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A single nucleotide polymorphism in the 3'UTR of the *SNCA* gene encoding alpha-synuclein is a new potential susceptibility locus for Parkinson disease

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Abstract

In Parkinson disease, the second most common neurodegenerative disorder in humans, increased alpha-synuclein (*SNCA*) levels are pathogenic, as evidenced by gene copy number mutations and increased alpha-synuclein levels detected in some familial and sporadic PD cases, respectively. Gene expression can be regulated at the post-transcriptional level by elements in the 3' untranslated region (3'UTR) of mRNAs. The goal of this study was to determine whether the 3'UTR of human *SNCA* can affect gene expression. Comparative sequence analysis revealed very high conservation across the entire 3'UTR of human *SNCA* over millions of years, suggesting the presence of multiple functionally important domains. EST and RT-PCR analyses showed that four different polyadenylation events occur in the 3'UTR of human *SNCA*. Finally, using luciferase assays, we examined the effect of the minor allele of five naturally occurring single nucleotide polymorphisms (SNPs) in the 3'UTR of *SNCA* on gene expression. The minor allele of SNP rs17016074 increased luciferase expression by 32% in a transient transfection assay in SHSY5Y neuroblastoma cells. Understanding the role of the 3'UTR of human *SNCA* and identifying functionally important naturally occurring SNPs using reporter assays can complement disease association studies in humans, uncovering potential susceptibility or protective polymorphisms in Parkinson disease. Our findings demonstrate that the 3'UTR of human *SNCA*, as a whole, and rs17016074, in particular, are loci of potential clinical importance for Parkinson disease.

Keywords

Parkinson disease; alpha-synuclein; *SNCA* 3'UTR; SNP; rs17016074

Introduction

Parkinson disease (PD) is the second most common neurodegenerative disorder in humans. Approximately 90% of all PD cases are sporadic, with the remaining ~10% being familial. In the vast majority of PD cases, the exact etiology of the disease is not known [10]. In addition to the point mutations that cause some cases of familial PD, two lines of evidence suggest that,

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in the absence of point mutations, increased levels of wild-type alpha-synuclein are also pathogenic. First, in some sporadic PD cases, *SNCA* mRNA levels in the brain are increased [3,9,21,25]. Second, eight familial PD cases carrying multiple copies of *SNCA* have been identified [2,6,11,12,19,24]. Due to the absence of point mutations in any of the copies of *SNCA* in these patients, the cause of PD appears to be the mere increase in alpha-synuclein levels. Moreover, in support of a dosage effect, PD patients from families with two extra copies of *SNCA* have a more severe phenotype than PD patients with only one extra copy [6,12,22]. The pathogenicity of multiple *SNCA* gene copies and the apparent dosage effect of alpha-synuclein levels in both sporadic and familial PD highlight the clinical significance of the regulation of *SNCA* gene expression.

Regulation of gene expression can take place at the level of transcription, by controlling mRNA synthesis, as well as post-transcriptionally, by controlling mRNA stability and translation. Studies on the transcriptional regulation of *SNCA* have been primarily centered on the NACP repeat, which is believed to affect *SNCA* transcription [4]. Numerous association studies have established a link between increased risk of PD and NACP alleles that increase *SNCA* expression [13].

Increased *SNCA* expression could also result from misregulation of its post-transcriptional control. Post-transcriptional control of gene expression can be mediated by several elements, many of which are located in the 3'UTR of mRNAs [1,15,26]. Despite a series of studies that report an association of polymorphisms at the 3' end of *SNCA* with sporadic PD [16-18,23, 27], the precise function (and mature sequence) of the 3'UTR of human *SNCA* mRNA is not known.

The goal of this study was to determine whether the 3'UTR of human *SNCA* can affect gene expression. To find evidence of important regulatory elements, we first examined the degree of sequence conservation of the 3'UTR of *SNCA* across multiple species. We then performed experiments that were designed to identify the sequence that constitutes the functional 3'UTR of the human *SNCA* message. Finally, using luciferase reporter assays, we identified a single nucleotide polymorphism in the 3'UTR of human *SNCA* that has a significant impact on gene expression.

Methods

Sequence analyses

NM_000345 is the human *SNCA* mRNA sequence used as a reference throughout this study. The 3'UTRs of *SNCA* orthologs (Fig. 1) were obtained from the following NCBI entries L33860 (canary), NM_204673.1 (chicken), XM_001496904 (horse), XM_855879 (dog), NM_000345 (human), XM_1162591 (chimpanzee), XM_001095402 (rhesus monkey), NM_001034041.1 (cow), NM_001037145.1 (pig), NM_009221.2 (mouse), NM_019169.2 (rat), NM_001087154.1 (*Xenopus laevis*). Multiple sequence alignments were performed using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2>) with the gap extension penalty set to 0.05 and the gap distance penalty was set to 1. Sequence similarities were visualized using the BOXSHADE program (http://www.ch.embnet.org/software/BOX_form.html). The last 337 nucleotides shown in Fig. 2 were obtained from contig NT_016354 (NCBI). RepeatMasker (<http://www.repeatmasker.org>) identified repeats in the 3'UTR of *SNCA*.

Global sequence alignments of chicken-human ortholog pairs were performed using a version of FASTA (GRASTA: ktup = 3, opt cutoff = 100) on sequences previously identified [20]. Sequence pairs were used if the 3'UTRs of both orthologs were longer than 50 nucleotides in length after the trimming of terminal adenosine residues; The final data set consisted of 426 ortholog pairs. For local sequence alignments, chicken-human ortholog pairs from

HomoloGene (NCBI) were identified by text matching of gene symbols. We obtained 2653 pairs of orthologs for which both species had unambiguously associated, experimentally verified RefSeq mRNA records and where both of the paired sequences had trimmed 3'UTRs greater than 50 nucleotides in length. The sequences were subjected to pairwise BLAST alignment (bl2seq version 2.2.19); all masking and low-complexity filtering options off. Only non-overlapping, plus-strand alignments with E-values $< 1 \times 10^{-6}$ were accepted.

All expressed sequence tags (ESTs) were obtained from the Unigene cluster Hs271771 (NCBI). ESTs that did not align with fully-spliced *SNCA* mRNAs and did not contain a polyA tail were excluded.

RT-PCR analysis

RT-PCR analysis (Amersham Pharmacia, 27-9261-01) was performed on RNA extracted from the brain of [Tg(*SNCA*^{WT}); *Snca*^{-/-}] [8] mice. These mice are deleted for the murine *Snca* gene and contain a P1 artificial chromosome (PAC) that carries the entire *SNCA* including several kb of upstream and downstream genomic sequence. The mice were sacrificed following NIH guidelines (protocol number G-98-13). RNA was extracted from 14 days old mouse brain using Trizol (Invitrogen, 15596-026). Oligo(dT)₁₈-NotI was used for first-strand synthesis (Amersham Pharmacia 27-9261-01) and appropriate samples were treated with DNaseI (Gibco, 18068-015). PCR amplification was carried out using anchored oligo(dT)₂₀ (Invitrogen 12577-011) or TTAAGGAACCAAGTGCATACCAAAACACA (R1) and one of the following forward primers: CTACGAACCTGAAGCCTAAGAAAT (F1) or ATTTTATTTTATCCCATCTCACT (F2).

Luciferase reporter constructs and SNPs

The first 574 nucleotides of the 3'UTR of human *SNCA* was amplified using the primers TCTAGAGAAATATCTTTGCTCCCAGTTT and GGATCCTAAAGTGAGATGGGATAAAAATAAAAT from DNA extracted from human fibroblasts using standard protocols. The full length 3'UTR was cloned into the pGL3-Promoter (Promega E1761), after XbaI/BamHI excision of the SV40 late polyA signal. The open reading frame (ORF) of luciferase of the pGL3-Promoter was replaced with the ORF of Luc2CP obtained from pGL4.16 (Promega E6711).

All SNPs in the 3'UTR of human *SNCA* were obtained from dbSNP (NCBI). Haplotypes and linkage disequilibrium information across the 3'UTR of human *SNCA* were retrieved from the HapMap Genome Browser (www.hapmap.org) and the NIEHS Environmental Genome Project Web site (<http://egp.gs.washington.edu/>). The minor alleles of all SNPs were introduced into luciferase constructs using the QuikChange Multi Site-Directed Mutagenesis kit (Stratagene 200515). The mutagenesis primers used are shown below:

ATCAGCAGTGATGGAAGTATCTG TACCT (for rs10024743),
TATGAAATTTTACCATTTTGTGATGTG (for rs17016074),
TCCCTTTCACCTGAAGCGAATACAT GGTAGCAGG (for rs34825021),
TCCCTTTCACCTGAAGTGAATACATAGTAGCAGG (for rs35716318) and
TTATAAGATTTTATAGGTGTTTTTAATGATACTGTC (for rs35733299).

Cell culture, transfections and luciferase assays

SHSY5Y cells (ATCC CRL-2266) were transfected with 1 µg of luciferase construct and 100 ng of Renilla plasmid (Promega E2231) using FuGENE (Roche 11-814-443-001). Cell lysates were analyzed using the dual luciferase assay kit (Promega E1910) and the semi-automatic luminometer Lumat LB 9507 (EG&G Berthold).

Results

Stringent evolutionary constraints over hundreds of millions of years throughout the 3'UTR of *SNCA*

To gain an understanding of the overall functional significance of the 3'UTR of *SNCA*, we examined its degree of conservation throughout evolution. Alignment of the 3'UTR of *SNCA* from twelve species revealed sequence blocks that have remained unchanged over millions of years (Fig. 1).

To quantify the degree of *SNCA* 3'UTR conservation seen across species, we subjected the 3'UTRs of 426 chicken-human ortholog pairs to global sequence alignment. We determined that the 3'UTR of *SNCA* was the 24th most highly conserved 3'UTR of the sequences analyzed, yielding a 610-nucleotide alignment with 71.6% identity. To extend this analysis, we performed local sequence alignments on a larger set of ortholog pairs to delineate regions of unusually strong 3'UTR conservation between chicken and human. About 25% of 2653 orthologous pairs yielded one or more local alignments of 80% identity or greater, indicating that conservation between chicken and human orthologs in limited regions of the 3'UTR is not a rare occurrence. However, the *SNCA* 3'UTR sequences displayed an atypically high level of conservation, with three significantly scoring local alignments: 88.7% identity (106 nucleotides), 86.2% identity (87 nucleotides), and 91.2% identity (34 nucleotides). We considered alignment quality (E-value) and relative coverage of length in evaluating the local alignment results, and *SNCA* ranked in the top 10% of chicken-human orthologs analyzed with respect to both criteria. These results suggest that human *SNCA* contains several functional elements that are distributed throughout its 3'UTR.

Alternative polyadenylation in the 3'UTR of human *SNCA*

To determine the pattern of alternative polyadenylation in the 3'UTR of human *SNCA*, we identified all putative polyA signals present in the 3'UTR of human *SNCA* (Fig. 2). Of the eight polyA signals predicted, three are detected in a short stretch of 25 nucleotides and are referred to herein as the “polyA triplet”. The eighth polyA signal is located downstream of a LINE element that is also present in the 3'UTR of some human *SNCA* transcripts (Fig. 2).

Alignment of all ESTs of human *SNCA* obtained from various tissues revealed that the eight putative polyA signals mediate four polyadenylation events (Fig. 3). EST analyses showed that the 3'UTRs of 95% of *SNCA* mRNAs are up to 574 nucleotides long (Fig. 3 and Table 1). This suggests that the first 574 nucleotides of the 3'UTR of *SNCA* are the most relevant for human physiology.

RT-PCR analyses using total mouse brain RNA confirmed the polyadenylation pattern shown in Fig. 3 (data not shown). RT-PCR analyses additionally confirmed a much higher abundance of *SNCA* mRNAs that are 574 nt long (data not shown).

Table 1 summarizes the polyA signal usage in the 3'UTR of human *SNCA* and includes information on predicted GU-rich elements. GU-rich elements located downstream of polyA signals are known as downstream sequence elements (DSE) and can affect polyadenylation efficiency [5,7,14].

Single nucleotide polymorphisms in the 3'UTR of human *SNCA* and their effect on gene expression

Due to the clinical importance of increased alpha-synuclein expression in Parkinson disease, we retrieved all single nucleotide polymorphisms (SNPs) present in the first 574 nucleotides of the 3'UTR of human *SNCA* (Fig. 4a) and examined their effect on luciferase expression in

neuroblastoma SHSY5Y cells. Of the five SNPs examined, only rs17016074 resulted in an increase in luciferase expression over numerous experiments performed under diverse conditions (Fig. 4b). The average increase in luciferase activity caused by the presence of the minor allele of rs17016074 is 32.8% (Fig. 4b). A single nucleotide change at the polymorphic locus rs17016074 can therefore affect reporter gene expression in neuroblastoma SHSY5Y cells.

If rs17016074 can affect expression of endogenous SNCA in humans, it could also affect susceptibility to Parkinson disease. Due to an apparent lack of recombination events, the low frequency of the minor alleles of most of these five SNPs and their differing frequency in various human populations (data available through NCBI, HapMap, and the NIEHS Environmental Genome Project Web sites), haplotypes containing the minor allele of rs17016074 with more than one minor allele of the four other SNPs are not found. We conclude, therefore, that combinations of minor alleles at the other four SNPs are unlikely to affect *SNCA* expression *in vivo* in humans.

Discussion

In this study, we have shown that human *SNCA* mRNA molecules have any of four different 3'UTR sequences. We have also shown that the first 574 nucleotides of the 3'UTR of human *SNCA* are highly conserved and included in 95% of its mRNAs. Finally, we have identified a single nucleotide polymorphism, rs17016074, that can increase reporter gene expression in neuroblastoma SHSY5Y cells. The presence of a functional SNP in the 3'UTR of human *SNCA* qualifies the SNP and the entire *SNCA* 3'UTR as potential susceptibility loci for Parkinson disease.

Rs17016074 is located between the two most frequently used polyA sites in the 3'UTR of human *SNCA* (Fig. 4a). Rs17016074 is located immediately upstream of (but not in) a downstream sequence element, which could affect polyadenylation efficiency at the immediately upstream polyA signal (Table 1). The effect on reporter gene expression of rs17016074 suggests that a functionally important element is located between nucleotides 473 and 528 of the 3'UTR of human *SNCA*. Therefore, it contains elements that can indeed affect gene expression, a finding that is consistent with the biological importance suggested by the high conservation of the 3'UTR of *SNCA* over millions of years.

Knowing the 3'UTR sequence of *SNCA* mRNAs lays the framework for understanding the importance of genetic variation at the 3' end of *SNCA* in Parkinson disease. Several studies have reported an association of the 3' end of human *SNCA* with sporadic PD [16-18,23,27]. To date, the molecular details of these association studies were not clear, because the entire 3'UTR sequence of *SNCA* mRNAs in both cases and controls was not known. Of all the polymorphisms that are reported to be associated with sporadic PD in these studies, only rs356165 is actually present in the 3'UTR of *SNCA* mRNAs. Rs356165 is located in the LINE element of the 3' UTR of human *SNCA* (Fig. 2 and Fig. 3) and is therefore included in only 5% of *SNCA* mRNAs (Fig. 3). The minor allele of rs356165 could, therefore, have a direct effect on *SNCA* expression only by affecting a small minority (about 5%) of *SNCA* mRNAs in which it is present. Alternatively, it is possible that the association between rs356165 and sporadic PD is driven by other SNPs in *SNCA* that are functionally important and in linkage disequilibrium with rs356165. The minor allele of rs17016074 is unlikely to be such a polymorphism, since none of the published association studies included PD patients of African descent, where the minor allele of rs17016074 is detected. It is noteworthy that Parkinson disease is inadequately studied in people of African descent (African Americans and sub-Saharan Africans). Rs17016074 is therefore a novel, clinically important polymorphism identified as a result of our studies.

In summary, we have determined the sequence of the 3'UTR present in *SNCA* mRNAs in humans and identified a single nucleotide polymorphism that is of potential clinical importance for Parkinson disease. Identifying polymorphisms that can increase alpha-synuclein expression may lead to an understanding of the etiology of sporadic Parkinson disease, which constitutes ~90% of all PD cases. As the total number of sporadic PD patients increases due to increase of the human lifespan, polymorphisms that stimulate alpha-synuclein expression become even more clinically significant.

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Fig. 1. Multiple sequence alignment of 12 alpha-synuclein orthologs

The alignment of sequences starts at the top left corner, proceeds down the left column, then continues in the column on the right. Only the first 574 nucleotides of the 3'UTR of human alpha-synuclein are shown. The most frequent ('consensus') nucleotide in each column is shown against a black background. Nucleotides most similar to the 'consensus' nucleotide in each column are indicated by a grey background. The functional polyA signals are enclosed by black rectangles.

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Fig. 3. Alignment of human alpha-synuclein ESTs

A diagram of the 3'UTR of human alpha-synuclein with the position of the functional polyA signals is shown above the alignment, with the 5' end of the 3'UTR on the left and the 3' end on the right. The numbers along the line that represents the 3'UTR of human alpha-synuclein indicate the nucleotide position of each functional polyA signal. Each colored horizontal bar represents a single EST. The tissue source of each EST is shown in the left column.

rs10024743 GAAATATCTTTGCTCCCAGTTTCTTGAGATCTGCTGACAGA
 TGTTCATCCTGTACAAGTGCTCAGTTCCAATGTGCCCAGT
 CATGACATTTCTCAAAGTTTTTACAGTGTATCTCGAAGTCT
 TCCATCAGCAGTGAT**T**GAAGTATCTGTACCTGCCCCACTC
 AGCATTTTCGGTGCTTCCCTTTCACTGAAG**T**GAATACAT**G**GT
 AGCAGGGTCTTTGTGTGCTGTGGATTTTGTGGCTTCAATCT
 ACGATGTTAAACAA**ATTAAAA**ACACCTAAGTGACTACCAC
rs35733299 TTATTTCTAAATCCTCACTATTTTTTTTGTGTGCTGTTGTTCA
 GAAGTTGTTAGTGATTTGCTATCATATATTATAAGATTTTT
 AGGT**G**T**C**TTTTAATGATACTGTCTAAGAATAATGACGTATT
 GTGAAATTTGTTAATATATATAATACTTAAAAATATGTGAG
 CATGAAACTATGCAC**C****TATAAA**TACTAAATATGAAATTTTA
 CCATTTT**C**GATGTGTTTTATTCACTTGTGTTTGTAT**TATAA**
rs17016074 ATGGTGAGAA**ATTAAAA**TAAACGTTATCTCATTTGCAAAAT

rs34825021
rs35716318

Fig. 4. All known SNPs in the first 574 nt of the 3'UTR of human alpha-synuclein and the effect of the minor allele of rs17016074 on luciferase gene expression

(a) Only the first 574 nt of the 3'UTR of human alpha-synuclein are shown. This gene segment includes the 3'UTR of up to 95% of alpha-synuclein mRNAs in humans. The minor allele of all five SNPs was introduced in luciferase reporter constructs and its effect on gene expression was examined.

(b) The luciferase activity of constructs carrying the full-length (574 nt) 3'UTR of *SNCA* with the major (black line) and minor allele (gray line) of rs17016074 is shown. The set of data depicted represent different experiments performed under a wide range of conditions (e.g., different plasmid preparations).

Table 1**Summary of polyA signals, polyA cleavage sites and downstream sequence elements in the 3'UTR of alpha-synuclein**

Starting on the left, all functional polyA signals in the 3'UTR of human alpha-synuclein are shown. "Position" is the nucleotide position of each functional polyA signal, starting from the 5' end of the 3'UTR of human alpha-synuclein in NM_000345 as shown in Fig. 2. "% ESTs" is the percentage of human ESTs detected for each polyA signal. The numbers in parentheses are the actual number of ESTs detected for each polyA signal out of a total of 37 ESTs that met our inclusion criteria. All cleavage sites detected in the EST analyses for each polyA signal are shown. The slash indicates the exact cleavage site. The two nucleotides located upstream and downstream of each cleavage site are also shown (see also Fig. 2). The predicted downstream sequence elements (DSEs) associated with each polyA signal are shown in the leftmost column. Note: The sixth and seventh polyA signal predicted in the 3'UTR of human alpha-synuclein (Fig. 2) were never detected as functional in any of our multiple EST (Fig. 3) and RT-PCR analyses (data not shown).

PolyA signals	Position	% ESTs	Cleavage site	DSE
ATTAAA	261-267	14 (5/37)	CC/AC (3/5; 285nt) CT/AA (2/5; 295nt)	UUUUUUUGUU...UGUUGUU
TATAAA	467-473	27 (10/37)	TT/AC (1/10; 490nt) TT/TA (8/10; 491nt) TT/TT (1/10; 497nt)	UGUGUUUUA...UGUGUUUGU
TATAAA...ATTAAAATAAA	528-553	54 (20/37)	GC/AA (14/20; 568nt) AT/AT (6/20; 574nt)	UUUUU
AATAAA	1053-1059	5 (2/37)	TG/AA (1/2; 1074nt) AT/AA (1/2; 1077nt)	GUGGGUU