup>c</sup>	OR	95% CI	P-value <sup>c</sup>
Number	354	375				663	268	745						
Ethnic background	German	German				German	Serbian	German						
Gender M:F ratio	2.18	1.01			1.3	1.47	1.27							
Age (years ± SD)	67.53 (± 10.76)	45.15 (± 13.16)			65.56 (± 12.43)	59.51 (± 10.94)	62.14 (± 11.83)							
Age of onset	59.45 (± 12.23)				49.81 (± 14.58)	50.52 (± 10.32)								
Positive family history	5.10%				13%	NA								
rs145304567 (heterozygous)	7	1	7.5	(0.95; 340.38)	0.033	3	0	16	0.21	(0.04; 0.72)	0.009	0.6	(0.26; 1.50)	0.334
c.1004_1008delTTTT (heterozygous)	2	0	inf	(0.20; inf)	0.23	1	0	3	0.38	(0.01; 4.67)	0.627	1.1	(0.15; 8.24)	1

Abbreviations: NA, not available; inf, infinite.

<sup>a</sup>Without Serbian patients, for lack of matched controls.<sup>b</sup>With Serbian patients.<sup>c</sup>P-values were computed with Fisher's exact for 2 × 2 contingency tables – We used the implementation in R (The R Project for Statistical Computing, <http://www.r-project.org/>).

Case-control association analysis of rs356165 was performed with Armitage's test for allelic trend.<sup>11</sup> To this purpose and also to test for deviations from Hardy-Weinberg equilibrium, we used the implementation in the genetic analysis software INTERSNP.<sup>12</sup> For the analysis of deletions, we used Fisher's exact test for 2 × 2 contingency tables and its implementation in R (The R Project for Statistical Computing, <http://www.r-project.org/>). All variants analyzed in this study were in Hardy-Weinberg equilibrium.

### RNA isolation and qRT-PCR

RNA was isolated from pbm of eight patients (four patients with/without the rs145304567 polymorphism each, collected in Bonn) with RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. For reverse transcription, 1 µg total RNA and m-MLV Reverse Transcriptase, RNase H Minus (Promega, Mannheim, Germany) were used with oligo dT and random hexamer primers according to the manufacturer's instruction. Quantitative PCR was performed in triplicate with SYBR Green JumpStart Taq ReadyMix (Sigma, Hamburg, Germany) and SNCA-primers (hSNCA-qF2\_ GGACCAGTTGGGCAAGAATG and snca-r\_ CCACAGCACACAAGACC CTGCTACC) on an Applied Biosystems (Darmstadt, Germany) 7500 Fast Real-Time PCR System using 2 µl of 1:10 diluted cDNA for each reaction.

### Vector construction, cell culture and transfection

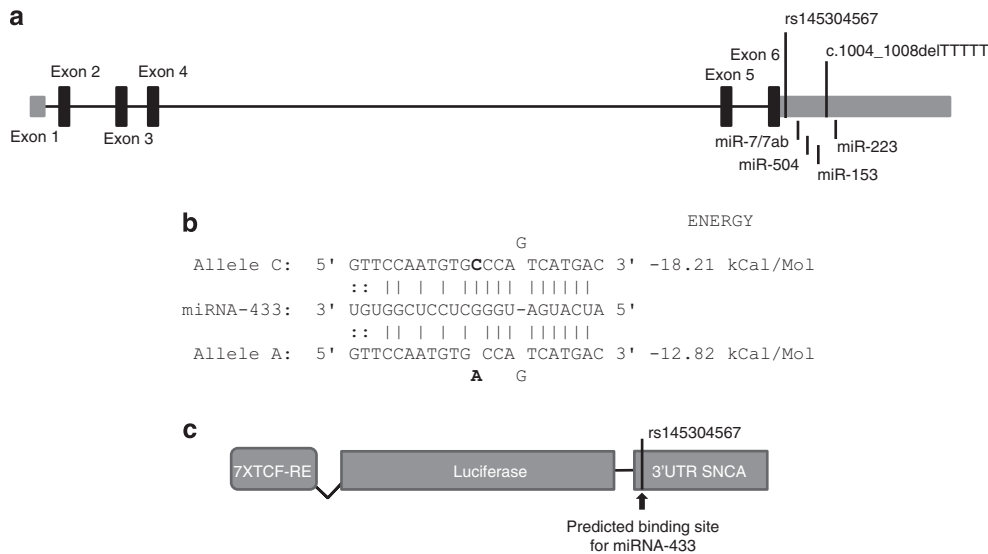
Neuro2A and SK-N-SH cells were cultured in DMEM (PAA, Coelbe, Germany) supplemented with 10% fetal bovine serum (FBS Gold, PAA), 100 U/ml Penicillin and 100 µg/ml Streptomycin (PAA) at 37 °C and 5% CO<sub>2</sub>. A total of 1.5 × 10<sup>5</sup> cells per well were seeded on 24 well plates and transfected with the indicated luciferase reporter constructs using Lipofectamine 2000 Reagent (Invitrogen, Darmstadt, Germany) according to the instructions of the manufacturer.

To delineate the 3'UTR of SNCA, we searched for putative polyA signals within the predicted 3'UTR of SNCA and also searched for mRNA supporting this polyA signals. Thus, we defined a region of 1100 bp after the stop codon of SNCA as the 3'UTR. This segment was amplified and introduced in the vector topflash directly after the stop codon of the firefly luciferase cDNA, thereby replacing the 3'UTR of the firefly luciferase. Both allele possibilities were generated by site-directed mutagenesis. The dual-luciferase reporter assay for both allele variants within the SNCA 3'UTR was performed following the protocol described by Chung *et al*,<sup>13</sup> with the exception that experiments were normalized using the topflash vector containing the luciferase with its own 3'UTR. To activate the wingless pathway, Neuro2A cells were transiently transfected with LRP5, Norrin, FZD4, Topflash and Renilla.<sup>14</sup> The firefly luciferase activity was measured and normalized to Renilla luciferase activity (to control for transfection efficiency) 30 h after transfection. The experiment was repeated three times, with each transfection performed in sixuplicate. The mean luciferase activity for each sixuplicate was used for the statistical analysis.

### RESULTS

The stage 1 of sequencing included 354 PD patients of German origin. In this sample, we identified a novel variant in a heterozygous state in seven patients (Table 1). This variant changes a cytosine for an adenine at position 500 of the SNCA mRNA (c.\*500C>A; Figure 1a). Analysis of the 1000 genome database showed that this polymorphism is a known single nucleotide polymorphism (SNP; rs145304567). In addition, we discovered a new five thymidine insertion/deletion variant at position 1004 of SNCA mRNA (c.\*1004\_1008delTTTTTT) in two PD patients. Sequencing of 375 healthy individuals of German origin detected rs145304567 in one person but not the c.\*1004\_1008delTTTTTT. Association analysis of both SNPs using this case-control sample revealed genetic association with PD only for rs145304567 and PD (OR = 7.52; P = 0.033, Table 1).

This finding prompted us to add a second set of patients and controls in our screening (replication analysis). Thus, we sequenced 663 additional PD patients from Germany and 268 Serbian PD patients. We also analyzed the sequencing results of 745 additional



**Figure 1** (a) Gene Structure of *SNCA*. Exons are denoted by blocks; coding regions are shown in black, and 5' and 3'UTRs are shown in grey. Locations of SNPs found within the 3'UTR in this study are indicated with black lines. Putative-binding sites for different miRNAs are also shown with small black lines. (b) The predicted binding site for miRNA-433 at 3'UTR of the *SNCA* gene. At rs145304567, allele C base paired with G in Watson–Crick mode (as shown with a solid line), whereas allele A wobble base paired with G (as shown with a blank space). (c) The topflash construct with 7 × LEF/TCF response elements (7 × TCF-RE), the luciferase cDNA and the *SNCA*-3'UTR containing either the allele C or A.

healthy individual of German origin. For rs145304567, the second round of sequencing detected 3 more German PD patient and 16 German controls carrying this variant in heterozygous state. In the Serbian patients, we did not identify any carrier of this polymorphism and therefore decided not to screen additional Serbian controls. For c.\*1004\_1008delTTTTT, we discovered one additional German PD patient and three controls with this insertion/deletion in heterozygous state. No individual homozygous for each variant was identified. Thus, minor allele frequencies for both SNPs were <0.01. In the replication sample, genetic association analysis resulted in a significant association between rs145304567 and PD (Table 1). However, in this case the minor allele showed a protective effect (Table 1). The joint analysis of both samples revealed no association for rs145304567 and PD. This negative result was independent of the inclusion of the Serbian population (Table 1). Thus, our sequencing results did not support a contribution of either variant to PD. We did not detect additional variants within this conserved region of the 3'UTR of *SNCA* in our PD sample. To exclude the possibility that *SNCA* may be no susceptibility gene in our PD sample, we performed an association study using the entire German sample included in this study. To this end, we selected rs356165 that has been previously associated with PD and it is located within the 3'UTR of *SNCA*.<sup>15</sup> This analysis revealed an association between our PD sample and rs356165 (Table 2, OR = 1.36; 95% CI = 1.20–1.54;  $P = 1.85 \times 10^{-6}$ ). Consistent with previous reports, the minor allele G was significantly more frequent in the patient group than in controls (0.44 versus 0.37, respectively).<sup>15</sup>

Although rs145304567 showed no association with PD, the imbalanced distribution of heterozygous between patients and control (10 versus 17) led us to explore a possible functional relevance for this rare SNP. We detected that rs145304567 is located in a putative-binding site for the miRNA-433. Interestingly, genetic variants located in the miRNA-433-binding site of FGF20 have been associated with increased risk for PD due to overexpression of  $\alpha$ -synuclein.<sup>16</sup> In a similar manner, we sought to analyze the functional relevance of this putative miRNA-433-binding site on *SNCA* mRNA stability. We were

**Table 2** *SNCA* rs356165 (3'UTR) genotypic and allelic frequencies in patients and controls

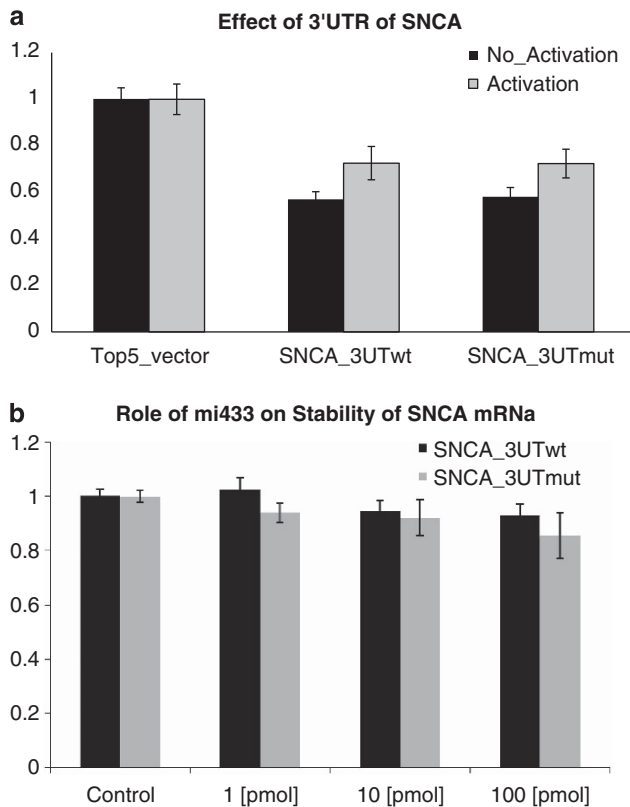
	German patients (N = 980)	German controls (N = 1005)
AA	290 (29.6%)	401 (39.9%)
AG	510 (52.0%)	464 (46.2%)
GG	180 (18.4%)	140 (13.9%)
Allele-A	1090 (0.56)	1266 (0.63)
Allele-G	870 (0.44)	744 (0.37)

Allele-G vs Allele-A:  $P = 1.85 \times 10^{-6}$ ; OR = 1.36; 95% CI = 1.20–1.54.

also interested in the ability of rs145304567 to modify the binding of miRNA-433 to this site (Figure 1b), which might thus contribute to PD susceptibility. We overexpressed luciferase constructs carrying the 3'UTR of *SNCA* containing either the wt (C) or the mutant (A) allele in different cell lines (Figure 1c). In Neuro2A cells, the expression of luciferase carrying the 3'UTR of *SNCA* was reduced when compared to the luciferase without the 3'UTR *SNCA*. This reduction was similar for both wild type and mutant (Figure 2a). Likewise, the mRNA levels of both constructs were similar in human SK-N-SH cells (Figure 3a). As both constructs are under the control of seven LEF/TCF response elements, we also analyzed their expression upon activation of the canonical wingless (Wnt) pathway by cotransfection with plasmids carrying cDNA sequences for Norrin, Fzd4 and LRP5 proteins.<sup>14</sup> The expression of both constructs increased to similar levels in Neuro2A cells (Figure 2a).

Next, we explored the role of miRNA-433 on the mRNA stability of both constructs. To this end, we added increasing amounts of miRNA-433 to the transfection mixture. These experiments revealed only a marginal effect of miRNA-433 at higher concentrations for both reporter constructs (Figure 2b).

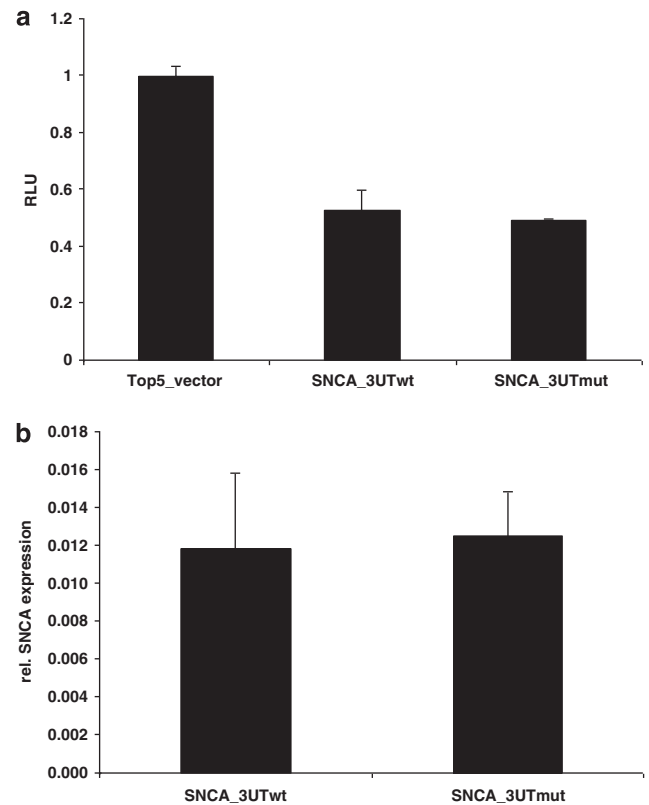
Furthermore, we did not observe differences in *SNCA* mRNA in pbm from PD patients and controls, carrying either the wt (C) or the mutant (A) alleles (Figure 3b).



**Figure 2** (a) Expression of luciferase constructs carrying either the C (SNCA\_3UTwt) or A (SNCA\_3UTmut) allele within the 3'UTR of *SNCA* in Neuro2A cells. The graph depicts the relative luciferase activities (average and standard deviation) of Top5\_control vector (topflash with native 3'UTR), allele C and allele A. Black bars show the activity of firefly luciferase without stimulation of Wnt pathway. Grey bars show the activity of luciferase upon activation of Wnt pathway using Norrin, Fzd4 and LRP5. Firefly luciferase activity was measured and normalized to Renilla luciferase activity (to control for transfection efficiency). As expected, this activation increased the expression of both constructs; however, in both the activation was similar. The experiment was performed three different times, in sixplicate each time. (b) Effect of growing concentration of miRNA-433 (1–100 pmol) on the expression of luciferase carrying either the C allele (SNCA\_3UTwt) or A allele (SNCA\_3UTmut). All experiments were performed under activated conditions. Luciferase activity under different miRNA-433 concentrations was normalized to the control situation without miRNA-433. This experiment revealed minimal effect of miRNA-433 on the stability of both constructs. The experiment was performed three different times, in sixplicate each time.

## DISCUSSION

Modulation of *SNCA* expression levels has been proposed as an important pathogenic mechanism in PD: duplication and triplication of the gene are responsible for familial forms of PD with high penetrance.<sup>3</sup> Interestingly, the levels of *SNCA* are also critical for idiopathic PD as indicated by expression analysis in dopaminergic neurons of the substantia nigra, which revealed higher levels of *SNCA* mRNA in PD patients compared with healthy individuals.<sup>17</sup> In line with this, several association studies in PD have found positive signals in variants within the promoter region and the 3'end of *SNCA*, both regions, which modulate expression of *SNCA*.<sup>15,18</sup> Remarkable, two recent GWASs in PD showed association between variants located in the 3'end of *SNCA* and PD.<sup>5,6</sup> Yet, the nature of these regulatory variants/mutations remains to be elucidated. We sought to identify variants in the 3'UTR



**Figure 3** Influence of the (C/A) mutation on *SNCA* mRNA expression. (a) Luciferase expression of *SNCA* 3'UTR constructs in SK-N-SH cells. Depicted are the mean relative light units (RLU  $\pm$  SD, normalized to Renilla luciferase activity to control for transfection efficiency) of three independent transfections of either the C (SNCA\_3UTwt) or A (SNCA\_3UTmut) allele compared with the empty vector (Top5\_control, topflash with native 3'UTR). (b) qRT-PCR analysis of *SNCA* expression in PD patients' pbm, carrying either the wt (C, four individuals) or the mutant (A, four individuals) allele (mean  $\pm$  SD of three independent amplifications).

of *SNCA* that might be responsible for a differential regulation in PD patients. Consequently, we sequenced the evolutionary most conserved region of the 3'UTR of *SNCA* in PD patients and controls. The analysis of 2570 chromosomes of PD patients and 2240 control chromosomes identified two rare polymorphisms, which showed no genetic association with PD. However, the association of rs356165 with PD in our sample confirmed that *SNCA* is a susceptibility gene in our PD sample.

The regulatory role of the 3'UTR of *SNCA* was suggested in recent reports showing post-transcriptional regulatory roles of miRNA-7 and miRNA-153 on *SNCA* mRNA level in human and murine expression systems.<sup>8,9</sup> This regulatory effect was mediated by its binding to the 3'UTR of *SNCA*. Interestingly, the rare variant rs145304567 is located in a putative miRNA-433-binding site affecting its putative affinity for this site. Another binding site of miRNA-433 in the 3'UTR of the fibroblast growth factor 20 gene (*FGF20*) has been reported to be associated with PD.<sup>16,19</sup> The variant (rs12720208) altered the binding of miRNA-433, thereby, increasing the translation of *FGF20* protein. This correlated with increased expression of *SNCA* in PD patients carrying the risk allele.<sup>16</sup> We therefore sought to determine whether the effects of miRNA-433 might have been mediated through direct modulation of *SNCA* expression.

However, our experimental data revealed no effect for rs145304567, neither under basal conditions nor in the presence of miRNA-433. As

expected, the presence of the A allele did not influence SNCA expression in PD patients at the mRNA level. Thus, although we cannot rule out the involvement of rare mutations affecting miRNA-binding sites in familial forms of PD, our study excludes common variants within the binding site of miRNA-7 and miRNA-153 as risk factors for idiopathic PD.

The fact that this region shows reduced variability could reflect a function of the respective mRNA. However, our results indicate that this genomic fragment does not carry mutations associated with the 3' signals found in association studies on PD.<sup>5,6,20</sup> Interestingly, detailed analysis of the SNCA locus revealed evidence for an additional independent association signal located upstream of exon 1 of SNCA.<sup>20</sup> Thus, several linkage disequilibrium (LD) blocks may exist within the SNCA locus carrying the association signals. Identification of these LD blocks will help to determine which regions of SNCA carries the causative variant for PD.

In summary, we found no consistent evidence associating common variants within the highly conserved 3'UTR of SNCA with idiopathic PD. Furthermore, it seems unlikely that this region carries the risk variants detected in the recent GWASs. Additional sequencing studies covering the entire SNCA locus may be needed to identify the causative variants linked to the association signals reported in SNCA.

#### CONFLICT OF INTEREST

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